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Effects of miR-155 on hypertensive rats via regulating vascular mesangial hyperplasia

D. XU, R. LIAO, X.-X. WANG, Z. CHENG

Department of Cardiology, The First Branch, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China

Abstract. – OBJECTIVE: Vascular smooth muscle cell (VSMC) excessive proliferation is related to hypertension. The cell cycle inhibitory factor (p27) can arrest cell cycle, while its down-regulation is associated with hypertension. It is found that microRNA-155 (miR-155) plays a regulatory role in VSMC proliferation, while its relationship with hypertension is still unclear. Bioinformatics analysis reveals the targeted relationship between miR-155 and the 3'-UTR of p27 mRNA. This study aims to explore the role of miR-155 in regulating p27 expression, VSMC proliferation and apoptosis, and the pathogenesis of hypertension.

MATERIALS AND METHODS: Dual luciferase reporter gene assay confirmed the relationship between miR-155 and p27. MiR-155, p27, α-smooth muscle actin (α-SMA), and Ki-67 expressions in the thoracic aorta media of rat hypertension model were detected. VSMCs were cultured in vitro and divided into five groups, including anti-miR-NC, anti-miR-155, pIRES2-blank, pIRES2-p27, and anti-miR-155 + pIRES2-p27 groups. Cell cycle was evaluated by using flow cytometry. Cell proliferation was detected with EdU staining. Hypertension rats were randomly divided into antagomir-155 and antagomir-control. Caudal artery systolic and diastolic pressures were measured.

RESULTS: MiR-155 targeted suppressed p27 expression. MiR-155 and Ki-67 expressions significantly enhanced, while p27 and α-SMA levels reduced in the tunica media from hypertension rats compared with control. Down-regulation of miR-155 and/or up-regulation of p27significantly declined cell proliferation and arrested cell cycle in G1 phase. Antagomir-155 injection markedly decreased systolic and diastolic pressures, elevated p27 and α-SMA expressions in media, and reduced the thickness of tunica media.

CONCLUSIONS: MiR-155 promoted VSMC proliferation by targeting p27. MiR-155 enhancement was related to hypertension. MiR-155 played a therapeutic effect on hypertension.

Key Words:
miR-155, p27, VSMC, Hypertension, Proliferation, Cell cycle.

Introduction

Hypertension is a kind of chronic cardiovascular disease characterized as systemic arterial blood pressure elevation. Numerous factors may lead to hypertension, such as heredity, psychentonia, poor dietary habit, age, and drugs[1-3]. Vascular smooth muscle cell (VSMC) is the main component of tunica media that plays an important role in maintaining the integrity of vascular wall structure and function, regulating angiotasis, and sustaining blood pressure fluctuation within normal physiological range[4]. VSMC excessive proliferation under pathological condition may migrate to subintima, thus playing a role in narrowing the blood vessel lumen, thickening the vascular wall, and elevating peripheral vascular resistance and blood pressure[5].

P27 is a type of cyclin dependent kinase inhibitor (CDKI) that plays a key role in regulating cell cycle, especially in G1 phase[6]. P27 blocks cell enter S phase from G1 phase by inhibiting the activation of G1 kinase complex, such as cyclin E-CDK2 and cyclin D-CDK4, to arrest cell cycle and inhibit cell proliferation[7,8]. Scholars[9-11] revealed that p27 down-regulation was associated with aberrant proliferation of VSMC and participated in the pathogenesis of hypertension and pulmonary arterial hypertension. MicroRNA(miR) is a kind of small non-coding single strand RNA at the length of 21-24 nt in eukaryotes. It plays a critical role in VASM proliferation and apoptosis, endothelial cell function, and cardiovascular development by complete or incomplete complementary binding with

Corresponding Author: Dan Xu, MD; e-mail: shuogang088@163.com

the 3'-UTR of mRNA to degrade mRNA or inhibit translation[12,13]. Evidence revealed that the expression and function of miRNA are associated with hypertension[13]. It was showed that down-regulation of miR-155 plays a regulatory role in VSMC proliferation[14,15]. However, its relationship with hypertension remains unclear. Bioinformatics analysis reveals the targeted relationship between miR-155 and the 3'-UTR of p27 mRNA. We explore the role of miR-155 in regulating p27 expression, VSMC proliferation and apoptosis, and the pathogenesis of hypertension.

Materials and Methods

Main Reagents and Materials

Dulbecco's modified eagle medium (DMEM) medium was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Mediatech-Cellgro (Miami, FL, USA). Type II collagenase was derived from Sigma-Aldrich (St. Louis, MO, USA). RNeasy MiNi Kit was purchased from Qiagen (Hilden, Germany). ReverTra Ace quantitative PCR (qPCR), reverse transcription (RT) Kit and SYBR were purchased from Toyobo Life Science (Tokyo, Osaka, Japan). DharmaFECT transfection reagent was presented by Dharmacon RNA Technologies (Lafayette, CO, USA). MicrOFF™ antagomir-155, micrOFF™ antagomir-control, miR-155 mimic, anti-miR-155, miR-NC, and EdU flow cytometry detection kit were derived from RiboBio (Guangzhou, China). Mouse anti p27 primary antibody was obtained from Abcam Biotech. (Cambridge, MA, USA). Mouse anti α-SMA and Ki-67 primary antibodies were purchased from GeneTex Inc. (Irvine, CA, USA). Mouse anti β-actin primary antibody, horseradish peroxidase (HRP) labeled secondary antibody, pGRE-luc luciferase reporter plasmid, and cell cycle detection kit were obtained from Beyotime Biotech. (Shanghai, China). Luciferase activity detection kit was acquired from Promega (Madison, WI, USA). Annexin V/propidium iodide (PI) apoptosis detection kit was bought from Multi Sciences (Hangzhou, China). pIRES2 over-expression plasmid was got from Tuoyan Biotechnology Co., Ltd., (Shanghai, China).

Abdominal Aorta Constrictive Type Hypertension Model Establishment

Male Sprague-Dawley (SD) rat (6-8 weeks old, 20 ± 20 g), derived from Medical Animal Experiment Center of Chongqing Medical University (Chongqing, China) was anesthetized by pentobarbital intraperitoneal injection. An incision was made to expose the abdominal cavity. The abdominal aorta was isolated above the bilateral aorta ascendens. The abdominal aorta was ligated by suture together with a syringe needle. The needle was then removed and the abdominal cavity was closed after the blood flow was expedited. The abdominal cavity was exposed without abdominal aorta ligation in sham group. The blood pressure was measured by carotid artery intubation on the 6th week after operation. The rat was anesthetized by pentobarbital intraperitoneal injection. An incision was made on the right neck to expose right carotid artery (RCA). Next, the distal part of RCA was ligated and the urodynamic catheter was put into the RCA to ascending aorta. After 20 min, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by multichannel physiologic recorder. The mean arterial pressure (MAP) was calculated (MAP = DBP + 1/3 (SBP-DBP)). This study was approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (Chongqing, China).

Hypertension Rats Grouping and Treatment

The hypertension rats were randomly divided into micrOFF™ antagomir-155 group and micrOFF™ antagomir-control group. The rats received caudal vein injection at 20 mg/kg once per three days for five times. Caudal artery SBP and DBP were measured before and at the 7th 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 by pentobarbital tail vein injection. The thoracic aorta was extracted under aseptic condition. After removing the fat and connective tissues around the blood vessel, the tunica media was conserved to extract RNA and protein or VSMC cultivation. The media was digested in type II collagenase at 37°C for 2 h, and further digested in 0.05% trypsin for 10 min. After infiltration, the cell was re-suspended in Dulbecco's Modified Eagle Medium (DMEM) medium containing 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were cultured at 37°C and 5% CO_2 and passaged at 1:4. The cells in the 6-7th generation were used for experiment.

Luciferase Reporter Gene Assay

The full-length fragment of p27 3'-UTR was connected to pGRE luciferase reporter vector to form pGRE-P27-wt. The mutation of p27 3'-UTR was used to construct pGRE-P27-mut. DharmaFECT was applied to co-transfect 1 µg pGRE-P27-wt or pGRE-P27-mut with 50 nm/l miR-155 mimic to HEK293T cells. Dual luciferase activity was tested after 48 h.

Cell Transfection and Grouping

MiR-NC, anti-miR-155, pIRES2-blank, or pIRES2-P27 was transfected to VSMC from hypertension rats *in vitro* using the DharmaFECT. VSMCs were divided into five groups, including anti-miR-NC, anti-miR-155, pIRES2-blank, pIRES2-p27, and anti-miR-155 + pIRES2-p27 groups. The cells were collected after 48 h for the following experiment.

qRT-PCR

Total RNA was extracted by using Rneasy MiNi Kit and reverse transcribed to complementary DNA (cDNA) using ReverTra Ace qPCR RT Kit. The RT reaction system contained 1 µg total RNA, 2 µl 5×RT buffer, 0.5 µl oligo dT+Random primer Mix, 0.5 µl RT Enzyme Mix, 0.5 µl RNase inhibitor, and ddH_2O. The reverse transcription condition was 37°C for 15 min and 98°C for 5 min. The PCR reaction system contained 5 µl 2×SYBR Green Mixture, 1.0 µl primer (miR-155, forward: 5′-GGAGGTTAATGCTAATTGTGATAG-3′, reverse: 5′-GTGCAGGGTCCGA GGT-3′, actin, forward: 5'-CTCTTCCAGCCTTCCTTCCT-5', reverse: 5'-TCATCG TACTCCTGCTTGCT-3') at 0.5 µm/l, 1.0 µl cDNA, and ddH_2O. The PCR reaction was composed of 95°C pre-denaturation for 5 min, followed by 40 cycles of 95°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.

Western Blot

Total protein was extracted by 500 µl RIPA. A total of 50 µg protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 3 h and transferred to membrane. Next, the membrane was blocked in 5% skim milk at room temperature for 60 min and incubated in primary antibody at 4°C for 12 h (p27, Ki-67, α-SMA, and β-actin at 1:200, 1:300, 1:300, and 1:800, respectively). The membrane was incubated in secondary antibody (1:10000) for 60 min after washed by phosphate-buffered saline and Tween-20 (PBST) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL) chemiluminescence.

Flow Cytometry

The cells were digested by trypsin and fixed by 70% ethanol. After that, the cells were stained by PI with RNase A at 37°C avoid of light for 30 min. At last, DNA content was measured on flow cytometry.

EdU Staining

VSMC cells were added with EdU solution at 5 µM in logarithmic phase. After incubated for 48 h, the cells were digested by trypsin and collected. After fixed in paraformaldehyde, the cells were neutralized in glycine. Next, the cells were incubated in 0.5% Triton X-100 and re-suspended in phosphate-buffered saline (PBS). At last, the cells were stained by 500 µl Apollo at room temperature for 10 min and tested on Gallios flow cytometry (Beckman, Krefeld, Germany).

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 (Media Cybernetics, Inc., Bethesda, MD, USA). Five sections were selected in each rat and three fields of view were randomly selected on each section.

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation (SD). Student's *t*-test was utilized for the statistical analysis between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among groups. $p<0.05$ was considered as statistical significance.

Results

MiR-155 Targeted Regulated p27 Expression

Bioinformatics analysis showed the complementary binding site between miR-155 and the 3'-UTR of p27 mRNA (Figure 1A). Dual luciferase assay revealed that miR-155 mimic and anti-miR-155 significantly declined and enhanced relative luciferase activity in HEK293 cells (Fig-

Table I. Tunica media thickness and MAP comparison.

	Thoracic aorta tunic media (μm)	MAP (mmHg)
Sham group	90.4±5.1	112.3±12.7
Hypertension group	108.2±6.6*	199.4±16.5*

*$p<0.05$, compared with Sham group.

Table II. The thickness of tunica media, SBP, and DBP comparison.

	Tunica media thickness (μm)		SBP (mmHg)		DBP (mmHg)	
Treatment	Pre-injection	Post-injection	Pre-injection	Post-injection	Pre-injection	Post-injection
Antagomir-control	109.3±14.7	111.4±16.9	186.1±16.5	187.1±15.8	103.7±8.1	101.3±9.1
Antagomir-155	107.2±13.3	96.2±8.8*	188.5±17.1	142.2±13.9*	104.2±8.4	91.2±7.6*

*$p<0.05$, compared with Sham group.

ure 1B), indicating the regulatory relationship between miR-155 and p27 mRNA.

MiR-155 Up-Regulated, Tunica Media Thickened, While α-SMA and p27 Declined In Hypertension Rat

The thickness of tunica media and MAP significantly increased in hypertension rat, suggesting successful establishment of animal model (Table I). qRT-PCR exhibited that miR-155 expression markedly increased in the tunica media from hypertension rat compared with sham group (Figure 2A). Western blot demonstrated that α-SMA and p27 protein significantly declined, while Ki-67 level significantly up-regulated in hypertension rat compared with control (Figure 2B). It was indicated that VSMC proliferation aberrantly increased and transformed from differentiated type to dedifferentiated type, which may be related to miR-15 elevation and p27 reduction.

Down-Regulation of miR-155 Inhibited VSMC Proliferation, Arrested Cell Cycle, and Reduced Blood Pressure

Anti-miR-155 and/or pIRES-p27 over-expression plasmid significantly enhanced p27 expression, increased α-SMA level (Figure 3A), arrested cell cycle (Figure 3C), and restrained cell proliferation (Figure 3D) in VSMC from hypertension rat. MicrOFF™ antagomir-155 injection significantly increased p27 and α-SMA expressions, while reduced Ki-67 level in tunica media (Figure 3B). The thickness of tunica media, SBP, and DBP significantly reduced in hypertension rat injected by micrOFF™ antagomir-155 (Table II).

Discussion

P27 is an important cancer suppressor gene that can regulate cell cycle and inhibit cell division. Its gene locates in chromosome 12p13

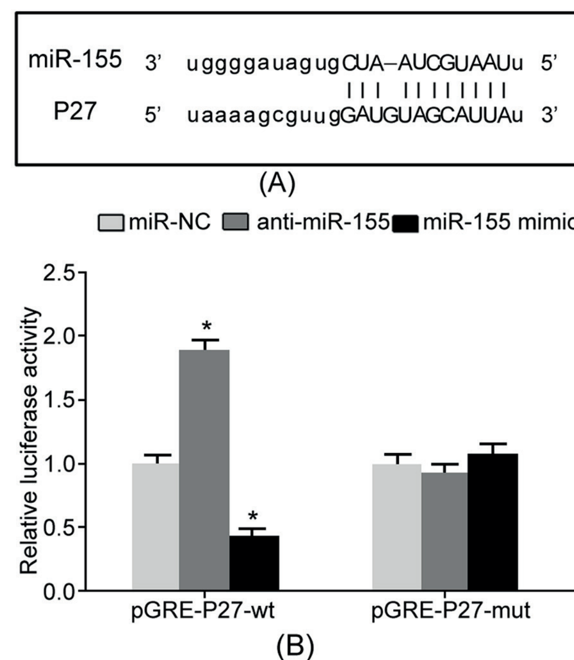

Figure 1. MiR-155 targeted regulated p27 expression. ***(A)*** The binding site between miR-155 the 3'-UTR of p27 mRNA. ***(B)*** Dual luciferase assay. *$p<0.05$, compared with miR-NC.

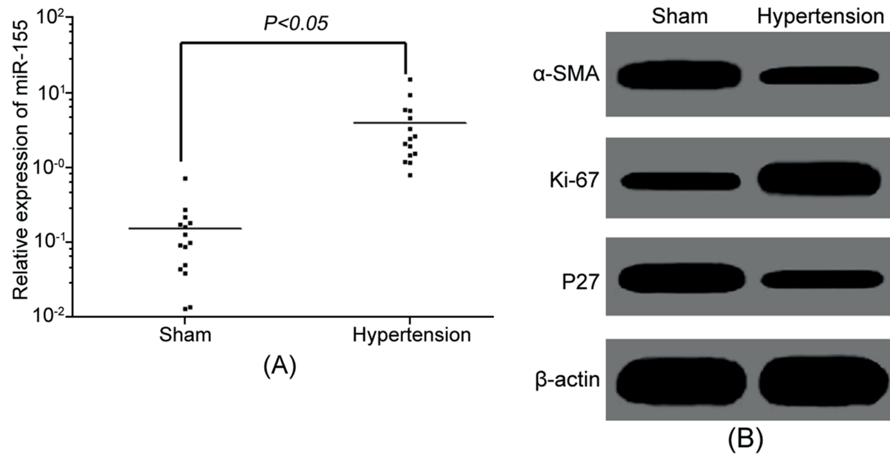

Figure 2. MiR-155 up-regulated, tunica media thickened, while α-SMA and p27 declined in hypertension rat. ***(A)*** qRT-PCR detection of miR-155 expression. ***(B)*** Western blot detection of protein expression in tunica media.

with two exons and two introns, which encodes the protein with molecular weight at 27 kd containing 198 amino acid[16]. P27 belongs to CDKI that plays an inhibitory effect on CDK from two aspects, suppressing the activation of Cyclin-CDK complex by restraining the CDK binding to Cyclin. On the other hand, it exerts its influence via directly inhibiting the kinase activity of CDK[17]. P27 arrested cell cycle from G1 phase to S phase to block cell mitosis mainly through restraining the activation of G1 phase kinase complex, such as cyclin E-CDK2 and cyclin D-CDK4[7]. In addition, it was found that p27 plays a critical role in inducing cell apoptosis[18,19]. Several investigations[9-11] revealed that down-regulation of p27 was associated with VSMC aberrant proliferation and participated in the pathogenesis of hypertension and pulmonary arterial hypertension. MiR-155 was reported to regulate VSMC proliferation[14,15], while its relationship with hypertension was still unclear. Bioinformatics analysis reveals the targeted relationship between miR-155 and the 3'-UTR of p27 mRNA. We explore the role of miR-155 in regulating p27 expression, VSMC proliferation and apoptosis, and the pathogenesis of hypertension. Dual luciferase assay revealed that miR-155 mimic and anti-miR-155 significantly declined and enhanced relative luciferase activity in HEK293 cells, indicating the regulatory

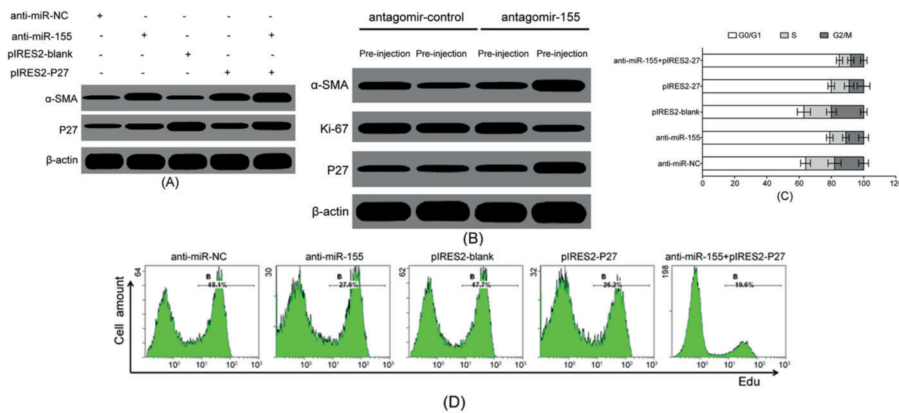

Figure 3. Down-regulation of miR-155 inhibited VSMC proliferation, arrested cell cycle, and reduced blood pressure in hypertension rat. ***(A)*** Western blot detection of protein expression in VSMC. ***(B)*** Western blot detection of protein expression in tunica media. ***(C)*** Flow cytometry detection of cell cycle. ***(D)*** EdU staining detection of cell proliferation.

relationship between miR-155 and p27 mRNA. We established rat hypertension model by ligating abdominal aorta. The thickness of tunica media and MAP significantly increased in hypertension rat, suggesting successful establishment of animal model. MiR-155 and Ki-67 expressions significantly enhanced, while p27 level reduced in the tunica media from hypertension rats compared with control showing that VSMC proliferation increased in tunica media from hypertension rat. Up-regulation of miR-155 mediated p27 reduction may play a role in promoting VSMC proliferation in tunica media from hypertension rat. α-SMA is the major protein to perform contraction in VSMC, thus could be treated as the biomarker to reflect VSMC contractile phenotype (differentiated type). In this study, α-SMA significantly declined in the tunica media from hypertension rat, revealing the transformation from differentiated type to dedifferentiated type, which was in accordance with the enhancement of VSMC proliferation. Liu et al[20] reported that miR-155 significantly increased in pulmonary blood vessel from pulmonary arterial hypertension model. Ceolotto et al[21] found that miR-155 was involved in the regulation of blood pressure in hypertension patients. Yang et al[22] revealed that miR-155 up-regulation is associated with vascular inflammation, intimal hyperplasia, and vascular wall remodeling by inhibiting mammalian sterile 20-like kinase 2 (MST2) expression and enhancing the interaction between Raf-1 and mitogen-activated extracellular signal-regulated kinase (MEK). Zhang et al[15] indicated that miR-155 level was significantly higher in the plaque tissue in blood vessel from atherosclerotic patients compared with healthy control, suggesting that up-regulation of miR-155 was associated with vascular wall remodeling. Zhu et al[23] discovered that miR-155 elevation was related to the pathological remodeling in atherosclerotic rat and coronary artery lesion patients. In this study, miR-155 was over-expressed in tunica media from hypertension rat and related to VSMC phenotype transformation and vascular wall remodeling, which was similar with Liu et al[20], Ceolotto et al[21] and Yang et al[22]. Hao et al[24] reported that p27 reduction in pulmonary artery smooth muscle cells was related to the enhancement of cell proliferation and played a role in facilitating the pathogenesis of pulmonary arterial hypertension. Liu et al[25] presented that p27 level significantly declined in VSMC from restenosis hyperplastic intima tissue after angiopoiesis, suggesting that down-regulation of p27 was associated with VSMC proliferation and blood vessel thickening. In this study, p27 expression significantly reduced in the thickened tunica media of hypertension rat, revealing that p27 was associated with cell proliferation, vascular thickening, and hypertension, which was in accordance with Hao et al[24] and Liu et al[25] findings. Anti-miR-155 and/or pIRES-p27 over-expression plasmid significantly enhanced p27 expression, arrested cell cycle, and restrained cell proliferation in VSMC from hypertension rat. Antagomir is the microRNA inhibitor with special chemical modification. It suppresses the function of miRNA through competitive binding with mature miRNA to block its complementary pairing with target mRNA. Antagomir exhibited relative high stability and inhibitory effect both *in vivo* and *in vitro*. As a miRNA inhibitor widely applied in animal model, antagomir can overcome the obstacle of cell membrane and tissue to enrich in target cell[26]. MicrOFF™ antagomir-155 injection markedly increased p27 and α-SMA expressions. The thickness of tunica media, SBP, and DBP significantly reduced in hypertension rat injected by micrOFF™ antagomir-155. It suggested that down-regulation of miR-155 up-regulated p27 expression, suppressed VSMC proliferation and phenotype transformation, alleviated vascular wall thickening, and reduced blood pressure. Zhang et al[15] revealed that over-expression of miR-155 promoted human aortic smooth muscle cell proliferation and migration, while suppressed cell apoptosis by targeted inhibiting endothelial nitric oxide synthase expression. Yang et al[22] presented that elevating and decreasing miR-155 markedly accelerated and restrained VSMC cell proliferation, respectively. MiR-155 up-regulation further aggravated hyperplasia in tunica intima. We also confirmed the regulatory role of miR-155 on VSMC cell proliferation, similarly to Zhang et al[15] and Yang et al[22] results. Liu et al[25] demonstrated that reduction of p27 significantly weakened VSMC cell proliferation and inhibited vascular wall thickening and remodeling after angiogenesis. Hao et al[24] showed that up-regulation of p27 significantly suppressed VSMC proliferation and facilitated VSMC transformation from synthetic phenotype and contractile phenotype to alleviate pulmonary arterial hypertension. Though it has been showed that miR-155 was related to

cardiovascular disease, most studies revealed the regulatory role of miR-155 in endothelium inflammation, oxidative stress, and atherosclerosis. This research suggested the role of miR-155 in regulating VSMC cell proliferation and hypertension through targeting p27.

Conclusions

We found that miR-155 promoted VSMC proliferation by targeting p27. MiR-155 enhancement was related to hypertension. Down-regulation of miR-155 played a therapeutic effect in hypertension.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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