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Original Article

MicroRNA-130a overexpression promotes proliferation, migration, and phenotypic modulation of vascular smooth muscle cells via enhancing phosphorylation of NF-κB p65

Shuning Guo

Department of Internal Medicine, Sports Rehabilitation Hospital of Nanjing Sport Institute, No. 8, Linggu Temple Road, Xiaolingwei Street, Xuanwu District, Nanjing City 210014, Jiangsu Province, China

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Abstract: Purpose: This study aimed to evaluate the effects of upregulated microRNA-130a (miRNA-130a) on proliferation, migration, and phenotypic modulation of vascular smooth muscle cells (VSMCs), and reveal the potential regulatory mechanisms. Methods: VSMCs were transfected with 50 nM miRNA-130a mimics for 48 h, and then treated with 10^{-6} M Ang II for 48 hours (miRNA-130a mimics + Ang II). Expression of miRNA-130a was detected by quantitative real-time PCR (qRT-PCR). Proliferation, cell cycle and migration of VSMCs were analyzed by MTT, flow cytometry, and wound healing assay, respectively. Expression of α-SMA, osteopontin (OPN), and phosphorylated NF-κB p65 (p-NF-κB p65) in VSMCs were detected by Western blot. Results: After transfected with miRNA-130a mimics, miRNA-130a was overespressed in VSMCs. Compared with Ang II group, significantly higher proliferative and migratory ability was exhibited in miRNA-130a mimics + Ang II group, higher percentage of cells in S stage, and lower percentage of cells in G0/G1 stage were also examined ($P < 0.05$). Expression of α-SMA was significantly lower, and expression of OPN was significantly higher in miRNA-130a mimics + Ang II group than in Ang II group ($P < 0.05$). Furthermore, miRNA-130a overexpression significantly increased the expression of p-NF-κB p65 in Ang II-treated VSMCs. Conclusions: MicroRNA-130a overexpression might promote the proliferation, migration, and phenotypic modulation of VSMCs via enhancing phosphorylation of NF-κB p65.

Keywords: microRNA-130a, angiotensin II, nuclear factor κB p65, vascular smooth muscle cells

Introduction

Vascular smooth muscle cells (VSMCs) are the most important components of blood vessel wall that play critical roles in the regulation of blood vessel volume and local blood pressure [1]. Activated proliferation and migration of VSMCs contribute to the reparation of vascular wall [2]. However, excessive proliferation of VSMCs contributes to the development of vascular remodeling in diverse vascular disorders, such as atherosclerosis, pulmonary hypertension (PH), and essential hypertension [3]. The remodeled arterial structure with smaller lumen and increased media may lead to severe cardiovascular complications, which seriously threatening human lives [4]. Until now, various factors have been confirmed to be involved in the regulation of VSMCs proliferation, such as Angiotensin II (Ang II), norepinephrine, endothelin-1, insulin-like growth factor 1, epidermal growth factor, and platelet-derived growth factor [5]. However, the molecular mechanisms underlying VSMCs proliferation are still not fully revealed.

MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNAs, that participate in post-transcriptional regulation of mRNA [6]. miRNAs play important regulatory roles in diverse cellular processes, such as proliferation, differentiation, and apoptosis [7]. Various miRNAs are associated with the proliferation of VSMCs, such as miRNA-146a [8], miRNA-155 [9], miRNA-221/222 [10], miRNA-365 [11], and miRNA-599 [12]. miRNA-130 is a regulator of

multiple pro-proliferative pathways in PH, which promotes PH-associated phenotypic transformation of VSMCs [13]. Importantly, the promotive role of miRNA-130a on the proliferation of VSMCs has also been identified. It has been reported that miRNA-130a was upregulated in the remodeled aorta and superior mesenteric artery of spontaneously hypertensive rats, and miRNA-130a mimic significantly promoted the proliferation of VSMCs [14]. miRNA-130a is upregulated in murine model of hypoxia-induced PH, and miRNA-130a transfection promotes proliferation of pulmonary artery smooth muscle cells (PASMCs) [3]. However, related research on the specific roles of miRNA-130a on the migration and phenotypic modulation of VSMCs are still limited.

Nuclear factor κB (NF-κB) is a key transcription factor that regulates the expression of various genes involved inflammatory response [13]. NF-κB is also involved in the regulation of VSMCs proliferation. It has been reported that activated NF-κB p65 promotes the proliferation of VSMCs via upregulating miRNA-17 [15]. Transfection of miRNA-146 inhibitor inhibits the proliferative and migratory ability of VSMCs, probably by downregulating NF-κB p65 [16]. Knockdown of CREB binding protein inhibits Ang II-induced proliferation of VSMCs via downregulating the transcriptional activity of NF-kB [17]. However, it is still unclear whether the regulatory effects of miRNA-130a on VSMCs are related with NF-kB.

In this study, Ang II is used as a stimulator to mimic vascular disorders *in vitro*. The effects of miRNA-130a overexpression on the proliferation, migration, and phenotypic modulation of VSMCs were evaluated. In addition, the potential regulatory effects of miRNA-130a on NF-κB p65 were evaluated. The findings may reveal the specific role of miRNA-130a in vascular remodeling, and provide new insights into the underlying mechanisms responsible for vascular remodeling in vascular disorders.

Methods

Cell culture and transfection

Rat VSMCs, purchased from China Center for Type Culture Collection (Wuhan, China), were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum. Cells were maintained in an incubator at 37°C with 5% CO_2. Lgarithmic growth phase cells were used for transfection. VSMCs were transfected with 50 nM miRNA-130a mimics (Ribobio, Guangzhou, China) using lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). VSMCs transfected with miRNA-130a negative control (miRNA-130a NC) were considered as control. After 48 hours of transfection, transfected cells were used for further assay.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from VSMCs of different groups using TRizol reagent (Thermo Fisher Scientific), and reverse transcripted using RevertAidTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). qRT-PCR was performed on ABI7500 (Thermo Fisher Scientific) using special primers (miRNA-130a, forward: 5'-TTGCGATTCTGTTTTGTGCT-3'; reverse: 5'-GTGGGGTCCTCAGTGGG-3'). U6 was used as an internal control (U6, forward: 5'-CTCGCTTCGGCAGCACATATACT-3'; reverse: 5'-ACGCTTCACGAATTTGCGTGTC-3'). The PCR program included 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 34 s. The relative expression of miRNA-130a was calculated using the $2^{-\Delta\Delta Ct}$ method [18].

Ang II treatments

Ang II at a concentration of 10^{-6} M was used to treat VSMCs of different groups. VSMCs were randomly divided into four groups, including normal control (NC) (normal VSMCs without treatment), Ang II (normal VSMCs treated with 10^{-6} M Ang II), miRNA-130a mimics + Ang II (miRNA-130a mimics-transfected VSMCs treated with 10^{-6} M Ang II), and miRNA-130a NC + Ang II (miRNA-130a NC-transfected VSMCs treated with 10^{-6} M Ang II). After 48 h of treatment, cells were used for further assay.

MTT assay

MTT was performed to detect the proliferative ability of VSMCs. Simply, 100 μl VSMCs of different groups were seeded in 96-well plates at a density of 2.5×10^4/well. Then 20 μg MTT (Sigma, USA) was added into each well. After 4 hours of culturing, the medium was removed, and 150 μl DMSO was added into each well. Optical density (OD) at 490 nm was detected by a Microplate Reader (Thermo Fisher Scientific).

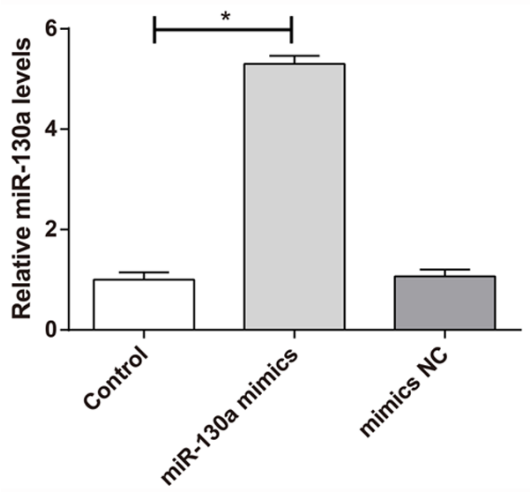

Figure 1. Expression of miRNA-130a in vascular smooth muscle cells (VSMCs) transfected with the miRNA-130a mimics or the miRNA-130a negative control (miRNA-130a NC) detected by quantitative real-time PCR (qRT-PCR). *, P < 0.05 vs. miRNA-130a NC.

Cell cycle assay

Flow cytometry was performed to detect the cell cycle progression of VSMCs. Simply, VSMCs of different groups were washed with PBS, and fixed in 70% ethanol. Then Muse Cell Cycle Reagent (Millipore, USA) was added into cells, and incubated for 30 minutes in the dark. The percentage of cells in different cell cycle stage was analyzed on MUSE cell analyzer (Millipore).

Cell migration assay

Wound healing assay was performed to detect the migratory ability of VSMCs. VSMCs of different groups were cultured in culture dishes (60 mm) until 100% confluence. A wound track was scored in each dish with sterile pipette tip, and cell debris was removed by washing with PBS. After 48 hours of treatment with Ang II, migrated cells in the wounded area was visualized and photographed.

Western blot

Expression of α-SMA, osteopontin (OPN), and phosphorylated NF-κB p65 (p-NF-κB p65) in VSMCs were detected by Western blot. Total proteins of VSMCs of different groups were extracted by lysis buffer. After centrifugation, proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidenefluoride membrane (Millipore). Then the membrane was blocked with 5% skim milk for 2 hours, and incubated with primary antibodies (anti-α-SMA, -OPN, -p-NF-κB p65, -GAPDH, rabbit anti-human, 1:1000, Abcam, USA) overnight at 4°C. After washed with TBST for three times, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Goat anti-rabbit, 1:5000, Cwbio, China) for 1 hour at 25°C. Protein brands were visualized using Gel imaging system (Thermo Fisher Scientific).

Statistical analyses

All experiments were performed with three replications. All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by SPSS version 17.0 (SPSS Inc., Chicago, IL). Comparison between different groups was determined by one-way ANOVA. A *p*-value less than 0.05 was considered to be significantly different.

Results

miRNA-130a was over-expressed in VSMCs transfected with miRNA-130a mimics

VSMCs were transfected with miRNA-130a mimics for 48 hours. qRT-RCR showed that the expression of miRNA-130a was significantly higher in VSMCs transfected with miRNA-130a mimics than those transfected with miRNA-130a NC (P < 0.05) (**Figure 1**). This phenomenon indicated that miRNA-130a was successfully transfected into VSMCs.

miRNA-130a over-expression promoted Ang II-induced proliferation of VSMCs

MTT was performed to evaluate the proliferative ability of VSMCs. As shown in **Figure 2**, the proliferative ability of VSMCs was significantly increased with the treatment of Ang II in a time-dependent manner (*P* < 0.05). Then the effect of miRNA-130a over-expression on the proliferation of VSMCs was evaluated at 48 hour post-treatment. MTT showed that the proliferative ability of VSMCs was significantly higher in miRNA-130a mimics + Ang II group than in Ang II group (P < 0.05). No significantly different was revealed on the proliferative ability of VSMCs between miRNA-130a NC + Ang II group and Ang II group (**Figure 2**).

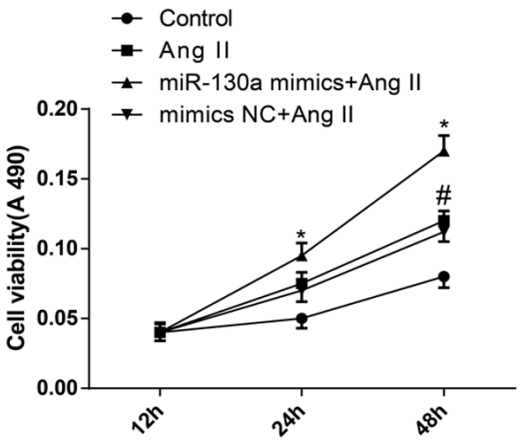

Figure 2. Proliferative ability of vascular smooth muscle cells (VSMCs) detected by MTT. Ang II, normal VSMCs treated with 10^{-6} M Ang II; Control, normal VSMCs without treatment; miRNA-130a mimics + Ang II, miRNA-130a mimics-transfected VSMCs treated with 10^{-6} M Ang II; miRNA-130a negative control (miRNA-130a NC) + Ang II, miRNA-130a NC-transfected VSMCs treated with 10^{-6} M Ang II. *, $P < 0.05$ vs. Control; #, $P < 0.05$ vs. Ang II.

miRNA-130a over-expression promoted Ang II-induced cell cycle progression of VSMCs

Flow cytometry was performed to evaluate the cell cycle progression of VSMCs. Ang II treatment significantly increased the percentage of cells in S stage, and reduced the percentage of cells in G0/G1 stage ($P < 0.05$). Then the effect of miRNA-130a over-expression on cell cycle progression of VSMCs was evaluated at 48 hours post-treatment. Flow cytometry showed that the percentage of cells in S stage was significantly higher in miRNA-130a mimics + Ang II group than in Ang II group ($P < 0.05$). Furthermore, the percentage of cells in G0/G1 stage was significantly lower in miRNA-130a mimics + Ang II group than in Ang II group ($P < 0.05$). No significant differences were revealed on the percentages of cells in S and G0/G1 stage between miRNA-130a NC + Ang II group and Ang II group (**Figure 3**).

miRNA-130a over-expression promoted Ang II-induced migration of VSMCs

Wound healing assay was performed to evaluate the migration of VSMCs. As shown in **Figure 4**, the migratory ability of VSMCs was significantly increased with the treatment of Ang II ($P < 0.05$). Then the effect of miRNA-130a over-expression on the migration of VSMCs was evaluated 48 hours post-treatment. Wound healing assay showed that the migratory ability of VSMCs was significantly higher in the miRNA-130a mimics + Ang II group than in the Ang II group ($P < 0.05$). No significantly different was revealed on the migratory ability of VSMCs between miRNA-130a the NC + Ang II group and the Ang II group (**Figure 4**).

miRNA-130a over-expression promoted Ang II-induced phenotypic modulation of VSMCs

The phenotypic modulation of VSMCs was evaluated according to the expression of α-SMA and osteopontin. As shown in **Figure 5**, the Ang II group exhibited significantly lower expression of α-SMA, and higher expression of OPN when compared with the NC ($P < 0.05$). This phenomenon indicated the phenotypic modulation of VSMCs from contractile to synthetic phenotype. Then the effect of miRNA-130a over-expression on the phenotypic modulation of VSMCs was evaluated 48 hours post-treatment. The results of Western blot showed that expression of α-SMA was significantly lower, while expression of OPN was significantly higher in the miRNA-130a mimics + Ang II group than in the Ang II group ($P < 0.05$). No significant differences were revealed on the expression of α-SMA and OPN between the miRNA-130a NC + Ang II group and the Ang II group (**Figure 5**).

miRNA-130a over-expression enhanced Ang II-induced phosphorylation of NF-κB p65

In order to reveal the potential regulatory mechanisms of miRNA-130a on VSMCs, phosphorylation of NF-κB p65 was detected. Western blot showed that expression of p-NF-κB p65 was significantly increased in VSMCs by the treatment of Ang II ($P < 0.05$). Moreover, expression of p-NF-κB p65 was significantly higher in the miRNA-130a mimics + Ang II group than in the Ang II group ($P < 0.05$). No significant difference was revealed on the expression of p-NF-κB p65 between the miRNA-130a NC + Ang II group and the Ang II group (**Figure 6**).

Discussion

Excessive proliferation of VSMCs in arterial walls is an important pathogenic factor of vascular disorders [19]. Increasing evidence has proven that miRNAs are involved in the regula-

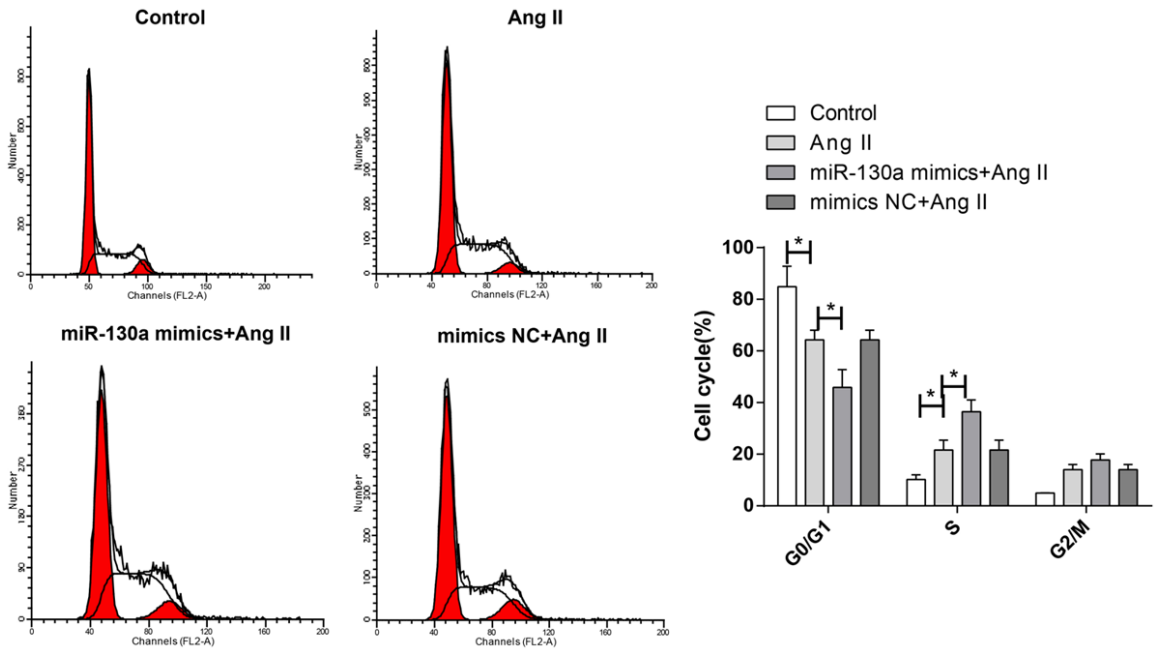

Figure 3. Cell cycle progression of vascular smooth muscle cells (VSMCs) detected by flow cytometry. Ang II, normal VSMCs treated with 10^{-6} M Ang II; Control, normal VSMCs without treatment; miRNA-130a mimics + Ang II, miRNA-130a mimics-transfected VSMCs treated with 10^{-6} M Ang II; miRNA-130a negative control (miRNA-130a NC) + Ang II, miRNA-130a NC-transfected VSMCs treated with 10^{-6} M Ang II. *, P < 0.05 vs. Control; #, P < 0.05 vs. Ang II.

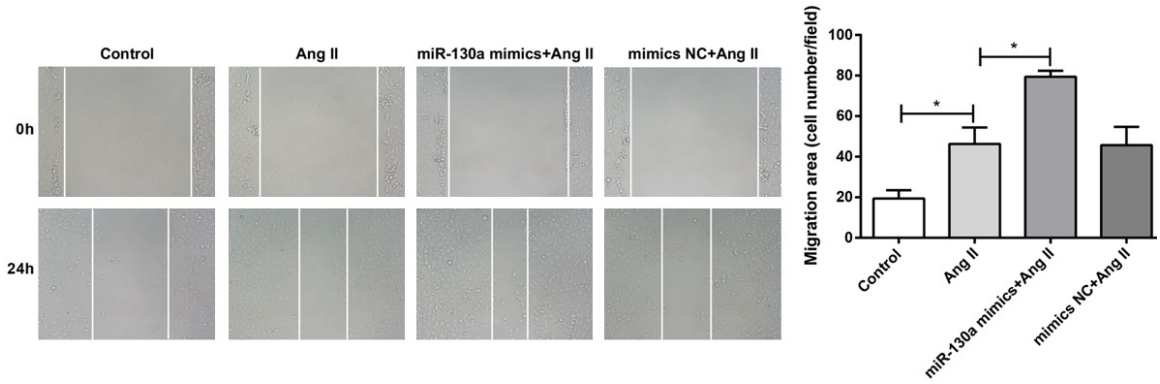

Figure 4. Migratory ability of vascular smooth muscle cells (VSMCs) detected by wound healing assay. Ang II, normal VSMCs treated with 10^{-6} M Ang II; Control, normal VSMCs without treatment; miRNA-130a mimics + Ang II, miRNA-130a mimics-transfected VSMCs treated with 10^{-6} M Ang II; miRNA-130a negative control (miRNA-130a NC) + Ang II, miRNA-130a NC-transfected VSMCs treated with 10^{-6} M Ang II. *P < 0.05 vs. Control; #P < 0.05 vs. Ang II.

tion of VSMCs proliferation and vascular remodeling [20, 21]. However, the specific role of miRNA-130a on VSMCs and related regulatory mechanisms are still unclear. In this study, we found that miRNA-130a overexpression significantly promoted the proliferation, migration, and phenotypic modulation of VSMCs. In addition, the regulatory effect of miRNA-130a on VSMCs was closely related with enhanced phosphorylation of NF-κB p65.

Ang II is an important vasoactive stimuli, which can induce vasoconstriction via acting on VSMCs [22]. The specific roles of Ang II on VSMCs have been identified by a large number of studies. For examples, Ang II promotes the proliferation of VSMCs in a concentration- and time-dependent manner [23, 24]. Ang II results a significant increase in the migration of VSMCs in a concentration-dependent manner [24]. Ang II induces the phenotype modulation of VSMCs

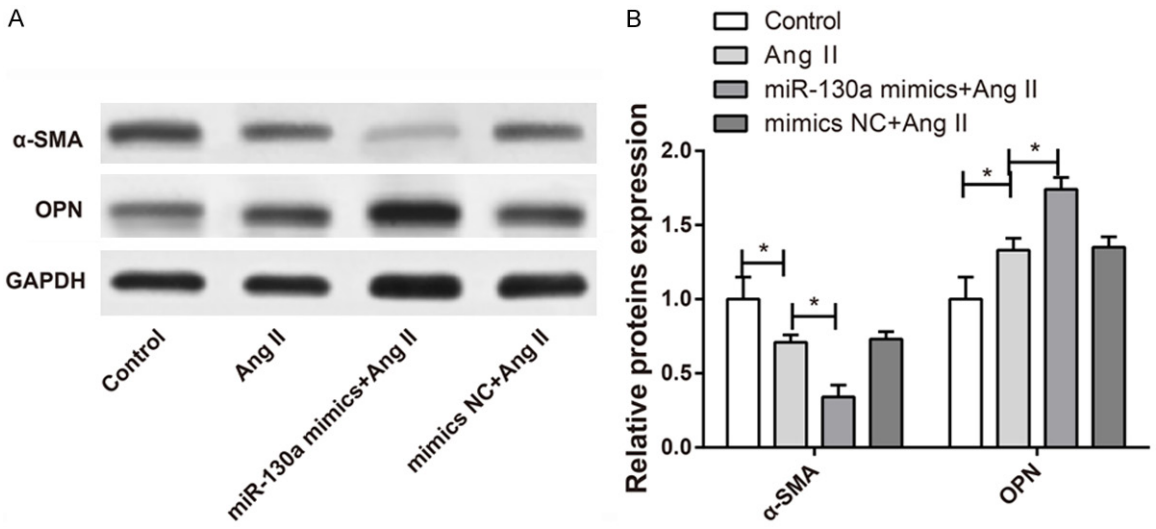

Figure 5. Expression of α-SMA and osteopontin (OPN) in vascular smooth muscle cells (VSMCs) detected by Western blot. Ang II, normal VSMCs treated with 10^{-6} M Ang II; Control, normal VSMCs without treatment; miRNA-130a mimics + Ang II, miRNA-130a mimics-transfected VSMCs treated with 10^{-6} M Ang II; miRNA-130a negative control (miRNA-130a NC) + Ang II, miRNA-130a NC-transfected VSMCs treated with 10^{-6} M Ang II. $^{*}P < 0.05$ vs. Control; $^{\#}P < 0.05$ vs. Ang II.

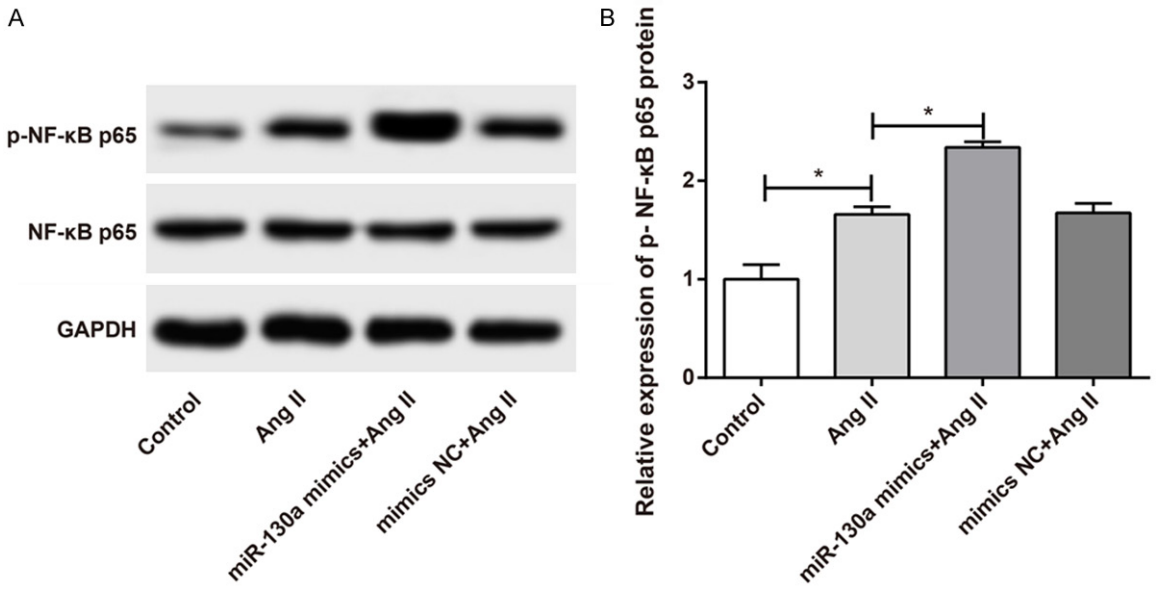

Figure 6. Expression of phosphorylated NF-κB p65 (p-NF-κB p65) in vascular smooth muscle cells (VSMCs) detected by Western blot. Ang II, normal VSMCs treated with 10^{-6} M Ang II; Control, normal VSMCs without treatment; miRNA-130a mimics + Ang II, miRNA-130a mimics-transfected VSMCs treated with 10^{-6} M Ang II; miRNA-130a negative control (miRNA-130a NC) + Ang II, miRNA-130a NC-transfected VSMCs treated with 10^{-6} M Ang II. $^{*}P < 0.05$ vs. Control; $^{\#}P < 0.05$ vs. Ang II.

from contractile to synthetic phenotype [25]. In this study, VSMCs treated for 48 hours of 10^{-6} M Ang II exhibited significantly enhanced proliferative and migratory ability, as well as significantly increased cells in S stage, and reduced cells in G0/G1 stage. Meanwhile, Ang II significantly down-regulated the expression of α-SMA and up-regulated the OPN level. Consistent with previous studies, Ang II contributed to excessive proliferation of VSMCs and vascular remodeling in vascular disorders.

miRNA-130a is a novel regulator of VSMCs proliferation, which contributes to vascular remodeling in vascular disorders [13]. Previous studies have proved that miRNA-130a transfection

significantly promotes the proliferation of HPASMCs and VSMCs [3, 14]. Consistent with previous studies, the proliferative ability of VSMCs was significantly higher in the miRNA-130a mimics + Ang II group than in the Ang II group. In addition, significantly higher number of cells in S stage, and lower number of cells in G0/G1 stage were observed in the miRNA-130a mimics + Ang II group than in the Ang II group. CDKN1A is a major regulator of cell cycle [26]. A previous study has proven that miRNA-130a transfection significantly decreases the expression of CDKN1A in HPASMC [3]. CDKN1A is a direct target of miRNA-130a, thereby, miRNA-130a may promote the proliferation of VSMCs via down-regulating CDKN1A.

In this study, the migratory ability of VSMCs was significantly higher in the miRNA-130a mimics + Ang II group than in the Ang II group. The promotive effects of miRNA-130a on the migration of VSMCs are rarely reported. A previous study has proved that miRNA-130a mimic significantly decreases the expression of GAX at both mRNA and protein levels in VSMCs [14]. GAX, a growth arrest-specific homeobox mediates the proangiogenic effect of miRNA-130a in vascular endothelial cells [27]. GAX exhibits obvious inhibitory effects on the proliferation, differentiation, and migration of VSMCs [28]. Since GAX is a direct target of miRNA-130a, we suspected that the promotive effects of miRNA-130a on the proliferation and migration of VSMCs are closely related with the down-regulation of GAX.

Proliferation of VSMCs always complies with its phenotype modulation. In this study, we found that the expression of α-SMA was significantly lower, and the expression of OPN was significantly higher in the miRNA-130a mimics + Ang II group than in the Ang II group. The downregulated α-SMA and upregulated OPN illustrate the phenotype modulation of VSMCs from contractile to synthetic phenotype. It has been reported that miRNA-21 induced by platelet-derived growth factor BB promoted the conversion of saphenous veins smooth muscle cells to a synthetic phenotype [29]. miR-181a/b was involved in VSMCs differentiation through upregulating synthetic marker genes and downregulating contractile genes [30] miRNA-130a may exert similar functions with miRNA-21 and miRNA-181a/b, which can also promote the phenotype modulation of VSMCs to synthetic phenotype. The phenotype modulation induced by miRNA-130a contributes to vascular remodeling in vascular disorders.

NF-κB is a key transcription factor involved in multiple biological processes, including inflammation, proliferation, and cancer development [31]. The regulatory relationship between MiRNA-130a and NF-κB p65 has been identified by various studies. It has been reported that miRNA-130a over-expression significantly promoted the proliferation and migration of human keratinocyte HaCaT cells via upregulating NF-κB p65 [32]. miRNA-130a accelerates the proliferation of HeLa and CaSki cells via targeting the phosphatase and tensin homolog [33]. NF-κB p65 is activated during hepatic inflammation [34]. In this study, expression of p-NF-κB p65 was significantly higher in the miRNA-130a mimics + Ang II group than in the Ang II group. The findings are consistent with previous studies, and further indicate that the regulatory effects of miRNA-130a on VSMCs are associated with enhanced phosphorylation of NF-κB p65. It is thus suspected that miRNA-130a may promote the proliferation, migration, and phenotype modulation of VSMCs via enhancing phosphorylation of NF-κB p65.

In conclusion, miRNA-130a over-expression might promote the proliferation, migration, and phenotypic modulation of VSMCs via enhancing phosphorylation of NF-κB p65. Inhibition of miRNA-130a may be used as a therapeutic target in suppressing vascular remodeling. However, this study is still limited in cellular level. Further research on the roles and related regulatory mechanisms of miRNA-130a on vascular remodeling in vascular disorder models is still needed.

Disclosure of conflict of interest

None.

Address correspondence to: Shuning Guo, Department of Internal Medicine, Sports Rehabilitation Hospital of Nanjing Sport Institute, No. 8, Linggu Temple Road, Xiaolingwei Street, Xuanwu District, Nanjing City 210014, Jiangsu Province, China. Fax: +86-025-84755158; E-mail: guoshuning286@163.com

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