

## Original Article

# MiR-204 inhibits hypertension by regulating proliferation and apoptosis of vascular smooth muscle cells

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**Abstract:** Vascular smooth muscle cell (VSMC) abnormal proliferation and reduced apoptosis is related to hypertension. It is found that miR-204 is associated with pulmonary hypertension. Bioinformatics analysis reveals a relationship between miR-204 and the 3'-UTR of Bcl-2 mRNA. This study aimed to investigate the role of miR-204 in regulating Bcl-2 expression, VSMC proliferation and apoptosis in the pathogenesis of hypertension. Dual luciferase reporter gene assay was performed for analysis of the relationship between miR-204 and Bcl-2. MiR-204 and Bcl-2 expressions in the thoracic aorta media of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) were detected by RT-PCR and western blot. VSMCs were cultured in vitro and divided into five groups, including mimic NC, miR-204 mimic, si-NC, si-Bcl-2, and miR-204 mimic + si-Bcl-2 groups. Cell apoptosis was evaluated by flow cytometry. Cell proliferation was detected by EdU staining. SHR rats were randomly divided into miR-204 agomir and miR-204-control. Caudal artery systolic and diastolic pressures were measured. Bcl-2 expression in the media and the thickness of media were tested. MiR-204 suppressed Bcl-2 expression. MiR-204 expression was significantly reduced, while Bcl-2 level was elevated in the vascular media from SHR rats compared with those from WKY rats. Upregulation of miR-204 and/or downregulation of Bcl-2 obviously declined cell proliferation and facilitated cell apoptosis. MiR-204 agomir injection markedly decreased the systolic and diastolic pressures, declined Bcl-2 expression in media, and reduced the thickness of vascular media. In conclusion, MiR-204 reduction was related to hypertension and it plays a therapeutic effect in hypertension through inhibiting Bcl-2 expression, restraining VSMC proliferation, and accelerating cell apoptosis.

**Keywords:** MiR-204, Bcl-2, VSMC, hypertension, proliferation, apoptosis

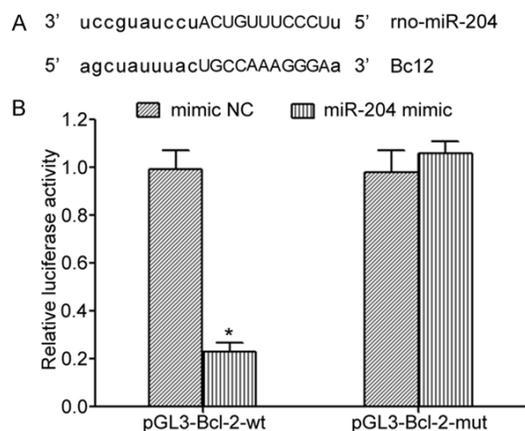
## Introduction

Hypertension is a kind of clinical syndrome characterized by systemic arterial blood pressure elevation, associated with the functional or physical damages of heart, brain, kidney and other organs [1, 2]. Vascular structure and function play a crucial role in maintaining normal blood pressure. The elevation of peripheral vascular resistance and abnormal vasomotor activity are the direct causes of high blood pressure, of which the vessel wall reconstruction is an important pathophysiological process in the development of hypertension. Current studies suggest that hypertension is a kind of disease mainly featured as abnormal hyperplasia of vascular smooth muscle cell (VSMC). Excessive VSMC proliferation and reduced

apoptosis narrow the blood vessel lumen and thicken the vascular wall under hypertension condition, leading to vascular remodeling, peripheral vascular resistance elevation, and eventually resulting in hypertension [3, 4].

B-cell lymphoma-2 (Bcl-2) is an important anti-apoptotic factor that can inhibit mitochondrial dependent apoptosis pathway by inhibiting the release of mitochondrial Cyt C [5, 6], affecting the transmembrane transport of calcium ion, and suppressing apoptotic protease activating factor-1 (Apaf-1) activation [7]. Numerous studies showed abnormal Bcl-2 upregulation is closely associated with excessive VSMC proliferation and reduced apoptosis [8, 9], suggesting it might play a key role in the pathogenesis of hypertension [10].

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**Figure 1.** MiR-204 targeted regulated Bcl-2 expression. (A) The binding site between miR-204 the 3'-UTR of Bcl-2 mRNA; (B) Dual luciferase assay. \* $P < 0.05$ , compared with mimic NC.

MicroRNA is kind of small noncoding single strand RNA with the length of 18-22 nt in eukaryotes. It participates in multiple biological processes, such as proliferation, apoptosis, and migration, by completely or incompletely complementary binding to the 3'-UTR of mRNA to degrade mRNA or inhibit translation [11, 12]. More and more evidences revealed that abnormal expression and function of mi-145 (Trigger pulmonary arterial hypertension), miR-204 (Antiproliferative and promote apoptotic of SMC) and miR-193 (Inhibit SMC proliferation) is associated with hypertension [13, 14]. It was showed that downregulation of miR-204 plays a role in the pathogenesis of pulmonary arterial hypertension (PAH) [15, 16]. However, its role in hypertension remains unclear. Bioinformatics analysis demonstrates the targeted relationship between miR-204 and the 3'-UTR of Bcl-2 mRNA. This study explores the role of miR-204 in regulating Bcl-2 expression, VSMC proliferation and apoptosis in the pathogenesis of hypertension.

### Materials and methods

#### Main reagents and materials

DMEM medium, FBS, penicillin-streptomycin, and type II collagenase were purchased from Gibco (USA). Rneasy MiNi Kit and QuantiTect SYBR Green RT-PCR Kit were bought from Qiagen (Germany). FuGENEHD Transfection Reagent was got from Roche (USA). MicrON™ miR-204 agomir, micrON™ agomir-control, miR-

204 mimic, miR-NC, and EdU flow cytometry detection kit were derived from RiboBio (Guangzhou, China). Mouse anti Bcl-2,  $\beta$ -actin, and HRP labeled secondary antibodies were obtained from Abcam (USA). Annexin V/PI apoptosis detection kit pGL3 luciferase reporter plasmid, and luciferase activity detection kit were acquired from Promega (USA).

#### Experimental animals

Male SPF grade spontaneously hypertensive rats (SHR) and male Wistar-Kyoto rats (WKY) weighted  $225 \pm 20$  g at 10-week old were purchased from Kay biological technology co., Ltd (Shanghai, China). The rats were raised in the environment of good light with the temperature at 22-25°C and relative humidity at 50-60%. The rats had free access to food and water. Animal raising and manipulation were approved and abided by the ethics principle of experimental animal.

#### SHR rats grouping and treatment

The SHR rats were randomly divided into micrON™ miR-204 agomir group and micrON™ agomir-control group. The rats received caudal vein injection at a dose of 20 mg/Kg once per five days for four times. Caudal artery systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured before and on the 7<sup>th</sup> day after last injection. The pressure was measured for three times to calculate the mean value.

#### Rat thoracic aorta VSMC isolation and cultivation

The rat was anesthetized through pentobarbital tail vein injection. The thoracic aorta was extracted under aseptic condition. After removing the fat and connective tissues around the blood vessel, the tissue was digested in 0.1% type II collagenase at 37°C for 30 min. After removing the adventitia, the vessel was opened on longitude to scrape the intima. The vascular media was collected to extract RNA and protein or VSMC cultivation.

The media was digested in 0.1% type II collagenase at 37°C for 2 h, and further digested in 0.05% trypsin for 10 min. After infiltration, the cell was resuspended in DMEM medium containing 20% FBS and 1% penicillin-streptomycin.

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**Table 1.** Vascular media thickness and blood pressure comparison

	Thickness of vascular media ( $\mu\text{m}$ )	SBP (mmHg)	DBP (mmHg)
WKY rat	89.6 $\pm$ 3.3	115.2 $\pm$ 9.1	76.3 $\pm$ 6.5
SHR rat	109.5 $\pm$ 4.1*	194.8 $\pm$ 18.7*	101.6 $\pm$ 11.4*

\* $P < 0.05$ , compared with WKY rat.

cin. The cells were cultured at 37°C with 5% CO<sub>2</sub> and passaged at 1:4. The cells in the 4-5<sup>th</sup> generation were used for experiment.

### Luciferase reporter gene assay

The full length fragment of Bcl-2 3'-UTR was connected to pGL3 luciferase reporter vector to form pGL3-Bcl-2-wt. The mutation of Bcl-2 3'-UTR was used to construct pGL3-Bcl-2-mut. FuGENEHD Transfection Reagent was applied to co-transfect 1  $\mu\text{g}$  pGL3-Bcl-2-wt or pGL3-Bcl-2-mut with 50 nm/L miR-204 mimic to HEK293T cells. Dual luciferase activity was tested after 48 h.

### Cell transfection and grouping

Mimic NC, miR-204 mimic, si-NC, or si-Bcl-2 was transfected into VSMC from SHR rats *in vitro* using the FuGENEHD Transfection Reagent. VSMCs were divided into five groups, including mimic NC, miR-204 mimic, si-NC, si-Bcl-2, and miR-204 mimic + si-Bcl-2 groups. The cells were collected after 48 h for the following experiment.

### qRT-PCR

Total RNA was extracted using Rneasy MiNi Kit and detected using QuantiTect SYBR Green RT-PCR Kit for one-step qRT-PCR. The reaction system contained 10.0  $\mu\text{l}$  2 $\times$ QuantiTect SYBR Green RT-PCR Master Mix, 1.0  $\mu\text{l}$  primer at 0.5  $\mu\text{M}$ /L, 2  $\mu\text{g}$  Template RNA, 0.5  $\mu\text{l}$  QuantiTect RT Mix, and ddH<sub>2</sub>O. The RT condition was 50°C for 30 min. The PCR reaction was composed of 95°C pre-denaturation for 15 min, followed by 40 cycles of 94°C denaturation for 15 s, 60°C annealing for 30 s, and 72°C elongation for 30 s. Real-time PCR was performed on ABI ViiA™ 7 to test the relative expression. The primer sequences was designed as follows: miR-204 (Forward: 5'-GCCCGTCCCTTTGTCATC-3'; Reverse: 5'-TCCAGTGCAGGGTCCGAG-3'), U6 (Forward: 5'-GACACGCAAATTCGTGAAGCG-3'; Rever-

se: 5'-TCCAGTGCAGGGTCCGAG-3'), Bcl-2 (Forward: 5'-GTATGATAACCGGGAGATCG-3'; Reverse: 5'-AGCCAGGAGAAATCAAACAG-3'), GAPDH (Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3').

### Western blot

Total protein was extracted through adding 600  $\mu\text{l}$  RIPA on ice for 30 min and centrifuged at 4°C and 10000 g for 10 min. The supernatant was moved to a new Ep tube for quantification. A total of 50  $\mu\text{g}$  protein was separated by 10% SDS-PAGE and transferred to membrane. Next, the membrane was blocked with 0.5% skim milk at room temperature for 60 min and incubated with primary antibody at 4°C for 12 h (Bcl-2 at 1:200 dilution and  $\beta$ -actin at 1:800, respectively). Then the membrane was incubated with secondary antibody (1:8000) for 60 min after washed by PBST for three times. At last, the protein expression was detected by ECL chemiluminescence.

### Flow cytometry

The cells were collected and 100  $\mu\text{l}$  binding buffer was added. Then the cells were incubated with 5  $\mu\text{l}$  Annexin V-FITC and 5  $\mu\text{l}$  PI for 10 min under dark. Next, the cells were resuspended after adding 400  $\mu\text{l}$  binding buffer. Cell apoptosis was tested on Gallios flow cytometry (Beckman Coulter).

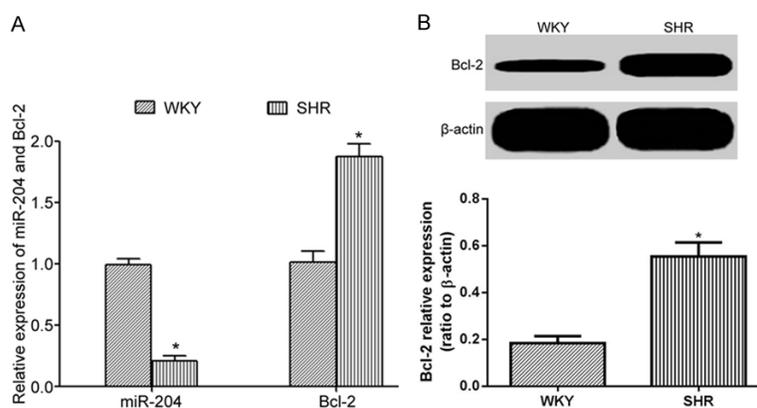
### EdU staining

VSMC cells were seeded in 6-well plate at 5000/cm<sup>2</sup> and EdU solution at 5  $\mu\text{M}$  was added to cells at logarithmic phase. After incubated for 48 h, the cells were digested by trypsin and collected. After fixed in 4% paraformaldehyde, the cells were neutralized in 2 mg/ml glycine for 5 min. Next, the cells were incubated with 0.5% TritonX-100 at room temperature for 10 min and resuspended in PBS. At last, the cell was tested on flow cytometry.

### Measurement of aorta media thickness

The thoracic aorta was prepared as paraffin section. The thickness of aorta media was measured using Image Pro Plus 6.0 software. Five sections were selected in each rat and

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**Figure 2.** MiR-204 reduced, while Bcl-2 upregulated in the vascular media tissue of SHR rat. A. qRT-PCR detection of miR-204 and Bcl-2 mRNA expression. B. Western blot detection of Bcl-2 protein expression. \* $P < 0.05$ , compared with WKY rat.

three fields of view were randomly selected on each section.

### Statistical analysis

All data analyses were performed on SPSS 18.0 software. The measurement data were depicted as mean  $\pm$  standard deviation (SD). T test was performed for comparison of difference between two groups and one-way ANOVA was performed for comparison among different groups.  $P < 0.05$  was considered as statistical significance.

## Results

### MiR-204 targeted regulated Bcl-2 expression

MicroRNA.org online target gene prediction showed the complementary binding site between miR-204 and the 3'-UTR of Bcl-2 mRNA (Figure 1A). Dual luciferase assay revealed that miR-204 mimic significantly declined the relative luciferase activity in HEK293T cells (Figure 1B), indicating the regulatory relationship between miR-204 and Bcl-2 mRNA.

### MiR-204 was reduced, while Bcl-2 was up-regulated in the vascular media tissue of SHR rat

The thickness of vascular media, SBP, and DBP was obviously increased in SHR rat compared with WKY rat (Table 1). qRT-PCR analysis exhibited that miR-204 expression was markedly reduced, whereas Bcl-2 mRNA level was apparently upregulated in the vascular media from

SHR rat compared with those from WKY rat (Figure 2A). Western blot demonstrated that Bcl-2 protein level was significantly higher in SHR rat than that in WKY rat (Figure 2B).

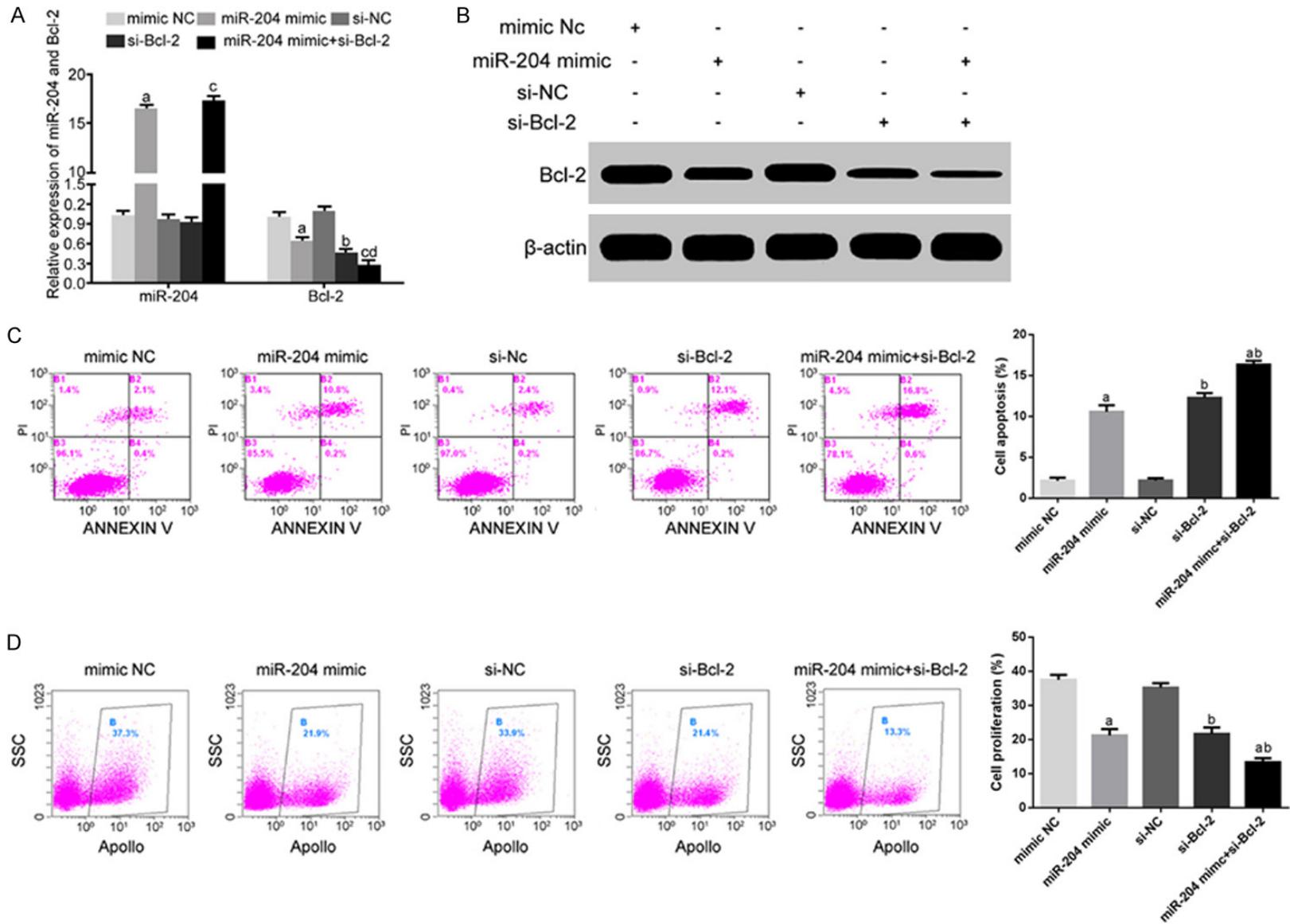
*Upregulation of miR-204 inhibited VSMC proliferation, promoted apoptosis, and reduced blood pressure in SHR rat*

MiR-204 mimic and/or si-Bcl-2 transfection obviously reduced Bcl-2 expression (Figure 3A and 3B), accelerated cell apoptosis (Figure 3C), and restrained cell proliferation in VSMC (Figure 3D). MicrON™ miR-204 agomir injection markedly declined Bcl-2 expression in media (Figure 3E), reduced the thickness of vascular media (Figure 3F), and decreased systolic and diastolic pressures (Figure 3G). However, micrON™ agomir-control injection showed no statistical differences on Bcl-2 expression, media thickness, SBP, and DBP.

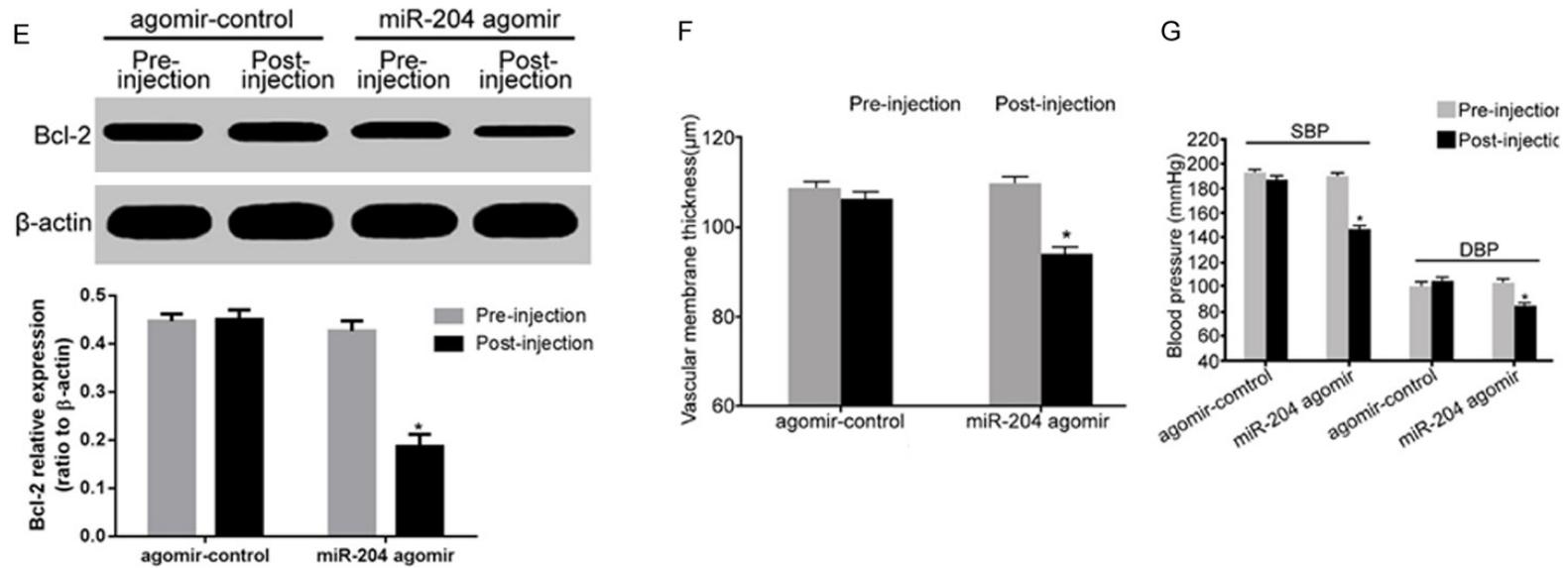
## Discussion

Hypertension is a kind of clinical syndrome characterized as systemic arterial blood pressure elevation, which is associated with the functional or physical damage of heart, brain, kidney and other organs that seriously threatens to human health and life [17, 18]. Vascular structure and function play a crucial role in maintaining normal blood pressure. The elevation of peripheral vascular resistance and abnormal vasomotor activity are the direct causes of high blood pressure, of which the vessel wall reconstruction is an important pathophysiological process in the development of hypertension. VSMC is located under the endothelial cells of vascular intima. It maintains the tension of vascular wall through chronic and slight contraction to regulate blood pressure within normal physiological ranges. Excessive proliferation of VSMC renders vascular media hyperplasia. VSMCs migrate to subintima, leading to thickening of vascular wall and narrowing of lumen, eventually triggering hypertension. Except hypertension, abnormal VSMC

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**Figure 3.** Upregulation of miR-204 inhibited VSMC proliferation, promoted apoptosis, and reduced blood pressure in SHR rat. (A) qRT-PCR detection of miR-204 and Bcl-2 mRNA expressions in VSMC; (B) Western blot detection of Bcl-2 protein expression in VSMC; (C) Flow cytometry detection of cell apoptosis; (D) EdU staining detection of cell proliferation; (E) Western blot detection of Bcl-2 expression in vascular media; (F) Measurement of thoracic aorta vascular media thickness; (G) Measurement of SBP and DBP. <sup>a</sup> $P < 0.05$ , compared with mimic NC; <sup>b</sup> $P < 0.05$ , compared with si-NC; <sup>c</sup> $P < 0.05$ , compared with miR-NC; <sup>d</sup> $P < 0.05$ , compared with miR-NC; \* $P < 0.05$ , compared with pre-injection.

proliferation also generates atherosclerosis, restenosis after percutaneous transluminal coronary artery angioplasty, and neointimal formation after vascular bypass surgery. Thus, inhibition of excessive VSMC proliferation and promotion of apoptosis may become a strategy for the treatment of cardiovascular and cerebrovascular diseases including hypertension.

Bcl-2 is an important anti-apoptotic factor that regulates mitochondrial dependent apoptosis pathway [19, 20]. Bcl-2 protein is located on the nuclear membrane, endoplasmic reticulum membrane, and mitochondrial membrane. It plays an anti-apoptotic role by inhibiting the release of Cyt C to cytoplasm, reducing free radical generation and lipid peroxidation, affecting the transmembrane transport of calcium ion, protecting cell from the damage of DNA cleavage agent, forming heterodimer with Bax and maintaining the intracellular distribution of pro-apoptotic factors, blocking the space displacement and activation of Apaf-1 [21]. Multiple studies reported that abnormal Bcl-2 upregulation is closely associated with excessive VSMC proliferation and reduced apoptosis [8, 9], thus plays a key role in the pathogenesis of hypertension [10]. It was showed that downregulation of miR-204 plays a role in the pathogenesis of pulmonary arterial hypertension (PAH) [15, 16]. However, its role in hypertension remains unclear. Bioinformatics analysis demonstrates the targeted relationship between miR-204 and the 3'-UTR of Bcl-2 mRNA. This study explores the role of miR-204 in regulating Bcl-2 expression, VSMC proliferation and apoptosis, during the pathogenesis of hypertension.

Dual luciferase reporter gene assay demonstrated that miR-204 mimic transfection significantly attenuated the relative luciferase activity in HEK293T cells, confirming the targeted relationship between miR-204 and Bcl-2 mRNA. Our results demonstrated that miR-204 expression was significantly reduced, while Bcl-2 level and vascular media thickness were obviously elevated in SHR rats compared with those in WKY rats, suggesting the role of miR-204 in regulating Bcl-2 expression, vascular media thickness, and hypertension. Courboulin reported that miR-204 expression was apparently decreased in the pulmonary artery smooth muscle cells (PASMC) from PAH patients and

animal model, and its level was closely related to PAH degree [15]. Courboulin further showed that downregulation of miR-204 promoted PASMC proliferation and reduced cell apoptosis by upregulating SHP2 level to facilitate the activation of Src kinase and transcription factor NFAT, resulting in facilitating PAH [15]. Bockmeyer reported that miR-204 content was obviously declined in the pulmonary blood vessel from PAH patients and related to the enhancement of cell proliferation [22]. Ruffenach presented that miR-204 level was abnormally decreased in pulmonary arterial vascular tissue from PAH patients and PASMC cultured in vitro [23]. Our study observed that miR-204 expression was markedly downregulated in the vascular media from SHR rats compared with that from WKY rats, consistent with previous studies conducted by Courboulin [15], Bockmeyer [22], and Ruffenach [23]. Rodriguez demonstrated that Bcl-2 expression was obviously enhanced, while Bax level was significantly declined in the cardiac tissue from hypertension rat compared with normal control, revealing the role of Bcl-2 in the regulation of the anti-apoptotic effect in promoting hypertension rat myocardial hypertension [10]. Cao found that Bcl-2 level was increased, while cell apoptosis was attenuated in the vascular tissue from SHR rat compared with WKY rat, indicating that reduction of apoptosis might be involved in hypertension [24]. Marchand discovered that Bcl-2 and VSMC amount was elevated, whereas Bax was reduced in VSMC from hypertension rats, suggesting that reduction of apoptosis and abnormal proliferation were related to hypertension [25]. Diez observed that Bcl-2 level in VSMC from SHR rat was obviously higher than that from WKY rat [26]. This study showed that Bcl-2 level in vascular media tissue from SHR rat was markedly higher than that from WKY rat, which was consistent with previous studies conducted by Rodriguez [10] and Cao [24]. Further investigation revealed that miR-204 mimic and/or si-Bcl-2 transfection obviously reduced Bcl-2 expression, accelerated cell apoptosis, and restrained cell proliferation in VSMC. In vivo experiment also displayed that micrON™ miR-204 agomir injection markedly declined Bcl-2 expression in media, reduced the thickness of vascular media, and decreased systolic and diastolic pressures. Ruffenach revealed that suppression of miR-204 apparently upregulated RUNX2 expression

in PASMC, accelerated PASMC proliferation, inhibited cell apoptosis, and promoted phenotype transition, as well as facilitated vascular remodeling and PAH [23]. Marchand demonstrated that drug treatment obviously declined Bcl-2/Bax ratio in VSMC, promoted cell apoptosis, reduced VSMC number and cross sectional area of vascular media, and declined blood pressure in mouse hypertension model, suggesting that Bcl-2 elevation-induced reduction of VSMC apoptosis and abnormal proliferation was related to the thickening of vascular media [25]. Downregulation of Bcl-2 may play a therapeutic role in mouse hypertension. Our study revealed that miR-204 was involved in hypertension possibly through regulating Bcl-2 expression, proliferation, and apoptosis of VSMC.

### Conclusion

Decreased miR-204 expression was associated with hypertension. MiR-204 plays a therapeutic effect in hypertension possibly by targeting Bcl-2 expression, restraining VSMC proliferation, and accelerating apoptosis.

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### Disclosure of conflict of interest

None.

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