The antagonistic effects and mechanisms of microRNA-26a action in hypertensive vascular remodeling

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Abstract

Background and Purpose: Hypertensive vascular remodeling (VR) is responsible for end-organ damage and is the result of increased extracellular matrix accumulation and excessive vascular smooth muscle cell (VSMC) proliferation. MicroRNA-26a (miR-26a), a non-coding small RNA, is involved in multiple cardiovascular diseases. We aimed to validate the effect and mechanisms of miR-26a in hypertensive VR. Experimental Approach: Spontaneously hypertensive rats (SHRs) were injected intravenously with recombinant adeno-associated virus-miR-26a. In vitro experiments, angiotensin II (AngII)-induced VSMCs were transfected with miR-26a mimic or inhibitor. Key Results: We found miR-26a downregulated in the thoracic aorta and plasma of SHRs. Overexpression of miR-26a inhibited extracellular matrix deposition by targeting connective tissue growth factor (CTGF) and mitigated VSMC proliferation by regulating the enhancer of zeste homolog 2 (EZH2)/p21 pathway both in vitro and in vivo. AngII-mediated Smad3 activation suppressed miR-26a expression, which in turn promoted Smad3 activation via targeted regulation of Smad4, leading to further downregulation of miR-26a. Conclusion and Implications: Our study reveals that AngII stimulates a Smads/miR-26a positive feedback loop, which further reduces miR-26a expression, leading to collagen production and VSMC proliferation and consequently, VR. MiR-26a has an antagonistic effect on hypertensive VR and can be a strategy for treating hypertensive VR.

Abbreviations

AngII: angiotensin II

Col: collagen

CTGF: connective tissue growth factor

CVF: collagen volume fraction

ECM: extracellular matrix

EZH2: enhancer of zeste homolog 2

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

H&E: hematoxylin-eosin

LD: lumen diameter

miR: microRNA

MT: media thickness

MuT: mutant-type

OD: optical density

PBS: phosphate buffered saline

PCNA: proliferating cell nuclear antigen

p-Smad3: phospho-Smad3

qRT-PCR: quantitative real-time PCR

rAAV: recombinant adeno-associated virus

SBP: systolic blood pressure

SHR: spontaneously hypertensive rat

siRNA: small interfering RNA

VR: vascular remodeling

VSMC: vascular smooth muscle cell

WKY: Wistar Kyoto rat

Introduction

Vascular remodeling (VR) persists throughout the course of hypertension and is characterized by chronic, continuous, and complex changes in vascular structure and abnormalities in vascular functions (Hayashi*et al.* , 2009; Lemarie *et al.* , 2010; Zhang *et al.* , 2019). VR, as an important indicator of the progression of hypertension, is closely related to the severity and prognosis of the disease (Mulvany, 2012; Magnussen, 2017; Brown *et al.* , 2018). Angiotensin II (AngII)-induced dysfunction of vascular smooth muscle cells (VSMCs) plays a critical role in hypertensive VR (Das *et al.* , 2018), but the potential molecular mechanisms remain poorly understood. Therefore, study of new therapeutic targets for hypertensive VR and exploring potential molecular mechanisms are of benefit.

MicroRNAs (miRs) are highly conserved, small non-coding RNAs that modulate the function of VSMCs, such as their proliferation, differentiation, and migration (Chen *et al.* , 2018a; Chen *et al.* , 2018b; Wang *et al.* , 2019a; Wang *et al.* , 2019b). Moreover, miR expression is highly related to vascular remodeling and angiogenesis (Henn *et al.* , 2019). Cai *et al.* found that miR-24 attenuated VR under diabetic conditions (Cai *et al.* , 2019). Downregulation of miR-224 aggravated VR in acute coronary syndrome (Xu *et al.* , 2019). MiR-1 overexpression mitigated pulmonary VR, thereby protecting against the progression of pulmonary hypertension (Sysol *et al.* , 2018).

MiR-26a, a highly conserved post-transcriptional regulator, can inhibit the proliferation and migration of VSMCs after vascular injury (Tan*et al.* , 2017) and protect VSMCs against oxidative stress (Peng*et al.* , 2018). Thus, miR-26a may be a therapeutic target for vascular dysfunction. Moreover, the serum level of mir-26a was found altered in patients with hypertension (Yang *et al.* , 2018). However, the role and molecular mechanisms of miR-26a in hypertensive VR need to be clarified.

Our research aimed to elucidate the role of miR-26a in hypertensive VR and to reveal the potential miR-dependent mechanisms. We present evidence that miR-26a plays a protective role in hypertensive VR. AngII-activated Smad3 inhibited miR-26a expression, which in turn promoted Smad3 activation by targeting Smad4, thereby forming a Smads/miR-26a positive feedback loop and further downregulating miR-26a. Downregulation of miR-26 further led to VR by promoting connective tissue growth factor (CTGF) expression and the enhancer of zeste homolog 2 (EZH2) /p21 pathway.

Materials and Methods

2.1 Animals and experimental protocols

Seven-week-old male spontaneously hypertensive rats (SHRs; 190±10 g) and Wistar Kyoto rats (WKYs; 200±10 g) were purchased from Beijing Wei Tong Lihua Experimental Animal Center. Ten-week-old male C57BL/6 mice (25±2 g) were purchased from the Laboratory Animal Center of Xi'an Jiaotong University. Animals were housed in a room with a controlled environment (12/12-h light/dark cycle, 22-25°C, 50-60% relative humidity) with free access to normal diet and water. After study, all animals were anesthetized by inhalation of 1-2.5% isoflurane, and then euthanized by cervical dislocation. All animal experimental protocols were approved by the local and national ethical committee and conformed to NIH guidelines and the recommendations made by the British Journal of Pharmacology.

After 1 week of adaptation, SHRs were randomly divided into 3 groups of 8 rats each: recombinant adeno-associated virus (rAAV)-miR-26a (150 μl, i.v.); rAAV-GFP (150 μl, i.v.) (a total dose of viral particles was 2×10^{11} v.g.; the rAAV vectors we used were rAAV of type-9; rAAV of miR-26a and vector controls was synthesized by Han Heng Biotechnology, China); and SHR-Ctrl (normal saline 150 μl, i.v.). Six WKY rats were normal controls (normal saline 150 μl, i.v.). Systolic blood pressure (SBP) of the caudal artery of rats was measured every 3 to 5 days by using a non-invasive BP analysis system (BP-2000 SERIESII, Visitech, USA). After 3 weeks, plasma and thoracic aortas of rats were collected.

For mice experiments, C57BL/6 mice (n = 6 per group) received AngII (Merck, USA, 2.0 mg/kg/d) or normal saline subcutaneously by implanted ALZET® 2002 minipumps under isoflurane anesthesia. Two weeks later, plasma and thoracic aortas of mice were collected.

2.2 Histology

Thoracic aorta sections were fixed in 4% paraformaldehyde, then embedded in paraffin. Morphological characteristics of thoracic aorta were assessed by hematoxylin-eosin (H&E) staining. The media thickness (MT) of thoracic aorta was defined as the region between the internal and external elastic laminae. The MT/lumen diameter (LD) represented the level of VR. Thoracic aorta fibrosis was assessed by Masson's trichrome staining. Collagen volume fraction (CVF) was quantified by measuring the ratio of blue fibrotic area to total thoracic aorta area.

2.3 Immunohistochemical staining

Aortic tissue sections (4 μm) were deparaffinized, and endogenous peroxidase activity was quenched with H_2O_2. The sections were incubated with anti-α-actin (dilution 1:200) and anti-proliferating cell nuclear antigen (PCNA; dilution 1:100) antibody at 4°C overnight, then with secondary antibody. After staining, each section was analyzed by confocal microscopy (DS-Fi1-Eclipse, Nikon).

2.4 Cell culture

VSMCs were prepared from thoracic aorta of 50-day-old female Sprague-Dawley rats. Aortic VSMCs were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) with 10% fetal bovine serum (BI, USA) and 1% penicillin/streptomycin at 37 degC in a 5% CO_2humidified incubator. Immunohistochemical staining revealed positivity for smooth muscle α-actin (Wanleibio, China) and negativity for CD105 (Wanleibio, China), indicating that primary VSMCs of rats were successfully isolated. Cells in passages 3-9 were used for all experiments.

2.5 Transfection

After 24-h incubation in serum-free medium, VSMCs were transfected with the miR-26a mimic (JTS Scientific, China) or miR-26a inhibitor (JTS Scientific, China) or a corresponding negative control (NC) or siRNA (Wanleibio, China) or SB431542 (MCE, China) according to the manufacturer's instructions. After 24- or 48-h transfection, cells were treated with AngII (10^{-7} mol/L for 24 h).

2.6 CCK-8 assay

VSMCs were cultivated in 96-well plates, with 4×10^3cells in each well, and 5 duplicate wells were designed for each group. A 10-μL amount of CCK-8 solution (Wanleibio, China) was added after 24 h and incubated

for 1 h under 5% CO_2 at 37. A microplate reader (BioTek, USA) was used to detect optical density (OD) values at 450 nm, which represents cell proliferation.

2.7 Cell cycle assay

Each group of VSMCs was washed twice with ice-cold phosphate buffered saline (PBS); cells were harvested by centrifugation and re-suspended in PBS, then fixed and permeabilized with 70% ethanol. Cells were washed twice in ice-cold PBS to remove ethanol, then treated with 500 μl propidium iodide (PI; Wanleibio, China) in the dark at 4°C for 30 min. Flow cytometry (ACEABio, NovoCyte, USA) was used to detect cell cycle.

2.8 Immunofluorescence cell staining

Cultured VSMCs were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% Triton-X-100 (Beyotime, China) for 30 min. Then cells were washed three times with PBS and incubated with anti-phospho-Smad3 (anti-p-Smad3) antibody (1:200 dilutions, ab52903, Abcam, England) overnight at 4°C, followed by secondary goat anti-rabbit IgG-Cy3 antibody (1:200 dilutions, A0516, Beyotime, China) incubated for 1 h at room temperature. Nuclear staining of the cells involved using 4'6-diamidino-2-phenylindole dihydrochloride (DAPI, Beyotime, China) for 5 min at room temperature. Images were captured by fluorescence microscopy (OLUMPUS, Japan).

2.9 ELISA

Levels of collagen I and III (Col I and III) were detected by using ELISA kits (Cloud Clone Corp., China) according to the manufacturer's recommendations, and the absorbance at 450 nm was read. The relative Col I and III levels were calculated according to the standard concentration-absorbency curve.

2.10 Quantitative real-time PCR (qRT-PCR)

Total RNA from plasma, tissues or cells was extracted with Trizol reagent (Thermo Fisher Scientific, USA). cDNA was synthesized by using the miRNA Reverse Transcription System kit (TaKaRa, Japan) or 2×SYBR real-time PCR premixture (Bioteke Corp., China), following the manufacturer's protocol. Real-time PCR was performed with the StepOnePLus Real-Time PCR System. The primer sequences and conditions for amplification are in Supplemental Table S2 and S2, respectively. U6 or β-actin was the internal reference. The relative expression of mRNAs and miR was calculated by the 2^{-t}method.

2.11 Western blot analysis

Protein was extracted from tissues or cells by using RIPA lysate (Hat Biotechnology, China). After quantification with bicinchoninic acid, equal amounts of protein were loaded and separated by SDS-PAGE, then transferred electrophoretically to polyvinylidene difluoride membranes, which were incubated with primary antibodies overnight at 4, followed by a 1:5000 dilution of goat anti-rabbit IgG antibody (EK020, Zhuangzhi Biotech, China) at room temperature. Primary antibodies were for CTGF (1:1000, ab6992), Smad3 (1:1000, ab40854), Smad4 (1:1000, ab40759), p-Smad3 (1:500, ab193297), cyclin D2 (1:1000, ab230883), p21 (1:500, ab109199; all Abcam, England); EZH2 (1:500, 5246; CST, USA); Col I (1:500, WL0088), Col III (1:500, WL0318), β-actin (1:500, WL01372; all Wanleibio, China), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000, AF7021, Affinity Biosciences, USA). Protein bands were visualized by using a potent ECL kit (KF001, Affinity Biosciences, USA) and the Gene Company imaging system (China). GAPDH or β-actin was an internal reference.

2.12 Chromatin immunoprecipitation (ChIP) assay

1% formaldehyde was used to cross-link VSMCs for 10 min at room temperature, then glycine (0.125M) was added for 5 min. The ChIP assay was performed with Smd3 or lgG antibody (Abcam, USA) and the ChIP kit (Wanleibio, China) according to the manufacturer's instructions. Bound DNA fragments were subjected to PCR. For PCR, 2 μL of DNA was amplified with 2×Taq PCR Master-mix (Bioteke Corp., China), using a primer pair specific to the binding region of the miR-26a promoter. The primer sequences and the condition

are in Supplemental Table S1 and S2, respectively. Finally, PCR products were visualized on 2% agarose gels.

2.13 Luciferase reporter assay

The mutant-type (MuT) 3'-UTR of EZH2, CTGF and Smad4 was constructed. The WT or MuT 3'-UTR of EZH2, CTGF and Smad4 were inserted into pMIRGLO vectors (Promega, USA). The miR-26a mimic or miR-negative control and WT or MuT 3'-UTR of EZH2, CTGF and Smad4 vector were co-transfected into HEK293T cells (Wanleibio, China). After 48 h, cells were lysed, and luciferase activity was determined by using a luciferase assay kit (Promega, USA) and multi-function microplate reader (TECAN, M200Pro, Switzerland).

2.14 Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis *et al.* , 2018). All data are expressed as mean +- SD and GraphPad Prism 6 software was performed to statistical analysis. To determine differences between groups with one factor, data were tested using unpaired Student's t-test for two group's comparison, and one-way ANOVA with Tukey's post hoc test analysis for multiple group comparisons. To determine differences between groups with multiple factors, data were tested using two-way ANOVA followed by Bonferroni's test. Post hoc tests were run only when F achieved $P < 0.05$. Differences were considered statistically significant at $P < 0.05$. All measurements were undertaken only for n [?] 5.

Results

3.1 Ang II regulated miR-26a expression both in vivo and in vitro

MiR-26a expression was reduced in AngII-treated VSMCs dose- and time-dependently as compared with the control (Fig. 1a, b), so AngII negatively regulated the expression of miR-26a. In vivo, the relative level of miR-26a was lower in the thoracic aorta and plasma of AngII-treated mice than the control (Fig. 1c), which indicated Ang II regulated miR-26a expression both in vivo and in vitro.

3.2 miR-26a had a protective effect on hypertensive VR

To evaluate the role of miR-26a in hypertensive VR, rAAV vectors were constructed to overexpress miR-26a in SHRs. As expected, rAAV-miR-26a transfection significantly increased the expression of miR-26a in aortas and plasma of SHRs (Fig. 2a). Meanwhile, rAAV-miR-26a prevented systolic blood pressure (SBP) from continuously increasing in SHRs (Supplemental Fig. S1). To evaluate VR, thoracic aortas underwent H&E staining. As compared with normal WKYs, in SHRs, the media of the thoracic aorta was significantly thickened. However, miR-26a overexpression could reduce the medial thickening of the thoracic aorta caused by hypertension. The MT/LD ratio declined profoundly with miR-26a overexpression (Fig. 2b, c; rAAV-miR-26a vs rAAV-GFP and SHR-Ctrl was 5.430 +- 0.2943 vs 6.793 +- 0.4130 and 7.575 +- 1.584; $P<0.05$). Immunohistochemistry for smooth muscle α-actin showed well-distributed smooth muscle and intact intima in WKY rats. However, VSMCs formed intima hyperplasia and parts of the vascular walls protruded into the aortic lumen in the SHR-Ctrl and rAAV-GFP groups. rAAV-miR-26a treatment significantly improved smooth muscle alignment and integrity (Fig. 2b).

3.3 miR-26a ameliorates extracellular matrix (ECM) production in vivo and in vitro.

ECM is mainly composed of collagen, so we observed and quantified CVF in vivo by Masson's trichrome staining. Trichrome staining and CVF were reduced in SHRs transfected with miR-26a as compared with rAAV-GFP and SHR-Ctrl groups (Fig. 3a, b; 31.26 ± 3.902 vs 54.25 ± 5.987 and 56.17 ± 7.218, $P<0.05$). Then, we transfected miR-26a mimic and inhibitor into AngII-induced VSMCs to evaluate the effect of miR-26a on collagen production in vitro. On qRT-PCR, miR-26a was significantly elevated by miR-26a mimic treatment and markedly inhibited by miR-26a inhibitor treatment in AngII-treated VSMCs; neither of their negative controls (NCs) promoted or suppressed miR-26a expression (Fig. 3c). As expected, miR-26a mimic overexpression significantly suppressed and miR-26a inhibition enhanced the mRNA and protein expression

of Col I and Col III according to ELISA, qRT-PCR and western blot analysis (Fig. 3d-f). In short, miR-26a inhibited collagen deposition both in vivo and in vitro.

3.4 miR-26a inhibits VSMC proliferation in vivo and in vitro.

The development of hypertensive VR is associated with the excessive proliferation of VSMCs. To determine whether miR-26a affected VSMC proliferation, immunohistochemistry for PCNA was used to test VSMC proliferation in thoracic aortas of SHRs and WKY rats. Cells with brown staining were defined as PCNA-positive cells. The proportion of PCNA-positive cells (ratio of number of PCNA-positive cells to total number of sampled cells) was lower with rAAV-miR-26a overexpression than in vector- and vehicle-treated controls (Fig. 4a, b; 0.08801 ± 0.01623 vs 0.4440 ± 0.04262 and 0.4496 ± 0.01413, $P<0.05$). We used CCK-8 assay and flow cytometry to measure the proliferation of VSMCs in vitro. Upregulation of miR-26a reduced the proliferation of AngII-induced VSMCs and retained cells in the G1 phase (Fig. 4c-e). miR-26a inhibitor treatment had the opposite effect, promoting cell proliferation.

3.5 miR-26a suppresses ECM deposition by directly targeting CTGF.

CTGF is essential for the synthesis of collagen (Liang *et al.* , 2014). We hypothesized that miR-26a attenuates ECM accumulation by regulating CTGF. In vivo data shown in Figure 5a support this hypothesis: CTGF protein level was significantly downregulated in SHR thoracic aortas with rAAV-miR-26a overexpression. The effect of miR-26a was further confirmed in vitro: miR-26a mimic suppressed CTGF expression in AngII-induced VSMCs and miR-26a inhibitor increased CTGF protein and mRNA levels in vitro (Fig. 5b, c). Bioinformatics analysis by using Targetscan (www.targetscan.org) predicted CTGF as a potential target gene of miR-26a (Fig. 5d). By using a reporter construct with the putative 3'-UTR miR-26a binding site of CTGF downstream of the luciferase gene, we found that miR-26a reduced luciferase activity, which provides experimental validation of CTGF as a target for miR-26a. Accordingly, after mutation of the binding site, the activity of luciferase was not altered (Fig. 5e), which confirms that the binding of miR-26a to the 3'-UTR site was necessary for silencing CTGF

3.6 EZH2/p21 pathway mediates miR-26a dysregulation-induced VSMC proliferation.

To explore the mechanisms of miR-26a-inhibited proliferation of VSMCs, we investigated the regulation of miR-26a of proliferation-related genes. Considering that rAAV-miR-26a overexpression decreased EZH2 protein level in the thoracic aorta of SHRs (Fig. 6a), we assumed that miR-26a inhibiting VSMC proliferation is mediated by EZH2. Consistent with in vivo findings, in AngII-induced VSMCs, the expression of EZH2 at both the protein and mRNA levels was significantly decreased after transfection with miR-26a mimic (Fig. 6b, e). Furthermore, Targetscan revealed that the miR-26a binding site within the 3'UTR of EZH2 mRNA is highly conserved (Fig. 6f). We further performed luciferase reporter assay and found reduced luciferase activity with miR-26a mimic transfection. However, miR-26a did not affect activity of the binding site mutation, which confirms that EZH2 is a target of miR-26a (Fig. 6g).

p21 belongs to a cell-cycle regulator that contributes to cell growth inhibition, and the expression of p21 is indirectly inhibited by EZH2 (Lu *et al.* , 2011; Li *et al.* , 2019). In our study, p21 expression was negatively related to the expression of EZH2 both in vitro and in vivo (Fig. 6a, c), which suggests that miR-26a upregulated p21 level by directly suppressing EZH2 expression. In other words, miR-26a inhibited VSMC proliferation by regulating the EZH2/p21 pathway.

miR-26a can also regulate cell proliferation by directly targeting cyclin D2 (Zhou *et al.* , 2016). To explore whether miR-26a regulated cyclin D2 expression in VSMCs, we transfected the miR-26a mimic and inhibitor in AngII-induced VSMCs. The expression of cyclin D2 was markedly upregulated in AngII-induced VSMCs on transfection with miR-26a inhibitor and was reversed by transfection with the miR-26a mimic (Fig. 6d). Thus, miR-26a mitigates excessive VSMC proliferation by targeting the EZH2/p21 pathway and cyclin D2.

3.7 AngII regulates miR-26a expression via Smad3 activation.

PROMO analysis revealed three potential Smad3 binding sites in the miR-26a promoter (Fig. 7a). ChIP

assay with VSMC lysates and anti-Smad3 antibody validated that Smad3 directly binds to the promoter of miR-26a to suppress its expression. Additionally, Smad3 exhibited stronger binding to the miR-26a promoter in AngII-induced VSMCs as compared with controls (Fig. 7b, c). Thus, AngII promoted Smad3 binding to the miR-26a promoter, thereby inhibiting miR-26a transcription. To further elaborate whether miR-26a downregulation was mediated by Smad3 in VSMCs, we used loss-of-function studies. We first constructed three siRNAs fragments of Smad3 to transfect VSMCs. Both qRT-PCR and western blot analysis confirmed that siRNA-3 silenced Smad3 the best (Supplemental Fig. S2a, b). Next, we used siRNA or SB431542, which inhibited Smad3 expression or activation, respectively, and observed increased miR-26a expression in AngII-induced VSMCs (Fig. 7d-f), which indicates that Smad3 negatively regulates miR-26a expression. These results indicate that AngII regulates miR-26a expression via Smad3 activation.

3.8 miR-26a inversely regulates the activation of Smad3

We transfected AngII-induced VSMCs with an miR-26a mimic, which showed high-transfection efficiency (Fig. 8a). Also, transfection of an miR-26a mimic significantly decreased p-Smad3 protein expression (Fig. 8b). Additionally, upregulation of miR-26a inhibited nuclear translocation of p-Smad3 (Fig. 8c). These results implied that miR-26a can also inversely inhibit Smad3 activation.

3.9 miR-26a inhibits Smad3 activation by targeting Smad4

We next sought to determine how miR-26a regulates Smad3 activation. MiRanda (www.microRNA.org) analysis indicated that Smad4 contains a binding site for miR-26a (Fig. 9a). We confirmed that Smad4 was a direct target of miR-26a by luciferase reporter assay (Fig. 9b). Consistently, Smad4 expression was significantly downregulated with miR-26a mimic overexpression, whereas miR-26a inhibitor treatment increased the protein level of Smad4 in AngII-induced VSMCs (Fig. 9c, d).

To explore the effect of Smad4 on Smad3 activation, we examined siRNA-mediated knockdown of reduced level of Smad4 in VSMCs. We selected siRNA with the best silencing effect on Smad4 to transfect AngII-treated VSMCs (Supplemental Fig. S2c, d). Smad4 expression was highly relevant for the expression and nuclear translocation of p-Smad3. Inhibition of Smad4 could reduce p-Smad3 protein expression and decreased the nuclear translocation of p-Smad3 (Fig. 9e-h). Therefore, Smad4 promoted the activation of Smad3. In summary, miR-26a regulated Smad3 activation by targeting Smad4.

Combined with previous results (Fig. 7-9), a positive feedback loop between miR-26a and Smad3/4 may be involved in hypertensive VR (Fig. 10).

Discussion

In this study, we evaluated the role and mechanisms of miR-26a in controlling hypertensive VR (Fig. 10). MiR-26a was significantly downregulated in the thoracic aorta and plasma of SHRs with VR. Overexpression of miR-26a effectively reduced ECM deposition and VSMC hyperproliferation both in vivo and in vitro. Also, AngII potentiated Smad3 binding to the miR-26a promoter, inhibiting miR-26a transcription, which in turn promoted Smad3 activation by upregulating Smad4, thereby further downregulating miR-26a. These findings reveal a possible new positive feedback loop between miR-26a and Smad3/4, which may be related to hypertensive VR. Moreover, miR-26a mitigated ECM accumulation and excessive VSMC proliferation in AngII-induced VSMCs by targeting CTGF and the EZH2/p21 pathway, respectively. Our study suggests that miR-26a is a protective molecule in hypertensive VR and can be considered a promising target for treating hypertensive VR.

The crucial finding of this study is that miR-26a plays a protective role in hypertensive VR. Previous studies have shown that miR-26a is closely related to cardiovascular diseases. Li *et al.* found low expression of miR-26a in plasma of patients with acute myocardial infarction as compared with healthy people (Li *et al.* , 2015). Zhang *et al.* showed that miR-26a plays a role in the process of myocardial fibrosis after acute myocardial infarction by inhibiting phosphatase and tensin homolog expression, enhancing matrix metalloproteinase 9 level and promoting the PI3K/AKT pathway (Zhang*et al.* , 2018). Chiang *et al.* discovered that miR-26a ameliorates cardiac dysfunction and fibrosis in myocardial infarction (Chiang *et al.* , 2020). In this study, we

first observed that miR-26a expression was downregulated in the thoracic aorta and plasma of SHRs. We next used rAAV-miR-26a to overexpress miR-26a in the thoracic aorta and plasma of SHRs. The overexpression of miR-26a reduced media thickening of the thoracic aorta and diminished the severity of VR induced by hypertension. However, due to the limitations of this experiment, we have not been able to obtain data on the extent of SHR aortic remodeling before miR-26a treatment. Therefore, our conclusion was based on the hypothesis that there was no difference in VR of SHRs in each group before intervention. Meanwhile, we found that overexpression of miR-26a appears to have an effect on blood pressure, it is unknown whether miR-26a directly reduces VR or if it is secondary to reduced blood pressure, which can continue to study in future.

One of the characteristics of VR is pathological remodeling of ECM, which is often accompanied by increased ECM release and collagen deposition (Ricard-Blum *et al.* , 2019). ECM is a structural scaffold of the blood vessel wall, which controls cellular functions in the pathological environment. When stimulated or injured, the deposition of ECM protein, especially Col I, alters the collagen/elastin ratio and hemodynamics, leading to increased vascular stiffness and affecting blood vessel function (Lee *et al.* , 2015). We found the CVF of the thoracic-aorta media layer reduced in miR-26a-overexpressed SHRs. In vitro studies showed that miR-26a mimic treatment inhibited but miR-26a inhibitor treatment promoted Col I and III expression in AngII-induced VSMCs. CTGF belongs to matricellular protein expressed by various cells in response to stimuli and is an important molecule prominently implicated in increased deposition of ECM (Li *et al.* , 2016; Petrosino *et al.* , 2019). Our results showed that that CTGF is a downstream target gene of miR-26a and the level of miR-26a was inversely associated with CTGF expression both in vivo and in vitro, so miR-26a may diminish ECM deposition by targeting CTGF. Additionally, the Col I is a downstream target gene of miR-26a (Wei *et al.* , 2013). MiR-26a may directly inhibit collagen synthesis to reduce ECM deposition, which needs further study.

Another pathological manifestation of hypertensive VR is excessive VSMC proliferation, which is determined by the cell cycle (Deniset *et al.* , 2018; Lu *et al.* , 2018; He *et al.* , 2019). EZH2 accelerates the cell cycle, whereas p21 arrests the cell cycle (Karimian*et al.* , 2016; Dai *et al.* , 2018). EZH2/p21 has been found a critical signaling pathway to regulate cell proliferation (Lu *et al.* , 2011; Li *et al.* , 2019). We found that transfection with rAAV-miR-26a suppressed PCNA and EZH2 expression, but p21 was upregulated in the thoracic aorta of SHRs. Similarly, in vitro experiments showed that miR-26a inhibited the progression of the cell cycle accompanied by downregulated protein level of EZH2, whereas p21 level was correspondingly increased in AngII-induced VSMCs; miR-26a inhibitor treatment had the opposite result. Furthermore, EZH2 is a target gene of miR-26a, so miR-26a participates in VSMC proliferation by targeting the EZH2/p21 pathway. Cyclin D2 can promote the G1/S transition (Gul *et al.* , 2018; Pei *et al.* , 2018), and previous studies with luciferase reporter assay showed that cyclin D2 is also a target gene of miR-26a (Zhou *et al.* , 2016). Consistently, we found that miR-26a suppressed cyclin D2 expression in AngII-induced VSMCs. Thus, miR-26a may be involved in excessive VSMC proliferation by directly regulating cyclin D2 apart from targeting the EZH2/p21 pathway.

Our study confirmed that AngII regulates miR-26a level dose- and time-dependently. However, how AngII regulates miR-26a expression is unclear. Some studies demonstrated that miRs are subject to sophisticated regulation, and superposition of subtle changes in the expression of several molecules may be responsible for miRs exerting their effects (Krol *et al.* , 2010; Liang *et al.* , 2014). Previous studies determined that miR-26a inhibits TGFβ-dependent Smad signaling (Leeper *et al.* , 2011). We focused on TGFβ-independent Smad signal regulatory mechanisms of miR-26a. In this study, we validated that Smad3 could directly suppress miR-26a expression, and AngII could promote this effect; AngII regulates miR-26a by activating Smad3. Previous studies reported a functional crosstalk between miR-26a and other molecules via a feedback loop (Jiang *et al.* , 2018; Liu*et al.* , 2018). We found that miR-26a downregulation enhanced Smad4 expression via targeted regulation and then induced Smad3 activation and p-Smad3 nuclear translocation. Smad3 activation caused further downregulation of miR-26a, thereby forming a Smads/miR-26a positive feedback loop. Once the loop is activated, it will run repeatedly, eventually leading to increased ECM accumulation, excessive VSMC proliferation and VR.

To sum up, miR-26a plays a protective role in hypertensive VR. The effect of miR-26a on VR was mediated by activation of a Smads/miR-26a positive feedback loop. In addition, miR-26a inhibited ECM deposition by targeting CTGF and attenuated VSMC proliferation by regulating the EZH2/p21 pathway. We clarify the functions and mechanisms of miR-26a in hypertensive VR and suggest that miR-26a may be a novel therapeutic target of hypertensive VR.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

D.G. conceived and designed this study. W.Z., Q.Z., X.X., L.Y., M.X., C.C., R.W. and W.C. conducted the experiments and analysed the data. D.G. and W.Z. drafted the manuscript and coordinated manuscript revisions. Prof. X. N. provided suggestion for the experiments. The manuscript has been reviewed and approved by all authors.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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