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Supplemental Information

A Human Pluripotent Stem Cell-Based Screen for Smooth Muscle Cell Differentiation and Maturation Identifies Inhibitors of Intimal Hyperplasia

Jue Zhang, Brian E. McIntosh, Bowen Wang, Matthew E. Brown, Mitchell D. Probasco, Sarah Webster, Bret Duffin, Ying Zhou, Lian-Wang Guo, William J. Burlingham, Craig Kent, Michael Ferris, and James A. Thomson

Supplemental Figures

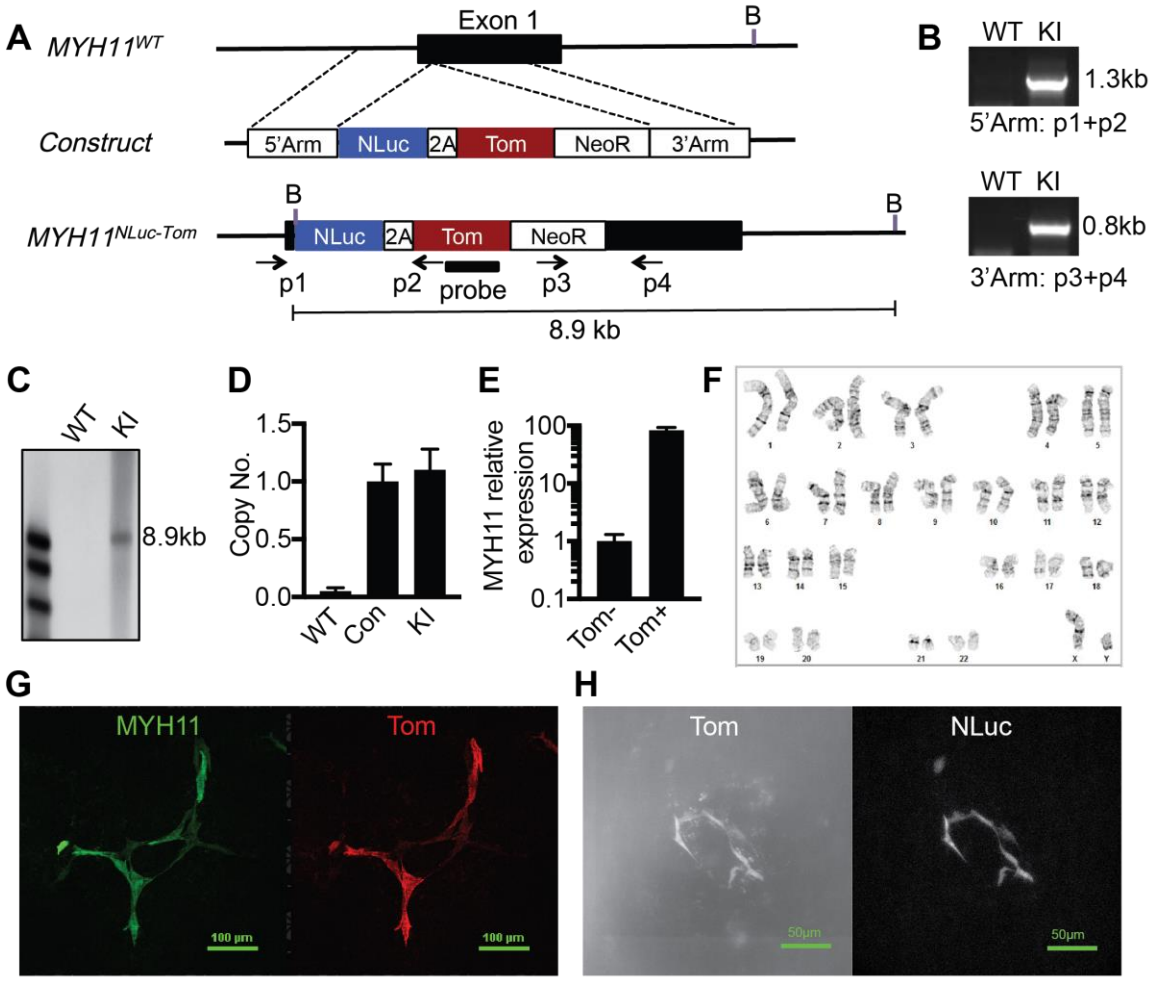

Figure S1. Generation and characterization of the reporter cell line. Related to Figure 1.

(A) Schematic of wild type and targeted *MYH11-NLuc-tdTomato* allele. B, BamH I cutting site; NLuc, NanoLuc; P1-4, PCR primers; Tom, tdTomato.

(B) Junction PCR of 5' arm and 3' arm of *MYH11-NLuc-tdTomato* allele. KI: Knock-in cells; WT: wild type.

(C) Southern blot of *MYH11-NLuc-tdTomato* allele. KI: Knock-in cells; WT: wild type.(D) qPCR analysis of tdTomato copy number. Data are represented as mean ±SD. *n*=3 independent experiments; Con, control samples with one copy of tdTomato; KI: Knock-in cells; WT: wild type.

(E) qPCR analysis of MYH11 expression in cells sorted by flow cytometry. Data are represented as mean ±SD. *n*=3 independent experiments; Tom, tdTomato.

(F) Karyotyping of *MYH11-NLuc-tdTomato* cell line.

(G) Immunostaining showing the overlapped expression of endogenous MYH11 and Knock-in tdTomato. Tom, tdTomato. Scale bar=100 μM.

(H) Live imaging showing the overlapped expression of tdTomato and NanoLuc Luciferase. NLuc, NanoLuc; Tom, tdTomato. Scale bar=50 μM.

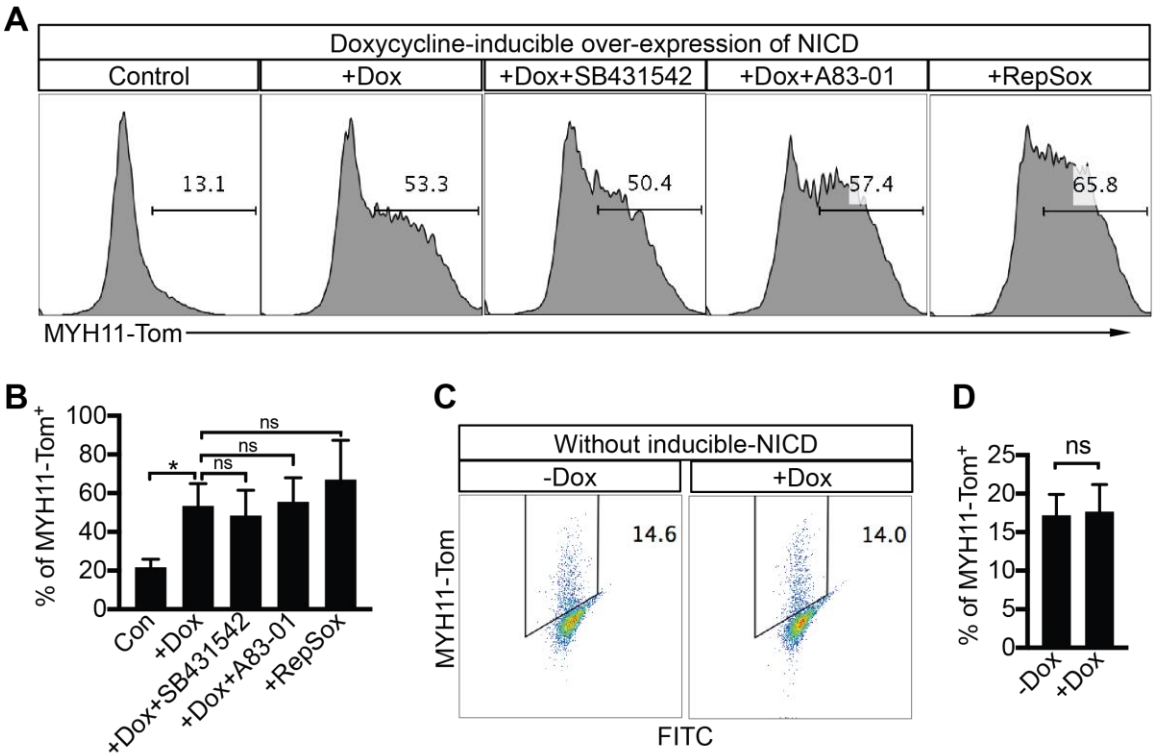
A
Doxycycline-inducible over-expression of NICD
Control
+Dox
+Dox+SB431542
+Dox+A83-01
+RepSox
13.1
53.3
50.4
57.4
65.8
MYH11-Tom
B
% of MYH11-Tom+
100
80
60
40
20
0
*
ns
ns
ns
Con
+Dox
+Dox+SB431542
+Dox+A83-01
+RepSox
C
Without inducible-NICD
-Dox
+Dox
14.6
14.0
MYH11-Tom
FITC
D
% of MYH11-Tom+
25
20
15
10
5
0
ns
-Dox
+Dox

Figure S2. Combination treatment of over-expression of NICD1 and inhibition of TGFβ signaling. **Related to Figure 2.**

(A) Flow cytometric analysis of MYH11-Tom$^+$ cells. The cells were treated with 1 μg/ml doxycycline (to induce the expression of NICD1), 10 μM doxycycline with SB431542 (a TGFβ receptor inhibitor), 2 μM doxycycline with A83-01 (a TGFβ receptor inhibitor), or 25 μM RepSox (from day 10-16 or day 12-16). Dox, doxycycline.

(B)Statistical data of MYH11-Tom$^+$ cells. Data are represented as mean ±SD. *n*=6 independent experiments; *, $p<0.05$; Student's t-test; ns, non-significant. Con, control; Dox, doxycycline.

(C) Flow cytometric analysis of MYH11-Tom$^+$ cells. RepSox is not included in the media. FITC, fluorescein isothiocyanate.

(D)Statistical data of MYH11-Tom$^+$ cells. Data are represented as mean ±SD. *n*=3 independent experiments; Student's t-test; ns, non-significant. Dox, doxycycline.

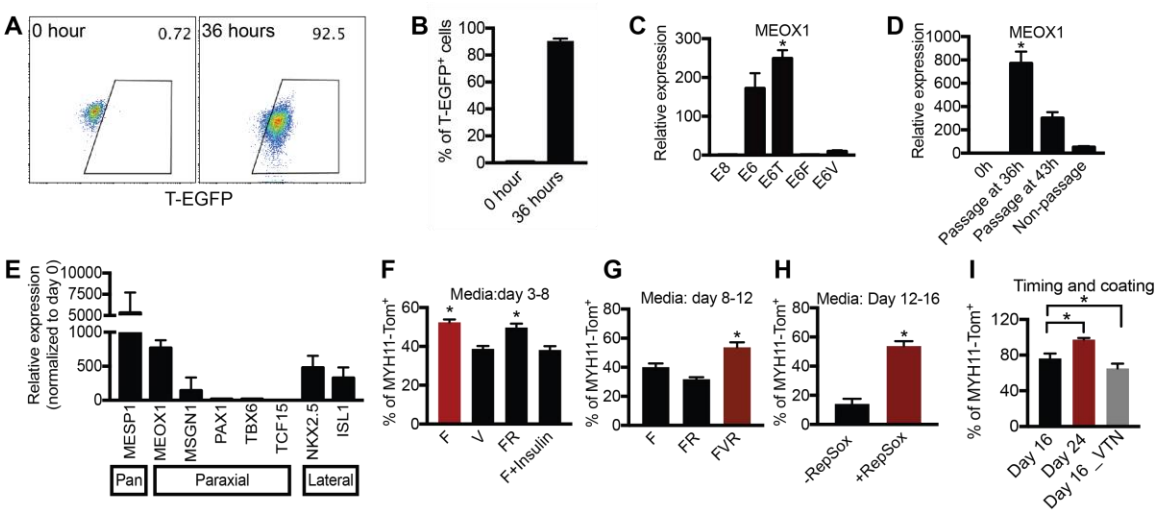
A
0 hour
0.72
36 hours
92.5
T-EGFP
B
% of T-EGFP+ cells
0 hour
36 hours
C
MEOX1
Relative expression
E8
E6
E6T
E6F
E6V
D
MEOX1
Relative expression
0h
Passage at 36h
Passage at 43h
Non-passage
E
Relative expression (normalized to day 0)
MESP1
MEOX1
MSGN1
PAX1
TBX6
TCF15
NKX2.5
ISL1
Pan
Paraxial
Lateral
F
Media:day 3-8
% of MYH11-Tom+
F
V
FR
F+Insulin
G
Media: day 8-12
% of MYH11-Tom+
F
FR
FVR
H
Media: Day 12-16
% of MYH11-Tom+
-RepSox
+RepSox
I
Timing and coating
% of MYH11-Tom+
Day 16
Day 24
Day 16 _ VTN

Figure S3. Optimization of SMC differentiation in xeno-free media. Related to Figure 3.

(A) Flow cytometric analysis of T-EGFP$^+$ cells. T-2A-EGFP reporter ES cell line (Chu et al., 2016) was cultured in E8BAC media (E8 media supplemented with 5 ng/mL BMP4, 25 ng/mL Activin A, and 1 μM CHIR99021) for 36 hours.

(B) Statistics of T-EGFP$^+$ cells measured by flow cytometry. Data are represented as mean ±SD. *n*=3 independent experiments.

(C) Regulation of MEOX1 expression (RT-qPCR analysis). Cells were cultured in E8BAC media for 36 hours and then treated with E6 (E8 media minus FGF2 and TGF-β1), E6T (E6+1.7 ng/ml TGF-β1), E6F (E6+100 ng/ml FGF2), or E6V (E6+50 ng/ml VEGFA) for another 2 days. Cells at day 4 were collected for RT-qPCR. Undifferentiated cells cultured in E8 media were used as the control. Data are represented as mean ±SD, *n*=3 independent experiments; *, $p<0.05$; Student's t-test.

(D) Passaging cells promotes MEOX1 expression. Cells were cultured in E8BAC media for 36 or 43 hours and then passaged or non-passaged as indicated. The cells were treated with E6T at 36 or 43 hours. Cells at day 4 were collected for RT-qPCR. Undifferentiated cells (0h) were used as the control. Data are represented as mean ±SD. *n*=3 independent experiments; *, $p<0.05$; Student's t-test.

(E) RT-qPCR analysis of mesoderm marker expression. Cells were cultured in E8BAC media for 36 hours then passaged in E6T media for another 18 hours.

The cells were then collected for RT-qPCR. Data are represented as mean ±SD. *n*=3 independent experiments.

(F) Optimization of the media used from day 3-8. Cells were cultured in E8BAC media for 36 hours then passaged and cultured in E6T media for another 18 hours. E5 (E8 media minus FGF2, TGF-β1, and insulin) was used as the base media from day 3-8. FGF (F), VEGFA (V), resveratrol (R), or insulin was added as indicated. Statistics of MYH11-Tom$^+$ cells measured by flow cytometry at day 16. FVR media was used from day 8-12. RepSox was included in the media form day 12-16. Data are represented as mean ±SD. Student's t-test; *, $p<0.05$; $n=3$ independent experiments.

(G) Optimization of the media used from day 8-12. E6 (E8 media minus FGF2 and TGF-β1) was used as the base media. FGF (F), VEGFA (V), or resveratrol (R) was added as indicated. RepSox was included in the media form day 12-16. Statistics of MYH11-Tom$^+$ cells measured by flow cytometry at day 16. Data are represented as mean ±SD. Student's t-test; *, $p<0.05$, $n=3$ independent experiments.

(H) Optimization of the media used from day 12-16. Cells were treated with or without RepSox. Statistics of MYH11-Tom$^+$ cells measured by flow cytometry at day 16. Data are represented as mean ±SD. Student's t-test; *, $p<0.05$; $n=3$ independent experiments.

(I) Statistics of MYH11-Tom$^+$ cells. Matrigel was used for coating in the first 2 experiments and vitronection (VTN) coating was used in the third experiment.

Cells were collected at day 16 or 24 for analysis. Data are represented as mean ±SD. Student's t-test; *, $p<0.05$; $n=3$ independent experiments.

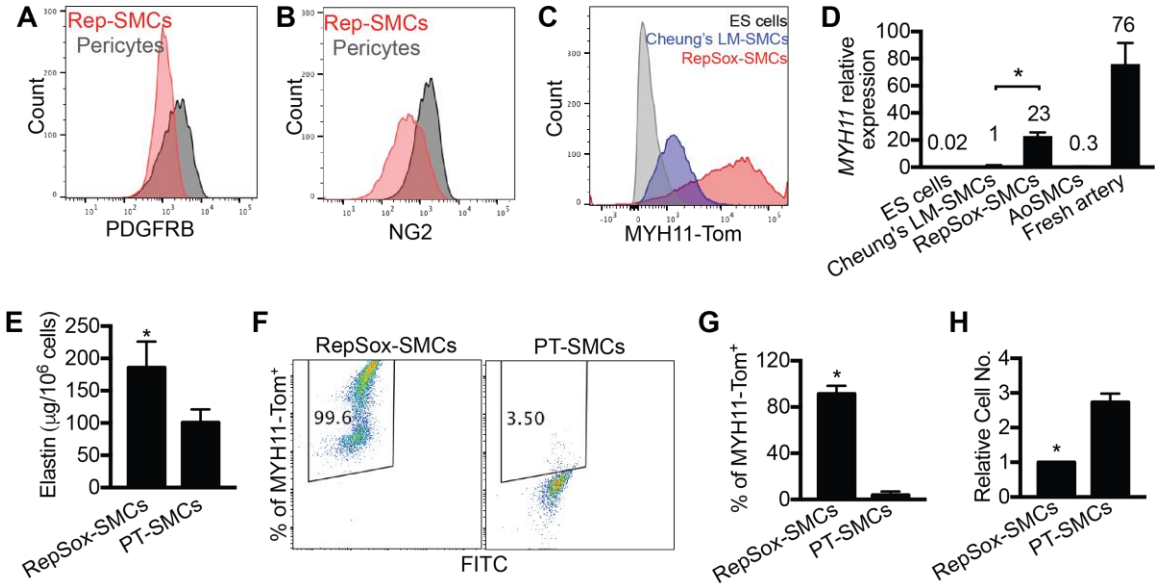
A
Rep-SMCs
Pericytes
Count
PDGFRB
B
Rep-SMCs
Pericytes
Count
NG2
C
ES cells
Cheung's LM-SMCs
RepSox-SMCs
Count
MYH11-Tom
D
MYH11 relative expression
0.02
1
23
0.3
76
*
ES cells
Cheung's LM-SMCs
RepSox-SMCs
AoSMCs
Fresh artery
E
Elastin (μg/10^6 cells)
*
RepSox-SMCs
PT-SMCs
F
RepSox-SMCs
PT-SMCs
% of MYH11-Tom+
99.6
3.50
FITC
G
% of MYH11-Tom+
*
RepSox-SMCs
PT-SMCs
H
Relative Cell No.
*
RepSox-SMCs
PT-SMCs

Figure S4. Characterization of RepSox-SMCs. Related to Figure 3.

(A-B) Flow cytometric analysis of PDGFRB and NG2 expression in primary human pericytes or RepSox-SMCs generated by the optimized 24-day protocol.

(C) Flow cytometric analysis of MYH11-Tom$^+$ cells derived from RepSox protocol or Cheung's protocol for LM-SMCs (Cheung et al., 2012).

(D) qPCR analysis of *MYH11* expression. Data are normalized to Cheung's LM-SMCs and represented as mean ±SD. *n*=3 independent experiments for all the samples except fresh artery (n=2); *, $p<0.05$; Student's t-test.

(E) Elastin production measured by Fastin Elastin Assay. *n*=3 independent experiments; *, $p<0.05$; Student's t-test.

(F) Flow cytometric analysis of MYH11-Tom$^+$ cells that were cultured for another 8 weeks after 24-day differentiation. FITC, fluorescein isothiocyanate.

(G) Statistics of MYH11-Tom$^+$ cells. Data are represented as mean ±SD. *n*=3 independent experiments; *, $p<0.05$; Student's t-test.

(H) Statistics of relative cell number. Cell number is counted 8 weeks after differentiation and normalized to Repsox-SMCs. Data are represented as mean ±SD. *n*=3 independent experiments; *, $p<0.05$; Student's t-test.

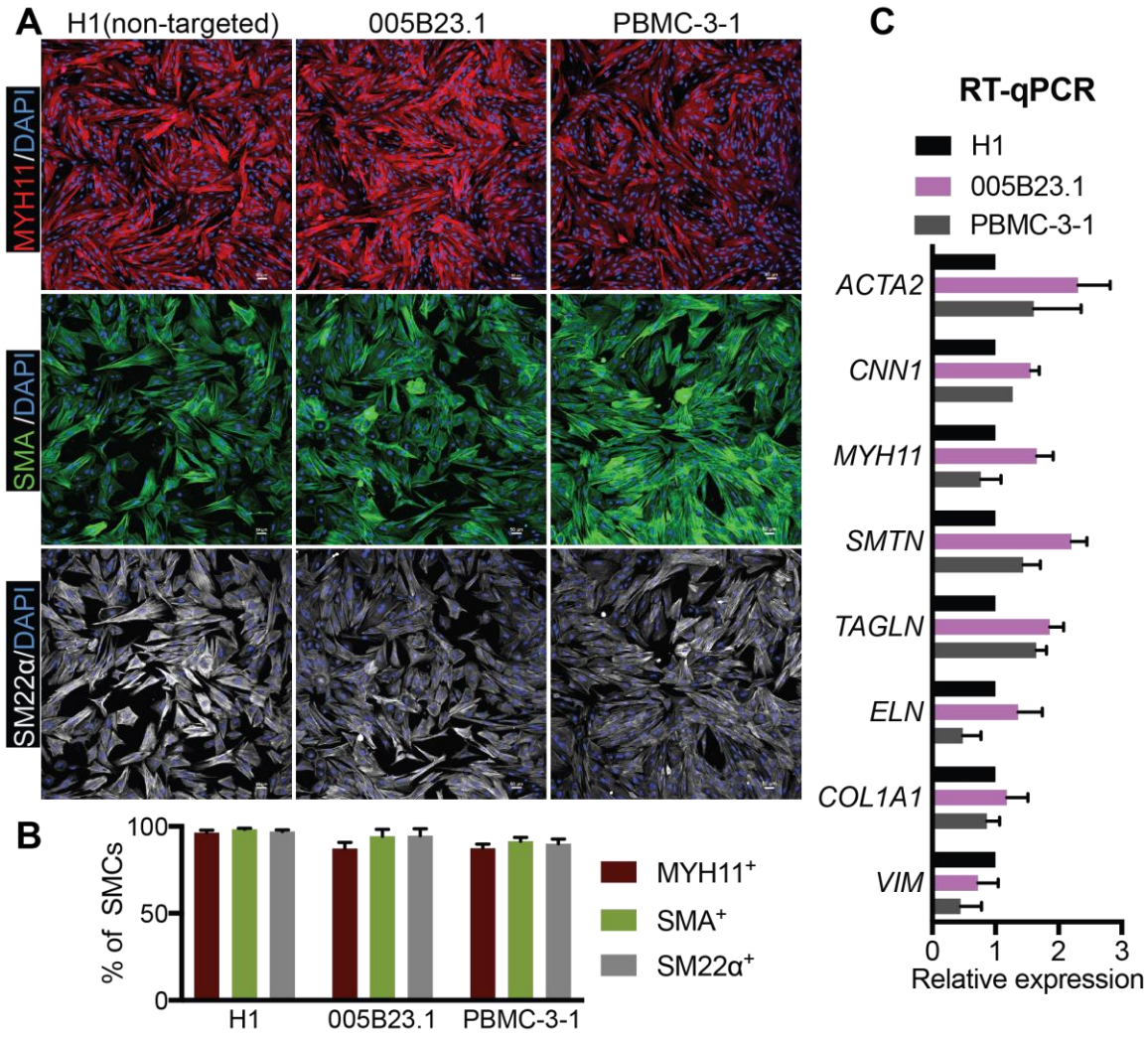
A
H1(non-targeted)
005B23.1
PBMC-3-1
MYH11/DAPI
SMA /DAPI
SM22α/DAPI
B
% of SMCs
100
50
0
H1
005B23.1
PBMC-3-1
MYH11+
SMA+
SM22α+
C
RT-qPCR
H1
005B23.1
PBMC-3-1
ACTA2
CNN1
MYH11
SMTN
TAGLN
ELN
COL1A1
VIM
0
1
2
3
Relative expression

Figure S5. Generation of SMCs from multiple pluripotent stem cell lines. Related to Figure 3.

(A) Immunostaining of MYH11, SMA, and SM22α. Scale bar=50 μM.

(B) Statistics of $MYH11^+$, SMA^+, and $SM22\alpha^+$ cells measured by Immunostaining. Data are represented as mean ±SD. *n*=3 independent experiments.

(C) RT-qPCR of SMCs. Data are normalized to H1-derived SMCs and represented as mean ±SD. *n*=3 independent experiments.

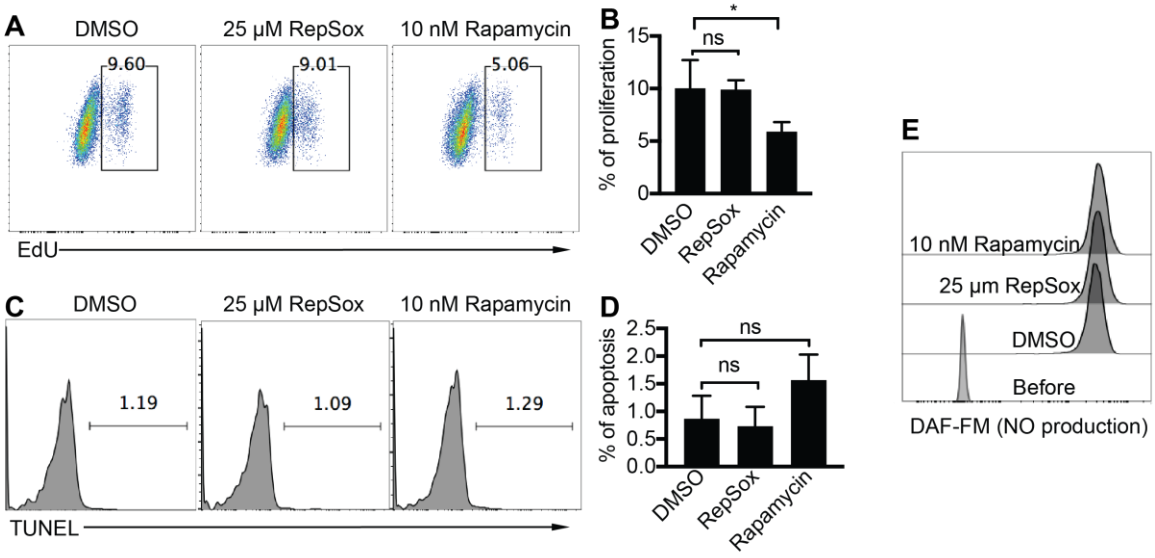
A
DMSO
25 μM RepSox
10 nM Rapamycin
9.60
9.01
5.06
EdU
B
% of proliferation
15
10
5
0
ns
*
DMSO
RepSox
Rapamycin
C
DMSO
25 μM RepSox
10 nM Rapamycin
1.19
1.09
1.29
TUNEL
D
% of apoptosis
2.5
2.0
1.5
1.0
0.5
0.0
ns
ns
DMSO
RepSox
Rapamycin
E
10 nM Rapamycin
25 μm RepSox
DMSO
Before
DAF-FM (NO production)

Figure S6. RepSox and arterial endothelial cells. Related to Figures 5 and 6.

(A) Flow cytometric analysis of cell proliferation which was determined by Click-IT-EdU Assay. Cells were treated with DMSO, RepSox, or Rapamycin for 24 hours.

(B) Statistical data of proliferative cells. Data are represented as mean ±SD. *n*=3 independent experiments; *, $p<0.05$; Student's t-test; ns, non-significant.

(C) Flow cytometric analysis of cell apoptosis which was measured by TUNEL assay. The cells were treated with DMSO, RepSox, or Rapamycin for 24 hours.

(D) Statistical data of apoptosis cells. Data are represented as mean ±SD. *n*=3 independent experiments; Student's t-test; ns, non-significant.

(E) Flow cytometric analysis of NO production. The human pluripotent stem cell derived arterial endothelial cells were used in the above assays (Zhang et al., 2017). Cells were treated with DMSO, RepSox, or Rapamycin for 24 hours.

Experimental procedures

Gene targeting on the H1 ES cells

The 5'- and 3'-homology arms of MYH11 targeting vector were synthesized by IDT (gBlock) and cloned into a NanoLuc and tdTomato containing vector. NanoLuc and tdTomato were inserted into the first exon of MYH11. To achieve the best electroporation efficiency, human ES cells (H1) were passaged with EDTA (1:4 split) and cultured to reach 80–90% confluency two days before the experiment. On the day of the experiment, ES cells were dissociated by Accutase, washed once with E8 (Chen et al., 2011) (Essential 8^{TM}) medium, and resuspended at densities of 4 x 10^6 cells/mL in E8 medium with 10 mM Hepes buffer (pH 7.2–7.5) (Life Technologies). For electroporation, 500 µL of cell suspension, 7.5 µg gRNA plasmid, 7.5 µg spCas9 plasmid, and 10 µg DNA template plasmid were mixed in a 4 mm cuvette (Bio-Rad) and immediately electroporated with a Bio-Rad Gene Pulser. Electroporation parameters were set at 250 V, 500 µF, and infinite resistance. Cells were then plated on a Matrigel-coated plate in E8 media (10 µM of Y27632 was added the first day. Geneticin (100 µg/ml) was added to the media when cells reached 20% confluency (usually 3–4 days after electroporation) and treatment continued for five days. Surviving colonies were picked 4–6 days after drug selection and expanded in E8 medium.

Karyotyping

Karyotyping was performed by the WiCell Research Institute.

Southern blot

PCR DIG Probe Synthesis Kit (Roche, Cat# 11 636 090 910) was used for synthesis of the probe. The southern blot was performed following the DIG Application Manual for Filter Hybridization from Roche.

High-throughput screening

The *MYH11-NLuc-tdTomato* reporter human ES cell line was differentiated into mesoderm by using E8BAC (E8 media supplemented with 5 ng/mL BMP4, 25 ng/mL Activin A, and 1 μM CHIR99021) media for two days. The cells were treated with 50 ng/ml FGF2 and 20 ng/ml BMP4 for another two days. The cells were passaged at day 4 and seeded on the 96-well plate for screening (2 x 10^6 cells/plate). The compounds were added from day 4-14. The Luciferase substrate (1000x dilution) (Nano-Glo Luciferase Assay system, Promega) was added to media for 15 minutes and luminescence was measured.

The Thomson Custom Stem Cell Modulator 1, GSK-kinase Inhibitor Library, SellekChem Kinase Inhibitor Library, Analyticon NATx Library, Prestwick Chemical Library, GlaxoSmithKline Protein Kinase Inhibitors, and part of Enamine Representative Diversity Library were used for screening.

Optimized smooth muscle cell differentiation

Human pluripotent stem cell cells (H1) were cultured in E8 media on a Matrigel-

coated plate (9 μg/cm^2, or 500 μg/plate). To achieve the best differentiation results, ES cells were split using EDTA at 1:4 ratios two days before differentiation. The cells reached 80–90% confluency two days later. At the day of differentiation, ES cells were dissociated by Accutase (Invitrogen) for 3-5 min at 37°C. The cells were plated on a Matrigel-coated plate at 1:4 ratios (1 x 10^5 cells/cm^2). The cells were cultured in E8BAC media (E8 media supplemented with 5 ng/mL BMP4, 25 ng/mL Activin A, and 1 μM CHIR99021) for 36 hours; at this time point, an increase of CHIR99021 concentration to 3 μM decreases the cell proliferation but increases the MYH11 expression. At 36 hours, the cells were passaged and seeded on a new Matrigel-coated plate (1.6 x 10^4 cells/cm^2), at which point they can be cryopreserved. Low cell density is critical for differentiation. We suggest testing 1.0-4.0 x 10^4 cells/cm^2 for different cell lines, or the same cell line with different culture history, so that 100% cell confluence will be achieved at day 6, allowing for the best differentiation efficiency. The cells were treated with E6T media (E8/Essential 8^{TM} media minus FGF2 but still containing 1.7 ng/ml TGF-β1) for 18 hours to induce the transient and medium level expression of MEOX1. E5F media (E8 media minus insulin and TGF-β1 but still containing 100ng/ml FGF2) was used to suppress MEOX1 expression for another 5 days (day 3-8). Next, the cells were treated with FVR media (E6 media+100 ng/ml FGF2, 50 ng/ml VEGFA, and 25 μM RESV, a NOTCH agonist) (E6 media is defined as E8 media minus FGF2 and TGF-β1) to induce SMC progenitors from day 8 to day 12. E6R media (E6 media +RESV) supplemented with RepSox (25 μM) was then used to further mature SMCs from day 12 to 24.

Cells were split ($1x10^5$ cells/cm^2) at day 16 and further differentiated until day 24. The cells can be cryopreserved at day 16 or day 24. For P-SMCs, T-SMCs, and PT-SMCs, RepSox was replaced by PDGF-BB (10 ng/ml), TGF-β1 (1.7 ng/ml), or a combination of PDGF-BB and TGF-β1 from day 12 to 24.

From day 0-4: media was changed every day.

From day 2-24: media was changed every other day.

For day 1 and day 8-24: 1.5 x volumes of media were used.

Thirty-six hours of E8BAC treatment can be 35-37 hours; further decreasing or increasing the time will reduce the differentiation efficiency.

Eighteen hours of E6T treatment can be 16-20 hours; further decreasing or increasing the time will reduce the differentiation efficiency.

RepSox-SMCs, P-SMCs, T-SMCs and PT-SMCs were maintained in E6R media supplemented with RepSox, PDGF-BB, TGF-β1, or PDGF-BB and TGF-β1, respectively. AoSMCs were maintained in SmGM2 (Lonza) media.

Alternative SMC differentiation protocol (1): the RESV can be withdrawn from the media from day 8-12.

Alternative SMC differentiation protocol (2): FVR media can be used from day 8-10 and then changed to E6R media supplemented with RepSox from day 10-24.

Alternative SMC differentiation protocol (3): vitronectin (0.9 μ g/cm^2 or 50 μ g/plate) can replace Matrigel for differentiation but with decreasing $MYH11^+$ cells.

RT-PCR analysis

See list of probes in the table below (Thermofisher). RNA was isolated with the RNAsesy Mini Kit (Qiagene). Reverse transcription was performed with SuperScript Vilo cDNA Synthesis Kit (Invitrogen). qPCR was performed with Taqman PCR Master Mix (Thermofisher, cat# 4440044) in ViiA7 Real-Time PCR System (Thermofisher).

Gene	**Cat#**
ACTA2	Hs00426835_g1
CNN1	Hs00154543_m1
MYH11	Hs00224610_m1
SMTN	Hs00199489_m1
TAGLN	Hs01038777_g1
ELN	Hs00355783_m1
COL1A1	Hs00164004_m1
VIM	Hs00958111_m1
GAPDH	Hs02786624_g1
CD31/PECAM1(Rat)	Rn01467262_m1
CD144/CDH5(Rat)	Rn01536708_m1
GAPDH(Rat)	Rn01775763_g1

Antibodies

Antibody	**Vendor**	**Cat#**
Human Nuclei	Millipore	MAB1281
CD31/PECAM1	BD Pharminger	550274
GAPDH	Millipore	MAB374
Ki67	Abcam	ab16667
MYH11	Dako	M3558
NICD1	Cell signaling	4147
NOTCH1	Cell signaling	3608
SM22α/TAGLN	Abcam	ab14106
SMA/ACTA2	Dako	M0851
pSMAD2	Cell signaling	3101

Immunostaining

Samples were fixed on the day indicated using 4% paraformaldehyde (PFA) for 10 min. Cells were then washed with PBS for 5 min (3 times), permeabilized with 0.2% Triton X-100 in PBS supplemented with 1% BSA for 10 min, and incubated in primary antibody (diluted in 1% BSA in PBS) for O/N at 4°C. In the second day, cells were then washed with PBS for 5 min (3 times) and incubated with fluorescence labeled secondary antibody (Invitrogen) for 2 hours at room temperature.

Western blot

Protein samples were loaded onto a PAGE gel (Bio-Rad, cat#456-1086) and run at 200V for 30 minutes. After electrophoresis, the PAGE gel, activated PVDF membrane, and filter paper were merged in Semi-dry transfer buffer for at least 5 minutes. The protein was transferred to PVDF membrane at 15V for 30 minutes by using a Bio-Rad Semi-dry transfer cell. The membrane was then blocked with 5% milk (in TBST buffer) for 1 hour at room temperature. Primary antibody was diluted in 5% milk-TBST and incubated with membrane overnight at 4°C. After washing with TBST (x3), the secondary antibody was applied and incubated for 1 hour at room temperature. The membrane was washed with TBST (x3). The protein was visualized by SuperSignal™ West Pico Chemiluminescent Substrate (Thermo-Fisher).

Cell Proliferation assay

Smooth muscle cells were seeded on a Matrigel-coated plate (3 x 10^4 cells/cm^2). The next day, 10 μM EdU was added to the media for three hours and cell proliferation was measured by Click-it EdU Kit (ThermoFisher, cat# C10425) according to the manufacturer's instructions.

TUNEL assay

Cell apoptosis was determined by *DeadEnd Fluorometic TUNEL System* (Promega, cat# G3250) according to the manufacturer's instructions

Cell Migration assay

Cell suspension (70 μl/well, 5 x 10^5 cells/ml) was seeded in the migration insert (culture-insert 2 well in μ-dish 35mm, ibidi). Next, inserts were removed and washed with fresh media twice. Cells were imaged at the indicated time point.

Cell contraction assay

Smooth muscle cells were seeded on a Matrigel-coated plate (1 x 10^4 cells/cm^2). The next day, 100 μM of carbachol was added to the media for 30 min. The cells were imaged before and after carbachol treatment. To facilitate the calculation of the cell surface change, the cells were stained with 5 μM CMFDA (green fluorescence) for 30 min before carbachol treatment. The cell surface change was measured by ImageJ.

Fastin Elastin Assay

Fastin Elastin Assay was performed according to the manufacturer's manual (Fisher Scientific, cat# NC9817729).

Kidney capsule

The experiments were performed under approval from the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health and the Health Sciences Institutional Review Board. NBSGW mice (McIntosh et al., 2015) were used for the experiments as previously described (Brown et al., 2018). The tissues were collected four weeks after surgery.

Rat carotid artery balloon injury model and periadventitial delivery

This animal study was conducted with approval from the University of Wisconsin-Madison Department of Surgery Animal Care and Use Committee. Briefly, 2 mg (2 animals/ group) or 10 mg (3 animals/group) of RepSox (or DMSO) dissolved in Regel (Tri-block gel) (Chen et al., 2017) was applied to the outside of the injured artery segment immediately after balloon injury of the rat carotid artery. Two weeks post-surgery, arteries were collected and sectioned. The ratio of intima area versus media area was measured by ImageJ.

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