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

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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 e[illegible] the role of miR-155 in regulating p27 [illegible]sion, VSMC proliferation and apoptosis, a[illegible]he pathogenesis of hypertension.

MATERIALS AND MATERIALS: Dual luc[illegible]ase reporter gene assay confirmed the re[illegible]tionship between miR-155 a[illegible] MiR-155, p27, α-smooth muscle acti[illegible] Si[illegible] and Ki-67 expressions in the tho[illegible]c aort[illegible]edia of rat hypertension model[illegible]e detec[illegible]SMCs were cultured in vitro[illegible]divi[illegible] groups, including a[illegible]mi[illegible]ti-miR-155, pIRES2-blank, pIR[illegible]p27, an[illegible]i-miR-155 + pIRES2-p27 grou[illegible]Cell cycle [illegible]valuated by using flow c[illegible]y. Cell proli[illegible]on was detected with[illegible]D [illegible]g. Hypertension rats were randomi[illegible]divided[illegible]antagomir-155 and antagomi[illegible]trol. Caudal [illegible]systolic and diastolic p[illegible]ures were meas[illegible]

RESULTS: MiR[illegible]55 targeted suppressed p27 expr[illegible]on. Mi[illegible]5 and Ki-67 expressions signif[illegible]enh[illegible]ed, whi[illegible]p27 and α-SMA levels reduc[illegible]tunica[illegible]dia from hypertension rats com[illegible]with[illegible]trol. Down-regulation of [illegible]55 an[illegible]ulation of p27significantly de[illegible]d cell p[illegible]ation and arrested cell cycle in G[illegible]hase. An[illegible]gomir-155 injection markedly de[illegible]stolic and diastolic pressures, ele-v[illegible]α-SMA expressions in media, and [illegible]uced the thickness of tunica media.

CONCLUSIONS: MiR-155 promoted VSMC p[illegible]ration by targeting p27. MiR-155 enhance-me[illegible]was related to hypertension. MiR-155 played a therapeutic effect on hypertension.

Key Words:
miR-155, p27, VSMC, [illegible]rtension, Proliferation, Cell[illegible]

[illegible]roduction

[illegible]rtensio[illegible]a kind of chronic cardiovascu[illegible]aracterized as systemic arterial blood pr[illegible]e elevation. Numerous factors may l[illegible]d to hypertension, such as heredity, psychentodietary habit, age, and drugs[1-3]. Vascular [illegible]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

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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). [illegible] Tra Ace quantitative PCR (qPCR), reverse scription (RT) Kit and SYBR were pur[illegible]ed from Toyobo Life Science (Tokyo, Osaka, Ja[illegible] DharmaFECT transfection reagent was presen[illegible] by Dharmacon RNA Technolo[illegible] (Lafayett[illegible] CO, USA). MicrOFF™ antago[illegible]icrOFF™ antagomir-control, miR-155 [illegible]nc, an[illegible]iR-155, miR-NC, and EdU flow [illegible]metry d[illegible]ion kit were derived from Ribob[illegible]ang[illegible] Mouse anti p27 prim[illegible] an[illegible]as obtained from Abcam Biot[illegible] (Cambri[illegible]MA, USA). Mouse anti α-SM[illegible]nd Ki-67 pr[illegible]antibodies were pur[illegible] GeneTex In[illegible] (Irvine, CA, USA). M[illegible]e anti [illegible]n primary antibody, horseradis[illegible]roxidase (H[illegible]beled secondary antibod[illegible]RE-luc luciferas[illegible]porter plasmid, and c[illegible]ycle d[illegible]tion kit were obtained from Bey[illegible]e Biot[illegible] (Shanghai, China). Luciferase acti[illegible]te[illegible]n kit w[illegible]cquired from Promega (Ma[illegible]WI, U[illegible] Annexin V/propidium [illegible] (PI) [illegible]to[illegible]detection kit was bought fro[illegible]ulti Sc[illegible] (Hangzhou, China). pIRES2 over[illegible]pression [illegible]asmid was got from Tuoyan Bi[illegible]Co., Ltd., (Shanghai, China).

[illegible]dominal Aorta Constrictive Type [illegible]rtension Model Establishment

[illegible] 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 [illegible]to-barbital intraperitoneal injection. An i[illegible] made to expose the abdominal cav[illegible] he abd[illegible] minal aorta was isolated above t[illegible]lateral aorta ascendens. The abdominal aorta [illegible]ted by suture together with a syringe needle. T[illegible]dle was then removed and the abdo[illegible]d cavity [illegible]se after the blood flow wa[illegible]pedited. The a[illegible] nal cavity was expose[illegible]thout [illegible]ominal a[illegible]a ligation in sham gr[illegible] The h[illegible] press[illegible]e was measured by carotid a[illegible]bation [illegible]e 6th week after oper[illegible]. The [illegible]s ane[illegible]ized by pentobarbital [illegible]peritoneal i[illegible]n incision was made [illegible]ht neck to e[illegible]e right carotid artery[illegible]A). [illegible] the distal part of RCA was ligated and the urod[illegible]ic catheter was put into the R[illegible] ascending a[illegible]fter 20 min, systolic bl[illegible]ssure (SBP) and d[illegible]stolic blood pressure [illegible]P) were measured by multichannel physiologic [illegible]order. The m[illegible] arterial pressure (MAP) was [illegible]lated (MAP [illegible]DBP + 1/3 (SBP-DBP)). This s[illegible]was appr[illegible] by the Ethics Committee of The[illegible]d Hospital of Chongqing Medical Univer[illegible] (Chongqing, China).

[illegible]nsion Rats Grouping [illegible]atment

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 μ[illegible] RNA, 2 μl 5×RT buffer, 0.5 μl oligo dT+[illegible] primer Mix, 0.5 μl RT Enzyme Mix, 0.5 μl [illegible]se inhibitor, and ddH_2O. The reverse transcri[illegible] condition was 37°C for 15 min and 98°C for 5 m[illegible] The PCR reaction system contain[illegible] 5 μl 2×SYB[illegible] Green Mixture, 1.0 μl primer [illegible] forward: 5′-GGAGGTTAATGCTA[illegible]TGT[illegible]AG-3′, reverse: 5′-GTGCAGGG[illegible]GA GC[illegible] actin, forward: 5'-CTCTTCC[illegible]CTT[illegible] reverse: 5'-TCATCG[illegible]TC[illegible]TGCT-3') at 0.5 μm/l, 1.0 μl cDN[illegible] and ddH_2[illegible] PCR reaction was compos[illegible]95°C pre-den[illegible]n for 5 min, followed [illegible]s of 95°C de[illegible]turation for 15 s, 60°C [illegible]nealin[illegible]30 s, and 72°C elongation for [illegible] Real-time P[illegible]as performed on ABI Vii[illegible] to test the relativ[illegible]pression.

We[illegible]n Blo[illegible]

To[illegible]ot[illegible]was ex[illegible]ted by 500 μl RIPA. A total [illegible]g prot[illegible]were separated by 10% [illegible]m do[illegible]-polyacrylamide gel electro[illegible]esis (S[illegible]GE) for 3 h and transferred to n[illegible]brane. Ne[illegible]t, the membrane was blocked in [illegible] at room temperature for 60 min [illegible]ncubate[illegible]n primary antibody at 4°C for 12 h [illegible]7, Ki-67, α-SMA, and β-actin at 1:200, 1:300, [illegible] and 1:800, respectively). The membrane wa[illegible]ubated 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 [illegible]en-hanced chemiluminescence (ECL) [illegible] nescence.

Flow Cytometry

The cells were digested by tryp[illegible]d fixed by 70% ethanol. After that[illegible] cells we[illegible]ne by PI with RNase A at [illegible] avoid of light[illegible] min. At last, DNA co[illegible] was p[illegible]ared on [illegible]w cytometry.

EdU Staining

VSMC cel[illegible]ere added w[illegible]olution at 5 μM in l[illegible]ic phase. Af[illegible]ncubated for 48 h, the[illegible]s w[illegible]gested by trypsin and collected. After fixed [illegible] formaldehyde, the cells were[illegible]lized in gly[illegible] Next, the cells were i[illegible]ed in 0.5% Triton X[illegible]00 and re-suspended [illegible]hosphate-buffered saline (PBS). At last, the [illegible]s were staine[illegible] 500 μl Apollo at room tem[illegible]ture for 10 m[illegible]nd tested on Gallios flow cyto[illegible] (Beckm[illegible] Krefeld, Germany).

Measurement of Aorta Media Thickness

The thoracic aorta was prepared as paraffin [illegible] The thickness of aorta media was mea[illegible]red[illegible]ng 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

*p<0.05, compared with Sham group.

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

	Tunica media thickness (μm)		SBP (mmHg)			mmHg)
Treatment	Pre-injection	Post-injection	Pre-injection	Post-inj	Pre-inj	Po jection
Antagomir-control	109.3±14.7	111.4±16.9	186.1±16.5	18	103.7±8.1	101.3±9.1
Antagomir-155	107.2±13.3	96.2±8.8*	188.5±17.1	13	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 in- ficantly increased in hypertension rat, sugge successful establishment of animal model (Ta I). qRT-PCR exhibited that miR-155 expressio markedly increased in the t dia from hypertension rat compared sham oup (Figure 2A). Western blot de strated SMA and p27 protein significa clin 67 level significantly reg n hypertension rat compared w control (2B). It was indicated that VS proliferation ntly increased and tra from differen ted type to dedifferen d type h may be related to miR-15 ele ion and p27 ion.

Do Regul n of miR-155 Inhibited VS ol ation, ested Cell Cycle, and R d Bloo ressure

ti-mi a pIRES-p27 over-expression smid ntly enhanced p27 expression creased α- MA level (Figure 3A), arrested cel 3C), and restrained cell prolife- n (Fig 3D) in VSMC from hypertension MicrOFF™ antagomir-155 injection signifi- increased p27 and α-SMA expressions, wh duced Ki-67 level in tunica media (Figure 3B). The thickness of tunica media, SBP, and DBP

ificantly red d in hypertension rat injected icrOFF™ an mir-155 (Table II).

Discussion

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

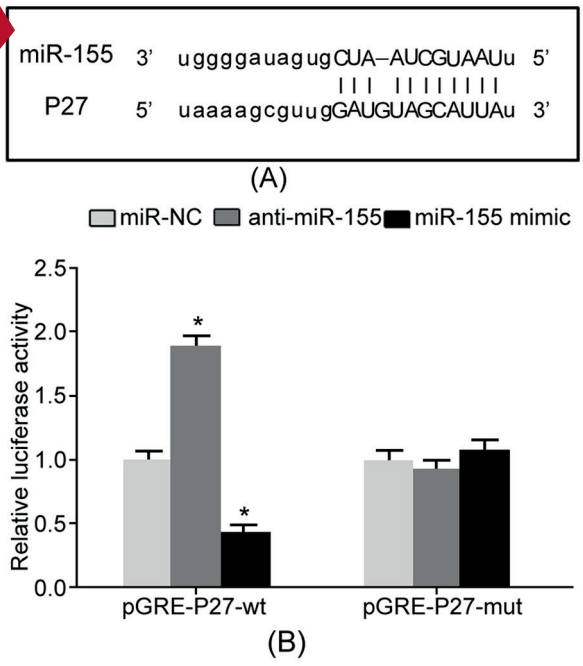

Figure 1. MiR-155 targeted regulated p27 expression. ***(A)*** The binding site between miR-155 the 3'-UTR of p27 mRNA. ***(B)*** Dual luciferase assay. *p<0.05, compared with miR-NC.

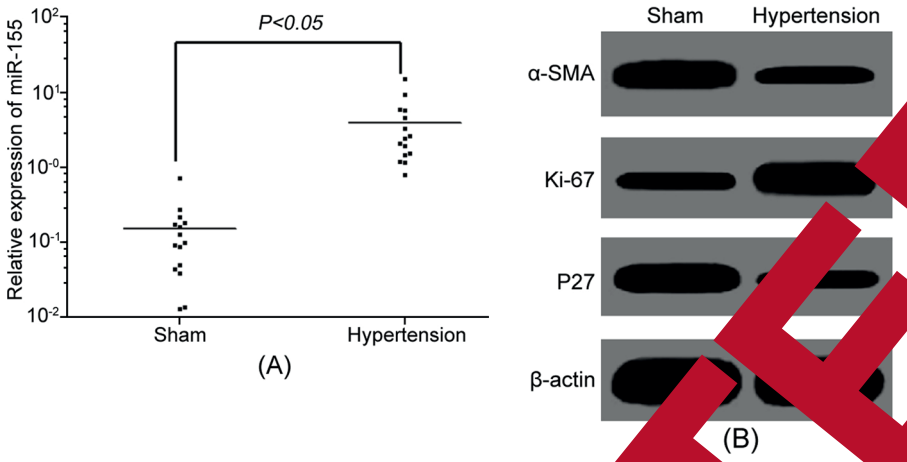

Figure 2. MiR-155 up-regulated, tunica media thickened, while α-SMA and ... decreased in hypertension rat. ***(A)*** qRT-PCR detection of miR-155 expression. ***(B)*** Western blot detection of protein expression in tunica ...

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 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 activity 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 ... tosis[18,19]. Several investigations revealed that down-regulation of p27 was associated with VSMC aberrant proliferation and participated in the pathogenesis of hypertension and pulmonary 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

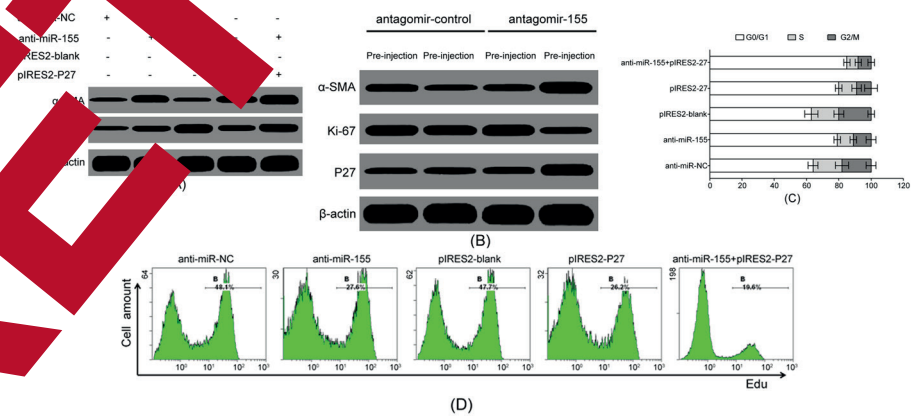

Figure 3. Down-regulation of miR-155 inhibited VSMC proliferation, arrested cell cycle, and reduced blood pressure in hypertension rat. ***(A)*** Western blot detection of protein expression in VSMC. ***(B)*** Western blot detection of protein expression in tunica media. ***(C)*** Flow cytometry detection 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. Ce[illegible]to et al[21] found that miR-155 was involve[illegible] regulation of blood pressure in hypert[illegible]on patients. Yang et al[22] revealed that mi[illegible] up-regulation is associated with vascular [illegible] flammation, intimal hyperplasi[illegible]d vascul[illegible] wall remodeling by inhibitin[illegible]lian sterile 20-like kinase 2 (MS[illegible] expr[illegible]on and enhancing the interactio[illegible]etween[illegible]1 and mitogen-activated extra[illegible]r [illegible] ted kinase (MEK). [illegible]g [illegible]icated that miR-155 level wa[illegible]gnificant[illegible]her in the plaque tissue in[illegible]d vessel fro[illegible]roscleroticpatients [illegible] with healthy control, suggesting t[illegible]up-reg[illegible]on of miR-155 was associated[illegible]th vascular [illegible]emodeling. Zhu et al[23] [illegible]vered that miR[illegible] elevation was relate[illegible]the pat[illegible]logical remodeling in atherosclle[illegible] rat a[illegible]ronary artery lesion patients. In t[illegible]R-155 [illegible]over-expressed in tunica me[illegible]m hyp[illegible]nsion rat and related to [illegible]C ph[illegible]e[illegible]nsformation and vascular wa[illegible]modeli[illegible]hich was similar with Liu et a[illegible]Ceolotto [illegible] al[21] and Yang et al[22]. Hao et al[illegible]t p27 reduction in pulmonary [illegible]y [illegible] muscle cells was related to the [illegible]ancement of cell proliferation and played a [illegible] facilitating the pathogenesis of pulmonar[illegible]erial 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 p[illegible] and blood vessel thickening. In t[illegible]tudy, p2[illegible] expression significantly reduce[illegible] the thickened tunica media of hypertensi[illegible] revealing that p27 was associated with cell p[illegible]ration, vascular thickening, and [illegible]pertensi[illegible]ich was in accordance wit[illegible]o et al[24] and [illegible] al[25] findings. Anti-m[illegible]55 an[illegible] pIRES-p[illegible] over-expression pla[illegible] signi[illegible]ntly enhanced p27 expression, [illegible]res[illegible]cle, an[illegible]strained cell prolif[illegible]on in [illegible] fro[illegible]pertension rat. Ant[illegible]mir is the [illegible] inhibitor with speci[illegible]ical modifica[illegible] It suppresses the [illegible]tion[illegible]iRNA through competitive binding with [illegible] miRNA to block its com[illegible]tary pairi[illegible]th target mRNA. A[illegible] exhibited rela[illegible]e high stability and [illegible]bitory effect both *in vivo* and *in vitro*. As a [illegible]RNA inhibit[illegible]idely applied in animal mo[illegible]antagomir c[illegible]vercome the obstacle of cell m[illegible]ane and [illegible]ue to enrich in target cell[26]. Mic[illegible]gomir-155 injection markedly increased p27 and α-SMA expressions. The [illegible]hickness of tunica media, SBP, and DBP si[illegible]y reduced in hypertension rat injected [illegible]OFF™ 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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