Materials and Methods

Cell culture

Primary cultures of human aortic smooth muscle cells (HAoSMCs) were purchased from Vasculife and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2mM glutamine, 1mM sodium pyruvate and 100U/ml penicillin-streptomycin. Human mesenchymal stem cells (HMSCs) were purchased from Sciencell, and cultured in DMEM supplemented as above with 5% FBS. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza, and grown in EBM-2 supplemented with the bullet kit as recommended (Lonza). Primary cells between passages 7-8 were used for all experiments. For virus production, TN-293 cells were purchased from Stratagene and cultured in DMEM supplemented as above with 10% FBS. Mouse embryo fibroblasts (MEFs) were isolated from embryonic day 10.5 mouse embryos and cultured in DMEM supplemented as above with 5% FBS.[1] HEK293 cells and PAC1[2] cells were cultured in DMEM supplemented as above with 5% FBS. All cultures were maintained in humidified 5% CO_2 at 37°C. For coculture, $3x10^4$ mural cells were seeded in 12-well plates, and after adhesion, $3x10^4$ HUVECs were added. To separate endothelial cells from HAoSMCs and HMSCs, anti-PECAM1-conjugated Dynabeads (Invitrogen) were used according to manufacturer's instructions. We have demonstrated efficacy of this purification procedure previously.[3] The purity of the smooth muscle cells was verified by costaining the separated cells for PECAM1 and ACTA2 and counting cell number. The separated smooth muscle cell population was greater than 99% pure. All cell coculture experiments, unless indicated, were performed in media consisting of EBM-2 supplemented with the bullet kit. NOTCH inhibitor, DAPT (anyl-2-phenyl]glycine-1,1-dimethylethyl ester, Calbiochem) was added to specified wells at the time of plating at 10μM. BMP (ALK2/3) inhibitor, LDN193189 (Reagentsdirect) was added at 100nM, and TGFβ (ALK4/5/7) inhibitor, SB431542 (Reagentsdirect) was added at 1μM. For TGFβ1 treatment, cells were serum starved for 24 hours before TGFβ1 (Peprotech) was added at a 10ng/ml concentration. For conditioned media assays, after 24 hours conditioning, media from HUVECs or HAoSMCs was transferred to HAoSMCs. For transwell assays, $4x10^4$ HAoSMCs were plated on 12-well plates, and 0.4μm pore-size transwell inserts (Corning Costar) were inserted containing $2x10^4$ HUVECs or HAoSMCs.

Primary mouse aorta smooth muscle cell culture

Mice were euthanized at 4–5 weeks of age and a midsternal thoracotomy was performed. The thoracic aorta was isolated and adventitia was carefully removed in cold PBS (Phosphate Buffered Saline). Aorta was digested with 1mg/ml Collagenase II (Sigma, C6885) and 100μg/ml Elastase (Sigma, E0127) at 37°C for 40 minutes. After digestion, cells were pelleted and plated in DMEM with 10% FBS. The next morning, cells were washed with PBS 3-4 times, followed by media refresh every 48 hours. Primary cells at passage 2 were used for experiments.

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated using TRIzol reagent following manufactures' instructions (Invitrogen). Mouse tissue was first homogenized using TissueLyzer II (Qiagen). RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) to generate cDNA. Real-time PCR was performed using a StepOne PCR system (Applied Biosystems) with SYBR Green. Taqman assays were performed to detect mature microRNAs using a Taqman microRNA assay kit (Life technologies # 4427975).

Immunoblotting

Equivalent amounts of protein were run on 10% SDS-PAGE gels, transferred to nitrocellulose membranes (Millipore), and subjected to incubation using primary antibodies to TGFBR2 (Cell Signaling, 3713), SERPINE1 (BD Transduction, 612025), Fibronectin 1 (FN1) (BD, 610078), Collagen, type I, alpha 1 (COL1A1) (Abcam, ab292), Elastin (ELN) (Abcam, ab77804), Calponin 1 (CNN1) (Sigma, C2687), smooth muscle α-actin (ACTA2) (Sigma, 1A4), Tubulin (TUBB2A) (Sigma, T7816) and GAPDH (Novus Bio, NB300-221). Phospho-Smad2 (Ser465/467)(Cell Signaling, 3101), Smad2 (Cell Signaling, 5339), Phospho-p38 MAPK (Cell Signaling, 9211), p38 MAPK (Cell Signaling, 9219).

RNA mimic, miRNA inhibitor, and siRNA transfection

HAoSMCs were plated in a 12-well plate at $3x10^4$ cells/well. After 12 hours, the cells were transfected with miR145 or control RNA mimic at 40nM using Lipofectamine RNAiMAX (Invitrogen). For miR145 inhibition, 200nM of miRVana inhibitor MH11480 (Life Technologies) was transfected into $6x10^4$ cells/well HAoSMCs using RNAiMAX as directed. For coculture experiments, HUVECs were added 24 hours after transfection.

Notch2 siRNA was purchased from QIAGEN (GS4853) and Notch3 siRNA was synthesized by IDT as follows: 5'-AAC UGC GAA GUG AAC AUU G. Control siRNA was purchased from Invitrogen. HAoSMC were plated in a 12-well plate at $6x10^4$ cells/well. After 24 hours, cells were transfected with 40 nM siRNA using RNAiMAX (Invitrogen). After 24 hours transfection, cells were cocultured with 6 $x10^4$ HUVEC for additional 96 hours and collected for qPCR analysis. siRNA to knockdown JAG1 in endothelial cells (Dharmacon M-011060-02) was used at 80 nM with RNAiMAX transfection reagent prior to coculture. Knockdown was verified qPCR and Western blot analysis.

Lentivirus Expression

Mouse NICD1 cDNA was cloned into pCDF1-MCS2-EF1-copGFP (System Biosciences) in front of the CMV promoter using BamHI and EcoRI sites. NICD2, NICD3 and DN-MAML constructs were made as described previously.[4] The lentiviral plasmids were transfected into TN-293 cells using Lipofectamine 2000 (Invitrogen), and the viral particles were amplified and purified as described.[5]

Plasmid Transfection and Luciferase Assays

psi-CHECK2-TGFBR2 3'UTR plasmid was obtained from Addgene plasmid #31882.[6] HEK293 or PAC1[2] cells were plated in a 12 well plate and transfected with 500ng plasmid and RNA mimics at 100nM concentration. 24 hours later, Dual luciferase assay was performed to measure the firefly luciferase conjugated to the 3'UTR normalized to Renilla luciferase activity followed the instructions of manufacturer (Promega). The miR145 target site in the TGFBR2 3'UTR was mutated from AACTGGAA to AAAAAAAA by PCR mutagenesis using the following primers; Forward: GGGTTATCAGCATAAAAAAAAATGTAGTGTCAGAGG; Reverse: CCTCTGACACTACATTTTTTTTTATGCTGATAACCC.

Collagen Secretion Assay

Cell culture medium was incubated with 25% (NH4)2SO4 at 4°C overnight. The secreted collagen was pelleted by centrifugation at maximal speed and resuspended in 950µl of 50µM Sirius Red at room temperature. The stained collagen was centrifuged down and dissolved in 0.1M KOH. The absorbance was determined in spectrophotometer of 540nm wavelength.

miR145 knockout animals and angiotensin II infusion

The mouse studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children's Hospital. *miR145* knockout mice, referred to here as *miR145*$^{-/-}$ were generated and generously provided by Dr. Eric Olson,[7] and maintained in C57Bl/6 background. *miR145*$^{+/-}$ mice were crossed to generate wildtype and *miR145*$^{-/-}$ mice. For Angiotensin II (Ang II) infusion, wild-type and *miR145*$^{-/-}$ mice (20 to 24 weeks old) were randomly divided into two treatment groups: one group (n = 6 per group) received vehicle (0.9% saline) and the other group was administered Ang II (1.4 mg/kg/day, Sigma, St. Louis, MO) via Alzet mini osmotic pumps (Durect Corporation, Model 2004, Cupertino, CA). Briefly, mini-pumps were filled with either vehicle or Ang II and allowed to prime for 48-hours prior to surgical implantation according to the manufacturer's instructions. Pumps were implanted subcutaneously under 2% isoflurane anesthesia using aseptic technique, after which they were given buprenorphine for pain in drinking water and monitored until ambulation. A subset of AngII-infused mice (n=6) were injected daily with TGFß receptor inhibitor (ALK4/5/7), SB431542 (Selleckchem) at 10 mg/kg/day in DMSO for 14 days. All other mice received DMSO vehicle injections as controls. After 14 days of treatment, mice were sacrificed and tissues were harvested for RNA isolation or histological analysis.

Ex vivo culture of mouse aorta

Thoracic aortas were dissected from 4-week old mice, and the endothelial layer was carefully removed by scraping with scalpel. After cutting into two equal halves, aorta pieces were cultured in EBM-2 with 10% FBS for 24 hours and then serum starved in DMEM with 0.25% FBS for additional 24 hours. After starvation, ex vivo cultured aortas were treated with or without TGFβ1 for 24 hours.

Immunohistochemistry and histology

After fixation in 4% paraformaldehyde, tissues were processed, embedded in paraffin, and sectioned at 8 μm. Sections were then incubated with primary antibodies, ACTA2 (1:1000, SIGMA, Cat: A2547), TGFBR2 (1:100, Santa Cruz, sc-400) overnight at 4 °C. Primary smooth muscle cells were cultured on chamber slides and fixed with 4% PFA at room temperature for 1 hour. Fluorescence from same area was quantified and

normalized to DAPI intensity. Masson's trichrome staining was performed on sections using a kit purchased from Sigma following kit instructions. Quantification of trichrome staining was performed using Image-Pro Plus software.

Statistical Analysis

Data analyses were performed using GraphPad Prism and comparisons between data sets were made using a Student's t test and ANOVA. Differences were considered significant if $P < 0.05$, and data are presented as mean ± standard deviation (SD). Data shown are representative of at least three independent experiments.

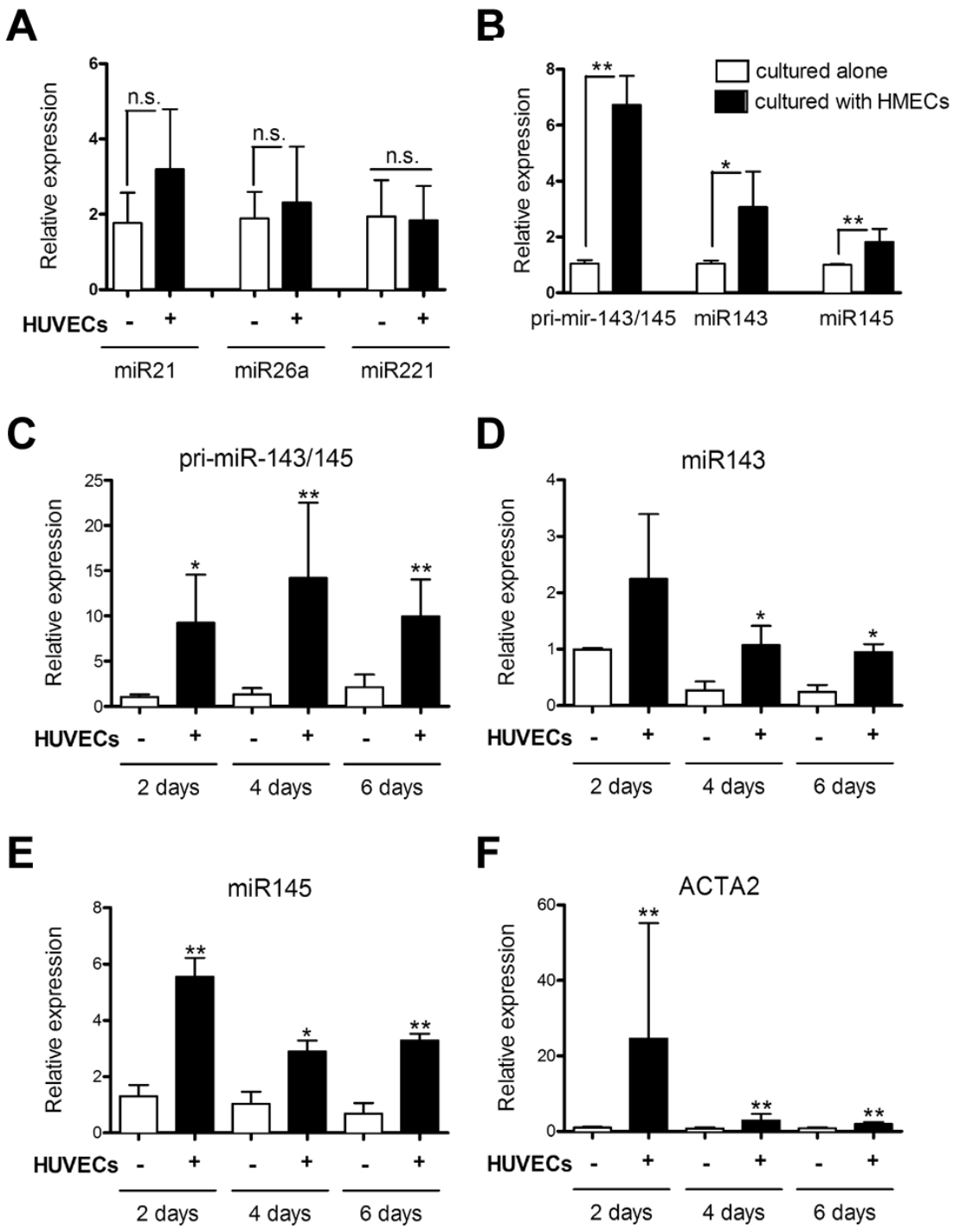
References

1. Wang Q, Zhao N, Kennard S, Lilly B. Notch2 and notch3 function together to regulate vascular smooth muscle development. *PLoS One*. 2012;7:e37365
2. Rothman A, Kulik TJ, Taubman MB, Berk BC, Smith CW, Nadal-Ginard B. Development and characterization of a cloned rat pulmonary arterial smooth muscle cell line that maintains differentiated properties through multiple subcultures. *Circulation*. 1992;86:1977-1986
3. Lilly B, Kennard S. Differential gene expression in a coculture model of angiogenesis reveals modulation of select pathways and a role for notch signaling. *Physiological genomics*. 2009;36:69-78
4. Zhao N, Liu H, Lilly B. Reciprocal regulation of syndecan-2 and notch signaling in vascular smooth muscle cells. *The Journal of biological chemistry*. 2012;287:16111-16120
5. Liu H, Kennard S, Lilly B. Notch3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed jagged1. *Circulation research*. 2009;104:466-475
6. Subramanyam D, Lamouille S, Judson RL, Liu JY, Bucay N, Derynck R, Blelloch R. Multiple targets of mir-302 and mir-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nat Biotechnol*. 2011;29:443-448
7. Xin M, Small EM, Sutherland LB, Qi X, McAnally J, Plato CF, Richardson JA, Bassel-Duby R, Olson EN. Micrornas mir-143 and miR145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. *Genes Dev*. 2009;23:2166-2178

Table I. Primer sequence for qPCR analysis. All sequences list rom 5'-end to 3'-end. For: Forward, Rev: Reverse.

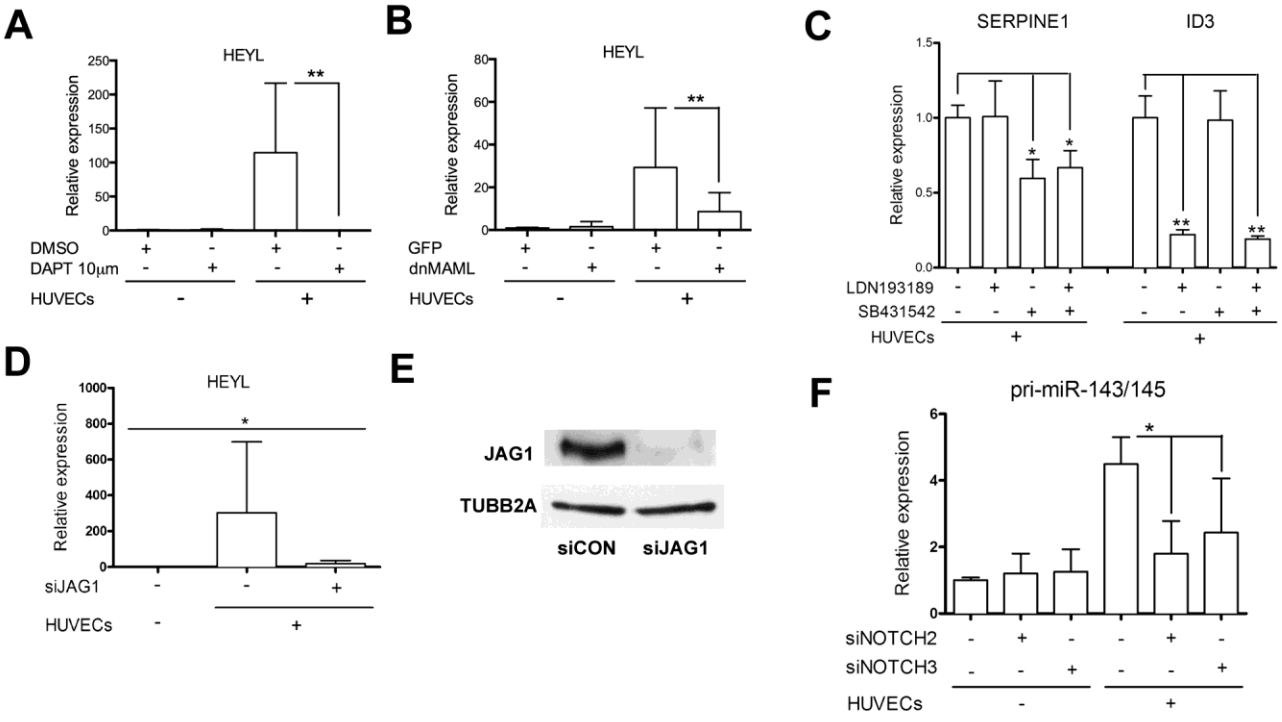
Human genes	
18S For	GTTGGTTTTCGGAACTGAGGC
18S Rev	GTCGGCATCGTTTATGGTCG
Pri-miR-143/145 For	CCCTGGAAAGCCACTAGTACTGA
Pri-miR-143/145 Rev	CTGACTCAAAGGCCAGAATTTTTAT
SM α-actin For	CAAGTGATCACCATCGGAAATG
SM α-actin Rev	GACTCCATCCCGATGAAGGA
PAI-1 For	CCGCCGCCTCTTCCA
PAI-1 Rev	GCCATCATGGGCACAGAGA
SMAD7 For	GGCCGGATCTCAGGCATT
SMAD7 Rev	CCCGTAAGATTCACAGCAACAC
TGFBR2 For	GGACAACGTGTTGAGAGATCGA
TGFBR2 Rev	TCAACGTCTCACACACCATCTG
INHBB For	AGCCTGTGGCTTTACCTGAAAC
INHBB Rev	GCCGGCTGCCCTTCTC
TGFB2 For	GCCGGAGGAGCCGAGTT
TGFB2 Rev	AGAGGAGGAAGTGGAGTTCAGTGT
ZFYVE9 For	GACTTTATCCATGCCCACTATTCA
ZFYVE9 Rev	TATGCCCCGTCTCTCCAAAC
CITED2 For	AAATCGCAAAAACGGAAGGA
CITED2 Rev	GCGCCCGTGGTTCATG
GDNF For	TGACCCCCCCCAACAACT
GDNF Rev	CCTTGGATCCTACAGCTTTTTTG
NRAS For	AAATACGCCAGTACCGAATGAAA
NRAS Rev	GCAATCCCATACAACCCTGAGT
TLL1 For	TGCTCTCTTGCAGTCAGTTGCT
TLL1 Rev	TCTACGCCGCGAGACCTTA
ACVR1B For	GGCTCAGTCTCTCTCGTATTTTGTC
ACVR1B Rev	AGGGCCCTAGAGCAAAAACC
SMAD2 For	GCCAGGATGGTCTCGATCTC
SMAD2 Rev	GGCGCGGTGGCTCAT
BMP3 For	GATGCTCCCATCATGCTCAGA
BMP3 Rev	TGAATTTGAGGGTCCATGCA
SMAD4 For	TTCTGGCCTTACTCCTGTACAGATATT
SMAD4 Rev	AAATCCCTGAAAACACTAGCAATTACT
SMAD3 For	GAGATTCGAATGACGGTAAGTGTTC
SMAD3 Rev	CAGTGTGGTGCTGCCTGAGA
SMAD5 For	CAGGAAGGTCTCCGAAGATTTGT
SMAD5 Rev	ACAATCGCTTTACTGCTGGACTAGT
FOXO1 For	GTGTTGCCCAACCAAAGCTT
FOXO1 Rev	CTCAGCCTGACACCCAGCTAT
ACVR2A For	CCGGAGATGGAAGTCACACA
ACVR2A Rev	CAGGATGTTGTAATAGGGTGGCTTA
ELASTIN For	CACGACCTCATCAACGTTGGT
ELASTIN Rev	TGCCCTGTGGATCTGCAA
LOX For	CACCAGGGCACAAGCTTACC
LOX Rev	TCCATAGCTCAGTTACAGCTCAACA

LOXL1 For	CCCAGGCTGCTATGACACCTA
LOXL1 Rev	TGCACGTCGGTTATGTCGAT
COL1A1 For	CAGACAAGCAACCCAAACTGAA
COL1A1 Rev	TGAGAGATGAATGCAAAGGAAAAA
MMP2 For	TGAGCTATGGACCTTGGGAGAA
MMP2 Rev	CCATCGGCGTTCCCATAC
COL3A1 For	TGGTCAGTCCTATGCGGATAGA
COL3A1 Rev	CGGATCCTGAGTCACAGACACA
FIBRONECTIN For	CACTTACCGAGTGGGTGACACTT
FIBRONECTIN Rev	GCAGGTACAGTCCCAGATCATG
Mouse genes	
GAPDH For	GACGGCCGCATCTTCTTGT
GAPDH Rev	CACACCGACCTTCACCATTTT
Pri-miR-143/145 For	GGACCGCAGCGAGAAGGT
Pri-miR-143/145 Rev	GGACTCTGGCCAGTGATTATTAAAA
PAI-1 For	CCGTGGAACAAGAATGAGATCAG
PAI-1 Rev	CTCTAGGTCCCGCTGGACAA
CTGF For	AAAGTGCATCCGGACACCTAA
CTGF Rev	TGCAGCCAGAAAGCTCAAACT
COL1A1 For	CTTCACCTACAGCACCCTTGTG
COL1A1 Rev	TGACTGTCTTGCCCCAAGTTC
ELASTIN For	CTTTGGACTTTCTCCCATTTATCC
ELASTIN Rev	GGTCCCCAGAAGATCACTTTCTC
LOX For	TGAGAGGTTGGCGAACAAGA
LOX Rev	GGTGCGTGCTCCTTGGTTT
LOXL1 For	CCGCAGCAGTTCCCCTATC
LOXL1 Rev	CGCGGGATCGTAGTTCTCAT
FN1 For	GTGTAGCACAACTTCCAATTACGAA
FN1 Rev	GGAATTTCCGCCTCGAGTCT
TGFBR2 For	CGTCCCGCTGCAATGC
TGFBR2 Rev	CGCACCTTGGAACCAAATG



Online Figure I. (A) HAoSMCs were cocultured with HUVECs for 2 days, followed by separation and qPCR analysis for miR21, miR26a and miR221. (B) HAoSMCs were cocultured with human microvascular endothelial cells (HMECs) for 2 days, followed by separation and qPCR analysis for pri-miR-143/145 transcript, miR143 and miR145. (C-F) Human mesenchymal stem cells (HMSCs) were cocultured with HUVECs for indicated days, separated using anti-PECAM1-conjugated dynabeads, and subjected to qPCR analysis for pri-miR-143/145 transcript, miR143, miR145 and ACTA2 expression. * $P < 0.05$, **$P < 0.01$, n.s. not significant, relative to control.

Online Figure I

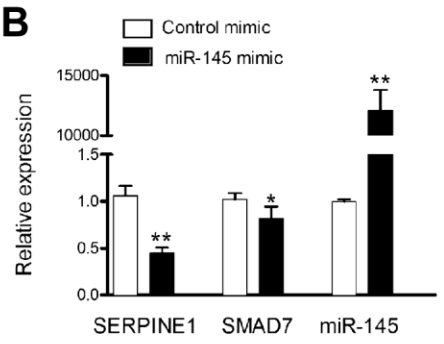


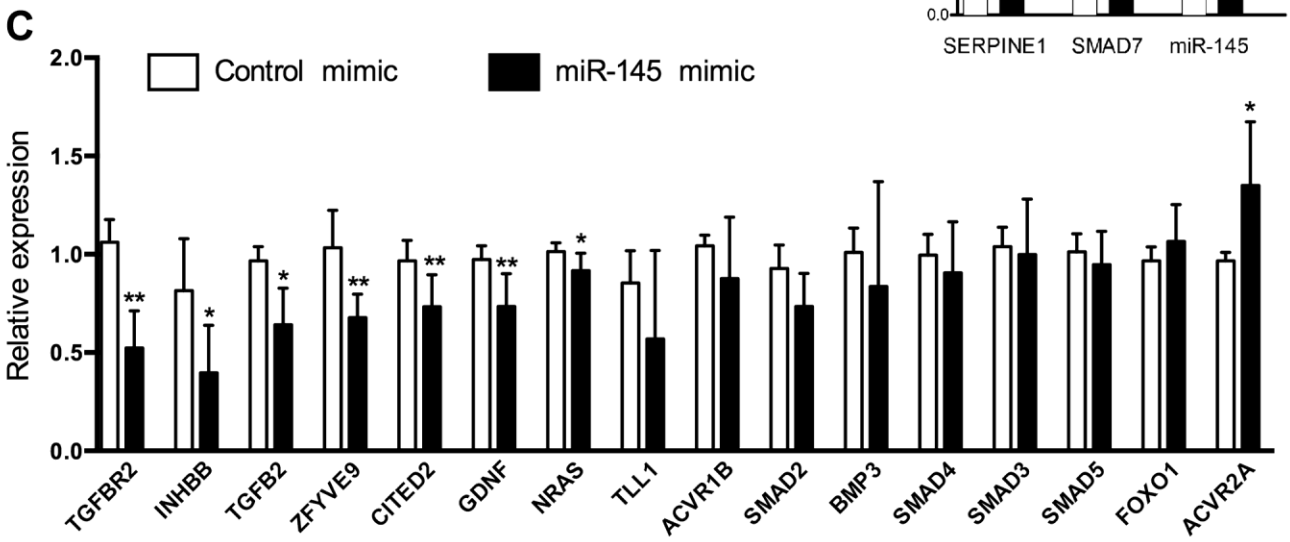
Online Figure II. Validation of inhibition of Notch, TGFß and BMP signaling by various inhibitors. (A) HAoSMCs were cultured with HUVECs in the presence or absence of Notch inhibitor DAPT for 48 hours, followed by separation and qPCR analysis for Notch target, HEYL. (B) HAoSMCs were lenti-virally transduced with GFP, as control, or dnMAML for 48 hours, and then cultured alone or with HUVECs for an additional 48 hours, followed by separation and qPCR analysis for HEYL. (C) HAoSMCs were cultured with HUVECs in the presence or absence of BMP receptor inhibitor (ALK2/3), LDN193189 and TGFß receptor inhibitor (ALK4/5/7), SB431542 for 48 hours, followed by separation and qPCR analysis for SERPINE1 (TGFß target) and ID3 (BMP target). (D) HAoSMCs cultured alone or with HUVECs, which were transfected with JAG1 siRNA., followed by separation and qPCR analysis for HEYL. (E) Western blot analysis for the JAG1 protein expression in HUVECs transfected with JAG1 siRNA. (F) HAoSMCs cultured alone or with HUVECs, which were transfected with control, NOTCH2 or NOTCH3 siRNA., followed by separation and qPCR analysis for pri-miR-143/145. * $P < 0.05$, **$P < 0.01$.

Online Figure II

A

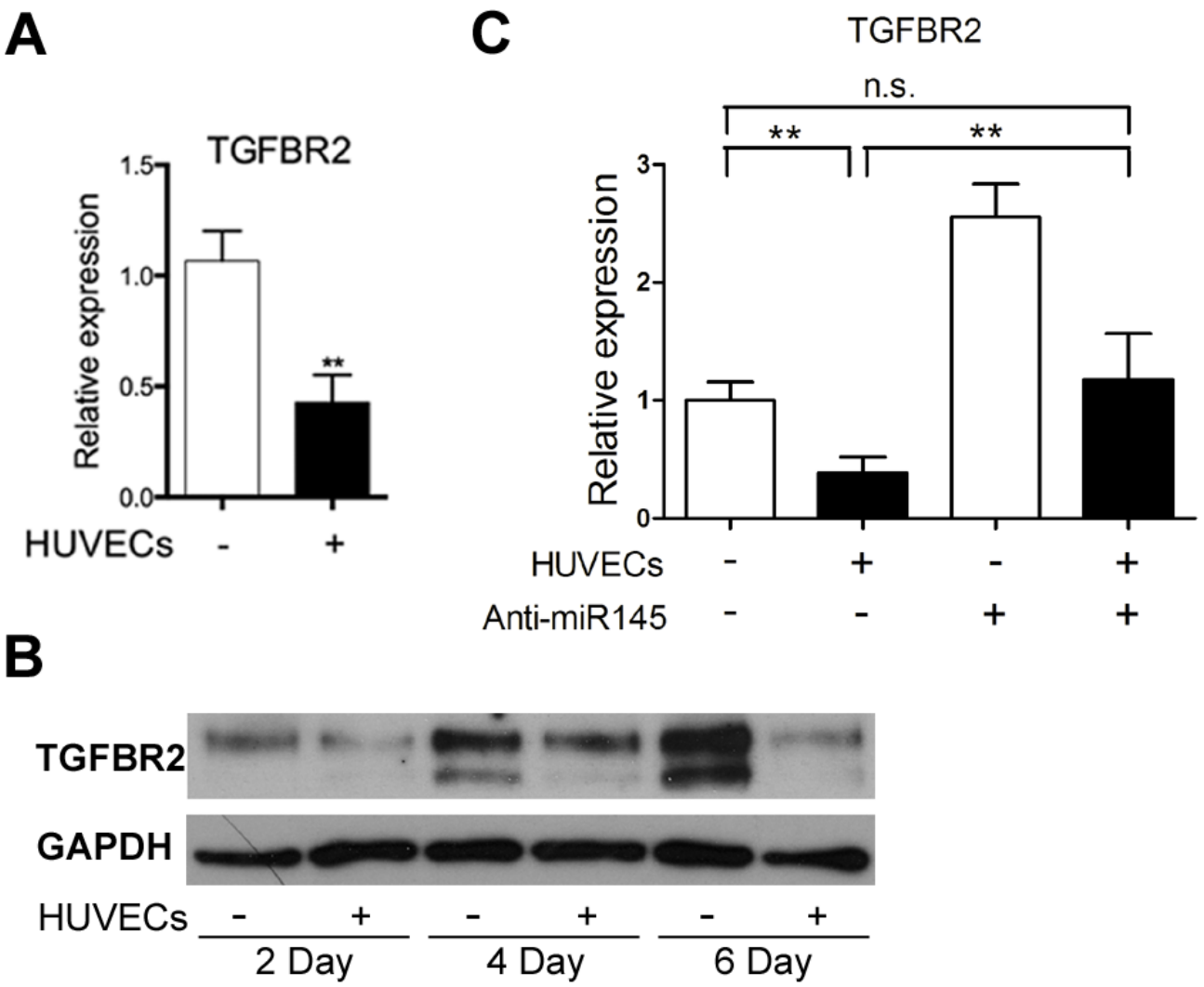
Pathways	# of predicted targets	# of expected targets	P value
PDGF signaling	23	5.33	9.62E-08
TGF-beta signaling	16	5.37	1.77E-06





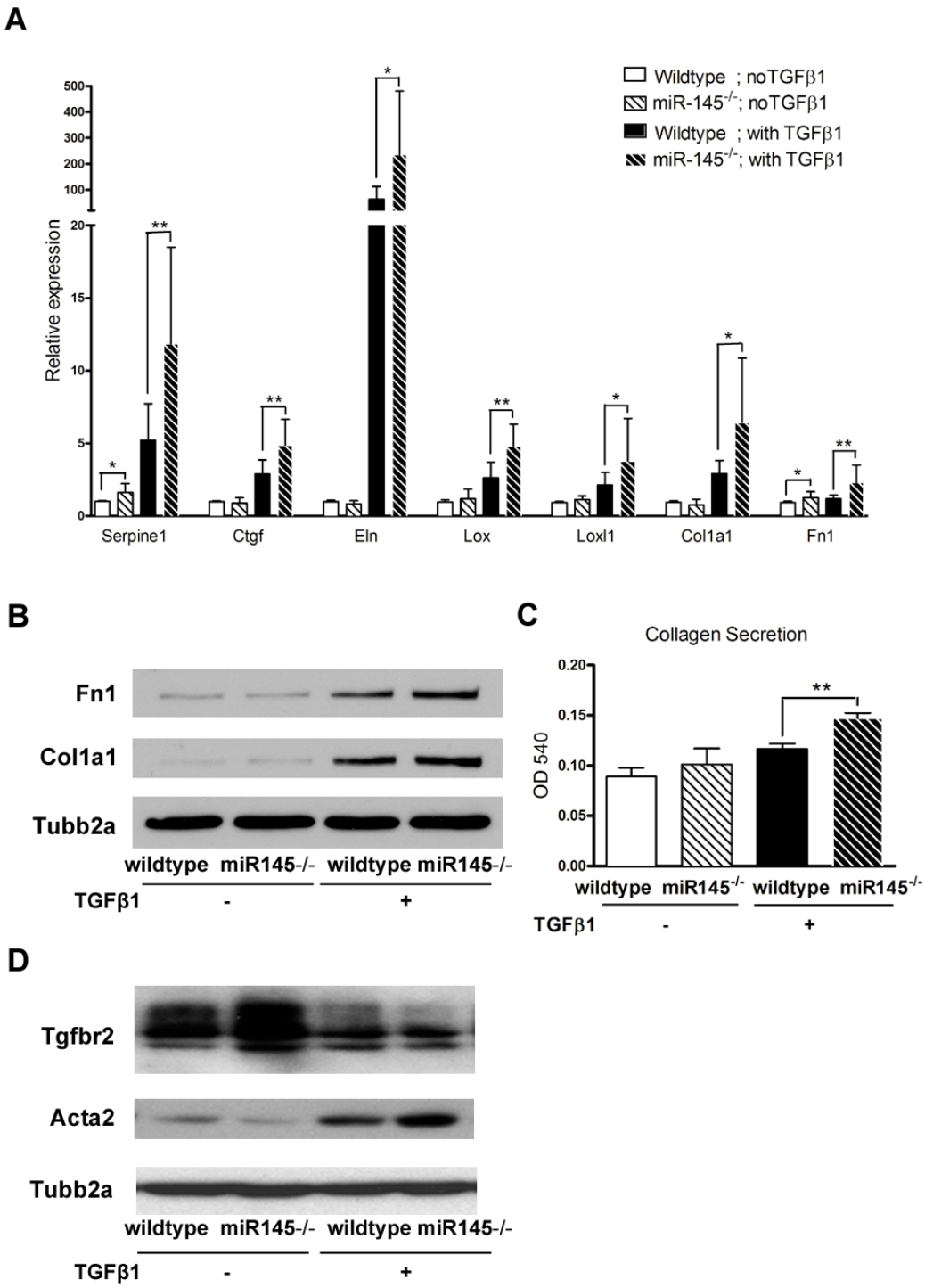
Online Figure III. Pathway analysis of miR145 targets. (A) 717 miR145 targets predicted by TargetScan were subjected to PANTHER pathway analysis to identify over represented pathways. (B) HAoSMCs were transiently transfected with control RNA mimic or a miR-145 mimic and isolated RNA was tested for expression of known TGFß-dependent genes SERPINE1, SMAD7 and miR-145 by qPCR. (C) The 16 predicted miR145 targets found in the TGFß signaling pathway were measured by qPCR following overexpression of the miR-145 mimic. * $P < 0.05$, ** $P < 0.01$.

Online Figure III



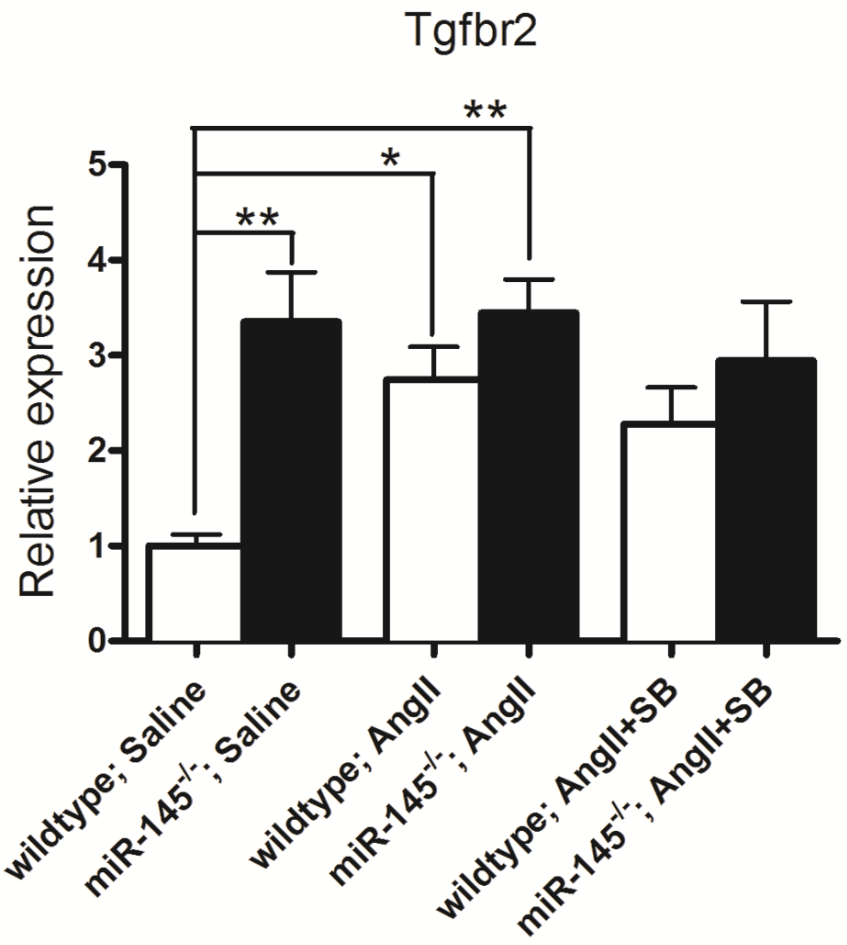
Online Figure IV. TGFβ receptor II (TGFBR2) is regulated by cocultured endothelial cells via miR145 activity. (A) HAoSMCs were cocultured with HUVECs for 2 days, followed by separation and qPCR analysis for TGFBR2. (B) HAoSMCs were cocultured with HUVECs for 2, 4 and 6 days, followed by separation and Western blot analysis for TGFBR2 protein level. (C) HAoSMCs were transfected with anti-miR-145 and followed by coculturing with HUVECs for 2 days. mRNA level of TGFBR2 was measured by qPCR analysis. **P < 0.01, n.s. not significant.

Online Figure IV



Online Figure V. Increased matrix synthesis in *miR145*-deficient smooth muscle cells. Aortic smooth muscle cells were isolated from wild-type and *miR145* mutant mice and treated with or without TGFß1 for 48 hours. (A) qPCR was performed to measure the expression of TGFß signaling downstream targets and matrix synthesis genes. (B) Western blot was performed to analyze Fn1 and Col1A1 protein expression. (C) Media was collected and collagen content was measured using a picosirius red assay. (D) Western blot was performed to analyze Tgfbr2 and Acta2 protein expression. * $P < 0.05$, **$P < 0.01$.

Online Figure V



Online Figure VI. Tgfbr2 mRNA expression is elevated by loss of *miR145* and by AngII infusion. qPCR was performed to measure the expression of Tgfbr2 from RNA derived from aortas of *miR145*-deficient mice infused with angiotensin II (AngII) or saline for 14 days. TGFß receptor inhibitor (SB)431542 was injected in a subset of mice. * $P < 0.05$, **$P < 0.01$.

Online Figure VI