Supplemental Material

Online Figures

Online Figure I: miR-663 is downregulated in human proliferating pulmonary artery smooth muscle (PASMCs)

Online Figure II: JunB is a target gene of miR-663 in VSMCs

Online Figure III: Alignment of potential binding site for miR-663 in 3'-UTR of Myl9 transcript

Online Figure IV: Knockdown of JunB attenuates anti-miR-663 medicated effect in VSMCs

Online Figure V: Expression of miR-663 and LacZ in mouse left carotid artery after transduction with Ad-miR-663 and Ad-LacZ

Online Figure VI: Effect of miR-663 on the expression of PDGF receptor and transcriptional factors in human VSMCs

Online Tables

Online Table I: miRNA expression in human aortic smooth cells after PDGF treatment

Online Table II: Summary of the real-time PCR Primer sets used

Supplemental Methods

Cell culture

Human aortic SMCs (AoSMCs) (Lonza, Walkersville, MD) were cultured in growth media SmGM-2 (Lonza) in 5% fetal bovine serum at 37℃ in a humidified 5% CO2 incubator. All cells used in this study were between passages 5 and 6. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). In an in vitro differentiation assay, the cells were grown in Medium 231 and smooth muscle differentiation supplement (Invitrogen) for 48 hrs with a daily change of freshly prepared differentiation medium.

Oligonucleotide transfection

For miR-663 overexpression, miR-663 mimic (QIAGEN) was added to the complexes at the final concentrations of 10, 20, 40 nM. For miR-663 knockdown, the miR-663 inhibitor (QIAGEN) was added to the complexes at final concentrations of 50 nM. Knockdown of JunB expression was performed using JunB siRNA (Sigma), with Allstars negative siRNA (QIAGEN) as a control. 2 hrs after seeded into the 6 well plates, cells were transfected using HiPerFect Transfection Reagent (QIAGEN) according to the manufacture's protocol. Transfection medium was replaced by regular cell culture medium after 24 hrs of transfection.

PCR analysis, RNA analysis by quantitative RT-PCR (qRT-PCR)

Total RNAs were extracted from human aortic VSMCs and mouse carotid arteries by using either miRNeasy Mini Kit (QIAGEN) or RNeasy kit (QIAGEN).

qRT-PCR for miRNA was performed on cDNA from 20 ng of total RNA by using miRCURY LNA™ Universal cDNA Synthesis kit and SYBR® Green Master Mix Kit (Exqion). PCR analysis of miR-663 was performed using the primers (forward: GAG AGG ATC CTG AGT TTG TGG CTG TG; reverse: GAG TAA GCT TGC AGA CAG GCA AGG G). qRT-PCR for matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), JunB, calponin, SM22α, SM α-actin (SMA), myocardin, myosin heavy chain gene (MYH11) were performed on the cDNAs generated from 250 ng of total RNA by HotStart-IT® SYBR® Green qPCR Master Mix with UDG (2X) Tested User Friendly™ kit (USB Corporation). Primer sequences used for the detection of human MMP-2, MMP-9, JunB, calponin, SM22α, SMA, myocardin, and MYH11 are listed in the Online Table II. The expression of miR-663 relative to U6 and the expression levels of MMP-2, MMP-9, JunB, calponin, SM22α, SMA, myocardin, MYH11 relative to 18S were determined using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Cells were lysed into Pierce® RIPA Buffer. After 30 min extraction at 4℃, insoluble material was removed by centrifugation. Cell lyses were then resolved by SDS/PAGE and transferred to nitrocellulose. Blots were blocked with 5% nonfat milk in PBS with 0.1% Tween20 (PBST) and developed with diluted antibodies to JunB (1:1000 dilution; Cat no: 3753S; Cell Signaling), SM22α (1:300 dilution; Santa Cruz Biotech), smooth muscle (SM) α–actin (1:800 dilution; Sigma), Myosin Light Chain 2 (MYL9; 1:1000 dilution; Cat no: 3672P; Cell

Signaling), α-tubulin (1:1000 dilution; Cell Signaling), and GAPDH (1:1000 dilution; Santa Cruz), followed by incubating with either IRDye 700 or 800 secondary antibodies and visualized using Odyssey Infrared Imaging System software (Li-Cor, Lincoln, NE).

Luciferase reporter assay

HEK293 cells were seeded in a 96-well plate and transfected at ~80% confluence. The fragment of the 3'-UTR of JunB mRNA containing the putative miR-663 binding sequence was cloned into a RenSP luciferase reporter and then co-transfected with either vehicle (vehicle control), miR-663 mimic, or a non-targeting mimic control into HEK293 cells following the instruction provided by Switchgear Genomics. 48 hrs after transfection, LightSwitch Luciferase Assay Reagent was added to measure luciferase activity.

Mutagenesis

Two miR-663 sites located at Jun-B 3'-UTR (site 663-1: CCCCGCC, starting at nt 300, and site 663-2: CCCCGCC, starting at nt 346) were mutated to M663-1 (CC**GGCGG**) and M663-2 (CC**GGCG**C) using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The following primers were used for the mutagenesis of the miR-663 target site-1 in the human JunB 3'-UTR (^{1st} forward: 5'-GGA GCT GGC CGG GCC GCC TGG TAC TC -3', ^{1st} reverse: 5'-GAG TAC CAG GCG GCC CGC CCA GCT CC-3'; ^{2nd} forward: 5'-GAG CTG GCC GGC GGG CCT GGT ACT CAA G -3', ^{2nd} reverse: 5'- CTT GAG

TAC CAG GCC CGC CGG CCA GCT C -3'); for the mutagenesis of the miR-663 target site-2 in the human JunB-3'-UTR (^{1st} forward: 5'-GGG AAG GGG ACC CGG GCC CCC TGC CCT CC-3', ^{1st} reverse: 5'-GGA GGG CAG GGG GCC CGG GTC CCC TTC CC-3'; ^{2nd} forward: 5'-GGA AGG GGA CCC GGC GCC CCT GCC CTC C-3', ^{2nd} reverse: 5'-GGA GGG CAG GGG CGC CGG GTC CCC TTC C-3').

Generation of recombinant adenovirus

The adenovirus expressing miR-663 (Ad-miR-663) and control viruses expression GFP (Ad-GFP) were made using RAPAd® Universal Adenoviral Expression System (CELL BIOLABS, INC) according to the manufacturer's instructions. Briefly, pacAd5 9.2-100 Ad backbone vector was cotransfected with the pacAd5 K-NpA shuttle vector containing the miR-663 sequence into Ad293 cells using FuGene 6 Transfection Reagent (Roche, Indianapolis, IN). The viruses were propagated on Ad293 cells and purified using Cscl2 banding followed by dialysis against 20 mmol/L Tris-buffered saline with 2% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech) according to the instructions of the manufacturer. For adenovirus-mediated GFP or miR-663 gene transduction, human aortic SMCs were grown to ~60% confluence and transduced with adenovirus at indicated multiplicity of infection (MOI).

Smooth muscle cell scratch wound assay

In gain of function experiment, human aortic SMCs were seeded in 6-well plates at a concentration of 2.5×10^5 cells per well and transduced with either Ad-miR-663 or Ad-GFP. In loss-of-function experiment, miR-663 inhibitors, JunB siRNA, or their negative controls were transfected with HiPerFect Transfection Reagent (QIAGEN) into SMCs seeded in 6-well plates at a density of 2.5×10^5 cells per well following the manufacturer's instructions. After incubation with starvation medium (0.5% FBS) for 48 hr, a linear wound was gently introduced in the center of the cell monolayer using 200 μl tip, followed by washing with PBS to remove the cellular debris. Cells were then subjected to stimulation with or without human PDGF-BB (PEPROTECH) at a final concentration of 20 ng/ml and monitored for additional 24 hr. Photos Images were captured using an Olympus IX 71 microscope equipped with a diagnostic instrument RT SPOT Digital Camera (Canon).

VSMC proliferation assay in vitro

VSMC proliferation in vitro was determined by either MTT assay using Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen™) or BrdU incorporation assay (Roche Applied Science). For MTT assay, SMCs were seeded at 1×10^4 cells per well in 96-well culture plates for 24 hrs. The cells were transfected with JunB siRNA or negative control siRNA for 24 hrs, and incubated with serum-deprived in 0.5% FBS for 48 hrs. Then MTT labeling reagent was added and incubated for 4 hrs, and solubilized in 0.01N HCl containing 10% SDS for 4 hr. Absorbance was measured at 570 nm on an ELISA plate reader. For BrdU incorporation

assay, BrdU was added to the culture medium for incorporation into cellular DNA. After 4 hrs of incubation, cells were fixed, and anti-BrdU antibody was added and incubated or 30 min. Finally, the absorbance was measured at 405 nm.

Adventitial gene transfer and carotid artery ligation injury

Briefly, Male C57BL/6N Mice (20-25g) were anesthetized with an intraperitoneal injection of Avertin (400 mg/kg). The left common carotid artery was ligated with a 6-0 silk suture so that the common carotid artery blood flow was completely disrupted. The common carotid artery was dissected free of the surrounding connective tissue and Ad-miR-663 (10^{10}pfu/ml) or Ad-LacZ (10^{10}pfu/ml) was suspended together in 50μl pluronic F127 gel (BASF, 25%wt/vol) and was applied around the carotid artery. Carotid arteries were harvested 2 weeks after ligation. Animals were anesthetized and perfused with 0.9% NaCl and fixed with 4% paraformaldehyde and embedded in paraffinum. Tissue was sectioned at 6um and stained with hematoxylin/eosin and were examined by a light microscope (Nikon) and the neointimal area was measured by the computer program ImageJ2x. This study was reviewed and approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University.

Immunofluorescence

For proliferating cells, the sections were incubated with anti-PCNA antibodies (1:50 dilution; Cell Signaling) followed by fluorescein conjugated secondary antibodies (1:300 dilution; Invitrogen). For JunB staining, the sections were

incubate with JunB antibody (1:50 dilution; Cell Signaling) followed by fluorescein conjugated secondary antibodies (1:300 dilution; Invitrogen). For smooth muscle (SM) α–actin staining, the sections were incubate with smooth muscle (SM) α–actin antibody (1:500 dilution; Sigma) followed by fluorescein conjugated secondary antibodies (1:300 dilution; Invitrogen). Cell nuclei were stained with DAPI.

In situ hybridization of miR-663 in human aortic SMC

Human aortic SMCs were seeded into 2 well glass slide chamber and grown to confluence of 60%, then starved in medium containing 0.5% FBS for 48 hrs. Cells were stimulated with PDGF-BB (20 ng/ml) for additional 24 hrs and then fixed in 4% paraformaldehyde. After washed with PBST, slides were digested with proteinase K (10 μg/ml) for 1 min, postfixed with 4% paraformaldehyde and followed by 3 washes in PBST. Slides were pre-hybridized in hybridization buffer (50 % Formamide, 5×SSC, 0.1% Tween-20, 50 μg/ml heparin and 500 μg/ml yeast tRNA) at 55 °C for 2 hrs and hybridized with hsa-miR-663 probes (LNA mercury probe, Exiqon) at 55°C overnight. Then sections were washed in 0.2% SSCT at 55 °C, followed by blocking at room temperature with PBST/5% horse serum for 1 hr. Subsequently, anti-digoxigenin-POD antibody (Roche) was added at dilution of 1:1000 and incubated at room temperature for 1 hr. After post-hybridization washes in PBST, the signals were detected using the tyramide signal amplification system (PerkinElmer). Slides were mounted in Prolong Gold containing DAPI (Invitrogen) and analyzed using a Nikon Eclipse 80i microscope

equipped with a CCD camera.

Statistical analysis

Data were expressed as mean±standard deviation (SD) and analyzed for statistical significance by unpaired Student's *t*-test or ANOVA using SPSS software (version 18.0). A *p* value less than 0.05 is considered statistically significant in all experiments.

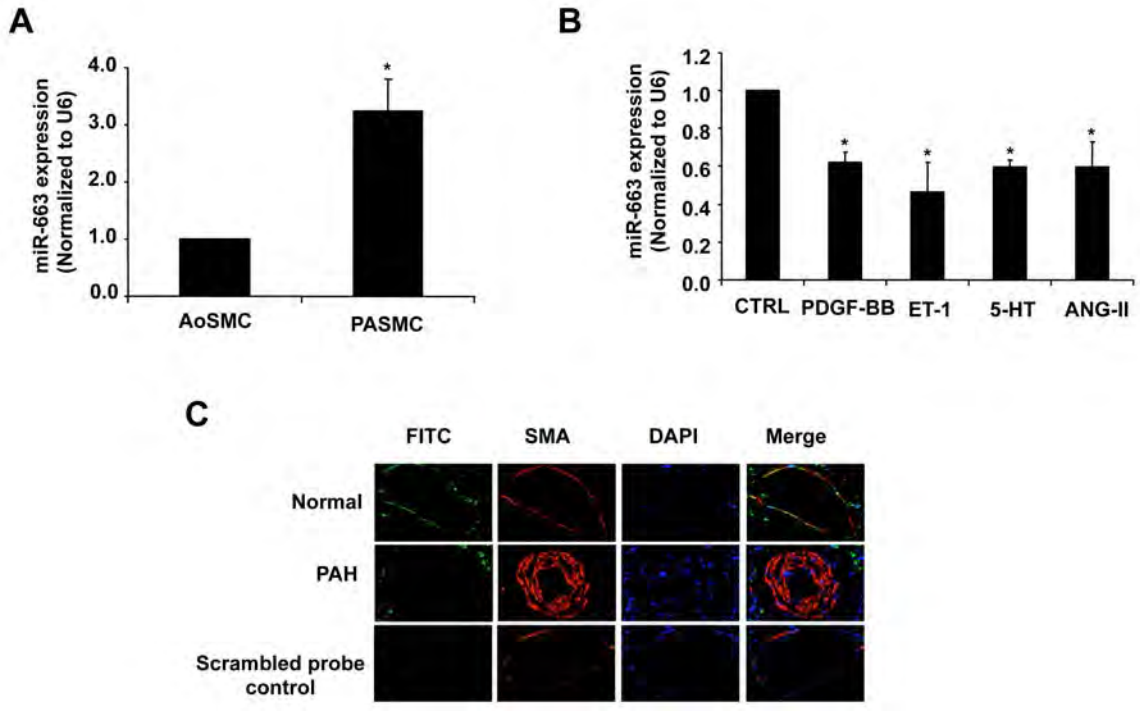

Figure I. miR-663 is downregulated in human proliferating pulmonary artery smooth muscle (PASMCs). (A) Expression of miR-663 in human aortic smooth muscle cells (AoSMCs) and PASMCs as determined by qRT-PCR (n=5). **P*<0.05 compared with AoSMC. (B) miR-663 was down-regulated in human PASMCs

after stimulation with PDGF-BB (20 ng/ml), ET-1 (100 nM), 5-HT (1000 nM), and ANG-II (100 nM) for 24 h, as determined by qRT-PCR (n=4). *P<0.05 compared with CTRL. (C) In situ detection of miR-663 expression in human normal lung vessels and vessels from patients with pulmonary artery hypertension.

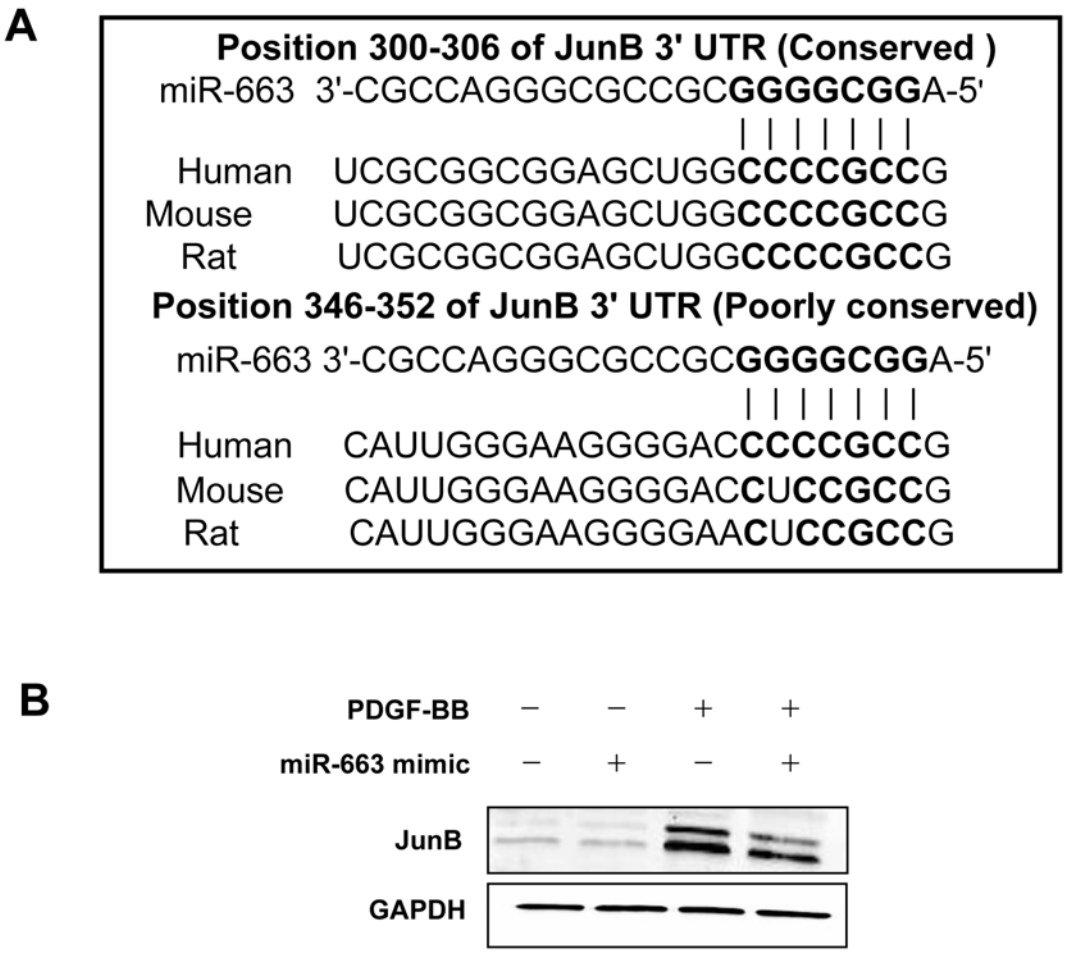

Figure II. JunB is a target gene of miR-663 in VSMCs. (A) Potential binding sites for miR-663 in 3'-UTR of JunB transcript. (B) Overexpression of miR-663 on the expression of JunB in mouse VSMCs as determined by western blot analysis.

Position 28-34 of Myl9 3'-UTR

miR-663 3′ CGCCAGGGCGCCGC**GGGGCGG**A 5′

Human 5′ CCUGACACCCCAGC**CCCCGCC**A 3′

Figure III. Alignment of potential binding site for miR-663 in 3'-UTR of Myl9 transcript.

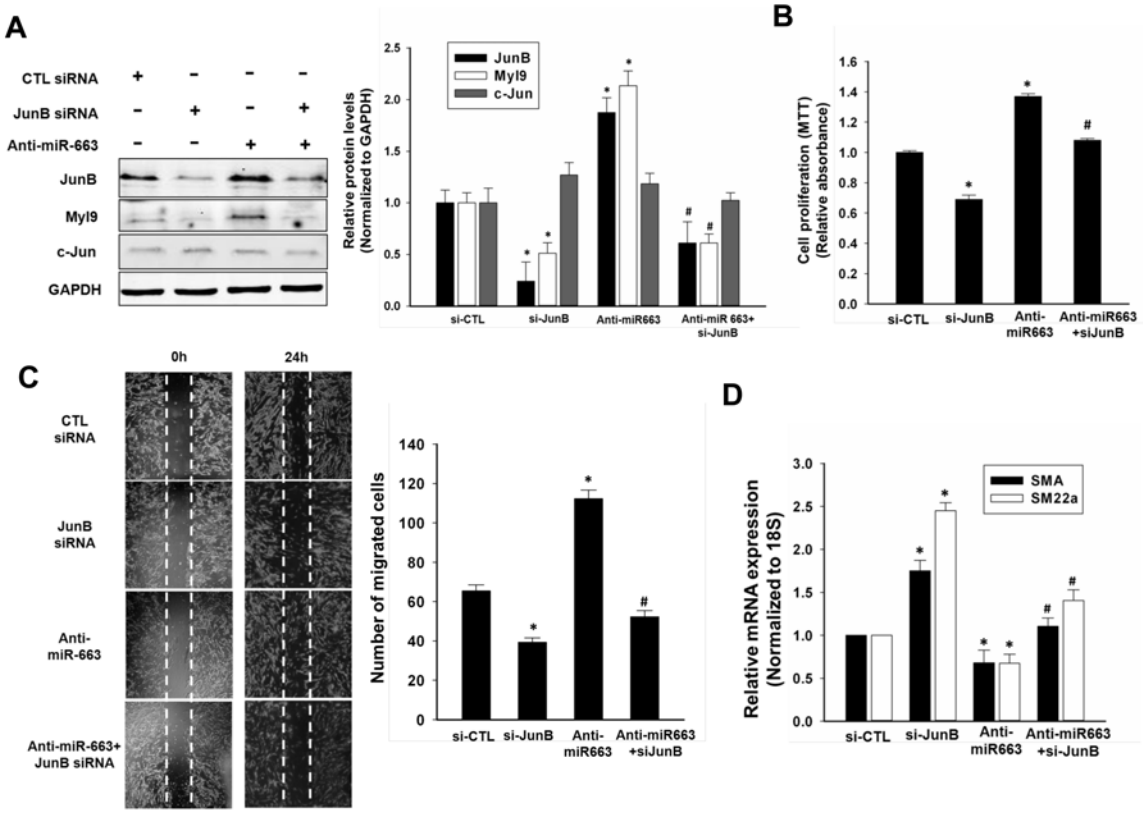

Figure IV: Knockdown of JunB attenuates anti-miR-663 mediated effect in VSMCs. (A) 72 hr after transfection of VSMCs with either CTL siRNA (50 nM), JunB siRNA (50 nM), anti-miR663 (50 nM) or combination of both JunB siRNA (50 nmol/L) and anti-miR663 (50 nM), the expression of JunB and Myl9 were detected by Western blot analysis. Densitometric analysis of JunB and Myl9 expression as determined by western blot (n=3). *P<0.05 vs CTL siRNA. $^{\#}P$<0.05

vs anti-miR-663; (B) 48 hr after transfection of VSMCs with either CTL siRNA (50 nM), JunB siRNA (50 nM), anti-miR663 (50 nM) or combination of both JunB siRNA (50 nM) and anti-miR663 (50 nM), VSMCs were starved and proliferation rate was analyzed by MTT assay after PDGF (2 ng/ml) stimulation for 24 hr. *P<0.05 vs CTL siRNA; $^{\#}P$<0.05 vs anti-miR-663. (C) 48 hr after transfection of VSMCs with either miR-663 mimic (50 nM), JunB siRNA (50 nM), anti-miR663 (50 nM) or combination of both JunB siRNA (50 nM) and anti-miR663 (50 nM). VSMCs were starved and cell migration was measured after PDGF (20 ng/ml) stimulation for 24 hr by scratch wound assay in the presence of mitomycin C (10 μg/ml). Migrated cells were quantitated. The data are means ± standard deviation of the number of migrated cells from 3 independent experiments. *P<0.05 vs CTL siRNA; $^{\#}$P<0.05 vs anti-miR-663. (D) 48 hr after transfection of VSMCs with either CTL siRNA (50 nM), JunB siRNA (50 nM), anti-miR663 (50 nM) or combination of both JunB siRNA (50 nmol/L) and anti-miR663 (50 nM), the expression of SMA and SM22α mRNA were detected by real-time PCR (n=3). *P<0.05 vs CTRL siRNA; $^{\#}$P<0.05 vs anti-miR-663.

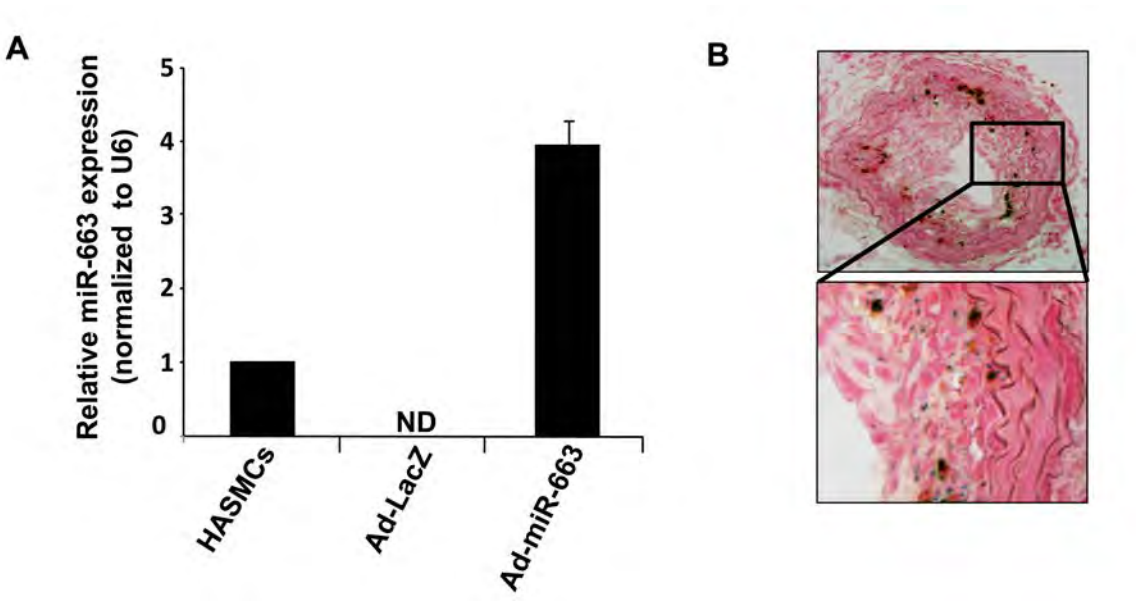

Figure V. Expression of miR-663 and LacZ in mouse left carotid artery after transduction with Ad-miR-663 and LacZ. (A) Ad-miR-663 (10^{10} pfu/ml) or Ad-LacZ (10^{10} pfu/ml) was suspended together in 50 μl pluronic F127 gel (BASF, 25%wt/vol) and was applied around the carotid artery. Carotid arteries were harvested 2 weeks after ligation. Total RNAs were then extracted and subjected to detect the expression of miR-663 by qRT-PCR (n=5). The expression of miR-663 in mouse carotid artery transduced with Ad-LacZ was undetectable (ND). (B) Ad-LacZ (10^{10} pfu/ml) was suspended in 50 μl pluronic F127 gel (BASF, 25%wt/vol) and was applied around the carotid artery. Carotid arteries were harvested 2 weeks after ligation and and sections stained with X-gal and nuclear fast red. Micrographs are representative of sections taken from 8 animals.

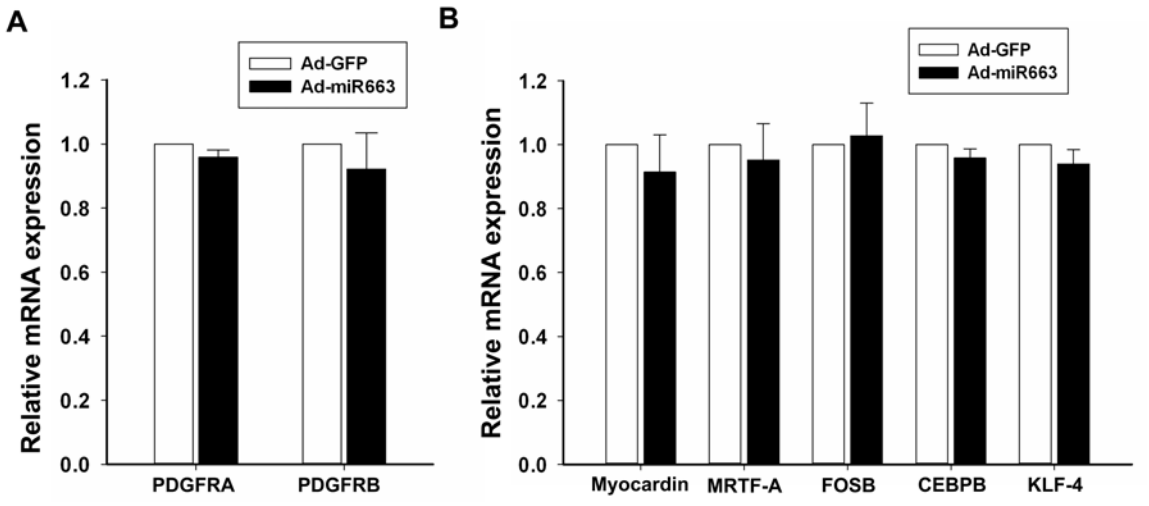

Figure VI. Effect of miR-663 on the expression of PDGF receptor and transcriptional factors in human VSMCs. Human aortic VSMCs were transduced with Ad-GFP or Ad-miR-663. 48 hr after transduction, total RNA was extracted and subjected to qRT–PCR analysis to detect the expression of PDGF receptor A and B (A) and transcription factors such as myocardin, MRTF-A, FOSB, CEBPB and KLF-4 (B). (n=3)

Supplemental data

Table 1. MiRNA expression in human aortic smooth muscle cell after PDGF-BB treatment

	Fold Change				Signal intensity
Hour	0	3	6	24	Baseline
Down-regulated					
hsa-miR-663	1	0.62	0.23	0.15	5,552
hsa-miR-638	1	0.60	0.40	0.19	35,390
hsa-miR-612	1	-	0.07	0.20	664
hsa-miR-103	1	0.66	-	0.64	5,408
hsa-miR-107	1	0.70	-	0.65	4,937
hsa-miR-191	1	0.73	1.3	0.74	7,213
hsa-miR-181a	1	0.81	-	0.74	5,564
hsa-miR-214	1	0.78	-	0.74	16,249
Up-regulated					
hsa-miR-368	1	1.68	-	4.32	782
hsa-miR-376a	1	2.43	-	3.84	262
hsa-miR-132	1	2.33	-	2.10	668
hsa-miR-29b	1	1.99	-	2.25	535
hsa-miR-30b	1	1.52	-	1.95	1,962
hsa-miR-98	1	1.52	-	1.91	3,321
hsa-miR-23b	1	1.18	-	1.37	27,680
hsa-miR-15a	1	1.87	-	1.88	724
hsa-miR-146b	1	1.99	-	1.79	1,544
hsa-miR-27a	1	1.23	-	1.43	14,681
hsa-miR-222	1	-	1.60	1.43	11,173
hsa-miR-23a	1	1.16	1.10	1.40	28,461
hsa-miR-221	1	-	1.23	1.25	11,195
hsa-miR-320	1		1.68	1.25	5,901
hsa-miR-125b	1	1.09	1.09	1.19	31,358
hsa-miR-199a	1	-	1.09	1.16	2,375
hsa-miR-24	1	-	1.14	1.15	16,035

Table II: Summary of the real-time PCR Primer sets used.

Primer	Sequence (5'-3')
SM α-actin	S: AATGCAGAAGGAGATCACGG AS: TCCTGTTTGCTGATCCACATC
Calponin	S: AACCATACACAGGTGCAGTC AS: GATGTTCCGCCCTTCTCTTAG
SM22α	S: TCCAGACTGTTGACCTCTTTG AS: TCTTATGCTCCTGCGCTTTC
MMP2	S: ACCCATTTACACCTACACCAAG AS: TGTTTGCAGATCTCAGGAGTG
MMP9	S: CGAACTTTGACAGCGACAAG AS: CACTGAGGAATGATCTAAGCCC
Myocardin	S: CCTCACTTTCTGCCCTCATC AS: GGAATTGAAAACTTTGGCCCC
MYH11	S: TGGAACTTCATCGACTTTGGG AS: ACAGCTTCTCCACGAAAGAC
PDGFR-A	S: ACAACCACACTCAGACAGAAG AS: CGTCATTCCTAGAGGTACAAAGG
PDGFR-B	S: GCTCACCATCATCTCCCTTATC AS: CTCACAGACTCAATCACCTTCC
MRTF-A	S: CTGAAGCAGGAGCTGAAGTT AS: AGGGCTGATTTGGTCTTGATAG
FOSB	S: CCTGACGGCTTCTCTCTTTAC AS: CAGGTGAGGACAAACGAAGA
CEBPB	S: CGCGACAAGGCCAAGAT AS: GCTGCTCCACCTTCTTCTG