MicroRNA miR145 Regulates TGFBR2 Expression and Matrix Synthesis in Vascular Smooth Muscle Cells

Ning Zhao, Sara N. Koenig, Aaron J. Trask, Cho-Hao Lin, Chetan P. Hans, Vidu Garg, Brenda Lilly

Rationale: MicroRNA miR145 has been implicated in vascular smooth muscle cell differentiation, but its mechanisms of action and downstream targets have not been fully defined.

Objective: Here, we sought to explore and define the mechanisms of miR145 function in smooth muscle cells.

Methods and Results: Using a combination of cell culture assays and in vivo mouse models to modulate miR145, we characterized its downstream actions on smooth muscle phenotypes. Our results show that the miR-143/145 gene cluster is induced in smooth muscle cells by coculture with endothelial cells. Endothelial cell–induced expression of miR-143/145 is augmented by Notch signaling and accordingly expression is reduced in Notch receptor–deficient cells. Screens to identify miR145-regulated genes revealed that the transforming growth factor (TGF)-β pathway has a significantly high number of putative target genes, and we show that TGFβ receptor II is a direct target of miR145. Extracellular matrix genes that are regulated by TGFβ receptor II were attenuated by miR145 overexpression, and miR145 mutant mice exhibit an increase in extracellular matrix synthesis. Furthermore, activation of TGFβ signaling via angiotensin II infusion revealed a pronounced fibrotic response in the absence of miR145.

Conclusions: These data demonstrate a specific role for miR145 in the regulation of matrix gene expression in smooth muscle cells and suggest that miR145 acts to suppress TGFβ-dependent extracellular matrix accumulation and fibrosis, while promoting TGFβ-induced smooth muscle cell differentiation. Our findings offer evidence to explain how TGFβ signaling exhibits distinct downstream actions via its regulation by a specific microRNA. (Circ Res. 2015;116:23-34. DOI: 10.1161/CIRCRESAHA.115.303970.)

Key Words: extracellular matrix ■ microRNAs ■ myocytes, smooth muscle ■ transforming growth factors

MicroRNAs have been cast as modulators of gene expression, whose fundamental function is to fine-tune cellular phenotypes in response to intrinsic signals or environmental stress. In the vasculature, the ability of cells within the vessel wall to adjust to a range of cues is critically important for maintaining proper flow and pressure. Although the endothelial cells serve as the primary sensor of blood vessels, smooth muscle cells act as the essential workhorse, by providing stability and contraction as needed. Smooth muscle cells are dynamic cells that can exist in a range of phenotypes. In addition to being contractile, these cells can be proliferative and exist in a synthetic state, where they secrete extracellular matrix that is needed for vessel wall stability. The ability of smooth muscle cells to undergo phenotypic transitions is essential for vascular development and remodeling associated with changes in blood flow; however, in vascular disease, phenotypic modulation can have a detrimental effect by contributing to disease pathology.

A host of mediators has been implicated in the control of phenotypic modulation. The transcription factor tandem, serum response factor, and Myocardin are master regulators of smooth muscle differentiation that drive cells toward a contractile phenotype. Notch signaling has been shown to be important for smooth muscle development and differentiation and plays a specific role in endothelial cell–dependent maturation. Two opposing growth factor signaling pathways, platelet-derived growth factor and transforming growth factor (TGF)-β have been shown to drive cells toward a proliferative and differentiated phenotype, respectively. In addition, the TGFβ pathway can induce matrix synthesis under certain conditions, suggesting that this pathway might have a dual role in smooth muscle cell programming. With the discovery of microRNAs that are enriched in smooth muscle cells, an obvious question is how they might contribute to phenotypic modulation by intervening with these established regulatory pathways. Not surprisingly,
several microRNAs have been associated with the regulation of smooth muscle phenotypes. For example, miR-1 was shown to be induced by Myocardin, can inhibit proliferation, and is reduced in a carotid artery ligation model. miR-21 also exhibits a prodifferentiation profile by being regulated by bone morphogenic protein and TGFβ and is important for TGFβ-mediated smooth muscle maturation. In contrast, miR-221 is induced by platelet-derived growth factor signaling and can drive vascular smooth muscle cells toward a dedifferentiated state, partially through downregulation of Myocardin.

In 2009, a series of publications highlighted the importance of the miR-143/145 microRNA cluster in the regulation of smooth muscle cell phenotypes. The results showed that microRNA-143/145 are highly expressed in contractile muscle cells promote the increase in miR145 expression through Notch signaling, consistent with a differentiated phenotype. Attempts to identify targets of this miR cluster revealed that they had a hand in the regulation of proliferation, actin remodeling, and contractility genes. Despite the consensus that miR-143/145 contribute to a differentiated phenotype, inconsistencies in the data using different experimental models strongly suggest that miR-143/145 function is context dependent.

In this study, we show that miR145 is induced in smooth muscle cells by endothelial cell signaling. Endothelial cells promote the increase in miR145 expression through Notch signaling, consistent with a differentiated phenotype. Examination of putative miR145 target genes revealed that miR145 regulates TGFβ receptor II (TGFBR2) expression and governs the expression of downstream matrix genes in smooth muscle cells. Our results suggest that miR145 functions to modulate TGFβ signaling in smooth muscle cells as a mechanism to suppress matrix gene expression, while sparing smooth muscle–specific differentiation genes. These actions of miR145 may have implications in disease progression, where suppression of detrimental matrix synthesis by miR145 could be used to alleviate fibrosis in a range of tissues.

### 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), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 100 U/mL penicillin-streptomycin. Human mesenchymal stem cells 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 (endothelial basal medium) supplemented with the bullet kit as recommended (Lonza). Primary cells between passages 7 and 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 were isolated from embryonic day 10.5 mouse embryos and cultured in DMEM supplemented as above with 5% FBS. HUVECs (human embryonic kidney 293) cells and PAC12 cells were cultured in DMEM supplemented as above with 5% FBS. All cultures were maintained in humidified 5% CO2 at 37°C. For coculture, 3×10⁶ mural cells were seeded in 12-well plates, and after adhesion, 3×10⁶ HUVECs were added. To separate endothelial cells from HAoSMCs and human mesenchymal stem cells, anti-PECAM1 (platelet endothelial cell adhesion molecule 1)–conjugated Dynabeads (Invitrogen) were used according to manufacturer’s instructions. We have demonstrated efficacy of this purification procedure previously.23 The purity of the smooth muscle cells was verified by obtaining the separated cells for PECAM1 and ACTA2 (smooth muscle alpha-actin) and counting cell number. The separated smooth muscle cell population was >99% pure. All cell coculture experiments, unless indicated, were performed in media consisting of EBM-2 supplemented with the bullet kit.

#### Primary Mouse Aorta Smooth Muscle Cell Culture

Mice were euthanized at 4 to 5 weeks, and a midsternal thoracotomy was performed. The thoracic aorta was isolated, and adventitia was carefully removed in cold PBS. Aorta was digested with 1 mg/mL Collagenase II (Sigma, C6885) and 100 μg/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× to 4×, followed by media refresh every 48 hours. Primary cells at passage 2 were used for experiments.

#### Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using TRizol reagent following manufacturers’ 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 polymerase chain reaction was performed using a StepOne polymerase chain reaction 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 TGFβ (1:1000 dilution, Cell Signaling, 5741S), SERPINE1 (serpine peptide inhibitor, 1:1000 dilution, Cell Signaling, 2237S), α-Actin (1:1000 dilution, Cell Signaling, 4970S), or HAoSMCs. For transwell assays, 4×10⁴ HAoSMCs were plated on 12-well plates, and 0.4-μm pore-size transwell inserts (Corning Costar) were inserted containing 2×10⁴ HUVECs or HAoSMCs.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>HAoSMCs</td>
<td>Human aortic smooth muscle cells</td>
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<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<td>TGFβ</td>
<td>Transforming growth factor β</td>
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<td>TGFBR2</td>
<td>TGFβ receptor II</td>
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<td>UTR</td>
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#### Nonstandard Abbreviations and Acronyms

- Ang II: Angiotensin II
- DMEM: Dulbecco’s Modified Eagle’s Medium
- FBS: Fetal bovine serum
- HAoSMCs: Human aortic smooth muscle cells
- HUVECs: Human umbilical vein endothelial cells
- TGFβ: Transforming growth factor β
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RNA Mimic, miRNA Inhibitor, and siRNA Transfection

HAoSMCs were plated in a 12-well plate at 3×10^4 cells per well. After 12 hours, the cells were transfected with miR145 or control RNA mimic at 40 nmol/L using Lipofectamine RNAiMAX (Invitrogen). For miR145 inhibition, 200 nmol/L of miRVana inhibitor MH11480 (Life Technologies) was transfected into 6×10^4 cells per well HAoSMCs using RNAiMAX as directed. For coculture experiments, HUVECs were added 24 hours after transfection. For siRNA knockdown, HAoSMCs were plated in a 12-well plate at 6×10^4 cells/well. Cells were transfected with 40 nmol/L of control, Notch2 or Notch3 siRNA using RNAiMAX (Invitrogen). After 24 hours, cells were cocultured with 6×10^4 HUVEC for additional 96 hours and collected for quantitative polymerase chain reaction analysis. siRNA to knockdown JAG1 in endothelial cells (Dharmacon M-011060-02) was used at 80 nmol/L with RNAiMAX transfection reagent before coculture. Knockdown was verified by quantitative polymerase chain reaction reaction and Western blot analysis (Online Figure II).24

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 (dominant negative mastermind-like) constructs were made as described previously.25 The lentiviral plasmids were transfected into TN-293 cells using Lipofectamine 2000 (Invitrogen), and the viral particles were amplified and purified as described.26

Plasmid Transfection and Luciferase Assays

psi-CHECK2-TGFBR2 3′ untranslated region (UTR) plasmid was obtained from Addgene plasmid 31882.27 HEK293 or PAC1 cells were plated in a 12-well plate and transfected with 500-ng plasmid and RNA mimics at 100 nmol/L concentration. Twenty-four 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 polymerase chain reaction mutagenesis.

Collagen Secretion Assay

cell culture medium was incubated with 25% (NH₄)₂SO₄ at 4°C over night. The secreted collagen was pelleted by centrifugation at maximal speed and resuspended in 950 μL of 50 μmol/L Sirius Red at room temperature. The stained collagen was centrifuged down and dissolved in 0.1 mol/L KOH. The absorbance was determined in spectrophotometer of 540-nm wavelength.

miR145 Knockout Animals and Angiotensin II Infusion

The mouse studies were performed 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 Olson18 and maintained in C57Bl/6 background. miR145⁺/⁺ mice were crossed to generate wild-type and miR145⁻/⁻ mice. For angiotensin II (Ang II) infusion, wild-type and miR145⁻/⁻ mice (20–24 weeks old) were randomly divided into 2 treatment groups: one group (n=6 per group) received vehicle (0.9% saline) and the other group was administered angiotensin II (1.4 mg/kg per day; Sigma, St Louis, MO) via Alzet mini osmotic pumps (Model 2004; Durect Corporation, Cupertino, CA). Briefly, minipumps were filled with either vehicle or angiotensin II and allowed to prime for 48 hours before 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 Ang II–infused mice were injected daily with TGFβ receptor inhibitor, SB431542 (Selleckchem) at 10 mg/kg per day in

Figure 1.

Endothelial cells induce miR-143/145 expression in vascular smooth muscle cells. Human aortic smooth muscle cells were cocultured with human umbilical vein endothelial cells (HUVECs) for 2, 4, and 6 days, separated using anti–PECAM1 (platelet endothelial cell adhesion molecule 1)-conjugated dynabeads, and smooth muscle-derived RNA was subjected to quantitative polymerase chain reaction analysis for (A) mature miR-143, (B) mature miR145, (C) pri-miR-143/145 transcript, and (D) ACTA2 (smooth muscle alpha-actin) expression. E, Western blot of ACTA2 protein samples isolated from alone and cocultured smooth muscle cells after separation. Tubulin (TUBB2A) was used as loading control. “**P<0.01 relative to control without endothelial cells.
dimethyl sulfoxide for 14 days. All other mice received dimethyl sulfoxide vehicle injections as controls. After 14 days of treatment, mice were euthanized 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 2 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; SantaCruz, sc-400) overnight at 4°C. Primary smooth muscle cells were cultured on chamber slides and fixed with 4% paraformaldehyde at room temperature for 1 hour. Fluorescence from same area was quantified and normalized to DAPI (4',6-diamidino-2-phenylindole) 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 t test or ANOVA. Differences were considered significant if P<0.05, and data are presented as means SD. Data shown are representative of ≥3 independent experiments.

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**Figure 2. Examination of miR-143/145 transcript expression in relation to Notch signaling.** A. Human aortic smooth muscle cells (HAoSMCs) were treated with conditioned media collected from HAoSMCs or human umbilical vein endothelial cells (HUVECs) for 48 hours. B, HAoSMCs were cultured with HUVECs seeded in a transwell insert as indicated for 48 hours. Cocultured cells were used as a positive control. C, HAoSMCs were cultured with HUVECs in the presence or absence of Notch inhibitor anly-2-phenylglycine-1,1-dimethyllethyl ester (DAPT) for 48 hours. D, HAoSMCs were lentivirally transduced with green fluorescent protein (GFP), as control, or DN-MAML (dominant negative mastermind-like) for 48 hours, and then cultured alone or with HUVECs for an additional 48 hours. E, HAoSMCs cultured alone or with HUVECs, which were transsected with JAG1 siRNA. F, HAoSMCs were cultured with HUVECs in the presence or absence of bone morphogenetic protein receptor inhibitor (ALK2/3), LDN193189, and transforming growth factor-β receptor inhibitor (ALK4/5/7), SB431542 for 48 hours. G, The Notch1 (NICD1), Notch2 (NICD2), or Notch3 (NICD3) intracellular domains were overexpressed in HAoSMCs by lentiviral infection for 96 hours. H, GFP-expressing virus was used as control. J, RNA was collected from mouse embryo fibroblasts isolated from embryos with mutations in the Notch2 and Notch3 genes. In all panels, expression of the pri-miR-143/145 transcript was analyzed by quantitative polymerase chain reaction. For coculture experiments, HAoSMCs were separated from endothelial cells before analysis. *P<0.05, **P<0.001. n.s. indicates not significant.
miR-143/145 Is Induced in Smooth Muscle Cells by Endothelial Cells

Endothelial cells can regulate the phenotype of smooth muscle cells and our laboratory previously demonstrated that cocultured endothelial cells promote vascular smooth muscle cell differentiation.\(^{26,29}\) To examine the extent of this modulation by endothelial cells, we measured the expression of the microRNA gene cluster miR-143/145, which has been linked to governing smooth muscle differentiation.\(^{14,16,18}\) Coculture of HUVECs with HAoSMCs caused an increase in precursor microRNA pri-miR-143/145 transcript as well as the individual mature microRNAs (Figure 1A–1C). The expression of both the precursor and mature microRNAs was observed at high levels after 48 hours of incubation and was sustained, albeit at reduced levels ≤6 days in coculture. Smooth muscle α-actin RNA and protein (ACTA2) showed a similar expression profile in cocultured aortic smooth muscle cells when compared with miR-143/145 (Figure 1D and 1E). Analysis of other smooth muscle–enriched microRNAs, \(^{8}\) miR-21, miR-26a, and miR-221 revealed that miR-143/145 were uniquely upregulated by cocultured endothelial cells (Online Figure 1A). The expression of miR-143/145 could also be induced in smooth muscle cells cocultured with microvascular endothelial cells and was also upregulated in cocultured smooth muscle precursors, mesenchymal stem cells (Online Figure 1B–1F). Thus, miR-143/145 shows an increase in expression consistent with the smooth muscle differentiation phenotype induced by endothelial cells.

miR-143/145 Is Regulated by Notch Signaling

To determine whether the induction of miR-143/145 required cell–cell contact between endothelial cells and smooth muscle cells, we first treated smooth muscle cells with conditioned media. miR-143/145 levels remained low in smooth muscle cells treated with HUVEC-conditioned media, similar to those treated with HAoSMC-conditioned media, whereas the control cocultured cells showed robust induction (Figure 2A). We also used a transwell assay to separate cocultured cells physically. Like the conditioned media experiments, smooth muscle expression of miR-143/145 was not increased by endothelial cells separated by a transwell membrane (Figure 2B). Our data suggested that miR-143/145 expression required cell–cell contact, and given that membrane-bound Notch signaling is known to be critical for endothelial cell–induced smooth muscle differentiation,\(^{26}\) we tested the role of Notch signaling in miR-143/145 expression.

Using the chemical inhibitor, DAPT and a lentiviral-delivered dominant-negative-mastermind-like 1 protein\(^{29}\) to block Notch signaling, we measured the induction of miR-143/145 in smooth muscle cells. Both strategies to block Notch signaling resulted in an almost complete absence of miR-143/145 induction by endothelial cells (Figure 2C and 2D; Online Figure II). Moreover, knockdown of the Notch ligand, JAGGED-1 (JAG1), on endothelial cells also caused a loss of miR-143/145 induction in smooth muscle cells (Figure 2E; Online Figure II). In contrast, receptor inhibitors to prevent TGFβ (ALK4/5/7) and bone morphogenetic protein (ALK2/3) signaling, both that have been previously shown to induce miR-143/145 expression, did not block the upregulation (Figure 2F; Online Figure II).\(^{30,31}\) We additionally targeted the NOTCH2 and NOTCH3 receptors by siRNA,\(^{24}\) in smooth muscle cells, and demonstrated that loss of either receptor attenuated the endothelial cell–induced upregulation of miR-143/145 (Online Figure II). Furthermore, overexpression of the intracellular domains of Notch1, Notch2, or Notch3 promoted robust expression of miR-143/145 in smooth muscle cells (Figure 2G). To test whether Notch signaling regulates miR-143/145 expression in vivo, we isolated ascending and descending aortas from Notch3-deficient mice\(^{12}\) and examined expression. The data show that miR-143/145 levels are decreased by =25% in the Notch3 null mice compared with heterozygous controls (Figure 2H). This was further confirmed using mouse embryonic fibroblasts deficient in both Notch2 and Notch3. Homozygous null mouse embryo fibroblasts cells exhibited >40% decrease in miR-143/145 expression compared with Notch2/Notch3 double heterozygotes (Figure 2I).\(^{21,33}\) These data are consistent with previous published in vitro findings\(^{34}\) and show that Notch signaling regulates miR-143/145 expression in vivo. The modest reduction of expression in the absence of Notch2 and Notch3 suggests that other mediators, such as Notch1 or Myocardin, might contribute to its expression as previously shown.\(^{16,18,34}\)

miR145 Targets the TGFβ Signaling Pathway

Expression and deletion analysis of miR-143/145 have suggested a role in the modulation of smooth muscle cell phenotypes and although targets for these microRNAs have been identified, the analysis is incomplete. In an attempt

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**Figure 3.** TGFβ receptor II (TGFBR2) is a direct target of miR145. A. The seed sequence (bold) of miR145 on TGFBR2 3′-untranslated region (UTR) is highly conserved across species. B. Wild-type or mutant 3′ UTR of TGFBR2 gene was cloned into the Renilla reporter plasmid. The TGFBR2-3′UTR constructs were transfected into HEK293 (human embryonic kidney 293) and PAC1 cells with control or miR145 mimic, followed by Renilla luciferase assays. C. Western blot analysis of TGFBR2 protein level in HAoSMCs transfected with a miR-145 or control (con) mimic. Graph shows quantification from 3 separate experiments. *P<0.05, **P<0.01. n.s. indicates not significant.
to identify key signaling pathways that miR-143/145 may influence we performed a computational screen with the PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system, using data derived from TargetScan. TargetScan identified 406 putative target genes for miR-143 and 717 possible targets for miR145. These target genes were screened as a group using PANTHER to identify pathways, which were preferentially targeted by these individual miRs. miR-143 targeted pathways unrelated to miR145 (not shown). Interestingly, miR145 preferentially targeted 2 opposing signaling pathways in smooth muscle, platelet-derived growth factor and TGFβ (Online Figure IIIA). The platelet-derived growth factor pathway had been previously shown to be a miR145 target and suppressor of miR145 expression, whereas TGFβ was shown to be upstream activator of miR145 expression, suggesting that miR145 may exist in a negative feedback loop to dampen TGFβ signaling.

Overexpression of a miR145 mimic in smooth muscle cells caused a decrease in TGFβ readout genes, SERPINE1 (PAI-1) and SMAD7, suggesting a decrease in TGFβ signaling (Online Figure IIIB). A cursory examination of the 16 putative target genes within the TGFβ pathway showed that 7 of the 16 exhibited a significant decrease in transcript expression in the presence of a miR145 mimic in smooth muscle cells (Online Figure IIC). The most significantly downregulated of these genes was the TGFBR2. Examination of the 3′ UTR of the TGFBR2 gene revealed a highly conserved miR145 target sequence (Figure 3). To test whether this element was a functional seed sequence, the 3′ UTR was cloned into a luciferase expression vector to evaluate its response to miR145. Cotransfection of the luciferase reporter with the miR145 mimic into HEK293 or PAC1 smooth muscle cells showed the 3′ UTR conveyed decreased expression, whereas a mutated version of the 3′ UTR that could not be recognized by miR145 was not affected (Figure 3B). The ability of miR145 to regulate TGFBR2 was further confirmed by Western blot, where overexpression of the miR145 mimic reduced TGFBR2 protein in smooth muscle cells (Figure 3C). Furthermore, we saw a reduction of TGFBR2 RNA and protein in smooth muscle cells cocultured with endothelial cells (Online Figure IVA and IVB), consistent with an increase in endogenous miR145 (Figure 1). Inhibition of miR145 by an antagonor revealed that TGFBR2 expression is attenuated by miR145 in smooth muscle cells cultured alone and additionally the downregulation of TGFBR2 by cocultured endothelial cells is dependent on miR145 function (Online Figure IVC).

**miR145 Regulates TGFβ-Pathway Genes and Matrix Gene Expression in Smooth Muscle Cells**

In smooth muscle cells, TGFβ signaling and miR145 are pro-differentiation mediators, yet, our data indicate that miR145 suppresses the expression of certain TGFβ pathway genes. Therefore, we sought to explore how miR145 would influence TGFβ-dependent smooth muscle gene expression. Overexpression of the miR145 mimic in smooth muscle cells in the presence and absence of TGFβ1 ligand showed, as before, a decrease in TGFBR2 and SERPINE1 RNA and protein expression (Figure 4A and 4D). As expected, the smooth

![Figure 4. miR-145 regulates transforming growth factor (TGF)-β-dependent matrix gene synthesis.](link)
muscle marker genes, ACTA2, calponin1 (h,-calponin), and TAGLN (transgelin; SM22α), all were increased with TGFβ stimulation and miR145 mimic alone, and together seemed to exhibit an additive response, suggesting independent mechanisms of action (Figure 4B and 4D). Together these data indicate that miR145 has a selective effect on TGFβ signaling and may facilitate unique downstream events. Deletion analysis of TGFBR2 in smooth muscle cells of mice revealed an important role in the regulation extracellular matrix genes, such as elastin, collagens, and the matrix crosslinking genes, Lox and Lox1.41,42 We examined the expression of these matrix genes in the presence of the miR145 mimic, and similar to TGFBR2 expression, we observed a decrease in expression at both RNA and protein level (Figure 4C and 4D). Interestingly, although fibronectin transcripts were decreased by miR145, we observed no significant effect on protein expression. These data demonstrate that in smooth muscle cells, miR145 selectively regulates TGFβ signaling and blocks matrix synthesis, while permitting expression of smooth muscle differentiation genes.

Figure 5. miR145 null mice have elevated Tgfbr2. A, Ascending aortas were isolated from wild-type and miR145 mutant mice and stained for Tgfbr2 (green), Acta2 (red), and DAPI (4',6-diamidino-2-phenylindole; blue). B and C, Intensity of Tgfbr2 and Acta2 was quantified and normalized to DAPI. D, Western blot to analyze the protein expression of Tgfbr2 and Acta2 in aortas and quantified relative to Tubb2a levels. E, Aortas were isolated from wild-type and miR145 mutant mice and cultured in the presence or absence of transforming growth factor (TGF)-β1. RNA was extracted after 24 hours and subjected to quantitative polymerase chain reaction analysis. F, Western blot to analyze levels of phosphorylated-Smad2 and phosphorylated-P38 MAP kinase in aortas. Amounts were quantified relative to total protein levels. *P<0.05, **P<0.01. n.s. indicates not significant.
miR145 Deficiency Increases TGFβ Signaling in Smooth Muscle Cells In Vivo

To investigate whether miR145 regulates TGFβ-dependent matrix gene expression in vivo, we used miR145-deficient (miR145−/−) mice.18 miR145 null mice are viable with deficits in smooth muscle function.14–18 We isolated aortas from adult wild-type and miR145−/− mice and performed immunostaining and Western analysis to detect Tgfbr2 and Acta2 expression (Figure 5A–5D). The expression of Tgfbr2 in the miR145−/− aortas was increased when compared with wild-type, whereas Acta2 expression showed slight but insignificant difference between wild-type and miR145−/− deficient mice. Aorta tissue was isolated and cultured ex vivo with or without TGFβ1 and matrix gene expression was measured by quantitative polymerase chain reaction (Figure 5E). Although there were no significant differences in the basal level of expression between wild-type and miR145 null mice, after TGFβ1 challenge there was a greater induction in the absence of miR145. We additionally isolated aortic smooth muscle cells from wild-type and miR145 null mice and measured matrix gene expression in cultured cells. Expression of some matrix and matrix synthesis genes was increased at basal levels in the absence of miR145, and all showed an increased level of expression in response to TGFβ1 (Online Figure VA and VB). Collagen secretion was also measured from the culture media and showed an increase in the miR145 null mice (Online Figure VC). Western blot on extracts from cultured cells to detect Tgfbr2 and Acta2 showed consistent results indicative of miR145 suppression (Online Figure VD). These findings demonstrate that miR145 functions to repress matrix gene expression and might likely act to govern aberrant extracellular matrix deposition. Given that downstream targets of the TGFβ pathway were altered by the loss of miR145, we measured whether TGFβ signaling mediators were changed. Analysis of phosphorylated-Smad2 and p38 MAPK in aorta extracts from wild-type and miR145-deficient mice revealed a trending but not significant increase in SMAD2 phosphorylation, whereas we observed a significant increase in phosphorylated-p38 MAPK (Figure 5F). Thus, these data indicate that miR145 attenuates TGFβ signaling and matrix synthesis, and the direct targeting of Tgfbr2 by miR145 likely contributes to these suppressive abilities.

Loss of miR145 Exacerbates Ang II–Induced Fibrosis

These data indicate that miR145 regulates TGFBR2 and matrix synthesis and this is most evident when the TGFβ pathway is robustly activated. Fibrotic diseases are associated with excess TGFβ signaling in activated fibroblasts, and given that miR145 also is expressed in cardiac fibroblasts,43–45 we hypothesized that it might act as a suppressor of pathological fibrosis. To test this, we infused Ang II into mice to induce TGFβ-dependent cardiovascular fibrosis.46,47 Infusion of Ang II (1.4 mg/kg per day)47 by osmotic pumps for 14 days in wild-type and miR145-deficient mice revealed data consistent with our previous results. Ang II caused interstitial and perivascular fibrosis within the hearts of wild-type mice, and this fibrosis was significantly increased in the miR145 knockout animals. Examination of the hearts of these mice by Masson trichrome staining revealed a much more pronounced collagen accumulation in the miR145 null mice (Figure 6). To determine whether the enhanced fibrosis observed in the miR145-deficient mice was dependent on TGFβ receptor signaling, we treated a subset of Ang II–infused mice with a TGFβ receptor inhibitor. Tgfbr2 requires Tgfbr1 to function,49 so we used a well-characterized Tgfbr1 inhibitor (ALK4/5/7; SB431562) to block receptor activity. The data show that blocking TGFβ receptor activity reversed the Ang II–induced fibrosis (Figure 6). Furthermore, examining vascular fibrosis of the coronary arteries by trichrome staining revealed a significant increase in collagen deposition in miR145 null mice compared

![Figure 6. Loss of miR145 exacerbates angiotensin II (Ang II)–induced fibrosis.](http://circres.ahajournals.org/figshare/circres.340503)
with wild-type mice, which was attenuated by the addition of the TGFβ inhibitor (Figure 7A–7C). Analysis of the aorta revealed no overt difference in collagen content by trichrome staining (not shown), and there was no significant difference in collagen I transcript expression in wild-type compared with miR145 null mice infused with Ang II (Figure 7D). However,
we did observe significant increases in the expression levels of Serpine1 and Ctgf between Ang II–infused miR145-deficient animals compared with control groups (Figure 7E and 7F). To assess whether the increase in fibrosis markers could be correlated to Tgfbr2 expression, we examined Tgfbr2 transcripts. Consistent with our previous data (Figure 5), Tgfbr2 expression in miR145-deficient aortas was higher than in wild-type mice, and there was a significant increase of expression in both wild-type and mutant mice infused with Ang II compared with the saline-treated wild-type control (Online Figure VI). However, we failed to observe a greater increase of Tgfbr2 expression in the miR145 null mice in the presence of Ang II, indicating that other factor likely contribute to the enhanced fibrosis phenotype in the miR145 mutant mice. Interestingly, the RNA expression of these TGFβ-dependent genes was not attenuated by the TGFβ receptor inhibitor, suggesting additional mediators contribute to their regulation. Together, these data demonstrate that miR145 has the capacity to regulate TGFβ-dependent responses in pathological conditions, and acts to regulate matrix synthesis independent of smooth muscle differentiation genes selectively.

Discussion

Previous studies demonstrated a role for miR145 in regulating smooth muscle differentiation.14-18 Collectively, the data indicate that miR145 acts to drive smooth muscle–specific gene expression in a vast regulatory loop that includes activation of Myocardin and inactivation of dedifferentiation mediator Kruppel-like factor-4.8 Our data support this prodifferentiation notion, as we show that miR-143/145 transcript is induced by endothelial cells during Notch-regulated smooth muscle differentiation. Previously, smooth muscle cells were shown to take up miR145 from exosomes secreted by endothelial cells, which was demonstrated to have an atheroprotective role.8 Thus, endothelial cells provide miR-143/145 to smooth muscle cells via multiple strategies that might be linked to a distinct function. TGFβ is a well-described smooth muscle differentiation inducer, and studies have shown that TGFβ activates miR145 expression.8,16 Our initial finding that miR145 preferentially targeted TGFβ signaling genes suggested that it might exist in a negative feedback loop. Although this may be the case, further analysis in this study revealed that miR145 targeted distinct subsets of TGFβ-dependent genes. We show that in smooth muscle cells, TGFβ activates both matrix genes and smooth muscle–specific genes, but miR145 specifically attenuated the expression of the matrix genes, leaving the smooth muscle–specific genes unaffected. This selective effect on TGFβ target genes implies that miR145 functions to control the actions of TGFβ and define smooth muscle cell phenotypes. A proposed model is illustrated in Figure 8, where an increase in TGFβ causes both matrix genes and smooth muscle differentiation genes. Under conditions that drive a contractile phenotype, miR145 expression is high causing reduced TGFβ to activate smooth muscle differentiation genes and to suppress matrix preferentially. Under conditions in which a synthetic phenotype is warranted, miR145 levels are reduced that allows for TGFβ-dependent matrix gene expression to ensue.

Our data demonstrate that TGFBR2 is a direct target of miR145 and this finding led us to examine matrix gene expression because of the established link to this receptor’s activity. Tgfbr2 is essential for normal vascular development, and mouse knockouts have shown a role in the regulation of matrix synthesis, elastogenesis, and aortic wall homeostasis.41,42 Human mutations found in this receptor cause Loeys–Dietz and Marfan syndrome type 2.51 The ability of miR145 to decrease TGFBR2 levels in smooth muscle cells is one mechanism through which it regulates matrix, but we expect other direct targets of the TGFβ pathway and matrix synthesis also contribute to this outcome. Indeed, we demonstrated that additional genes in the TGFβ pathway are decreased by miR145, but whether these are direct targets is currently under investigation. TGFβ signaling is a complex pathway that involves autoregulation of both a positive and negative nature and mutations in TGFβ components cause paradoxical increases in TGFβ signaling.7,52 Our attempts to quantify changes in TGFβ signaling under differing miR145 levels by measuring SMAD and p38 MAPK phosphorylation did reveal small increases in their activity in the absence of miR145. In addition, examination of promoter regions for SMAD binding elements of 5 matrix genes that were downregulated by miR145 mimic only found a potential SMAD binding elements in the collagen I promoter (not shown). We are presently unable to explain the exact mechanism through which miR145 suppresses matrix gene expression. Furthermore, it is unclear whether suppression of matrix genes by miR145 is solely through the TGFβ pathway and TGFBR2.

Finally, data presented here indicate that miR145 might serve as a critical checkpoint in the development of TGFβ-associated diseases. TGFβ signaling is linked to a range of cardiovascular diseases, many of which are based on inappropriate extracellular matrix deposition.52 Our results demonstrate that cardiac and perivascular fibrosis is increased in the absence of miR145, thus finding ways to increase or maintain miR145 in fibrotic diseases may have beneficial consequences. A recent report analyzing miR145 in cardiac fibroblasts indicated that just as in smooth muscle cells, miR145 promoted a contractile phenotype, and consistent with our results, loss of miR145 increased scarring in response to injury.43 Given that miR145 can preferentially downregulate TGFβ-dependent matrix synthesis while leaving other TGFβ-responsive pathways unaffected could serve as a valuable treatment strategy.
Sources of Funding
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Disclosures
None.

References


47. Williams B. Angiotensin II and the pathophysiology of cardiovascular remodeling. Am J Cardiol. 2001;87:10C–17C.


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**Novelty and Significance**

**What Is Known?**

- miR145 functions to modulate smooth muscle cell and fibroblast differentiation.
- miR145 favors a differentiated rather than a synthetic phenotype.
- Transforming growth factor (TGF)-β signaling has been linked to promoting both synthetic (fibrotic) and differentiated phenotypes.

**What New Information Does This Article Contribute?**

- The TGFβ signaling pathway is a target of miR145.
- miR145 selectively regulates downstream targets of TGFβ signaling.
- Loss of miR145 in mice increases cardiovascular fibrosis.

In this study, we found that microRNA miR145 can selectively suppress TGFβ-dependent matrix synthesis, while promoting smooth muscle–specific gene expression. In the absence of miR145, there was an increase in a synthetic or fibrotic phenotype. These findings suggest that miR145 acts to discriminate TGFβ targets, and this effect facilitates a differentiated phenotype. Thus, in the presence of miR145, TGFβ functions as a mediator of differentiation, and in the absence of miR145, TGFβ exhibits a synthetic and matrix-expressing phenotype. These results offer insight into how microRNAs modulate smooth muscle cells and fibroblasts through their unique regulation of TGFβ signaling and provide evidence for a mechanism in which TGFβ induces different downstream actions via regulation by miR145.
MicroRNA miR145 Regulates TGFBR2 Expression and Matrix Synthesis in Vascular Smooth Muscle Cells
Ning Zhao, Sara N. Koenig, Aaron J. Trask, Cho-Hao Lin, Chetan P. Hans, Vidu Garg and Brenda Lilly

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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. HEK293 cells and PAC1 cells were cultured in DMEM supplemented as above with 5% FBS. All cultures were maintained in humidified 5% CO2 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. 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 (anil-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. The lentiviral plasmids were transfected into TN-293 cells using Lipofectamine 2000 (Invitrogen), and the viral particles were amplified and purified as described.

Plasmid Transfection and Luciferase Assays
psi-CHECK2-TGFBR2 3’UTR plasmid was obtained from Addgene plasmid #31882. HEK293 or PAC1 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: CCTCTGACACTACATTTTTTTATGCTGATAACCC.

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, 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 TGFβ 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**

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
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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 II. Validation of inhibition of Notch, TGFβ 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 TGFβ receptor inhibitor (ALK4/5/7), SB431542 for 48 hours, followed by separation and qPCR analysis for SERPINE1 (TGFβ 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 III. Pathway analysis of miR145 targets. (A) 717 miR145 targets predicted by TargetScan were subjected to PANTHER pathway analysis to identify overrepresented pathways. (B) HAoSMCs were transiently transfected with control RNA mimic or a miR-145 mimic and isolated RNA was tested for expression of known TGFβ-dependent genes SERPINE1, SMAD7 and miR-145 by qPCR. (C) The 16 predicted miR145 targets found in the TGFβ signaling pathway were measured by qPCR following overexpression of the miR-145 mimic. * P < 0.05, **P < 0.01.
Online Figure IV. TGFβ receptor II (TGFB2) 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 TGFB2. (B) HAoSMCs were cocultured with HUVECs for 2, 4 and 6 days, followed by separation and Western blot analysis for TGFB2 protein level. (C) HAoSMCs were transfected with anti-miR-145 and followed by coculturing with HUVECs for 2 days. mRNA level of TGFB2 was measured by qPCR analysis. **P < 0.01, n.s. not significant.
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 TGFβ1 for 48 hours. (A) qPCR was performed to measure the expression of TGFβ 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 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. TGFβ receptor inhibitor (SB)431542 was injected in a subset of mice. * P < 0.05, **P < 0.01.