

Original Article

MicroRNA-21 inhibits platelet-derived growth factor-induced human aortic vascular smooth muscle cell proliferation and migration through targeting activator protein-1

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Abstract: Objectives: This study is to investigate whether microRNA (miR)-21 inhibits platelet-derived growth factor-induced human aortic vascular smooth muscle cell (VSMC) proliferation and migration through targeting activator protein-1 (AP-1). Methods: VSMCs were transfected with the miR-21 or miR-21 inhibitor. Cell proliferation was determined using methyl thiazolyl tetrazolium assay. Cell migration was detected by transwell assay. Luciferase reporter assay was used to study the interaction between miR-21 and AP-1. The levels of mRNA were determined using quantitative real-time polymerase chain reaction, while protein expression was measured using Western blotting assay. Results: Low expression of miR-21 significantly inhibited VSMC proliferation, invasion and migration. The mRNA levels and protein expression of α -SMA and AP-1 were down-regulated by low expression of miR-21. In addition, luciferase reporter assay demonstrated that AP-1 might be a direct target gene of miR-21 in VSMC initiation and development. Moreover, up-regulation of AP-1 was critical for miR-21-mediated inhibitory effects on platelet-derived growth factor-induced cell proliferation and migration in human VSMCs. Conclusions: In summary, miR-21 is a key molecule in regulating human VSMC proliferation and migration by targeting AP-1, suggesting that specific modulation of miR-21 in human VSMCs may become an attractive approach for the treatment of proliferative vascular diseases.

Keywords: microRNA-21, vascular smooth muscle cells, proliferation, migration, activator protein-1, platelet-derived growth factor

Introduction

Vascular smooth muscle cells (VSMCs) exhibit remarkable plasticity during postnatal development. Vascular injury initiates and perpetuates VSMCs dedifferentiation to a synthetic phenotype, which has been increasingly recognized to play a central role in neointimal hyperplasia during the pathogenesis of vascular proliferative diseases [1, 2]. Moreover, differentiated VSMCs demonstrate a very low rate of proliferation, appropriate contractility to contractile cues, and express smooth muscle cell (SMC)-specific proteins, such as smooth muscle α -actin, smooth muscle myosin heavy chain (SM-MHC), smooth muscle (SM) 22 α , and calponin.

In response to vascular injury or growth factor signaling, VSMCs dedifferentiate and adopt a synthetic phenotype, which is characterized by increased proliferation and migration, enhanced production of collagens and matrix metalloproteinases, and diminished expression of SMC-specific contractile markers [3-5]. Recent studies indicated that many microRNAs (miRNAs or miR) are highly expressed in vascular system and involved in the control of proliferation and differentiation of VSMCs [6, 7].

miRNAs are a novel class of regulatory non-coding RNAs that regulate gene expression at post-transcriptional level by binding to 3'-untranslated regions (3'-UTR) of target mRNAs [8, 9].

miR-21 inhibits VSMC by targeting AP-1

Recently, miRNAs are known to regulate the expression of target genes by degrading target mRNAs or repressing target mRNA translation [10, 11]. In recent years, growing pieces of evidence suggest that miRNAs are critical regulators of widespread cellular functions, such as differentiation, proliferation, migration, and apoptosis [12, 13]. Moreover, miRNAs are implicated in the development of a variety of important human diseases including cancer [14], cardiovascular diseases [15] and diabetes [16].

Growth factor signaling pathways are involved in the phenotype modulation of VSMCs [17, 18]. Growth factor signals, such as the transforming growth factor β and platelet-derived growth factor (PDGF), modulate cell growth and migration of VSMCs directly or indirectly by transcriptional regulation of the target gene expression [19, 20]. Recent reports demonstrated that miR-221 and miR-222 are also implicated in the modulation of VSMC differentiation [21, 22]. miRNA expression can be transcriptionally induced by PDGF signaling to mediate its action on VSMC phenotypic switching [23-27]. Moreover, bone morphogenic protein signaling represses the expression of miR-302-367 gene cluster for the autoregulatory mechanism of the bone morphogenic protein signaling pathway [28].

It has been shown that down-regulation of miR-15b decreased the expression of alpha-smooth muscle actin (α -SMA), a differentiation marker of VSMCs, suggesting that miR-15b was possibly functional in the regulation of VSMC differentiation and proliferation [29]. Moreover, miR-143 and miR-145 play important roles in the control of contractile phenotype of VSMCs and the response of the vascular wall to injury [30]. However, it is unclear whether miR-21 expression is modulated by PDGF, which is the essential signal for VSMC dedifferentiation. In this study, we investigate whether miR-21 is a key molecule in regulating VSMC proliferation and migration by targeting AP-1.

Materials and methods

Cells

Human aortic smooth muscle cell line was obtained from Shanghai Cell Bank, Chinese Academy of Sciences. The cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Calsbad, CA, USA) containing 10%

fetal bovine serum (Cell Application, Santiago, CA, USA), 100 IU/ml penicillin and 100 μ g/ml streptomycin in humidified 5% CO₂ at 37°C. For transfection, cells were grown up to 90% confluence and transfected with miRNA plasmids using Lipofectamine 2000 (Invitrogen, Calsbad, CA, USA) before incubation with DMEM for 4 h. The cells were then transferred into fresh endothelial basal medium-2 containing 10% fetal bovine serum.

Wound healing assay

The cultured cells were transfected with 50 nM miR-21 inhibitor, miR-21 mimic or negative control. Transfected VSMCs were seeded in 12-well plates and incubated at 37°C in 5% CO₂ until a complete monolayer was formed. Cells were co-transfected with a green fluorescent protein vector to track the migration of cells. The cells were then washed and allowed to migrate for 0 or 24 h. Photographs were taken at 0 and 24 hours to assess the level of migration in each group of cells. Migration was quantified by counting the total number of cells that migrated towards the original wound field. The experiment was repeated for three times.

Methyl thiazolyl tetrazolium (MTT) assay

Cell growth was determined using methyl thiazolyl tetrazolium spectrophotometric dye assay according to published protocols [31]. At 24 h after the transfection with miR-21 inhibitor, miR-21 mimic or negative control, VSMCs were seeded into 96-well plates (2×10^3 cells/well), and cell proliferation was documented every 24 h for 4 days. The absorbance of each plate was read at 490 nm using a plate reader (Thermo Scientific, Watertown, USA).

Colony formation assay

Cells were transfected with 50 nM of miR-21 inhibitor, miR-21 mimic or negative control, and cultured in media containing 10% fetal bovine serum. After incubation for 15 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted. Triplicate wells were measured for each group.

Migration and invasion assay

The cells were transfected with miR-21 inhibitor, miR-21 mimic or negative control. For tran-

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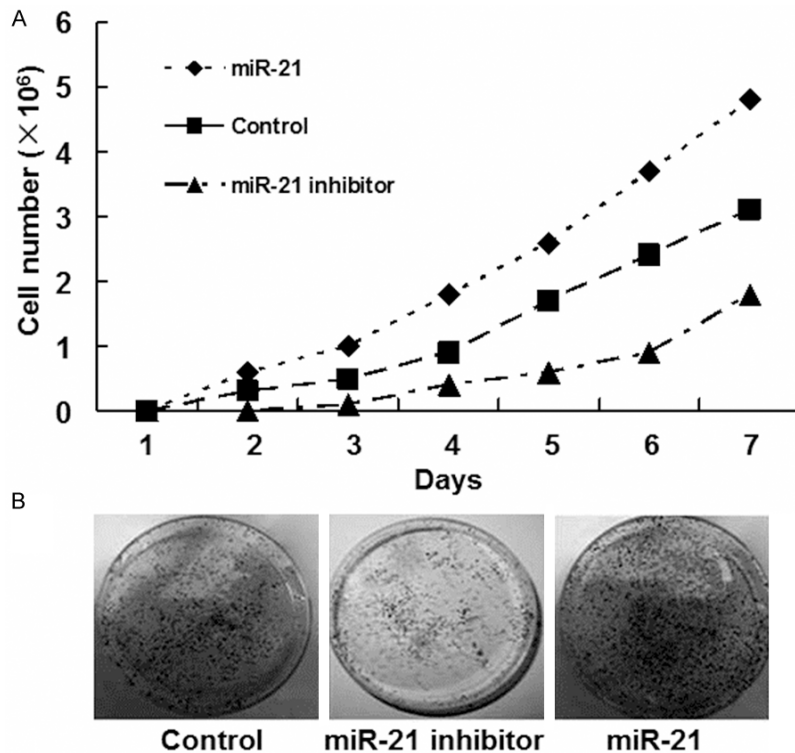


Figure 1. Vascular smooth muscle cell growth in control, miR-21 and miR-21 inhibitor groups. A. Proliferation curve of cells stably transfected with miR-21 mimics, miR-21 inhibitor or control mimics. B. Colony formation of cells transfected with miR-21 mimics, miR-21 inhibitor or control mimics.

swell migration assay, 1×10^5 cells were seeded into the top chamber containing a non-coated membrane. For transwell invasion assay, 3×10^5 cells were seeded into the top chamber containing a Matrigel-coated membrane. Transwell assay was performed using a 24-well cell transwell assay kit (Cell Application, San Diego, CA, USA) according to the manufacturer's manual.

Luciferase reporter assay

The cells were cultured in 6-well plates, and then transfected with $1 \mu\text{g}$ pMIR/activator protein (AP)-1 vector or pMIR/AP-1/mut vector containing firefly luciferase along with $0.05 \mu\text{g}$ pRL-TK vector (Promega, USA) containing Renilla luciferase and 50 nM miR-21 inhibitor or negative control. The cells were transfected in 24-well plates with wild-type or mutant reporter plasmids using Lipofectamine 2000. After transfection for 6 h, cells were transfected again with miR-21 inhibitor or negative control. Luciferase activity was measured using the dual luciferase assay system (Promega, USA) after 36 h incubation.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted and isolated from the cells using TRIzol method. The quality and quantity of the RNA samples were assessed by standard electrophoresis and spectrophotometric methods. After treatment with RNase-free DNase I to remove any genomic DNA contamination, the mRNAs were reverse-transcribed into cDNAs for qRT-PCR analysis as described previously [32].

Western blotting

Western blotting was performed as previously described [33]. Total protein was extracted from cells transfected with miR-21 and negative control. The lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gels

after protein quantification using bicinchoninic acid kit (Biotek, Beijing, China), followed by transfer to polyvinylidene difluoride (PVDF) membranes (Invitrogen, CA, USA), which was blocked for 1 h with blocking solution (Invitrogen, CA, USA) at room temperature with agitation and incubated with the primary antibody at 4°C for 12 h. After being blocked with 5% non-fat milk in Tris Buffered Saline with Tween 20 at 37°C for 1 h, the membrane was incubated with the primary antibodies for AP-1 (dilution factor 1:500) β -actin (1:500) was used as the internal reference. After incubation by 5% non-fat milk in Tris Buffered Saline with Tween 20 at 4°C overnight, the membrane was washed for 3 times of 10 min. After washing with wash solution for 3 times, the PVDF membrane was incubated with secondary antibodies (Invitrogen, CA, USA) at room temperature for 1 h. The blots were visualized using the electrochemiluminescence detection system.

Statistical analysis

All statistical analyses were performed and all graphs were plotted using SPSS 17.0 software

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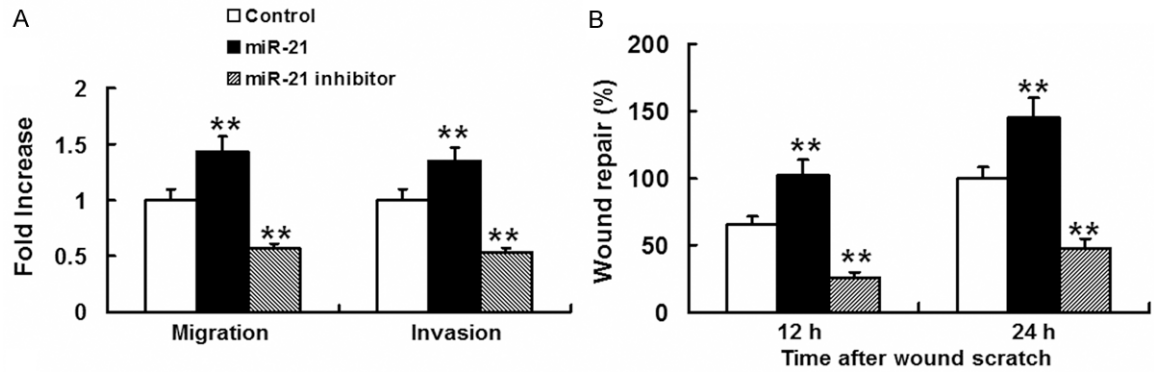


Figure 2. Effect of miR-21 on VSMC migration, invasion and wound repair. A. Migration and invasion of VSMCs transfected with miR-21 mimics, miR-21 inhibitor or control mimics. Transwell assay was performed. Data are means \pm SD. **, $P < 0.01$ compared with control. B. Wound repair of VSMCs transfected with miR-21 mimics, miR-21 inhibitor or control mimics after 12 or 24 h. Data are means \pm SD. **, $P < 0.01$ compared with control.

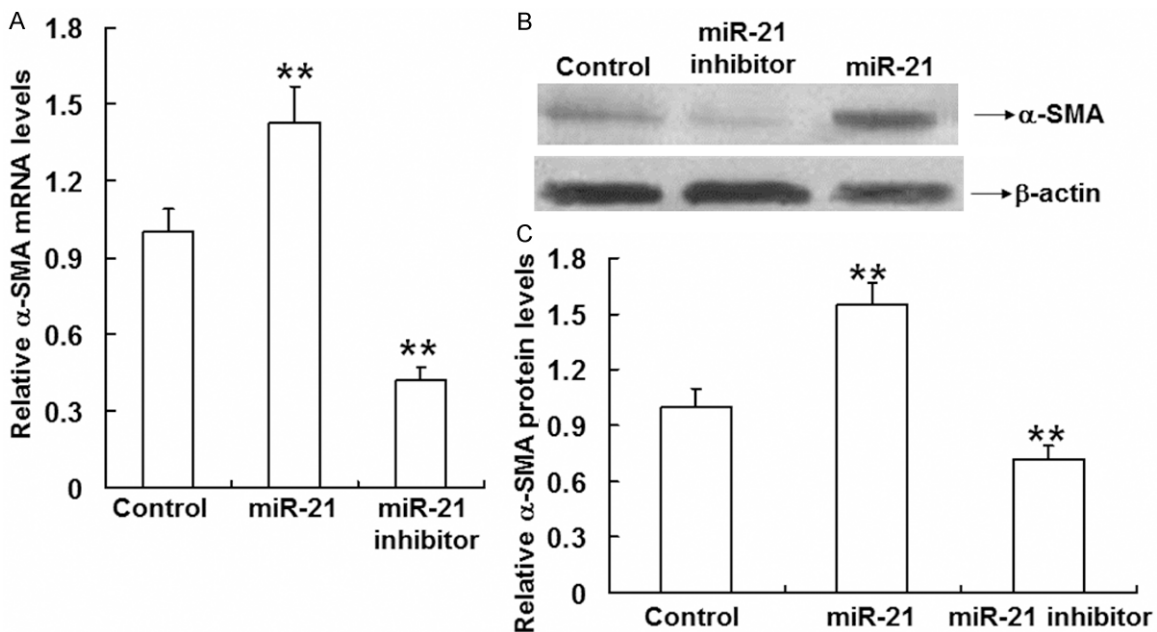


Figure 3. Effect of miR-21 on α -SMA expression in VSMCs transfected with miR-21 mimics, miR-21 inhibitor or control mimics. A. The level of α -SMA mRNA determined using qRT-PCR. B. α -SMA protein expression measured using Western blotting. C. Quantification of α -SMA protein expression. Data are means \pm SD. **, $P < 0.01$ compared with control.

(Chicago, IL, USA). Two-tailed student's t-test was used. All experiments were repeated for three independent times, and $P < 0.05$ was considered statistically significant.

Results

Low expression of miR-21 significantly inhibits VSMC growth

To test whether low expression of miR-21 in VSMCs affects cell growth, MTT assay was

used to investigate cell proliferation. The results showed that cells transfected with miR-21 inhibitor grew more slowly than the control group while cells treated with miR-21 grew faster than control (**Figure 1A**). Colony formation results showed that cells transfected with miR-21 inhibitor had lower level of proliferation compared with those treated with control or miR-21 (**Figure 1B**). These data suggested that low expression of miR-21 inhibited VSMCs growth *in vitro*.

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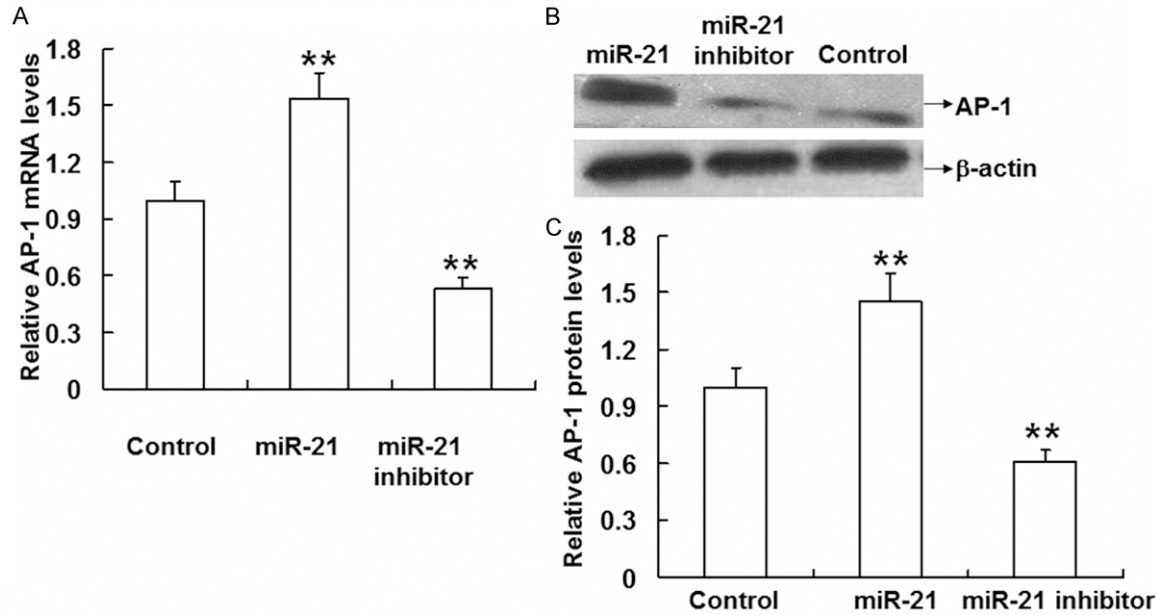


Figure 4. Effect of miR-21 on AP-1 expression in VSMCs transfected with miR-21 mimics, miR-21 inhibitor or control mimics. A. The level of AP-1 mRNA determined using qRT-PCR. B. AP-1 protein expression measured using Western blotting. C. Quantification of AP-1 protein expression. Data are means \pm SD. **, $P < 0.01$ compared with control.

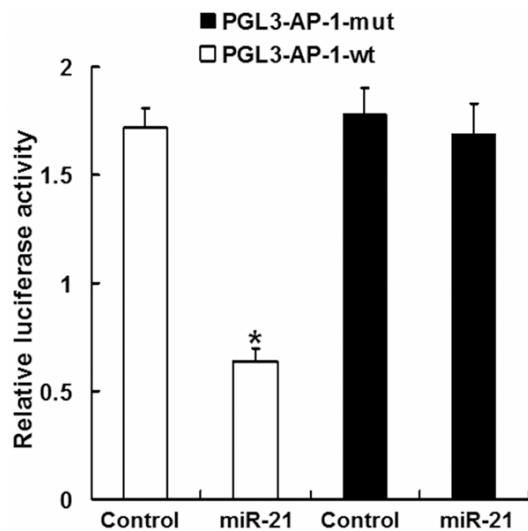


Figure 5. Relative luciferase activity in the detection of miR-21 binding to 3'-UTR of AP-1 mRNA in VSMCs. The miR-21 reporter plasmid was transfected into VSMCs together with AP-1 or negative control. **, $P < 0.01$ compared with control in PGL3-AP-1-wt.

Low expression of miR-21 significantly inhibits the migration and invasion of VSMCs

To investigate the effects of down-regulated miR-21 on cell migration and invasion, we conducted cell migration and invasion assays using VSMCs transfected by 50 nM miR-21 in

the presence of miR-21 inhibitor or negative control. The result showed that cells treated with miR-21 inhibitor showed lower levels of migration and invasion compared with those treated with control or miR-21 (Figure 2A). Wound healing assay showed that lower levels of wound repair were observed in cells treated with miR-21 inhibitor for 12 h or 24 h (Figure 2B). These data indicated that down-regulated expression of miR-21 significantly inhibited the migration and invasion of VSMCs.

Low expression of miR-21 significantly decreases α -SMA mRNA and protein levels in VSMCs

To test whether the miR-21 affects α -SMA mRNA or protein expression, qRT-PCR and Western blotting were performed. qRT-PCR analysis revealed that miR-21 increased α -SMA mRNA level by 43.00% compared with control ($P < 0.01$). By contrast, miR-21 inhibitor decreased α -SMA mRNA level by 58.00% compared with control ($P < 0.01$) (Figure 3A). Western blotting analysis revealed that miR-21 enhanced α -SMA protein expression by 55.00% compared with control ($P < 0.01$). By contrast, miR-21 inhibitor decreased α -SMA protein expression by 28.00% compared with control ($P < 0.01$) (Figure 3B and 3C). These data demonstrated

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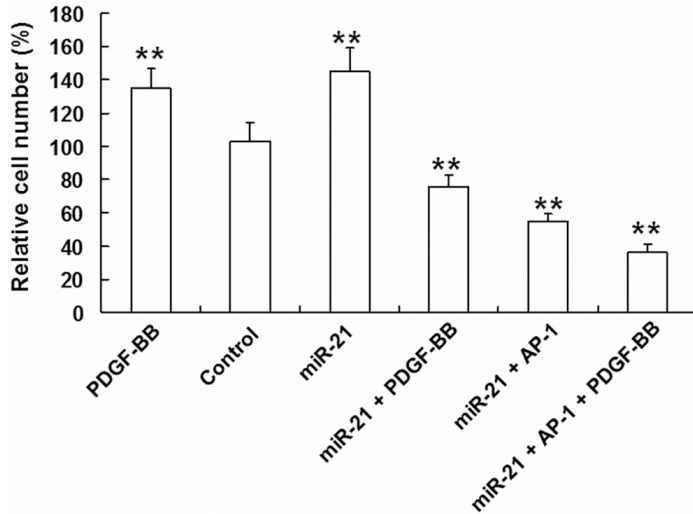


Figure 6. Proliferation of cells stably transfected with PDGF, control mimics, miR-21 mimics, miR-21+PDGF-BB, miR-21+AP-1, or miR-21+AP-1+PDGF-BB. Data are means \pm SD. **, $P < 0.01$ compared with control.

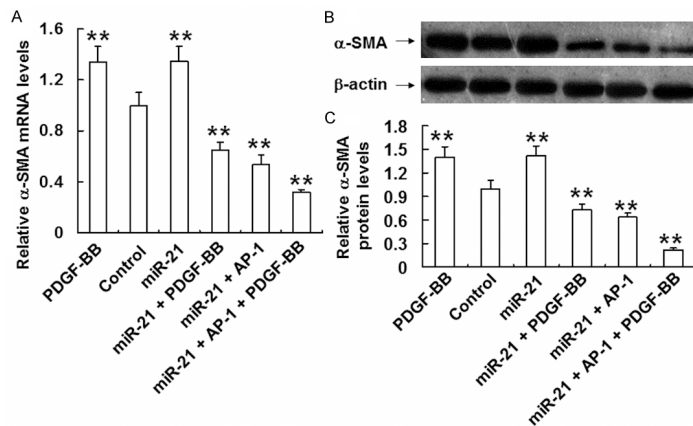


Figure 7. The α -SMA expression in the presence of transient AP-1 expression. VSMCs were transfected with the PDGF, control mimics, miR-21 mimics, miR-21+PDGF-BB, miR-21+AP-1, or miR-21+AP-1+PDGF-BB. A. The relative level of α -SMA mRNA determined using qRT-PCR. B. The α -SMA protein expression measured using Western blotting. C. Quantification of α -SMA protein expression. Data are means \pm SD. **, $P < 0.01$ compared with control.

that low expression of miR-21 decreased α -SMA mRNA and protein levels in VSMCs.

Low expression of miR-21 significantly decreases AP-1 mRNA and protein levels in VSMCs

To investigate the effect of miR-21 on AP-1 mRNA and protein levels, VSMCs transfected with miR-21 mimics, miR-21 inhibitor and con-

trol mimics were analyzed using qRT-PCR and Western blotting. qRT-PCR analysis revealed that miR-21 mimics increased AP-1 mRNA level by 54.00% compared with control ($P < 0.01$). By contrast, miR-21 inhibitor decreased AP-1 mRNA level by 47.00% compared with control ($P < 0.01$) (**Figure 4A**). Western blotting analysis revealed that miR-21 mimics increased AP-1 protein expression by 45.00% compared with control ($P < 0.01$). By contrast, miR-21 inhibitor decreased AP-1 protein expression by 39.00% compared with control ($P < 0.01$) (**Figure 4B** and **4C**). These results suggested that low expression of miR-21 decreased AP-1 mRNA and protein levels in VSMCs.

miR-21 modulates AP-1 expression by directly targeting 3'-UTR of AP-1 mRNA

To understand how miR-21 exerts its effect in VSMCs, we searched for its potential target genes. Among these genes, AP-1 plays a crucial role in the signaling pathway that regulates VSMC proliferation, apoptosis and migration. To experimentally test whether AP-1 is a target gene of miR-21 in VSMCs, wt or mutant reporter plasmids was co-transfected into VSMCs along with miR-21 or control. miR-21 significantly decreased the activity when co-transfected with the wt reporter plasmid compared with control (0.64 ± 0.06 vs 1.73 ± 0.09 , $P < 0.01$) (**Figure 5**). Meanwhile, when miR-21 and control were transfected with the mutant reporter plasmid, there was no significant difference

in relative luciferase activity between the two groups (1.78 ± 0.12 vs 1.69 ± 0.14 , $P > 0.05$) (**Figure 5**). These data indicated that miR-21 modulated AP-1 expression by directly targeting 3'-UTR of AP-1 mRNA.

miR-21 inhibits cell proliferation stimulated by PDGF

In order to investigate the effect of miR-21 on cell proliferation stimulated by PDGF, VSMCs

transfected with miR-21 mimics was analyzed using MTT assay. miR-21 inhibited cell proliferation stimulated by PDGF by 47.59% compared with miR-21 alone (76 ± 7 vs 145 ± 14 , $P < 0.01$). Meanwhile, co-transfection with miR-21 and AP-1 inhibited cell proliferation stimulated by PDGF by 34.55% (36 ± 5 vs 55 ± 4 , $P < 0.05$) (**Figure 6**). These results suggested that the miR-21 responsible for cell proliferation induced by PDGF.

Cell proliferation stimulated by PDGF inhibits the levels of α -SMA mRNA and protein in VSMCs

In order to test whether the effect of miR-21 on α -SMA is related to cell proliferation stimulated by PDGF, miR-21 was co-transfected with AP-1 in VSMCs. The results showed that α -SMA mRNA level in the presence of miR-21 and PDGF was decreased by 51.85% compared with that in miR-21 alone or PDGF alone ($P < 0.01$), with similar result being found that α -SMA protein expression in the presence of miR-21 and PDGF was significantly decreased by 48.59% compared with miR-21 alone or PDGF alone ($P < 0.01$). Meanwhile, the results showed that α -SMA mRNA levels in the presence of miR-21, AP-1 and PDGF were decreased by 53.70% compared with that in the presence of miR-21 and PDGF ($P < 0.01$) (**Figure 7A**). Similarly, α -SMA protein expression in the presence of miR-21, AP-1 and PDGF was significantly decreased by 65.63% compared with that in the presence of miR-21 and PDGF ($P < 0.01$) (**Figure 7B**). These data demonstrated that cell proliferation stimulated by PDGF inhibited the levels of α -SMA mRNA and protein in VSMCs.

Discussion

VSMC differentiation is an essential component of vascular development [33]. There is clear evidence that alterations in the differentiation state of the VSMCs play critical roles in the pathogenesis of atherosclerosis and intimal hyperplasia. miR-21 is well known in all kinds of cancer cells [34, 35]. However, little is known about miR-21 expression and function in VSMCs. One of the best ways to understand miRNA function is the elucidation of functional targets, which usually involves analysis of changes in target proteins following either gain or loss of function of the specific miRNA. In this study, three major findings were described.

First, we showed that low expression of miR-21 significantly inhibited VSMC growth, invasion and migration *in vitro*. Next, we showed that low expression of miR-21 decreased AP-1 and α -SMA expression at both mRNA and protein levels in VSMCs, and that AP-1 was a direct target of miR-21. Finally, we demonstrated that the effect of miR-21 on AP-1 was related to VSMC proliferation stimulated by PDGF. These results suggested that reduced miR-21-mediated suppression of AP-1 inhibited neovascularization.

It has been reported that miRNAs are important players involved in carcinogenesis. However, there is no report indicating the alteration of AP-1 expression in VSMCs. The results revealed that low expression of miR-21 inhibited VSMC proliferation, invasion and migration, which might be due in part to the negative regulation of AP-1 by miR-21. It has been shown that transcription factors and miRNAs are two large families of trans-acting gene regulators in multi-cellular genomes with extensive interactions on gene regulation [37, 38]. This dynamic interactive mechanism of gene regulation by transcription factors and miRNAs has been supported by a few recent studies [39-41]. Our results showed that low expression of miR-21 decreased the expression of AP-1 at both mRNA and protein levels. Furthermore, luciferase reporter assay found that AP-1 mRNA was the direct target of the miR-21. This suggests that low expression of miR-21 might affect its targets to generate diverse functions.

PDGF was identified as one of the most potent factors involved in angiogenesis [42, 43]. Recently, it has been proposed that growth factor signals regulate the expression of miRNA transcriptionally and/or post-transcriptionally and hence, modulating VSMC differentiation and proliferation [44, 45]. In this study, we found that the low expression of miR-21 inhibited VSMC proliferation stimulated by PDGF. More recently, some data showed that miRNAs could affect cell proliferation by targeting transcription factors in cancer or diabetic cells [46, 47]. Meanwhile, our previous work provided further evidence that miRNA could regulate endothelial cell proliferation via inhibiting signal transducer and activator of transcription 3 signaling pathway or transcription factor specificity protein 1 [48, 49]. In this study, we further demonstrated that low expression of miR-21

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suppressed α -SMA expression and decreased AP-1 expression in human VSMCs. These data suggested that miR-21 and AP-1 regulated α -SMA expression and VSMC proliferation.

In summary, we demonstrated that the down-regulation of miR-21 had a functional role in VSMC proliferation and migration via the post-transcriptional suppression/down-regulation of the unique miR-21 target, AP-1. Based on our findings, we speculate that miR-21 regulation of AP-1 will contribute to a better understanding of the molecular mechanisms involved in VSMC proliferation and vascular remodeling in cardiovascular disease, as well as the development of new therapies for vascular diseases.

Acknowledgements

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Disclosure of conflict of interest

All authors declare no financial competing interests.

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