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Supplemental Information

Recombinant Adeno-Associated Virus-Mediated Delivery of MicroRNA-21-3p Lowers Hypertension

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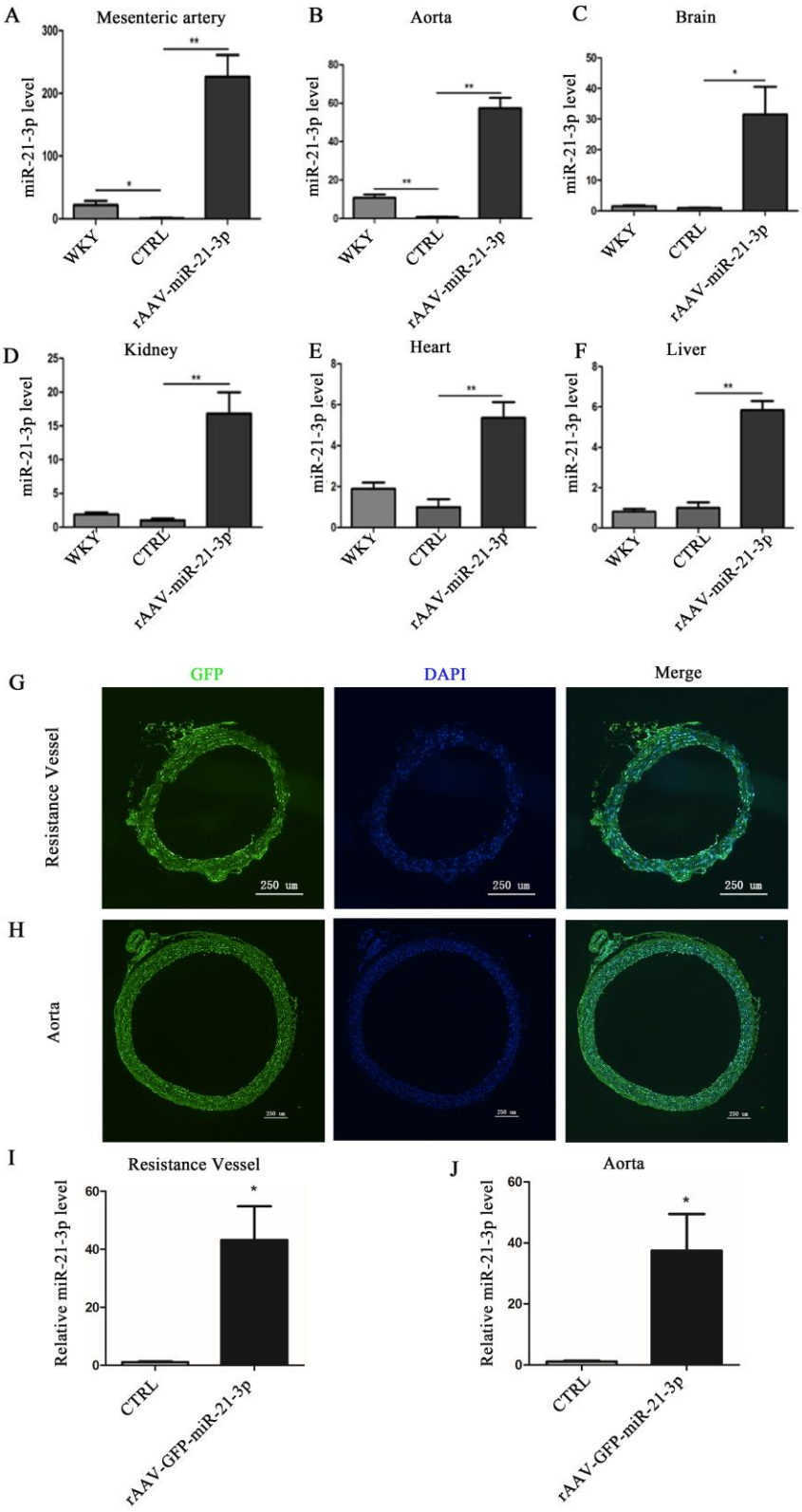
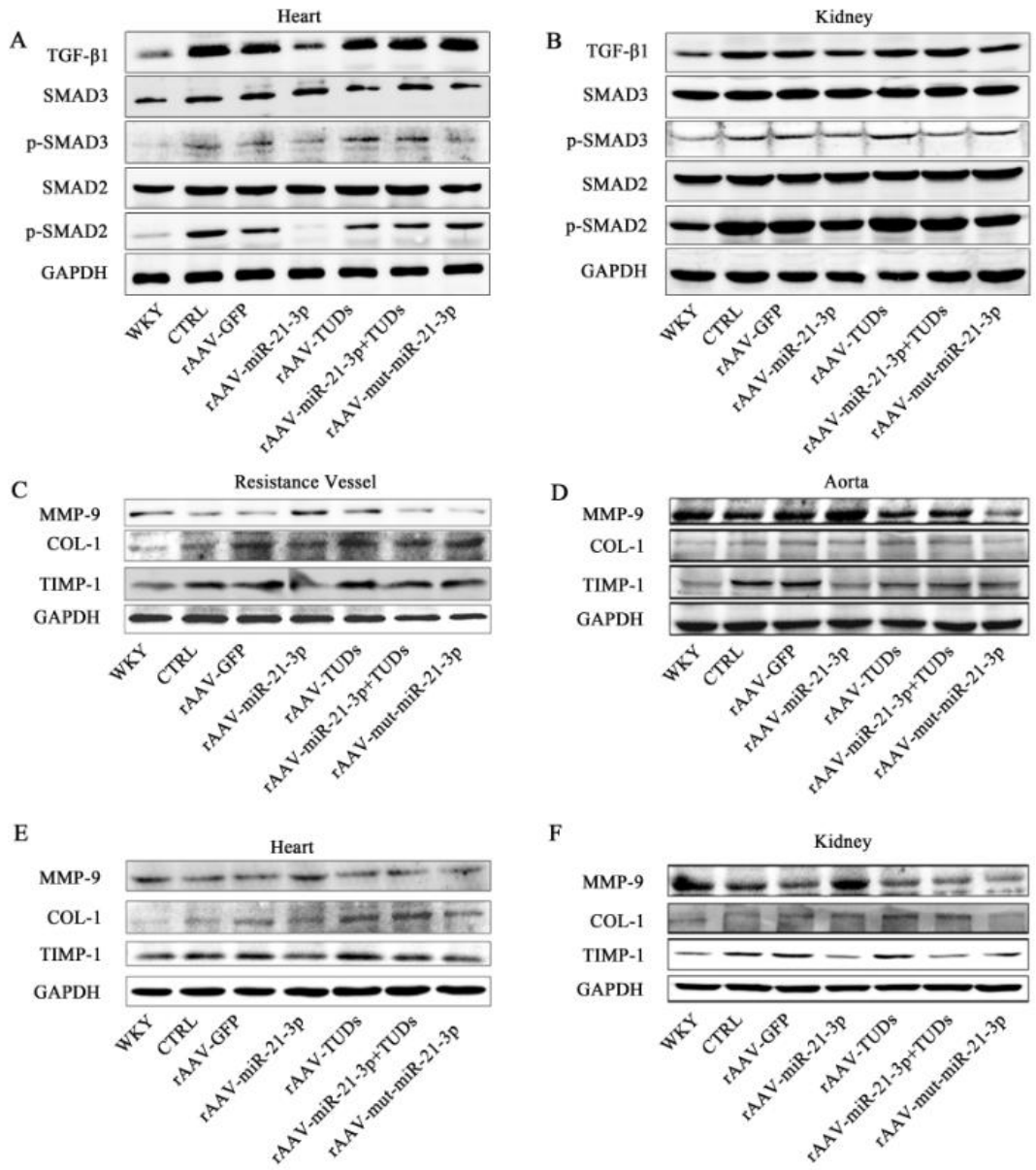
Supplemental Figure

Supplement Figure 1

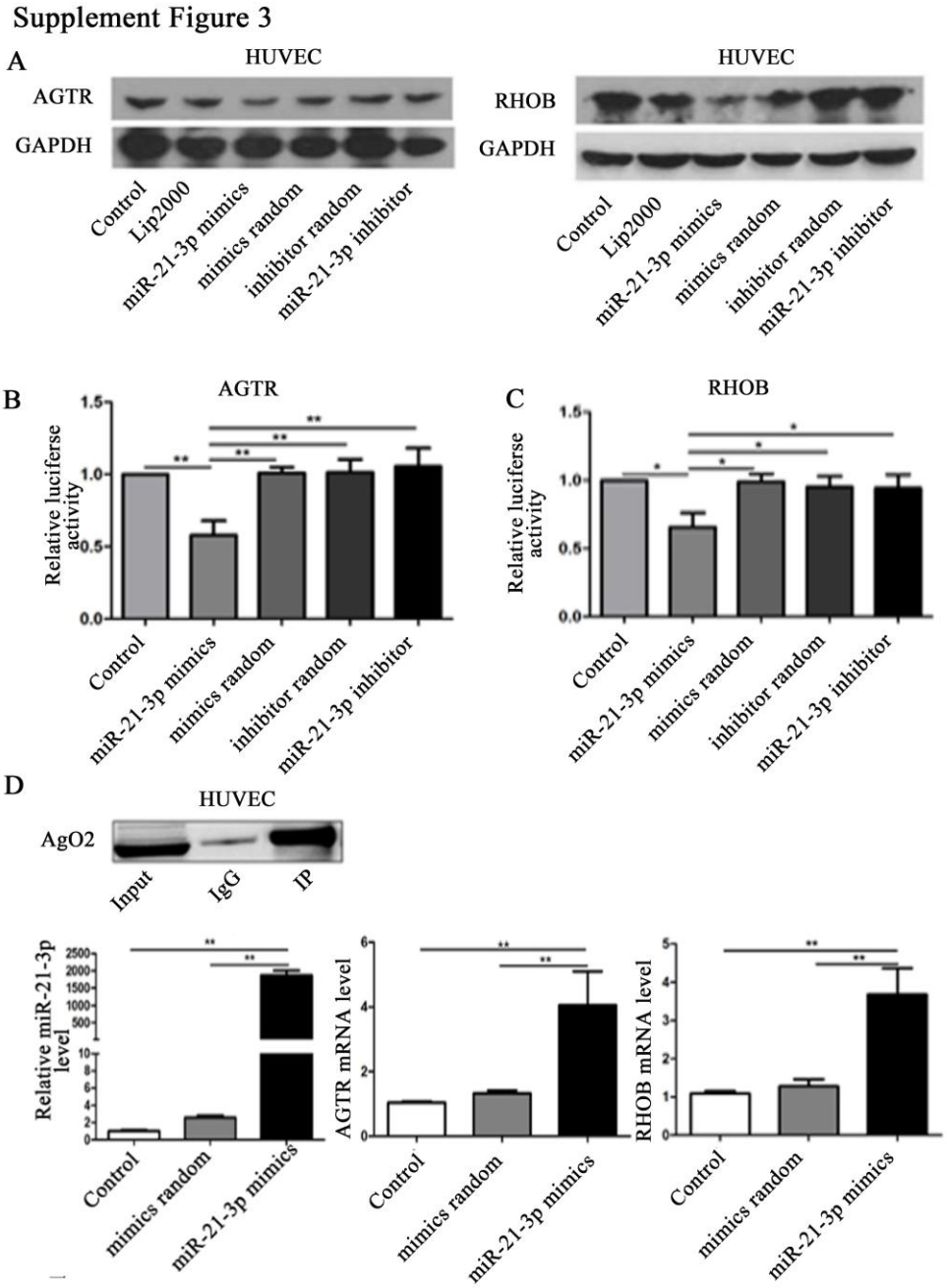


Supplemental Figure 1. The expression of miR-21-3p in various tissues from WKYs, SHRs control and SHRs treated with rAAV-21-3p. (A-F) The levels of miR-21-3p in resistance vessel, aorta, brain, kidney, heart, and liver from WKYs, SHRs control and SHRs treated with rAAV-21-3p, respectively; (G-H) Immunofluorescence staining of GFP in resistance vessel and aorta from SHRs treated with rAAV-GFP-miR-21-3p, respectively (GFP, green; nuclei, blue); (I-J)The expression of miR-21-3p in resistance vessel and aorta from SHRs treated with rAAV-GFP-miR-21-3p, respectively. WKY, Wistar-Kyoto rat; CTRL, SHR-Control (treated with normal saline). Data are presented as mean ± SEM ($n \geq 3$),*$p<0.05$, **$p<0.01$.

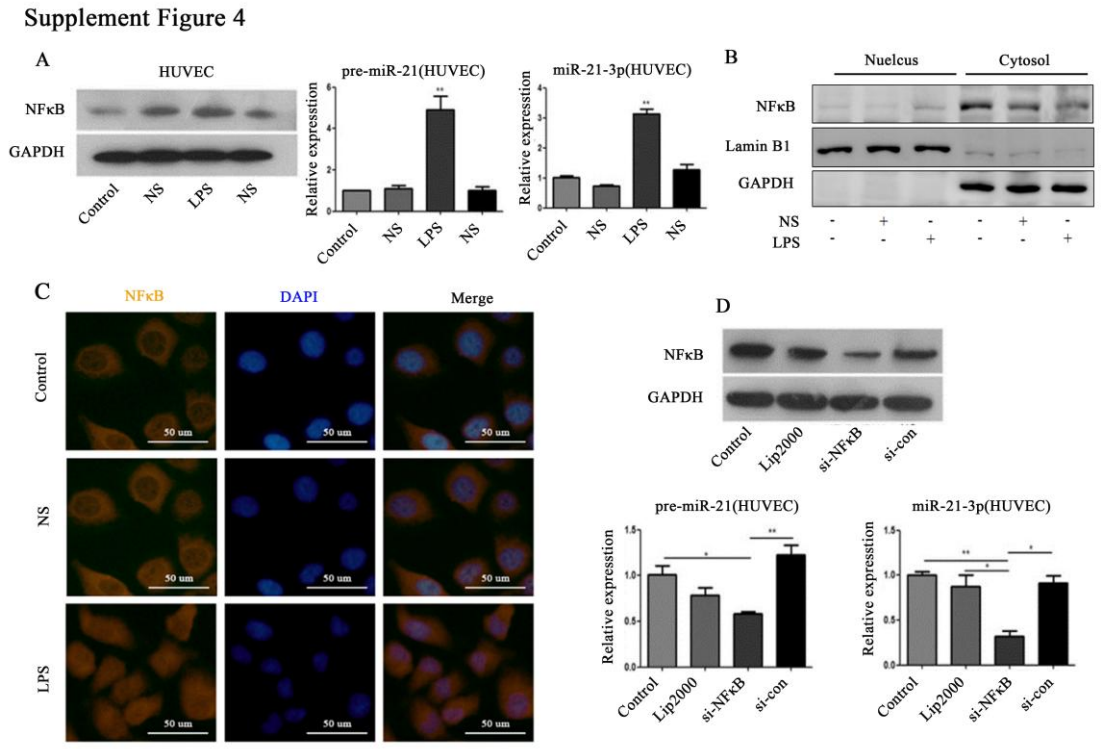
Supplement Figure 2



Supplemental Figure 2. Influence of miR-21-3p on tissues fibrosis in SHRs. (A-B) The protein expression of TGF-β1, SMAD2, p-SMAD2, SMAD3 and p-SMAD3 in heart and Kidney from WKYs and SHRs with different treated; (C-F) The protein levels of TIMP1, MMP9, and COL1 in resistance vessels, aorta, heart and kidney from WKYs and SHRs with different treated. WKY, Wistar-Kyoto rat; CTRL, SHR-Control (treated with normal saline). Data are presented as mean ±SEM (n≥3).



Supplemental Figure 3. miR-21-3p negatively regulates *AGTR1* and *RHOB* in human. (A)The expression of *AGTR1* and *RHOB* proteins in HUVEC after treated with miR-21-3p mimics; (B-C) The luciferase activity of pMIR-AGTR1 3'UTR and pMIR-RHOB 3'UTR plasmid in 239T cell after co-transfected with miR-21-3p mimics; (D) The levels of *AGTR1* and *RHOB* mRNAs captured by co-immunoprecipitation after administered with miR-21-3p mimics. lip2000, lipofectamine 2000; Data are presented as mean ± SEM(n≥3),*p<0.05, **p<0.01.



Supplemental Figure 4. NF-κB regulates the expression of miR-21-3p in HUVEC. (A) The expressions of NF-κB p65 protein, precursor-miR-21, and mature miR-21-3p in HUVEC after stimulated by LPS; (B) The nuclear translocation of p65 in HUVEC after stimulated by LPS; (C) Immunofluorescence staining of HUVEC treated with or without LPS (NF-κB, red; nuclei, blue); (D) The expressions of NF-κB p65 protein, precursor-miR-21, and mature miR-21-3p in HUVEC after co-transfected with *NF-κB* siRNA. NS, saline; Lip2000, lipofectamine 2000; si-con, siRNA-negative control. Data are presented as mean ±SEM (n≥3), *p<0.05, **p<0.01.

Supplemental Table 1. The baseline clinical characteristics of the hypertension patients

Characteristics	CTRL(n=32)	HYP1(n=33)	HYP2(n=15)	P value
Male/female (n/n)	22/9	15/18	7/8	0.089
Age (years)	53.0±11.1	56.9±9.6	59.6±9.9	0.102
BMI（kg/m^2）	23.4±2.4	23.8±2.2	23.2±2.8	0.785
SBP (mmHg)	120.1±1.8	149.7±2.7	123.9±1.7	<0.001
DBP (mmHg)	74.3±1.3	87.5±1.9	78.7±1.9	<0.001
Cr (mmol/L)	72.4±17.7	73.7±19.9	71.2±15.6	0.913
BUN（mmol/L）	5.5±1.3	6.0±1.2	6.3±2.6	0.224
Fasting glucose（mmol/L）	5.6±1.2	5.9±1.6	5.3±0.8	0.308
TG（mmol/L）	1.7±1.5	1.6±0.9	1.7±1.6	0.968
TC（mmol/L）	4.0±0.8	4.4±0.8	4.2±.9	0.148
HDL（mmol/L）	1.1±0.3	1.1±0.2	1.2±0.3	0.351
LDL（mmol/L）	2.4±0.8	2.5±0.7	2.2±0.7	0.300

BMI, Body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Cr, Creatinine; BUN, Blood urea nitrogen; TG, Triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; comparison among healthy control, hypertension patients with high blood pressure, and hypertension patients with normal blood pressure, Data are presented as mean ± SEM.

Supplemental Table 2. The details of synthetic miR-21-3p expression sequences, miR-21-3p TuDs sequences, and mutated miR-21-3p expression sequences

	The sequence of synthetic sense
MiR-21-3p	5′-GATCCACAGCCCATCGACTGGTGTTGTTCAAGAGACAACACCAGTCGATGGGCTGTCCGC-3′
MiR-21-3p TuDs	5′-GACGGCGCTAGGATCATCAACACAGCCCATCGATCTACTGGTGTTGCAAGTATTCTGGTCACAGAATACAA CACAGCCCATCGATCTACTGGTGTTGCAAGATGATCCTAGCGCCGTCTTTT-3′
Mutated miR-21-3p	5′-GATCCACAGCCCATCGACACCACAAGTTCAAGAGACTTGTGGTGTCGATGGGCTGTCCGC-3′

Supplemental Table 3. Echocardiographic characteristics of WKYs and SHRs administered with various constructed virus

			rAAV				
	WKY	**CTRL**	**GFP**	**miR-21-3p**	**TuDs**	**miR-21-3p + TuDs**	**mut-miR-21-3p**
HR (b.p.m.)	449±18	451±26	458±17	466±9	453±8	449±39	456±15
LVPW,d (mm)	2.59±0.23	3.38±0.25	3.28±0.28	2.62±0.15#&	3.70±0.15*	3.40±0.25*	3.51±0.11*
LVPW,s (mm)	3.44±0.27	4.54±0.33	4.42±0.25	3.85±0.18#&	5.03±0.16*	4.48±0.16*	4.42±0.10*
LVAW,d (mm)	2.33±0.30	3.22±0.03	3.16±0.10	2.53±0.12#&	3.25±0.17*	3.14±0.29*	3.10±0.13*
LVAW,s (mm)	3.12±0.38	4.29±0.11	4.23±0.25	3.59±0.15#&	4.36±0.12*	4.21±0.09*	4.30±0.20*
LVID,d (mm)	4.38±0.20	4.86±0.23	4.97±0.22	4.95±0.27	4.51±0.24	4.75±0.15	4.68±0.45
LVID,s (mm)	2.08±0.06	3.29±0.29	3.22±0.25	2.18±0.21#&	3.18±0.21*	3.13±0.43*	3.02±0.39*
FS (%)	74.6±1.25	53.8±2.85	58.2±4.16	65.2±1.48#&	44.3±2.52*	54.7±1.67*	56.4±1.32*
EF (%)	81.9±0.19	60.1±0.23	61.0±3.78	75.1±1.67#&	48.7±3.64*	59.6±6.93*	61.3±6.7*

Values represent mean ± SEM; n≥6 per group

HR, heart rate; LVPW,d, LV posterior wall thickness at diastole; LVPW,s, LV posterior wall thickness at systole; LVAW,d, LV anterior wall thickness at diastole; LVAW,s, LV anterior wallthickness at systole; LVID,d LV internal diameter at diastole; LVID,s, LV internal diameter at

systole; FS, fractional shortening.

$^{\#}p<0.05$ VS. CTRL, SHR-Control (treated with normal saline); $^{\&}p<0.05$ VS. rAAV-GFP; *$p<0.05$ VS. rAAV-miR-21-3p.

Supplemental Table 4. Primers for amplification promoters of miR-21-3p

	Primer sequence
p1-Forward 1	5′- TTATGCCAAACGAATCCAGC-3′
p2-Forward 2	5′- GAATGTGATGTTCAGCAGCAA-3′
p3-Forward 3	5′- GGAAAATCTGGTTGGTGTCTTAC-3′
Reverse	5′- GATGTCACGATGGTAGGCAAA-3′

	Primer sequence
f1-Forward 1	5'-CAAgCgATTCTCCTgCCTCA-3'
f2-Forward 2	5'-GTTATGCCAAACGAATCCAGC-3'
f3-Forward 3	5'-TCCTGCCCAACCCTTCCTC-3'
Reverse	5'-GGTCAGATGAAAGATACCAAAATGTC-3'

Supplemental Methods

Echocardiography analysis of WKYs and SHRs

At 24 weeks after rAAV delivery, WKYs and SHRs were anesthetized intraperitoneally with pentobarbital at a dose of 40 mg/kg body weight. Echocardiography was performed to assess the cardiac structure and functions of experimental rats using echocardiograph with VIVID 7 (General Electric, Milwaukee, WI), equipped with a 15-MHz linear array ultrasound transducer. The data of echocardiography was appropriately analyzed by 2 echocardiogram experts.

Prediction of miRNA Targets and Transcription factors

The microRNA databases and target prediction tools miRBase (http://www.mirbase.org/), miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org/), miRDB (http://mirdb.org/miRDB/) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) were used for predicting the potential targets of miR-21-3p. The transcription factors of miR-21-3p upstream were predicted via http://www.gene-regulation.com/pub/programs.html.

Cell Culture and Transfection

VSMCs, HUVEC and 293T cells were obtained from the American Type Tissue Collection, and were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) or Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI 1640, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), respectively. Cells were grown at 37℃ with an atmosphere of 5% CO_2. For the transfection of miRNAs, cells were treated with miR-21-3p mimics, mimics negative control, miR-21-3p inhibitors, and inhibitors

negative control at a final concentration of 100nM with lipofectamine 2000 (Invitrogen, Carlsbad, CA). 24-48 hours post-transfection, cells were harvested for RNA and protein, and functional assays were performed.

Plasmid Construction and Luciferase assay

The full length sequences of ADRA2B 3' UTR, AGTR1 3' UTR, and RHOB 3' UTR were amplified by PCR, then inserted into downstream of the luciferase reporter gene in the pMIR-REPORT^{TM} Luciferase Vector (Promega, Madison, WI) and were termed as pMIR-ADRA2B-3'UTR, pMIR-AGTR1-3'UTR, and pMIR-BOHB-3'UTR, respectively. 200 ng of pMIR reporter plasmid containing the 3'UTR sequences of miR-21-3p targets and empty pMIR reporter plasmid along with renilla luciferase plasmid were cotransfected into 293 cells with 100 nM miR-21-3p mimics, mimics negative control by lipofectamine 2000, respectively.

The expression sequence of ADRA2B was designed and cloned into pcDNA3.1 expression vector (Invitrogen) according to the manufacturer's protocol (5'-GGTAT CCCCG GACCT CCTA-3' and 5'-CCTGG CATAG ACAGC GAACA-3').

The expression sequence of SRF was designed and cloned into pcDNA3.1 expression vector (Invitrogen) according to the manufacturer's protocol (Human: 5'-CCCGTCCGCCCTCCTG-3'and5'-TGAAAAGGCAACAATAAATAAGTGGT-3') (Rat:5'-TTCCTCGCTGACTTGCCTGT-3'and5'-CTGCCCACTCCTTCTCCCC-3')

By promoter deletion, several promoter segments of miR-21-3p transcript with different lengths were amplified by PCR (primers were recorded in Supplemental Table 4), then inserted into Basic pGL3 Luciferase Reporter Vector.

Cells were collected at 48 hours after transfection for detecting luciferase activity using Dual-Luciferase reporter assay system (Promega, Madison, WI). Firefly

luciferase activity was normalized to renilla luciferase activity. All experiments were performed in triplicate with data from three independent experiments.

RNA isolation and detection

Total RNA was isolated from plasma or frozen tissue samples by TRIzol LS or TRIzol Reagent (Invitrogen) according to the manufacturer's protocol, respectively. Two microgram of total RNA was reverse-transcribed using a reverse transcription kit (RevertAid M-MuLV RT, Thermo scientific). The Bulge-Loop™ miRNA qRT-PCR Detection Kit (Ribobio Co., Guangzhou, China) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific) were used for qRT-PCR to examine the relative quantification of miRNAs according to the manufacturer's protocol with the 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City). U6 was used as endogenous control for normalizing the date of qRT-PCR.

Western blot analysis

Western blots were performed as previously described[1].

RNA Immunoprecipitation

VSMCs and HUVEC were treated with miR-21-3p mimics and inhibitors for 48 hours using lipofectamine 2000, respectively. Lysed cell extracts were immunoprecipitated with anti-Ago2 antibody (Santa Cruz, CA) by protein G Sepharose beads as previously described[2]. RNAs were extracted by Trizol LS reagent, and then miR-21-3p and its target mRNAs were detected by qRT-PCR after eluted from beads, respectively.

Functional experiments in superior mesenteric artery

Superior mesenteric arteries were carefully isolated and immersed in physiological salt solution (PSS) at 4℃, containing (in mM) 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$ H_2O, 1.17 MgSO4 H_2O, 25 $NaHCO_3$, 1.18 KH_2PO4, 0.027 EDTA, and 5.5 glucose, adjusted to pH 7.35-7.45. The bathing solution was gassed continuously with a mixture of 95% oxygen and 5% carbon dioxide at 37℃. Isometric tension was measured using a force displacement transducer connected to a Mac Lab recording system (ADI Instruments, Australia). Small arterial rings (3mm in length) were suspended on 2 intraluminal parallel wires, and progressively stretched to an optimal basal tension of 1g. Bathing solution was replaced every 15min during a 60min equilibration period, if needed, the resting tension was readjusted to 1g.

At the beginning of the experiment, vessel rings were repeatedly stimulated with KCl solution (high K^+, 60mM) for check their functional integrity. Subsequently, vessels were pre-contracted with norepinephrine (NE, 1nM-10uM) to stimulate vasoconstriction, and a cumulative relaxation respones to acetylcholine (Ach, 1nM-10uM) was performed to functionally evaluate the presence of endothelium. Thereafter, concentration-response curves to sodium nitroprusside (SNP 1nM-10uM) were recorded for determining the endothelium-independent vasodilatation.

Immunocytofluorescence

Cells and tissue sections were fixed in 4% paraformaldehyde for 10 min, and then soaked in 0.25% Triton-X100 for 10 min. After blocking with 5% BSA for 30 min, cells were incubated in primary antibody for 16h at 4℃, and then incubated with Cy3- or FITC conjugated secondary antibody for 2h at room temperature. Then, cells were visualized with DAPI (Boster) for nuclear staining.

References

1. Chen, F, Chen, C, Yang, S, Gong, W, Wang, Y, Cianflone, K, *et al.* (2012). Let-7b inhibits human cancer phenotype by targeting cytochrome P450 epoxygenase 2J2. *PloS one* **7**: e39197.
2. Goff, LA, Davila, J, Swerdel, MR, Moore, JC, Cohen, RI, Wu, H, *et al.* (2009). Ago2 immunoprecipitation identifies predicted microRNAs in human embryonic stem cells and neural precursors. *PLoS One* **4**: e7192.