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# miR-145-5p Inhibits Vascular Smooth Muscle Cells (VSMCs) Proliferation and Migration by Dysregulating the Transforming Growth Factor- $\beta$ Signaling Cascade

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
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**Background:** There is accumulating evidence demonstrating that microRNAs (miRNA) play essential roles in proliferation, migration, and invasion of vascular smooth muscle cells (VSMCs). However, the exact function of these molecules and the mechanisms involved are not fully understood. In this study, we defined the role of miR-145-5p in VSMCs.





**Material/Methods:** This study used the PDGF-bb-induced VSMCs proliferation model. Expression of miR-145-5p and its target, Smad4, were detected and measured by real-time PCR and Western blot analysis. The luciferase reporter of miR-145-5p was used to elucidate miRNA-target interactions. The functions of miR-145-5p in proliferation and migration were detected by CCK-8 assay, Transwell assay, and scratch test.

**Results:** This study demonstrates that miR-145-5p is downregulated in PDGF-mediated VSMCs in both time- and dose-dependent manners. The *in vitro* results suggest that overexpression of miR-145-5p results in a reduction in SMAD4 and an increase in SMAD2, Smad3, and TGF- $\beta$  at the mRNA and protein levels. Overexpression of miR-145-5p inhibited PDGF-induced VSMCs proliferation and migration. Moreover, SMAD4 was identified as a direct target of miR-145-5p and is involved in PDGF-mediated VSMC proliferation. Downstream factors such as Smad2, Smad3, and TGF- $\beta$  were also influenced by miR-145-5p.

**Conclusions:** We identify miR-145-5p as a novel regulator of VSMC. Moreover, miR-145-5p inhibits VSMCs proliferation and migration by directly targeting Smad4 and dysregulating the transforming growth factor- $\beta$  signaling cascade, including Smad2, Smad3, and TGF- $\beta$ .

**MeSH Keywords:** **MicroRNAs • Muscle, Smooth, Vascular • Transforming Growth Factor beta**

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## Background

Vascular smooth muscle cells (VSMCs) are recognized as an essential component of the vascular system, including arteries, veins, and microvessels, and are essential for vascular integrity, with a key role in maintaining vascular structure [1]. The endothelial layer in arterial vessels protects other tissues from harmful substances in the blood and regulates blood pressure. In pathophysiological conditions, for example, with endothelial dysfunction or damage, VSMCs attempt to migrate to the intima and proliferate to supplement neointimal lesions [2]. Primary culture of VSMCs is a pivotal technique in *in vitro* models, which displays considerable plasticity and can be used to study molecular mechanisms related to a specific physiopathological process at the cellular level, ranging from contraction [3] to proliferation [4]. The migration and proliferation of VSMCs occurs in both physiological and biological processes [5]. Proliferation and migration of VSMCs lead to structural remodeling, which has a significant effect on the pathological processes of hypertension development [3]. These responses are influenced by miRNAs in vascular smooth muscle cells and other signaling pathways [6].

MicroRNAs (miRNAs) are now receiving increased attention due to their roles in many biological processes in both pathological and physiological states. miRNAs are recognized as endogenous, non-coding, single-stranded RNAs whose function is to bind the messenger RNAs (mRNA) of expressed genes of approximately 22 nucleotides and target them by posttranscriptional mechanisms for reduction or inhibition of translation, resulting in reduced expressed protein levels [7,8]. miRNAs play a key role in gene expression, and dysregulation of their synthesis causes many serious chronic diseases [9]. miRNAs play important roles in many physiological and pathological cellular process, such as proliferation [10–12], differentiation [13,14], migration [15], and apoptosis [15,16]. In a previous study, miR-145-5p was confirmed as a tumor suppressor in different types of cancers, such as gastric [17], breast [18], and prostate [19] cancers, and is associated with cancer metastasis [20]. Potential mechanisms have been investigated as miR-145-5p regulates the proliferation and migration by modulating various signal pathways, such as inhibiting the MAPK and PI3K/AKT pathways [21,22].

Based on the description above, accumulating evidence have shown that miR-145 plays a critical role in various diseases. A previous study has indicated that miR-145 is downregulated in dedifferentiated VSMCs, and is also a critical modulator of VSMCs proliferation and migration [23]. Additionally, overexpression of miR-143/145 promotes cells differentiation and inhibits the proliferation of VSMCs [24].

Characterizing the role of miR-145-5p and TGF- $\beta$ -regulated genes associated with vascular smooth muscle cells proliferation and migration can identify therapeutic targets and diagnostic markers. However, there has been no research exploring the potential relationship between miR-145-5p and TGF- $\beta$  cascade in the proliferation and migration of VSMCs. Therefore, in the present study, we assessed the role of miR-145-5p, TGF- $\beta$  signaling, and TGF- $\beta$ -regulated genes on the development of proliferation and migration by using the PDGF-bb-induced VSMCs proliferation model.

## Material and Methods

### Cell culture and treatment

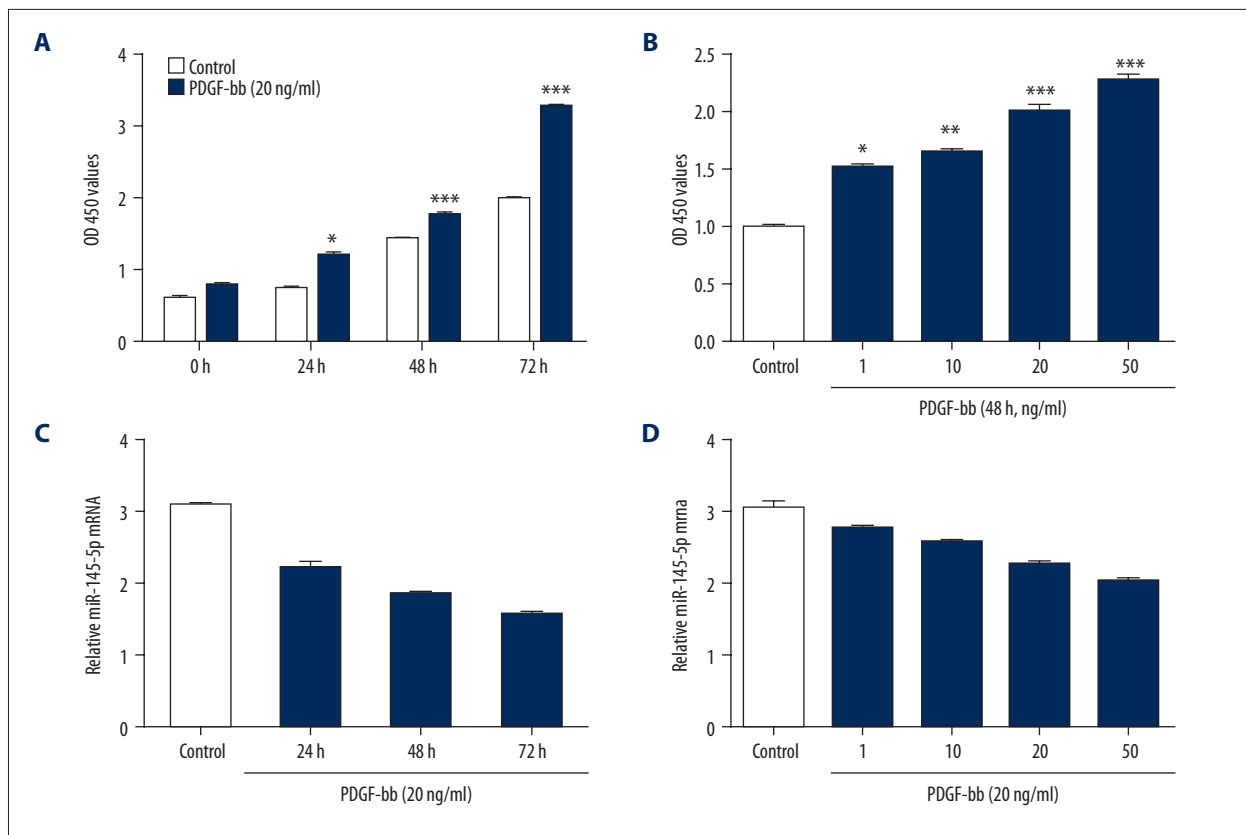
VSMCs were acquired from ATCC and cultured and maintained in DMEM supplemented with 10% fetal bovine serum (FBS). At the same time, 100 U/ml penicillin and 100  $\mu$ g/ml, streptomycin, epidermal growth factor 25 ng/ml, and basic fibroblast growth factor 10 ng/ml were added until confluent. VSMCs were grown to approximately 90% confluence in 75-cm<sup>2</sup> culture flasks. The culture temperature was maintained at 37°C in an incubator containing 5% CO<sub>2</sub>. The culture medium was changed every 3 days. For dissociation, the cells were dissociated with 0.025% trypsin and 0.1% EDTA every week. PDGF-bb (Promega) was added at 20 ng/mL concentration.

### Measurement of cell proliferation *in vitro*

Cell proliferation was measured by using CCK-8 cell viability assay. Briefly, approximately 5×10<sup>3</sup> VSMCs were distracted into each well of a 96-well plate, and cultured in an incubator at 37°C. The next day, VSMCs were transferred into 100  $\mu$ L medium containing 10% fetal bovine serum at indicated concentrations and incubated for 30 h. After that, 10  $\mu$ L CCK-8 reagent was added into each well of the 96-well plate and incubated at same temperature for 4 h. Finally, the 96-well plate plates were shaken for 10 min and the OD values were read at 450 nm.

### Measurement of cell migration *in vitro*

For measurement of migration, we used the wound healing assay and the Transwell migration assay. Wound healing assays were used as follows: VSMCs were cultured in 10% FBS-DMEM, then the serum in the medium were deprived for 18 h. Later, the confluent monolayer was scratched with a sterile pipette tip. After that, the VSMCs were washed with PBS and fresh medium was added. VSMCs were cultured and maintained in a stable and suitable environment for 24 h. Migration was measure by phase-contrast microscopy at different time points up to wound healing closure.



**Figure 1.** miR-145-5p is inhibited by PDGF-bb in VSMCs. (A) PDGF-bb induce VSMCs proliferation time-dependently. (B) PDGF-bb induced VSMCs proliferation dose-dependently. (C) The expression of miR-145-5p was measured by qRT-PCR, showing that miR-145-5p was downregulated. (D) The expression of miR-145-5p was measured by qRT-PCR, showing that miR-145-5p was downregulated. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

Transwell migration assay was conducted by following the manufacturer's protocol. Briefly, the cells migrating through the Transwell membrane were fixed with 3.7% paraformaldehyde. The images of the migrating cells stained with crystal violet were obtained using a Nikon Eclipse microscope with a Nikon DSR1 camera. The number of migrating cell was counted with ImageJ software.

### Plasmids and miRNA transfection

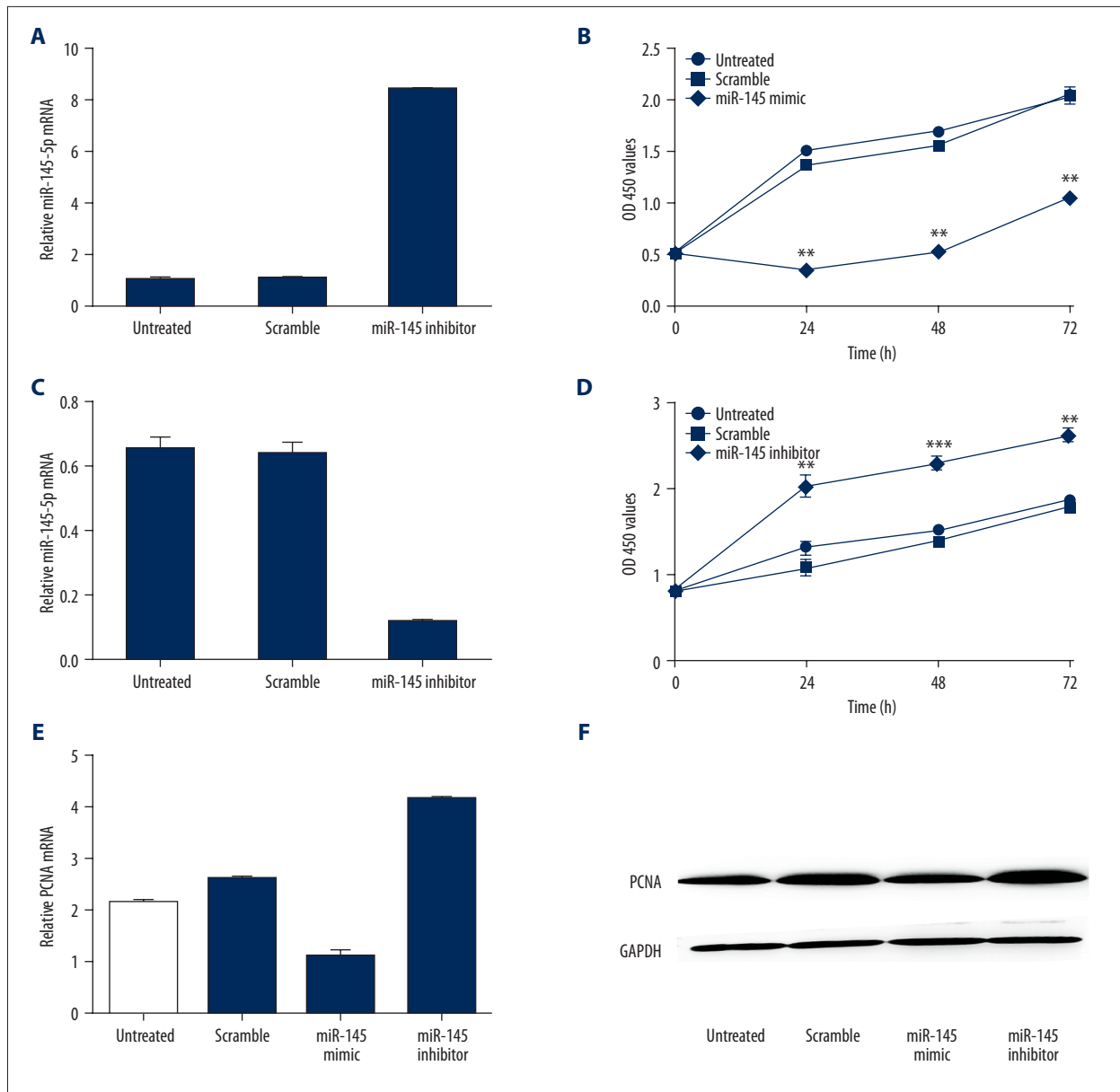
Potential target mRNAs of miR-145-5p were roughly searched on 3 websites including <http://www.targetscan.org/>, <http://www.microna.org/>, and <http://mirdb.org/>. miR-145-5p mimics and inhibitors were purchased from Ambion (mirVana miR-145-5p mimic: MC11051; mirVana miR-145-5p inhibitor: MH11051). After 24 h of transfection, total RNA and protein were extracted from the cells and assessed using qRT-PCR and Western blot analysis, respectively.

### Luciferase assay

We cultured  $1 \times 10^5$  HEK293 cells, then transfected them with Smad 4 R1-3'UTR-wt or Smad4-3'UTR-mt and mi-145-5p using Lipofectamine 2000 (Invitrogen, USA) according to the protocol previously described. Luciferase was measured 24 h after transfection by using the Dual Luciferase Reporter Assay System (Promega) and normalized to Renilla luciferase activity.

### Flow cytometry

VSMCs were incubated with culture medium for 24 h after transfection with miR-145-5p mimics, inhibitor, or negative control. For apoptosis measurements, VSMCs were collected, washed with PBS, resuspended in 100  $\mu$ l of 1 $\times$  binding buffer, and stained with 5  $\mu$ l Annexin V and 5  $\mu$ l of PI (Becton-Dickinson) at room temperature for 15 min in the dark. After that, we performed cell cycle analysis, in which VSMCs were washed with cold PBS and fixed with 70% ethanol overnight at  $-20^\circ\text{C}$ . Fixed cells were rehydrated in PBS for 10 min and subjected to PI/RNase staining. A flow cytometer was utilized to evaluate the apoptotic levels and cell cycle in each sample, following the manufacturer's protocol.

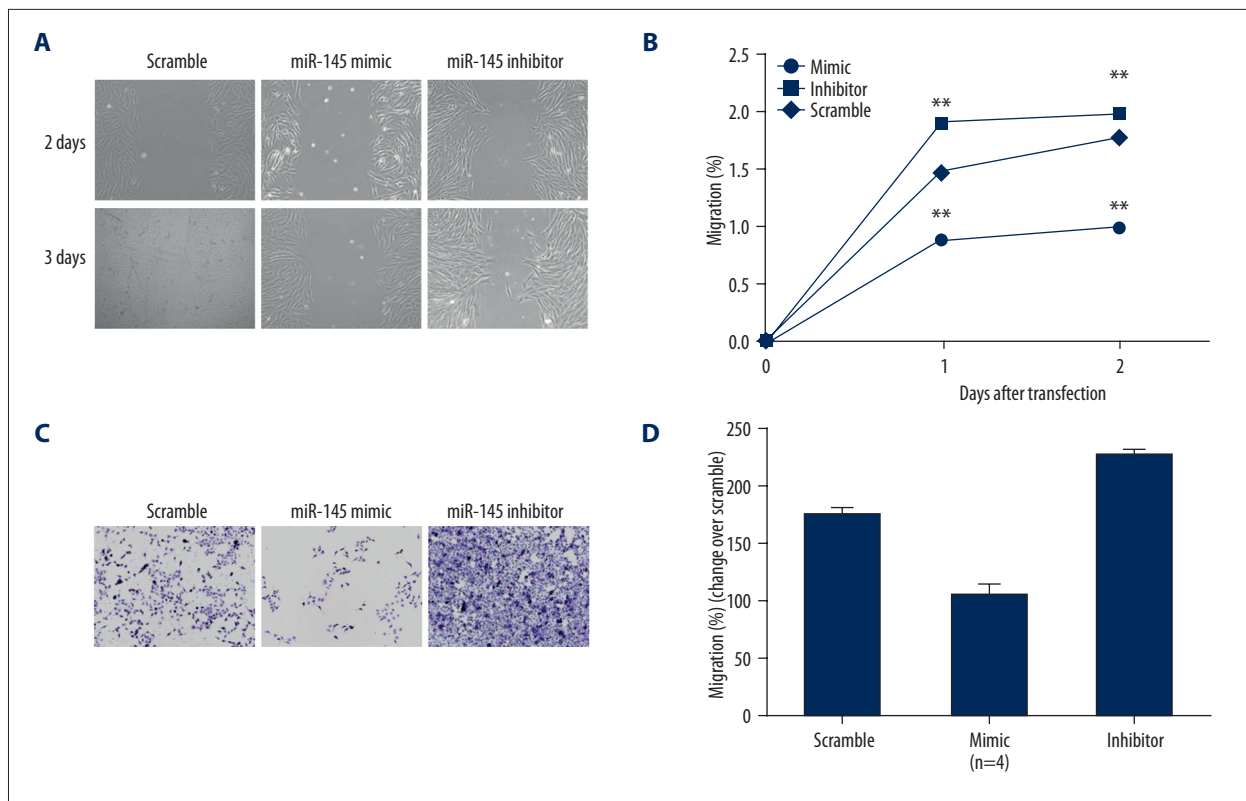


**Figure 2.** Overexpression of miR-145-5p diminishes the proliferation of VSMCs. **(A)** qRT-PCR analysis of miR-145-5p in VSMCs upon transfection of miR-145-5p mimics. U6 snRNA was used as internal control. **(B)** The CCK8 proliferation assay showed that miR-145-5p overexpression diminishes the VSMCs proliferation. **(C)** qRT-PCR analysis of miR-145-5p in VSMCs upon transfection of miR-145-5p inhibitor. **(D)** The CCK8 proliferation assay showed that miR-145-5p downregulation induced VSMCs proliferation. **(E)** qRT-PCR analysis of PCNA in VSMCs upon transfection of miR-145-5p mimics, inhibitor, scramble, or control. **(F)** Western blot analysis of PCNA in VSMCs upon transfection of miR-145-5p mimics, inhibitors, scramble, or control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

### RNA isolation and PCR

Total RNA was isolated from VSMCs samples using Trizol reagent (Invitrogen, USA) and quantified by absorbance at 260 nm. One microgram of RNA was used to perform the reverse transcription with a High-Capacity cDNA Archive kit (Applied Biosystems, USA). Finally, real-time PCR was performed on an

ABI Prism 7500 fast sequence detection PCR system (Applied Biosystems) according to the manufacturer's instructions, and after the process,  $\Delta\Delta Ct$  method was used to analyze the data.



**Figure 3.** miR-145-5p attenuated cell migration, whereas miR-145-5p inhibitor potentiated cellular movement. (A) The wound healing assay was performed. (B) Quantification results of the wound healing assay. (C) Transwell migration assay. (D) Quantification results. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

### Western blotting

Western blot methods were performed on protein extracts from VSMCs as previously described. Briefly, VSMCs were lysed with ice-cold RIPA buffer and supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Later, equal amounts of extracts (30  $\mu$ g) were electrophoresed on a sodium dodecyl sulfate (SDS) polyacrylamide gel and electroblotted to nitrocellulose filter membranes (Millipore). Then, membranes were immersed in blocking buffer with 5% nonfat milk powder for 1 h and incubated with antibodies against Smad3, Smad2, Smad4, PNCA, TGF $\beta$ , or GAPDH overnight at 4°C. Finally, they were incubated with horseradish peroxidase-conjugated secondary antibodies and the protein bands were visualized using the Super Signal chemiluminescent detection module.

### Statistical analysis

All data were analyzed using GraphPad Prism 5.1 software. Results are expressed as the mean  $\pm$  standard deviation. When only 2 groups were compared, the double-sided *t* test was used to assess statistical differences. Significant statistical differences between groups were analyzed using one-way ANOVA. *P* value less than 0.05 ( $p < 0.05$ ) was considered statistically significant.

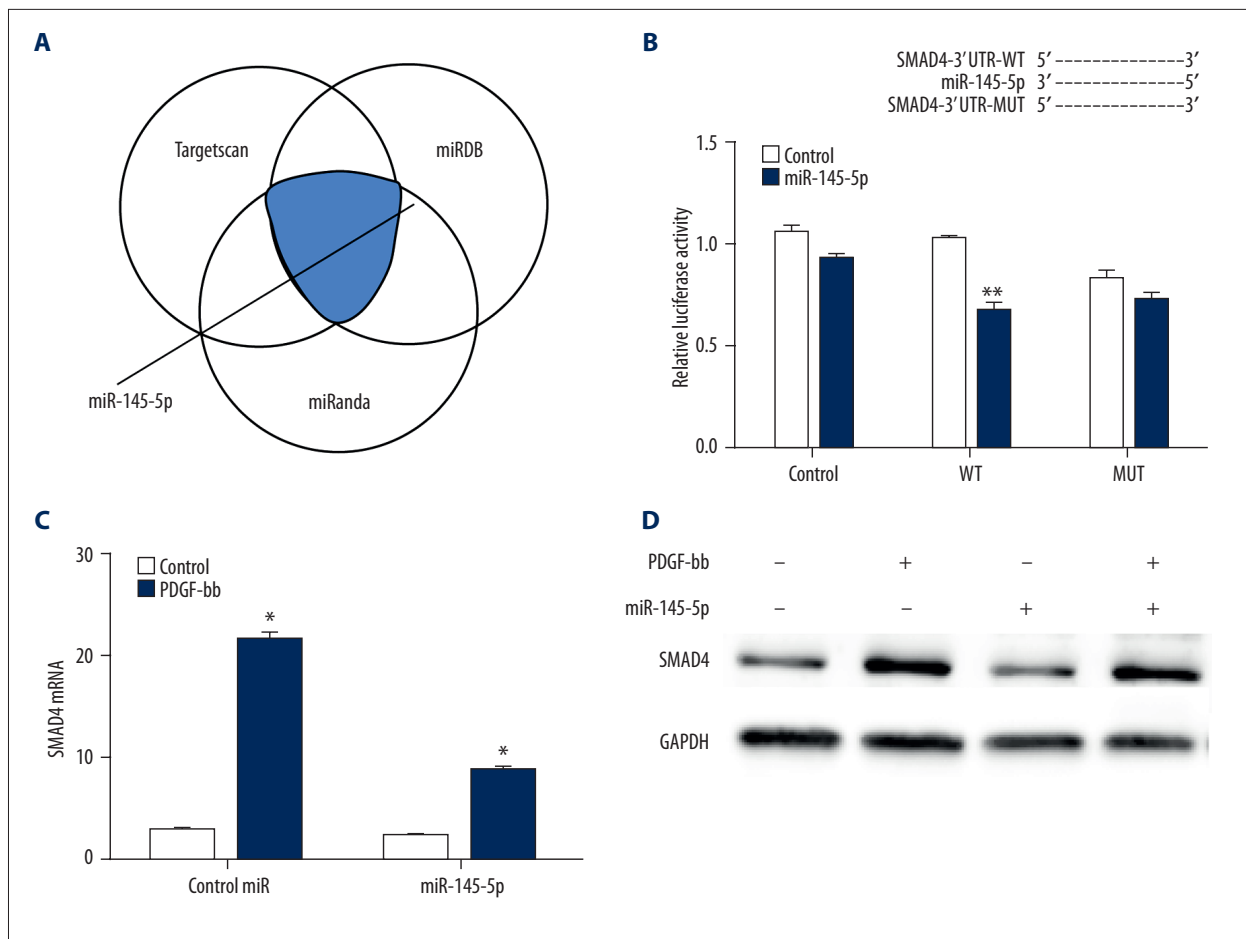
## Results

### miR-145-5p is suppressed by PDGF-bb in VSMCs

First, we examine the VSMCs proliferation using CCK8 assay. PDGF-bb induced VSMCs proliferation in time-dependent and dose-dependent manners (Figure 1A, 1B). Then, we assessed the miR-145-5p in VSMCs which treated by PDGF-bb. The results demonstrated that miR-145-5p was downregulated after using PDGF-bb (Figure 1C, 1D).

### miR-145-5p diminishes the PDGF-mediated VSMCs proliferation

To assess the function and molecular mechanism of miR-145-5p in VSMCs, we used miR-145-5p mimics and inhibitors. miR-145-5p expression was increased in VSMCs transfected with miR145-5p mimics (Figure 2A) and decreased in VSMCs transfected with miR-145-5p inhibitor (Figure 2C). For proliferation, the results of CCK-8 assay demonstrated that the growth rate of VSMCs was decreased in cells transfected with miR-145-5p mimics compared with cells transfected with scramble mimics or that were untreated (Figure 2B). In the meantime, miR-145-5p inhibitor enhanced the VSMCs



**Figure 4.** Smad4 is a direct target of miR-145-5p in VSMCs. (A) Venn diagrams show the number of genes identified as potential targets of miR-145-5p using 3 predicting search programs of TargetScan, miRanda and miRDB. (B) Relative luciferase activity was analyzed after wild-type or mutant 3'-UTR reporter plasmids were co-transfected with miR-145-5p in VSMCs. (C, D) qRT-PCR detection of Smad4 mRNA expression in VSMCs transfected with miR-145-5p mimics or miR-145-5p inhibitors. U6 was used as an internal control. (E) Western blot detection of Smad4 protein expression in VSMCs transfected with miR-145-5p mimics or miR-145-5p inhibitors. GAPDH was used as an internal control. \*  $p < 0.05$  and \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

proliferation compared with scramble and untreated groups (Figure 2D). We also confirmed that overexpression of miR-145-5p inhibited both mRNA and protein expression of PCNA in VSMCs (Figure 2E, 2F).

### Overexpression of miR-145-5p attenuates migration of VSMCs

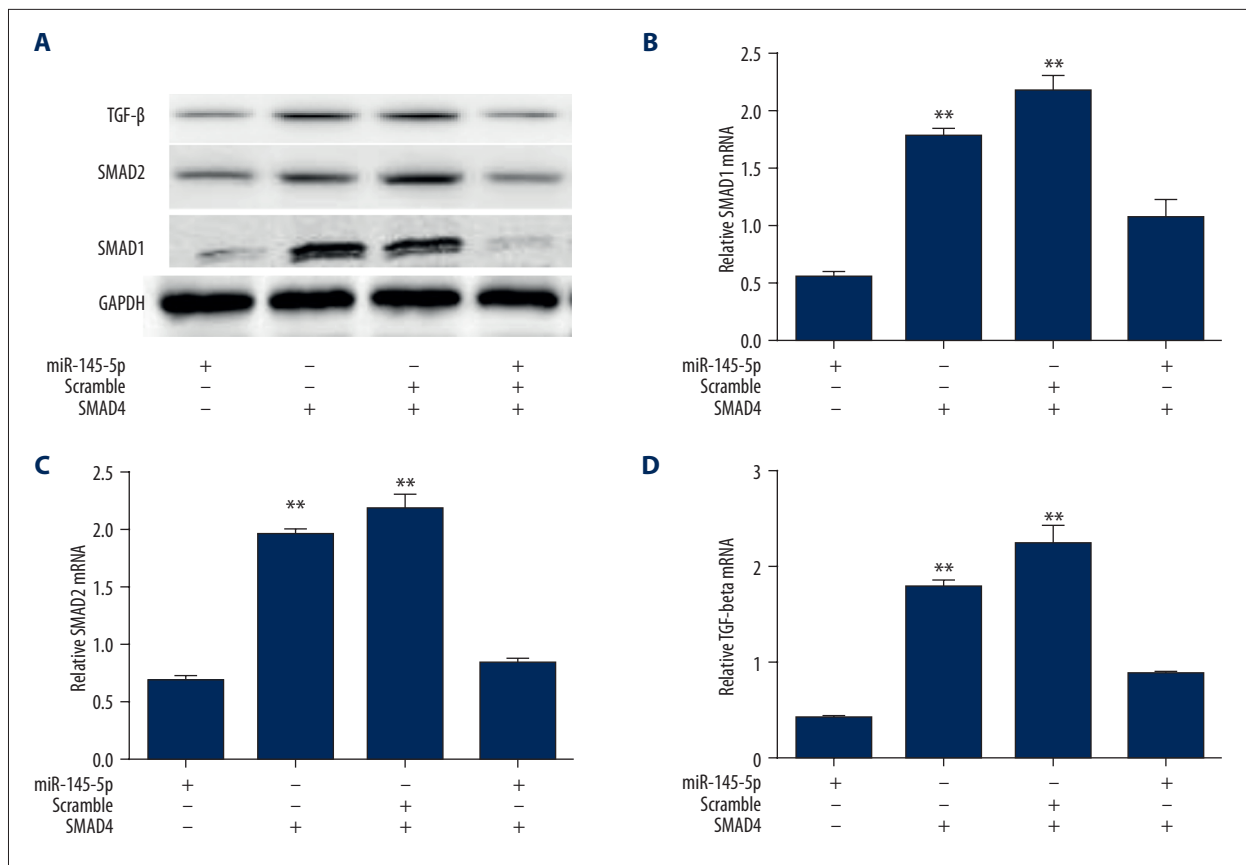
VSMCs migration is an essential component of atherogenesis and neointimal formation, in which the vessel wall is being remodeled [25]. Thus, we investigated whether miR-145-5p affected migration of VSMC. The wound healing assay and Transwell migration assay were used. Transfection of miR-145-5p mimic attenuated wound closure, which indicates reduced cell motility. In contrast, miR-145-5p inhibitor potentiated cellular movement (Figure 3A). The effects of miR-145-5p mimic and

inhibitor on cellular migration were quantified by measuring the cell-free area (Figure 3B). The results demonstrated that miR-145-5p attenuates migration of VSMCs. A Transwell migration assay also revealed that miR-145-5p negatively influences cell movements (Figure 3C, 3D).

### miR-145-5p targeted Smad4 in VSMCs

To evaluate the underlying further mechanisms of the suppressive effects of miR-145-5p on VSMCs proliferation, we used different bioinformatics methods to help find the target genes of miR-145-5p. Among the targets predicted by TargetScan, miRanda, and miRDB, Smad4 was the gene that was localized at the 3 main centers of the net-comprised genes (Figure 4A, 4B).





**Figure 5.** Overexpression of miR-145-5p repressed the Smad1, Smad2, and TGF- $\beta$  expression. (A) Expression of Smad1, Smad2, and TGF- $\beta$  were detected using Western blotting. GAPDH was used as a loading control. (B) The mRNA expression of Smad1 was detected using qRT-PCR. (C) The mRNA expression of Smad2 was detected using qRT-PCR. (D) The mRNA expression of TGF- $\beta$  was detected using qRT-PCR. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .

To confirm that miR-145-5p can regulate Smad4 expression by directly binding to the Smad4 3'-UTR, we generated luciferase reporter constructs containing specific mutations at the putative miR-145-5p binding site. As shown in Figure 4C, when cells were transfected with the wild-type Smad4 3'-UTR, co-transfection of miR-145-5p mimics inhibited luciferase activity. The result shows that miR-145-5p binds directly to the putative Smad4 3'-UTR regions predicted. Moreover, we found that both protein and mRNA levels of Smad4 increased in VSMCs transfected with miR-145-5p mimics and decreased in VSMCs transfected with miR-145-5p inhibitors (Figure 4C, 4D).

#### Overexpression of miR-145-5p suppressed growth factor production

We conducted Western blot and RT-PCR analyses to detect the downstream signaling molecules Smad3, SMAD2, and TGF- $\beta$  in the presence or absence of Smad4, scramble, or miR-145-5p, respectively. According to Western blot results, Smad3, Smad2, and TGF- $\beta$  protein expression decreased with the treatment of miR-145-5p compared with scramble (Figure 5A). The results

of RT-PCR showed that Smad4 enhanced the mRNA expression of Smad3, Smad2, and TGF- $\beta$ . However, after miR-145-5p treatment, Smad3, Smad2, and TGF- $\beta$  proteins and mRNA were significantly downregulated in the VSMCs (Figure 4B–4D).

#### Smad4 has a critical role in miR-145-5p-mediated VSMCs proliferation

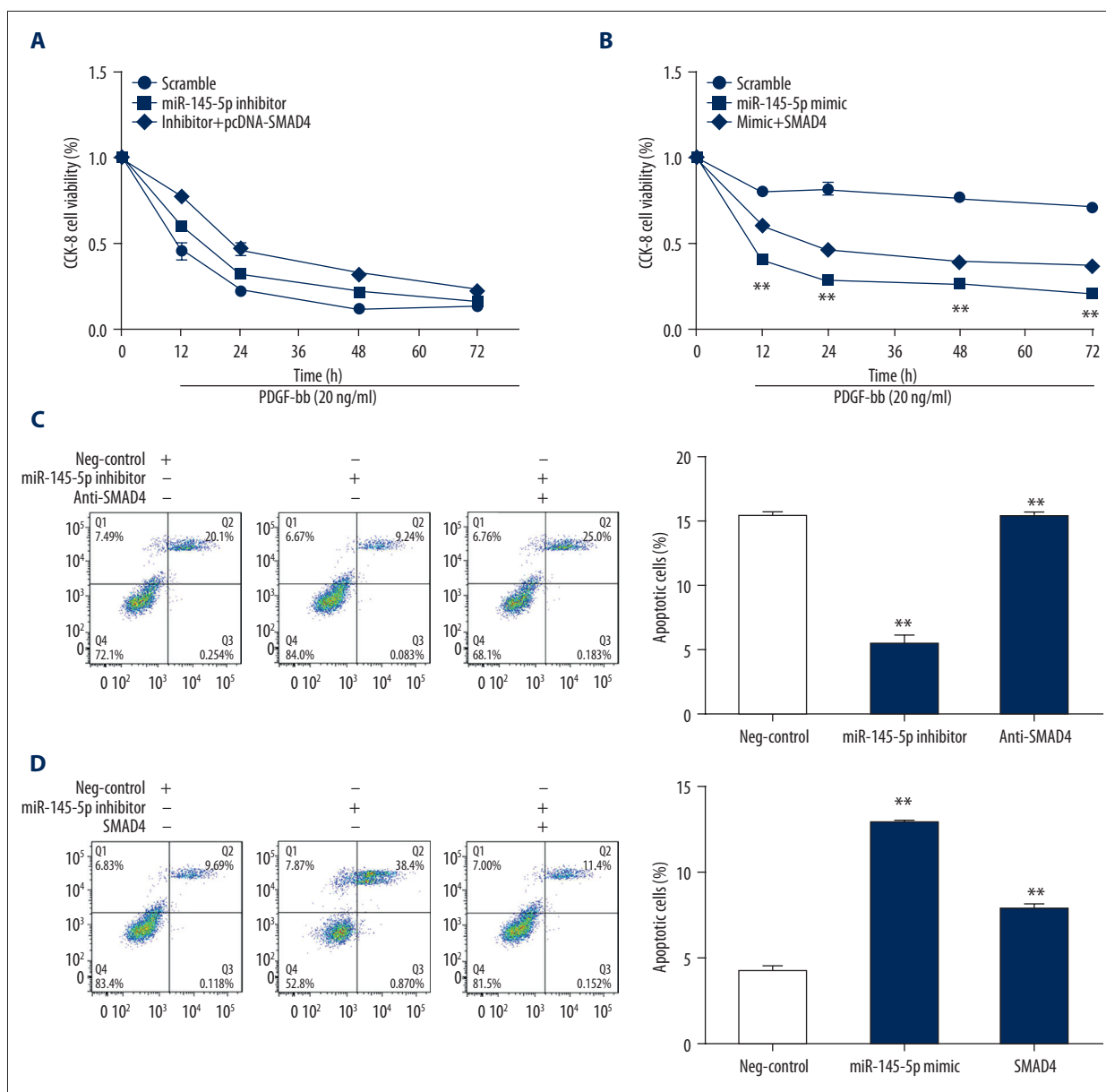
We further investigated whether Smad4 was responsible for VSMCs proliferation after regulating miR-145-5p expression. We used the VSMCs co-transfected with either miR-145-5p mimic or scramble and si-Smad4 or pcDNA empty vector, and CCK-8 proliferation assay, flow cytometric analysis, wound healing assays and Western blotting were also used. As shown in Figure 6A–6D, when miR-145-5p mimic and si-Smad4 were co-transfected into VSMCs, miR-145-5p expression significantly suppressed the VSMCs proliferation in CCK-8 proliferation assay, flow cytometric analysis, and wound healing assays. Furthermore, PCNA protein levels were correlated with both miR-145-5p and Smad4 (Figure 6E). These results indicate that Smad4 has a critical role in miR-145-5p-mediated VAMCs proliferation.

## Discussion

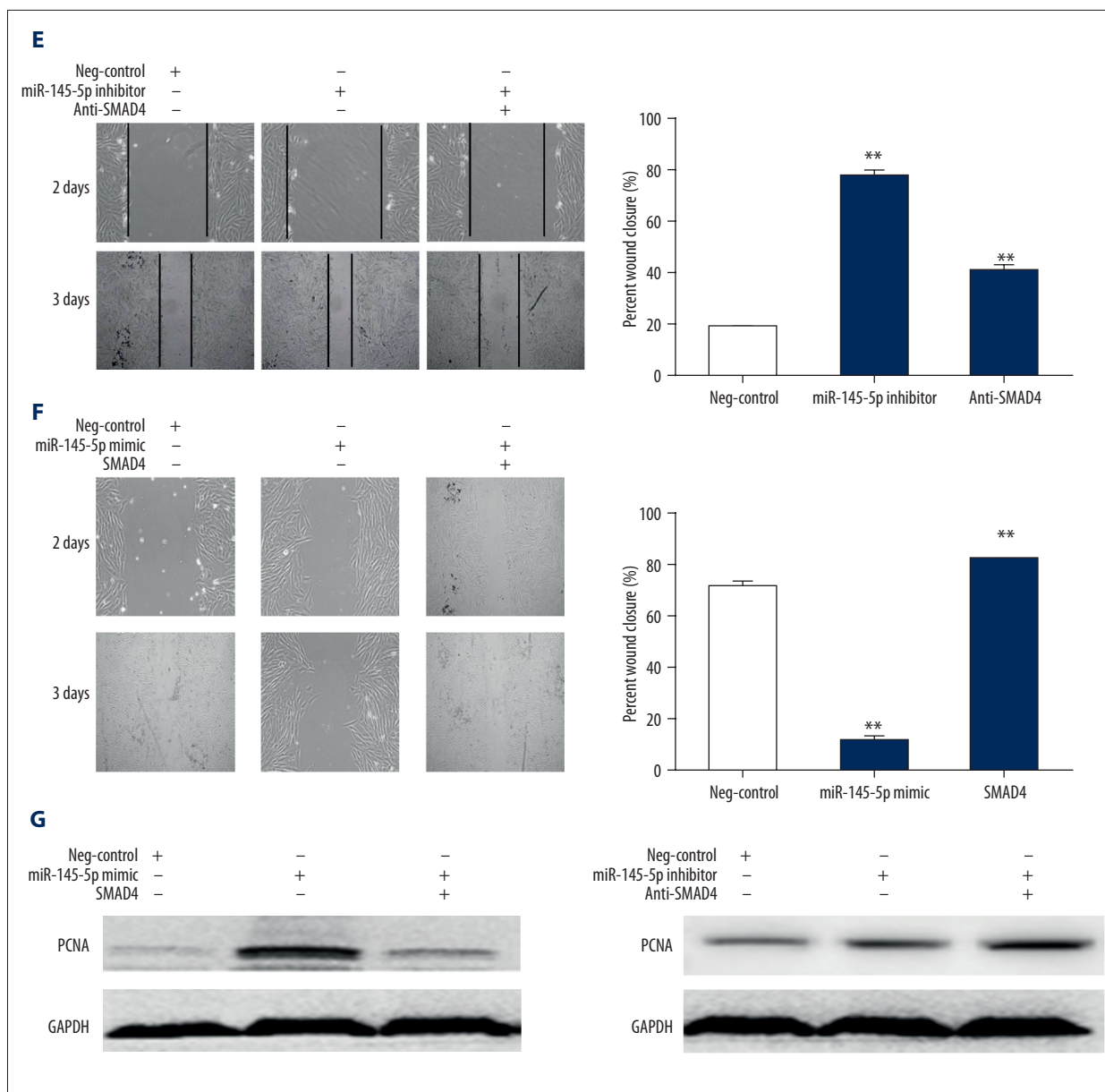
miR-145-5p is considered to be a tumor suppressor and a critical regulator of human stem cell growth and differentiation. Previous reports showed low levels of miR-145-5p in colon [26,27], breast [28], prostate [29], and ovarian [30] cancers. Furthermore, many miR-145-5p target genes have been confirmed, such as ISR-1, p70S1K1, ER, RTKN, and ZEB2. In the present study, we identified a novel role of miR-145-5p in the regulation of the transforming growth factor- $\beta$  signaling cascade, showing that overexpression of miR-145-5p led to a remarkable decrease in Smad4 gene expression, which is a key signaling molecule of the TGF- $\beta$ /Smads pathway. Exogenous overexpression of miR-145 effectively suppressed the high

glucose-induced excessive proliferation and migration of VSMCs and ROCK1 as a downstream target gene product of miR-145 [31].

This signaling pathway is found among patients with vascular disorders, indicating the essential role of TGF- $\beta$  pathways in vascular homeostasis. TGF- $\beta$  is an important member of the TGF- $\beta$  superfamily, which includes activins, inhibins, growth and differentiation factors, and bone morphogenetic proteins. For many diseases, such as colorectal [32], breast, prostate, and hepatocellular [33] cancers, TGF- $\beta$  plays an essential role in regulating cell proliferation and migration. The TGF- $\beta$  signaling pathway takes part in many physiological and pathological processes and plays a pleiotropic role in diverse functions







**Figure 6.** SMAD4 has a critical role in miR-145-5p-mediated VSMCs proliferation. (A) CCK analysis of cell proliferation in VSMCs with either miR-145-5p inhibitor or scramble and si- Smad4 or pcDNA empty vector. (B) CCK analysis of cell proliferation in VSMCs with either miR-145-5p mimic or scramble and si-Smad4 or pcDNA empty vector. (C) Flow cytometric analysis of apoptosis in VSMCs transfected with miR-145-5p inhibitors, miR-145-5p inhibitors/si-Smad4, and control. (D) Flow cytometric analysis of apoptosis in VSMCs transfected with miR-145-5p mimics, miR-145-5p mimics/Smad4, and control. (E) Wound healing assays in VSMCs transfected with miR-145-5p inhibitors, miR-145-5p inhibitors/si-Smad4 and control. (F) Wound healing assays in VSMCs transfected with miR-145-5p mimics, miR-145-5p mimics/Smad4, and control. (G) Western blot detection of PCNA protein in VSMCs upon transfection of miR-145-5p inhibitors, miR-145-5p inhibitors/si-Smad4, miR-145-5p mimics, miR-145-5p mimics/Smad4, and control. \* p<0.05, \*\* p<0.01, and \*\*\* p<0.001.

of diseases, including embryogenesis [34], immune responses [35], Fanconi anemia [36], and diabetes [37]. The association of TGF-β1 and Smad is well understood and is considered to be an important pathway in many pathophysiological processes [38]. However, a potential role for Smad transcription

factors in TGF-β1 cascade has never been investigated [39,40]. In the present study, we focused on the key regulator Smad4 in the TGF-β/Smads pathway. Smad4, as a member of the Smads family of signal transducers from TGF-β, mediates proliferation and apoptosis in many cell lines [41–43]. In VSMCs,

Zhang et al. indicating that Smad4-dependent TGF- $\beta$  signaling in VMSCs protects against aortic aneurysm formation and dissection [43]. Other members of the Smads family, including Smad3, Smad2, and Smad1, share similar structures. Despite their similarities, Smad2 and Smad3 have differential roles in TGF $\beta$  signal transduction. For example, Smad2 knockout mice die during gastrulation [44,45], and Smad3 knockout mice develop cartilage for several months after birth [46,47]. However, they both play essential roles in the transforming growth factor- $\beta$  signaling cascade.

In our study, firstly, we identified that miR-145-5p diminishes the PDGF-mediated VSMCs proliferation and migration by CCK-8 assay. To further identify the molecular mechanism by which miR-145-5p regulates VMSCs migration and proliferation, we searched for potential target genes of miR-145-5p via microRNA.org, TargetScan, and miRDB. The results of Luciferase assay demonstrated that Smad4 was a direct target of miR-145-5p. Western blotting assay and RT-PCR revealed that overexpression of miR-145-5p resulted in the downregulation of Smad4 at protein and mRNA levels. Many factors in the TGF- $\beta$ 1/Smad cascade, including Smad3, Smad2, and TGF- $\beta$  protein expression, were influenced by overexpression of miR-145-5p, indicating that miR-145-5p also plays an important

role in the TGF- $\beta$ 1/Smad cascade. Finally, we used si-SMAD4 and miR-145-5p mimic to co-transfect VSMCs, indicating that Smad4 has a critical role in miR-145-5p-mediated VAMCs proliferation. miR-145 is overexpressed in exercise, remodels arterioles in hypertension, and induces VSMCs to maintain a contractile phenotype, in which miR-145 appears to be involved by inversely regulating Akt signaling via its upstream signal [48].

## Conclusions

We demonstrated that miR-145-5p inhibited cell proliferation and suppressed migration and invasion in VMSCs *in vitro*. Furthermore, miR-145-5p performs a biological function through directly targeting Smad4 and influences the TGF- $\beta$ 1/Smad cascade. Thus, miR-145-5p plays suppressive roles in VMSCs. Our findings open new avenues for the study of the TGF- $\beta$ -family signaling cascade and miRNA biogenesis regulation and suggests that miR-145-5p/Smad4 is a promising prognostic and therapeutic target in vascular disease.

## Conflicts of interest

None.

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