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MiR-7-5p attenuates vascular smooth muscle cell migration and intimal hyperplasia after vascular injury by NF-kB signaling

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ABSTRACT

Background: Atherosclerosis (AS) is the primary cause of coronary artery disease, which is featured by aberrant proliferation, differentiation, and migration of vascular smooth muscle cells (VSMCs). MicroRNAs play crucial roles in AS, but the function of miR-7-5p in AS remains unclear. Here, we aimed to explore the effect of miR-7-5p on AS and VSMCs *in vitro* and *in vivo*.

Methods: The *in vivo* rat AS model and apoE−/− mouse model were established. The carotid artery injury was checked by immunohistochemistry staining. The RNA levels of miR-7-5p and p65 were measured by qPCR assay. Protein levels were checked by western blotting. Cell apoptosis was evaluated by flow cytometry. Cell migration was checked by Transwell assay and wound healing assay. The potential interaction between miR-7-5p with p65 was checked by luciferase reporter gene assay.

Results: MiR-7-5p was downregulated and NF-κB p65 was upregulated in injured carotid arteries in rat model. The carotid artery injury in the AS rats and the treatment of miR-7-5p attenuated the phenotype in the model. Immunohistochemistry staining and Western blot analysis revealed that PCNA levels were increased in injured carotid arteries of the model rats and miR-7-5p could reverse the levels. The cell viability of VSMCs was induced by PDGF-BB but miR-7-5p blocked the phenotype. PDGF-BB decreased apoptosis of VSMCs, while miR-7-5p was able to restore the cell apoptosis in the model. PDGF-BB-induced migration of VSMCs was attenuated by miR-7-5p. miR-7-5p mimic remarkably repressed the luciferase activity of p65 in VSMCs. The levels of p65 were inhibited by miR-7-5p in the cells. The PDGF-BB-promoted cell viability and migration of VSMCs was repressed by miR-7-5p and p65 overexpression reversed the phenotype.

Conclusion: We concluded that miR-7-5p attenuates vascular smooth muscle cell migration and intimal hyperplasia after vascular injury by NF-kB signaling.

1. Introduction

Atherosclerosis (AS) is a chronic disease of large arteries, and the primary cause of coronary artery disease [1,2]. Multiple pathological factors such as endothelial injury, inflammatory responses, and plaque rupture, are regarded as causes of AS [3]. Among them, inflammatory factors including the IL-6 and TNF-α activates the function of nuclear transcriptional factor NF-κB, and play critical roles in AS development [4,5]. Despite of the numerous researches on clinical therapy of AS, modest progress has been made over the past decades [6,7]. Hence, it is imperative to explore mechanisms involve in the AS progression. Intimal hyperplasia is an important factor of AS, which is featured by aberrant proliferation, differentiation, and migration of vascular smooth muscle

cells (VSMCs) [8,9]. VSMCs remain quiescent and stay in the media of blood vessel under normal condition, whereas under atherogenic factors stimulation, the VSMCs growth and migrate the intima, which eventually causes intimal hyperplasia [10]. Impeding proliferation and migration of VSMCs is a promising strategy for AS therapy.

Over the past decades, microRNAs (miRNAs) have been widely reported as pathological factors and promising targets for treatment of multiple diseases, especially the cardiovascular diseases [11–13]. MiR-NAs are short-length noncoding RNAs that could directly interact with the 3′-untranslated regions (UTR) of target mRNAs, hence modulate gene transcription and cause RNA degradation [14,15]. Owing to the large scale of targeted genes, miRNAs are found to participate in various cellular processes, including cell proliferation, migration, and apoptosis

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[16]. For example, inhibition of miR-495 alleviated intimal hyperplasia and notably decreased formation of atherosclerotic plaque, modulated macrophage influx, cholesterol level in plasma, and plaque stability [17]. MiR-374b stimulated the endothelial-mesenchymal transition during intimal hyperplasia by decreasing the expression of MAPK7 [18].

The miR-7-5p is reported to participate in the progression of several cancers, and frequently suggested as a tumor suppressor via inhibiting cell growth and migration [19–22]. However, the specific function of miR-7-5p in AS has not been determined yet. In this study, we constructed *in vitro* and *in vivo* model to mimic AS, and evaluated the expression of miR-7-5p in the established models. Study on molecular mechanisms suggested that miR-7-5p could suppress the proliferation and migration of VSMCs through directly targeting the NF-kB expression and function. Our study may provide novel regulatory mechanisms and potential therapeutic targets for AS.

2. Materials and methods

2.1. Rat model

All animal experiments were approved by Ethics Committee of The First affiliated Hospital of Northwest Minzu University. Male SD rats aged 8-weeks old were brought from Beijing Vital River Laboratory (China), and maintained in a standard environment with 12 h dark/12 light cycle. The *in vivo* AS model was established using unilateral carotid artery ligation, In brief, rats were anesthetized by chloralic hydras, then an incision was made on the neck to expose the bilateral common carotid arteries. The left artery was ligated by using a 6.0 suture, the right carotid artery was set as control. For treatment, rats were intravenously injected with agomir-7-5p or agomir-NC (as control) for 3 days before ligation. Rats in sham group received operation without ligation, and were injected with saline as control.

The 8-week-old apoE−/− mice and wild-type C57BL/6 mice were purchased from Beijing Vital River Laboratory (China). The apoE−/− mice were fed with a Western diet for 12 weeks and randomly divided into different treatment groups. The agomir-7-5p or agomir-NC (as control) were intravenously injected through tail vein once every 3 weeks from the start of Western diet. Mice in normal and control groups were injected with saline. The mice were sacrificed, and the aorta were collected after 12 weeks-treatment.

2.2. Histological analysis

The rats were anesthetized and perfused with PBS, followed by 4% PFA for 5 min via left ventricle. Then carotid arteries were isolated and made into paraffin-embedded slices. The morphology of arteries was analyzed using hematoxylin-eosin (HE) staining (Beyotime, China) as per manufacturer's instruction [23].

The expression of PCNA in tissues was examined by using immunohistochemical staining. Tissue sections were deparaffined, rehydrated, immersed with 3% H_2O_2 to eliminate endogenous peroxidase, followed by blocking in goat serum. After that, the tissue sections were incubated with anti-PCNA antibody (Abcam, USA) and biotin-labeled secondary antibody. The positive staining was visualized after incubation with DAB and observed under microscope (Leica, Germany).

2.3. Cell lines and treatment

Human aortic VSMCs were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (China), and were cultured in DMEM (Hyclone, USA) that added with 10% fetal bovine serum (FBS; Hyclone, USA) and 1% penicillin/streptomycin (Sigma, USA) at a humidified 37 °C incubator that filled with 5% CO_2.

The agomir-7-5p, negative control (NC), p65 overexpressing vectors (pcDNA-p65), and control vectors (pcDNA-3.1) were synthesized by Guangzhou RiboBio (China), and transfected into cells by using Lipofectamine 2000 reagent (Invitrogen, USA) in line with producer's protocol. PDFG-BB (20 ng/mL) was added to stimulate cells for 24 h after transfection.

2.4. Western blotting assay

Total proteins and nuclear proteins were extracted from tissues or VSMCs using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) following the producer's protocol. Proteins (50 μg) were divided in 8%–10% SDS-PAGE gel, transferred to NC membranes, and blocked with 5% non-fat milk for 1 h at room temperature. After that, the protein bands were incubated with primary antibodies against p65, Histone 3, PCNA, MMP-9 and GAPDH, respectively, at 4 °C overnight. Then the protein bands were hatched with anti-rabbit antibody (Invitrogen, USA), and reacted with ECL for visualization [24]. Antibodies were purchased from Abcam and used according to producer's protocol.

2.5. Quantitative PCR (qPCR) assay

Tissues and cells were homogenized by TRIzol reagent (Thermo, USA) in accordance with manufacturer's instruction. The cDNA was synthesized using a First Strand Synthesis Kit (TransGen, USA). The qPCR was conducted using a SYBR Green PCR kit (Takara, Japan). Relative level of miR-7-5p was calculated following $2^{-\Delta\Delta Ct}$ method and normalized to the level of U6.

2.6. Cell counting kit-8 (CCK-8) assay

Cell viability was evaluated using CCK-8 kit (Beyotime, China). In brief, VSMCs (1×10^4 cells per well) were placed into a 96-well plate and cultured for 48 h after indicated transfection. After that, 10 μl CCK-8 reagent was added into each well for 1 h incubation. Then absorbance values at 450 nm were checked by a microplate Reader (Thermo, USA).

2.7. Cell apoptosis

Cell apoptosis was measured using an Annexin V (AV)/PI kit. VSMCs were treated collected after indicated treatment, suspended in binding buffer that contains AV and PI (5 μl) and hatched for 30 min. The samples were then analyzed by flow cytometry (BD Biosciences, USA).

2.8. Cell migration

Cell migration was assessed by Transwell assay and wound healing assay. For Transwell assay, VSMCs suspended in FBS-free DMEM were seeded into an upper chamber of Transwell, whereas the lower chambers were filled with DMEM that contains 10% FBS. After incubation for 48 h, the chambers were collected, and stained with 1% crystal violet for 20 min. The stained cells were observed and photographed under a microscope (Leica, USA) [25].

For wound healing assay, VSMCs were cultured to form monolayer in a 6-well plate, scratched with 200 μl pipette tip, and washed with PBS. After incubation for 24 h, the wound was captured and the width of wound was measured using ImageJ software.

2.9. Luciferase reporter gene assay

The wild type (WT) and mutated (MUT) 3′UTR sequences of p65 were cloned into pGL3 control vectors to obtain luciferase reporter gene vectors. VSMCs were co-transfected with the constructed vectors and miR-7-5p mimics for 48 h and lysed. The luciferase activity was measured by using a dual-luciferase reporter assay system (Promega, USA) [26].
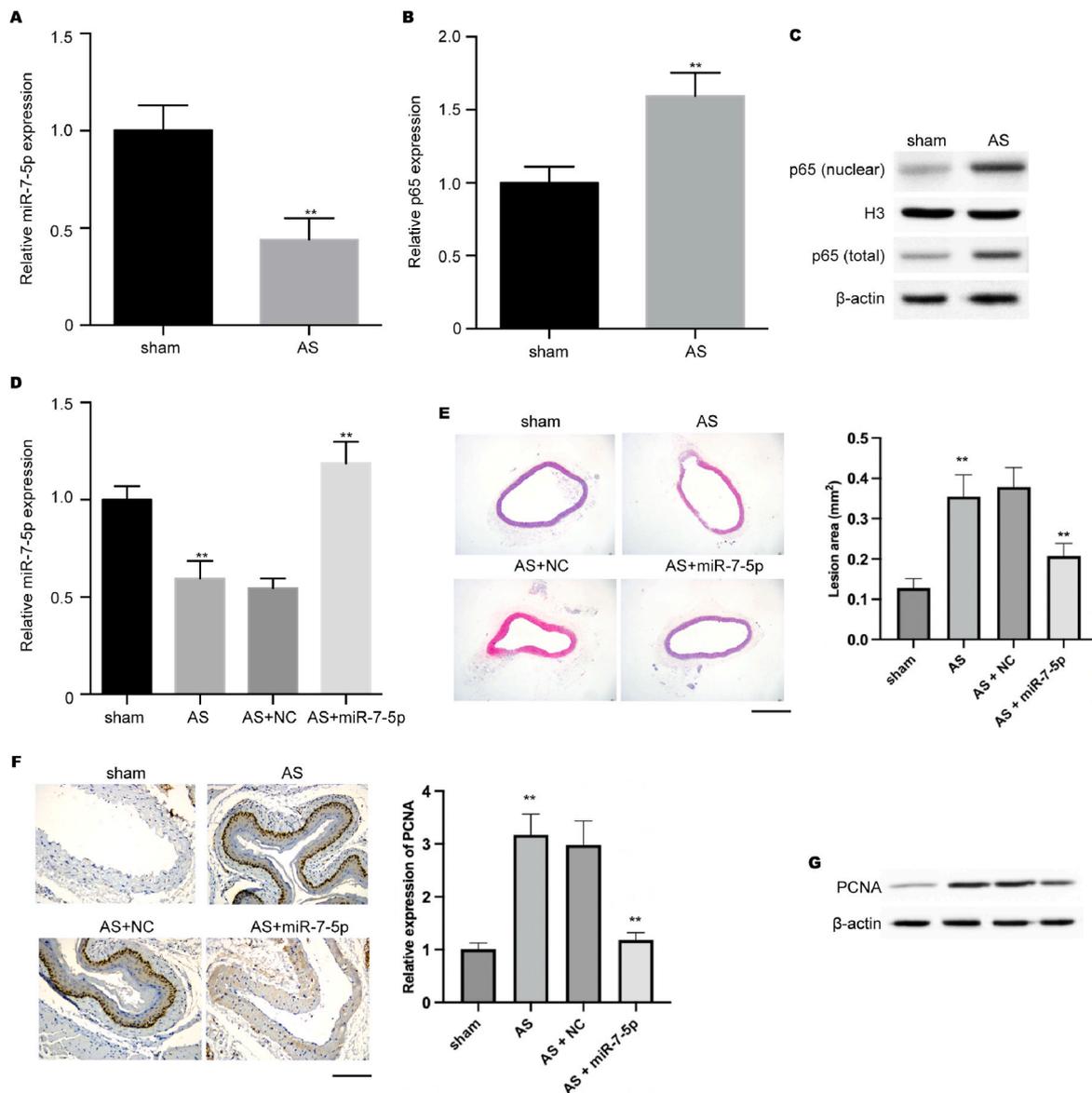
Fig. 1. MiR-7-5p relieves intimal hyperplasia in carotid artery injury rat model. (A–H) The AS rat model was constructed, and the rats were treated with miR-7-5p. (A–D) The expression of miR-7-5p and p65 was measured. (E) The injured carotid arteries were detected by Hematoxylin and eosin (HE) staining. (F and G) The levels of PCNA were analyzed immunohistochemistry and Western blot. Scale bar, 50 μm**$P < 0.01$.

2.10. Statistics

The statistical analysis was conducted using SPSS 19.0 software. Data were shown as mean ± SEM of three replicates. Student's *t*-test and one-way ANOVA followed by post-hoc tests were performed for comparison between two or more groups. *P* value < 0.05 was regarded as significant.

3. Results

3.1. MiR-7-5p relieves intimal hyperplasia in carotid artery injury rat model

To explore the function of miR-7-5p in AS pathogenesis, we established an *in vivo* rat model by using unilateral carotid artery ligation and transgenic apoE−/− mouse model. We observed notably decreased level of miR-7-5p and increased RNA level of p65 in the carotid arteries isolated from the model group, compared with that in the control rats (Fig. 1A and B). Results from western blotting assay indicated that the

total p65 protein was remarkably elevated in carotid arteries from AS model, compared with the sham group (Fig. 1C). Noteworthy, the localization of p65 was also notably elevated in AS model, indicating p65 activation as transcriptional factor. Further experiments demonstrated that treatment with miR-7-5p mimics successfully elevated the level of miR-7-5p in carotid arteries (Fig. 1D). Results from histological analysis showed that the miR-7-5p treatment notably reduced the neointimal thickness induced by ligation (Fig. 1E). Besides, the proliferative biomarker PCNA was dramatically upregulated in AS tissues, compared with the sham group, whereas the miR-7-5p treatment repressed this elevation (Fig. 1F and G). On the other hand, the decreased expression of miR-7-5p (Fig. S1A) and increased level of p65 (Figs. S1B and C) in the carotid arteries were observed in AS model. Treatment with significantly elevated the level of miR-7-5p in carotid arteries of apoE−/− mice (Fig. S1D). Histological analysis of carotid arteries indicated that miR-7-5p treatment notably decreased the tissue lesion caused by AS (Fig. S1E). The lipid drop accumulation was significantly induced in apoE−/− mice and alleviated by miR-7-5p treatment (Fig. S1F). These data indicated that miR-7-5p potentially protect carotid arteries from AS injury.
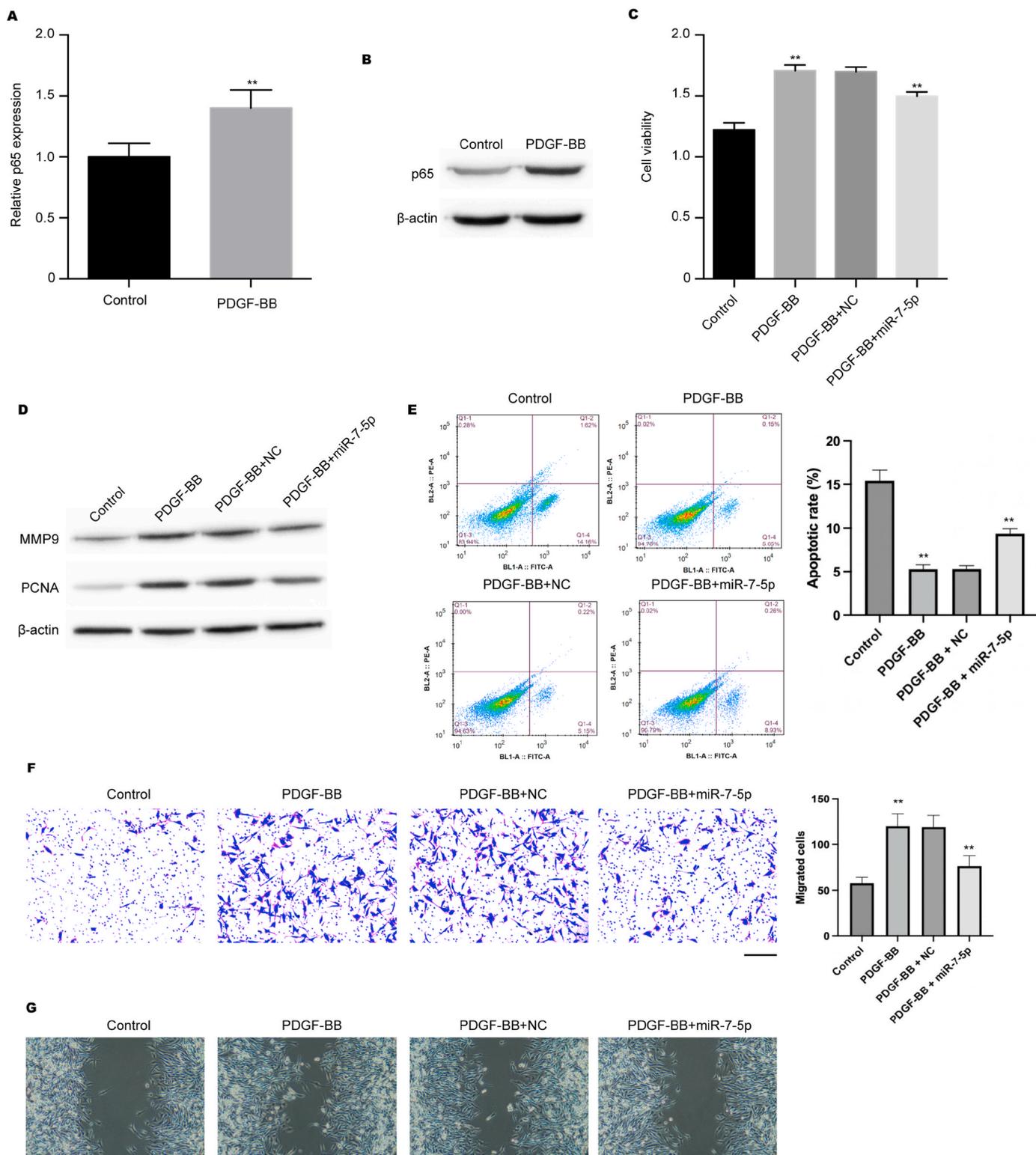
Fig. 2. MiR-7-5p attenuates proliferation and migration of VSMCs. (A–G) The VSMCs were treated with PDGF-BB and miR-7-5p. (A and B) The expression of miR-7-5p and p65 was determined. (C) Cell viability was measured by CCK-8 assays. (D) The expression of MMP9 and PCNA was tested by Western blot. (E) The cell apoptosis was measured by flow cytometry analysis. (F and G) Cell migration was analyzed by transwell and wound healing assay. Scale bar, 50 μm **$P < 0.01$.

3.2. MiR-7-5p attenuates proliferation and migration of VSMCs

We next established a PDGF-BB-stimulated cell model to mimic the damages of VSMCs in AS. Similar with the results from rat model, the p65 was significantly elevated in PDGF-BB-treated VSMCs at both RNA and protein levels (Fig. 2A and B). Moreover, miR-7-5p mimics remarkably suppressed the enhanced proliferation of VSMCs caused by PDGF-BB (Fig. 2C), along with inhibited expression of PCNA and MMP9 (Fig. 2D). The number of apoptotic cells was also increased after PDGF-BB treatment (Fig. 2E), confirming the decreased cell proliferation. In comparison with the PDGF-BB stimulation group, administration of miR-7-5p mimics dramatically decreased the number of migrated
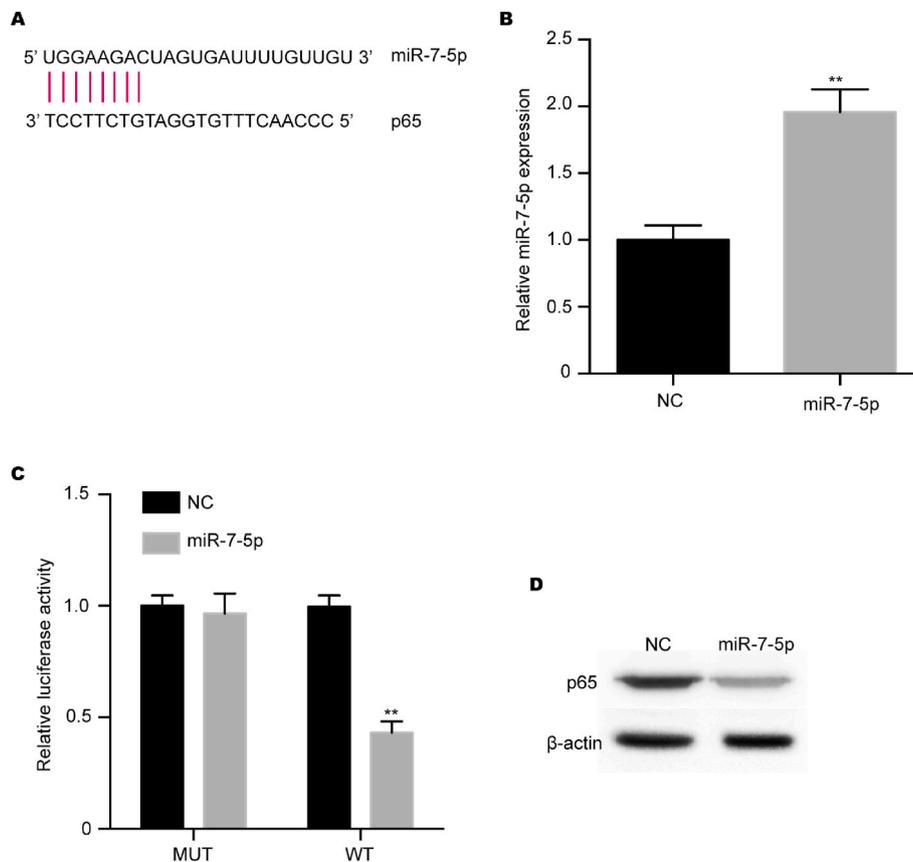
Fig. 3. MiR-7-5p represses NF-κB p65 in VSMCs. (A) The predicted binding site between miR-7-5p and p65. (B) The expression of miR-7-5p was measured by qPCR. (C) The luciferase activity of wild type p65 (WT) and p65mutant (MUT) was detected by dual-luciferase reporter assay. (D) The expression of p65 analyzed by Western blot. $**P < 0.01$.

VSMCs (Fig. 2F), as well as slowed down the confluence of cell scratch (Fig. 2G).

3.3. MiR-7-5p represses NF-κB p65 in VSMCs

Basing on the negative expression between miR-7-5p and p65 RNA level, we speculated that p65 may be a target of miR-7-5p. Prediction by TargetScan website indicated the potential binding site of miR-7-5p on p65 3′UTR region (Fig. 3A). Transfection with miR-7-5p mimics effectively elevated the miR-7-5p level in SMCs (Fig. 3B). Further luciferase reporter gene experiment demonstrated that miR-7-5p mimics notably suppressed the activity of luciferase reporter gene that contains wild type 3′UTR of p65 (WT), but not the vectors inserted with mutated sequences (MUT) (Fig. 3C). Results from western blotting assay confirmed that miR-7-5p mimics notably downregulated the protein level of p65 (Fig. 3D).

3.4. MiR-7-5p inhibits proliferation and migration of VSMCs by targeting NF-κB p65

To confirm that p65 mediate the miR-7-5p-modulated VSMCs proliferation ad migration, we performed co-transfection of miR-7-5p mimics and p65 overexpression vectors in VSMCs. Results from CCK-8 demonstrated that overexpression of p65 notably recovered the proliferation of PDGF-BB-stimulated VSMCs, compared with the miR-7-5p treatment (Fig. 4A), along with elevated expression of p65, MMP9 and PCNA (Fig. 4B) and suppressed cell apoptosis (Fig. 4C). Moreover, miR-7-5p-suppressed cell migration was recovered by p65 overexpression, manifested by increased portion of migrated cells (Fig. 4D) and enhanced cell confluence (Fig. 4E).

4. Discussion

Aberrant proliferation, differentiation, and migration of VSMCs play an important function in AS development. However, the molecular mechanism of VSMCs regulation is still obscure. In this research, we uncovered the critical role of miR-7-5p in AS and identified the potential mechanism.

miR-7-5p is involved in multiple disease models by regulating the targeted genes. It has been reported that miR-7-5p enhances activation of hepatic stellate cell by regulating fibroblast growth factor receptor 4 [27]. miR-7-5p modulates brain edema after intracerebral hemorrhage [28]. MiR-7-5p promotes cerebral ischemi/reperfusion injury by targeting sirt1 [29]. METTL14 induces cardiomyocyte ferroptosis by KCNQ1OT1/miR-7-5p/TFRC signaling [30]. MiR-7-5p inhibits metastasis of non-small cell lung cancer by regulating NOVA2 [20]. In this work, we observed the carotid artery injury in the AS rats and the treatment of miR-7-5p attenuated the phenotype in in vivo model and suppressed VSMCs migration *in vitro*. Besides, immunohistochemistry staining and Western blot analysis revealed that PCNA levels were increased in injured carotid arteries of the model rats and miR-7-5p could reverse the levels. The cell viability of VSMCs was induced by PDGF-BB but miR-7-5p blocked the phenotype. PDGF-BB decreased apoptosis of VSMCs, while miR-7-5p was able to restore the cell apoptosis in the model. PDGF-BB-induced migration of VSMCs was attenuated by miR-7-5p. These data suggest that miR-7-5p inhibits AS development *in vitro* and *in vivo*, providing new evidence of the role of miR-7-5p.

Moreover, it has been reported that lncRNA VINAS modulates AS by targeting MAPK and NF-κB signaling [31]. circ-Sirt1 regulates NF-κB activation by miR-132/212/SIRT1 in VSMCs [32]. Nicotine-mediated
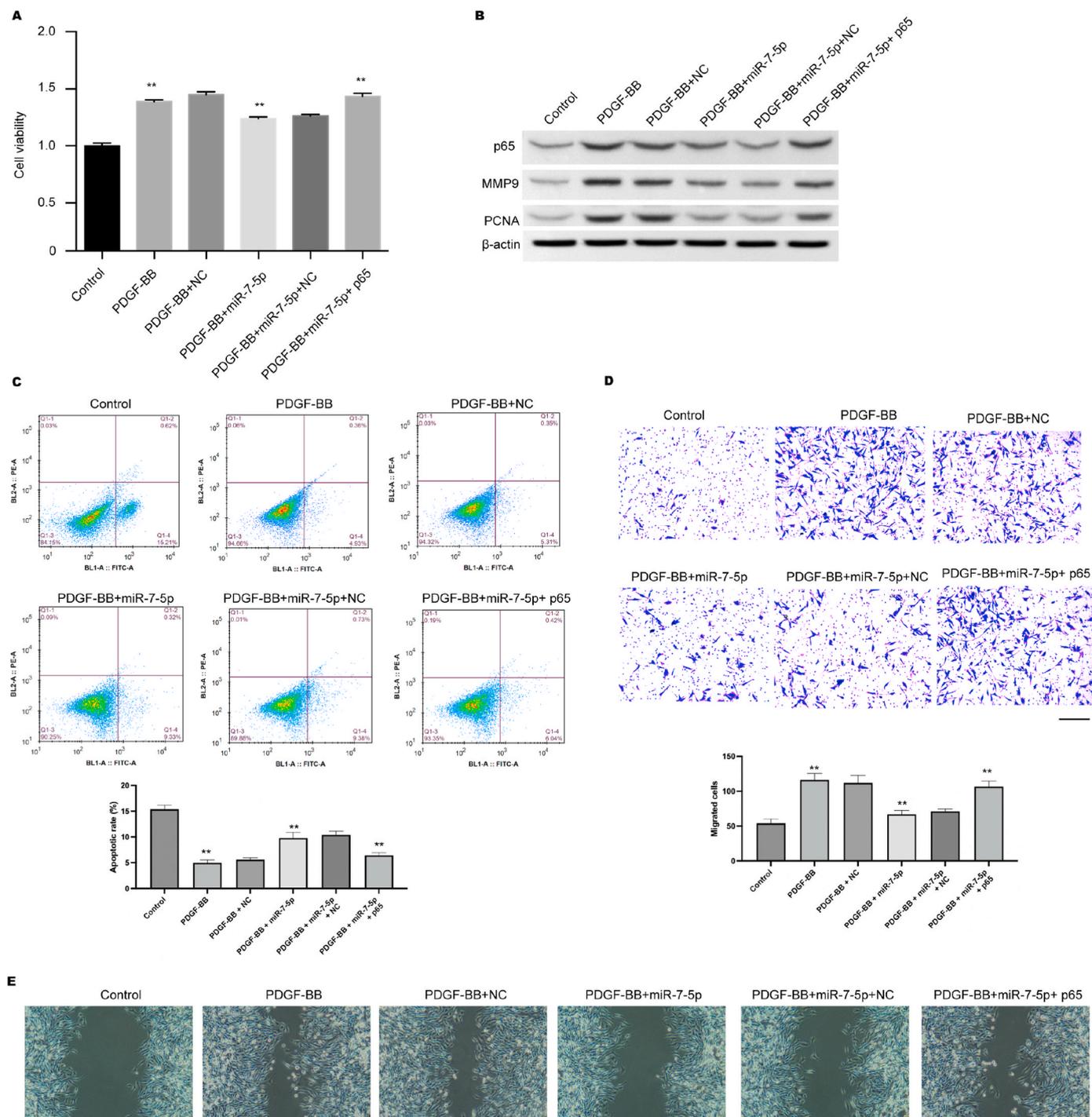
Fig. 4. MiR-7-5p inhibits proliferation and migration of VSMCs by targeting NF-κB p65. (A–E) The VSMCs were treated with PDGF-BB and miR-7-5p with or without p65 overexpressing plasmid. (A) Cell viability was measured by CCK-8 assays. (B) The expression of p65, MMP9 and PCNA was tested by Western blot. (C) The cell apoptosis was measured by flow cytometry analysis. (D and E) Cell migration was analyzed by transwell and wound healing assay. Scale bar, 50 μm **$P < 0.01$.

VSMCs autophagy promotes atherosclerosis by ROS/NF-κB signaling [33]. Here, in our work, we found that miR-7-5p mimic directly interact with the promoter region of p65 in VSMCs to suppress the expression levels of p65. Functionally, the PDGF-BB-promoted cell viability and migration of VSMCs was repressed by miR-7-5p and p65 overexpression reversed the phenotype. These data provide new insight into the mechanism by which miR-7-5p represses AS by targeting p65.

In summary, we concluded that miR-7-5p attenuates vascular smooth muscle cell migration and intimal hyperplasia after vascular injury by NF-kB signaling.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101394.

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