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Original Article

MiR-124-3p inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and migration through targeting STAT3

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Abstract: Aberrant vascular smooth muscle cells (VSMCs) proliferation and migration contribute significantly to the development of vascular pathologies, such as atherosclerosis. MicroRNAs (miR) have been reported to act as important gene regulators and play essential roles in the proliferation and migration of VSMCs in a cardiovascular disease. The aim of this study was to investigate the role of miR-124-3p in VSMCs. In the present study, we showed that platelet-derived growth factor (PDGF)-BB, as a stimulant, promoted VSMCs proliferation and suppressed the expression of miR-124-3p. Moreover, overexpression of miR-124-3p inhibited proliferation and also suppressed the proliferating cell nuclear antigen (PCNA) expression in PDGF-BB-induced VSMCs. In addition, we demonstrated that ectopic expression of miR-124-3p repressed the VSMCs migration. Furthermore, dual-luciferase assays identified that STAT3 is a direct target gene of miR-124-3p in VSMCs, and overexpression of miR-124-3p significantly down-regulated the mRNA and protein levels of STAT3. In addition, restored the expression of STAT3 in VSMCs could partially abolish the suppressive effects of miR-124-3p on the proliferation and migration. Collectively, these data suggest that miR-124-3p is a key molecule in regulating human VSMCs proliferation and migration by targeting STAT3 and suggest that specific modulation of miR-124-3p in human VSMCs may represent an attractive approach for the treatment of proliferative vascular diseases.

Keywords: miR-124-3p, proliferation, migration, STAT3, vascular smooth muscle cells (VSMCs)

Introduction

Cardiovascular disease is one of the leading causes of death worldwide. Despite technological advances in vascular angioplasty and stenting, the outcomes of these procedures are poorer in elderly patients than in younger patients [1]. Atherosclerotic plaques are complex lesions in which repair of tissue injury is associated with vascular smooth muscle cells (VSMCs) proliferation and migration, connective tissue formation and calcium deposition [2]. VSMCs are an important component of vascular walls and their proliferation plays a crucial role in atherosclerotic lesion formation [3].

MicroRNAs (miRNAs) are a class of endogenous and small (18-24 nucleotides) non-coding RNAs that negatively modulate gene expression through binding to the 3'-untranslated regions (3'-UTR) of target mRNAs to lead to protein translational repression [4, 5]. Recently, multiple lines of evidence suggest that miRNAs play pivotal roles in the control of VSMCs function and the response of the vascular injury through targeting transcriptional factors or key signaling molecules in SMC proliferation and migration [6, 7]. It has been reported that miR-133 has also been indicated to reduce VSMCs proliferation and migration by suppressing the expression of transcription factor Sp-1 [8]. A recent report also showed that knockdowns of miR-221 and miR-222 decrease VSMCs proliferation and migration in vitro by targeting cyclin-dependent kinase (CDK) inhibitors, p27 and p57, respectively [9]. Recently it has been shown that miR-638 is highly expressed in human VSMCs and inhibits PDGF-BB-induced cell proliferation and migration through targeting NOR1 [10]. However, the role of miR-124-3p in human VSMCs and proliferative vascular diseases remains completely unknown.

In the current study, we performed miRNA microarray analysis to identify the miRNAs involved in regulating human VSMCs proliferation. We, for the first time, demonstrated that miR-124-3p, which is abundantly expressed in VSMCs, was significantly down-regulated in proliferative VSMCs. Furthermore, we found that miR-124-3p inhibits proliferation and migration in PDGF-BB-induced VSMCs via directly targeting STAT3, which is a critical regulator implicated in proliferative vascular diseases.

Materials and methods

Cells culture and oligonucleotide transfection

The VSMCs cell line was purchased from Cascade Biologics (Portland, OR) and kept in DMEM/F12 medium (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA), supplemented with 10% FBS and 1% antibiotics (Gibco, MD, USA). Cells were cultured at 37°C in a humidified incubator under a 5% CO_2 atmosphere. VSMCs were seeded in complete medium for 24 h. Then, the cells were replaced with fresh serum-free media containing the 25 ng/mL of PDGF-BB for 24 h. miR-124-3p and scramble oligonucleotide was purchased from GenePharma (Shanghai, China) and transfected into the VSMCs used Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's information.

Quantitative real-time PCR

Total RNAs were isolated with the use of TRIZol reagent (Invitrogen, Grand Island, NY). miRNAs were assayed by real-time PCR. The cDNAs were produced from 100 ng purified total RNA with Taqman1 MicroRNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) in combination with Taqman1 MicroRNA Assays for quantification of specific miR-124-3p, according to the manufacturer's conditions. U6 was used as an endogenous control for data normalization. Real-time PCR analyses for amplification and detection of specific miRNAs were performed in a Light Cycler 480 II (Roche). The relative differences in expression levels of miRNA in VSMCs were calculated and presented as fold induction (2DDCt) after normalization to control U6.

Western blot analysis

The VSMCs were cultured in a 9 cm diameter dish, grown to 70-80% confluency, and then starved in serum-free medium for 24 h. The cells were lysed in radio immunoprecipitation assay (RIPA) buffer with protease and phosphatase cocktails. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked, and then incubated with various antibodies overnight, and then with the horseradish peroxidase-conjugated secondary antibody (Beijing TDY Biotech Co., Ltd.) (1:5,000) for 2 h. Specific protein expression levels were normalized to GAPDH for total protein analyses or to total proteins for phosphorylated protein measurements. The blots were analyzed using the ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiments were replicated a number of times.

Plasmid construction and luciferase reporter assays

The cells were seeded in triplicate in 24-well plates and allowed to settle for 12 h. The whole 3'-UTR of STAT3 gene was cloned and amplified. Mutation in 3'-UTR of STAT3 gene with miR-124-3p putative target binding site deleted was generated with the Quick Change Site-Directed Mutagenesis kit (Stratagene, CA, USA). Both the wild and mutant STAT3 genes were cloned into the pGL-3-vector (Promega, Wisconsin, USA) immediately downstream of the Renilla luciferase gene. A luciferase reporter construct containing the miR-124-3p consensus target sequence served as the positive control (PC) and the pRL-TK vector was used as positive and internal controls, respectively. Cells were co-transfected with pGL-3 firefly luciferase reporter (50 ng), pRL-TK Renilla luciferase reporter (10 ng) and miR-124-3p or negative control with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cell lysates were prepared using Passive Lysis Buffer (Promega, Wisconsin, USA) 48 h upon transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega, Wisconsin, USA). Results were normalized to the Renilla luciferase.

Cell proliferation assay

The proliferation of VSMCs was measured by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) assay. VSMCs were seeded in 96-well plates at a density of 1×10^4 cells/well in 200 μL culture

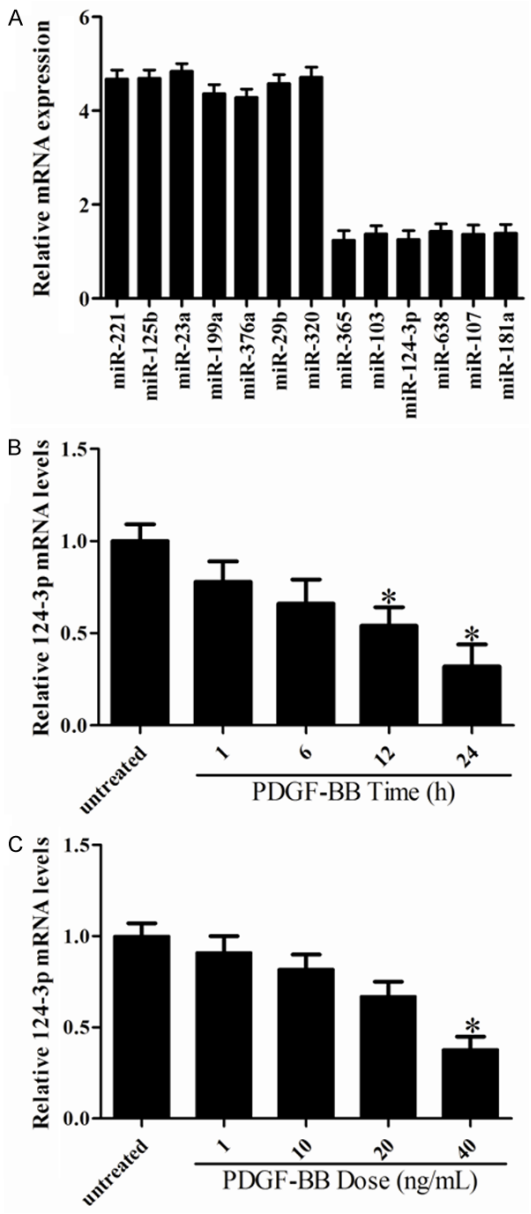

Figure 1. miR-124-3p is down-regulated in PDGF-BB-induced VSMCs. A. miRNAs expression in VSMCs after PDGF-BB treatment. B. PDGF-BB caused a time-dependent decrease in miR-124-3p expression in VSMCs, as demonstrated by qRT-PCR. C. PDGF-BB caused a dose-dependent decrease in miR-124-3p expression in human aortic VSMCs at 24 h after treatment, as demonstrated by qRT-PCR. *P<0.05 vs. untreated.

medium. After synchronization by DMEM with 0.1% FBS overnight, cells were stimulated with 20 ng/mL PDGF-BB and incubated for 24 h. After experimental treatment, 20 μl of the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was added to each well. The absorbance value was measured at 450 nm using a microplate reader (Perkin Elmer, Waltham, MA). Cell viability was assessed by trypan blue exclusion microscopy.

Cell migration assay

Cell migration activity was investigated with Transwell chamber system (Corning, Corning, NY) following the manufacturer's instruction. Briefly, cells were trypsinized, washed with PBS and suspension (2×10^4 cells) was added to the upper chamber. The lower chamber was filled with DMEM supplemented with PDGF-BB (20 ng/mL). The chamber was incubated at 37°C for 24 h. The cells on the upper surface of the filters were carefully removed while the cells on the lower surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Migrant cells were quantified by blind counting under a microscope (Nikon, Tokyo, Japan). The number of transmigrated cells were counted and averaged in eight random areas.

Statistics analysis

Results were displayed as mean ± SD. The significance between two groups was used Student's t test. The significance between more than two groups was used One-way ANOVA. A value of P<0.05 considered to indicate a statistically significant difference.

Results

MiRNA expression in PDGF-stimulated human VSMCs

To identify the miRNAs in the regulation of human VSMCs proliferation, we performed miRNA microarray in VSMCs after PDGF-BB stimulation (20 ng/mL). The results as shown in **Figure 1A**, seven miRNAs were up-regulated and six miRNAs were down-regulated in response to PDGF-BB stimulation. In agreement with previously published studies, miR-638 and miR-365 were found to be significantly down-regulated in response to PDGF-BB treatment. Among the six miRNAs that were down-regulated by PDGF-BB stimulation, miR-124-3p was found to be highly enriched in VSMCs, and its expression was markedly down-regulated in response to PDGF-BB stimulation. Thus, these findings suggest that miR-124-3p may be a critical regulator for VSMCs proliferation.

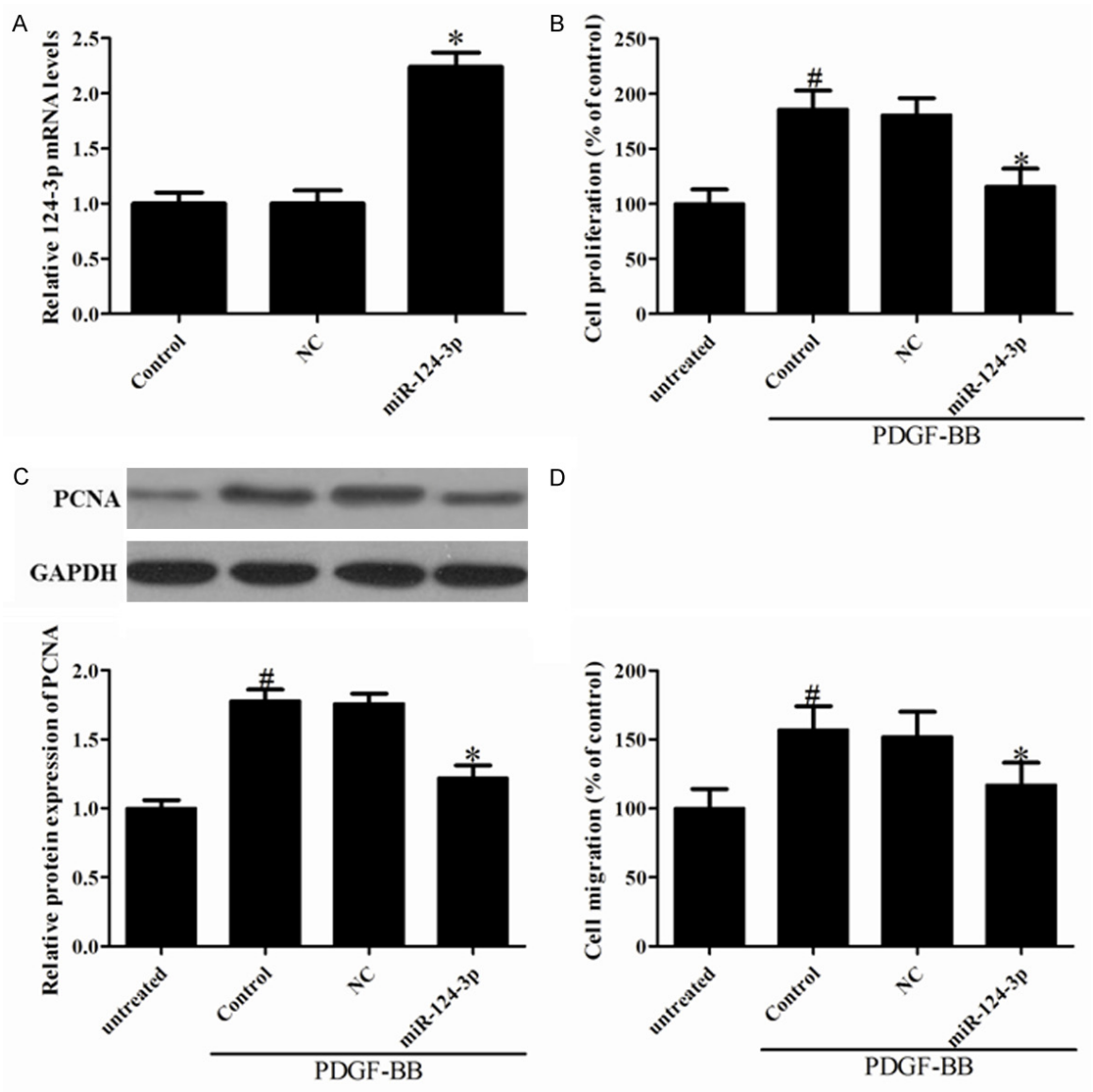

Figure 2. The effect of miR-124-3p in VSMCs proliferation and migration. VSMCs were transfected with Negative Control (NC) or miR-124-3p, and then treated with or without PDGF-BB (20 ng/ml). A. Transfection efficiency was detected by real-time PCR. B. Overexpression of miR-124-3p significantly decreased PDGF-BB-induced VSMCs proliferation, determined by CCK-8 assay. C. PCNA expression was measured using western blotting and protein level of PCNA was normalized by GAPDH. D. The effect of miR-124-3p on PDGF-BB-induced VSMCs migration was measured using a Transwell assay. #P<0.05 *vs.* untreated; *P<0.05 *vs.* control and NC group.

MiR-124-3p is inhibited by PDGF-BB in VSMCs

To further confirm the expression of miR-124-3p in proliferating VSMCs, qRT-PCR was performed in VSMCs stimulated by either PDGF-BB (20 ng/mL) at different time points. As shown in **Figure 1B**, miR-124-3p was substantially down-regulated in response to PDGF-BB treatment in a time dependent manner. In addition, PDGF-BB stimulation markedly reduced the-miR-124-3p expression in a dose dependent manner (**Figure 1C**). These results were consistent with changes seen in our microarray data. Taken together, these findings indicate an inverse relationship between miR-124-3p expression and VSMCs proliferation.

MiR-124-3p suppressed VSMCs proliferation and migration

PDGF-BB is a well-known growth factor of cell proliferation, and is a key factor in migration

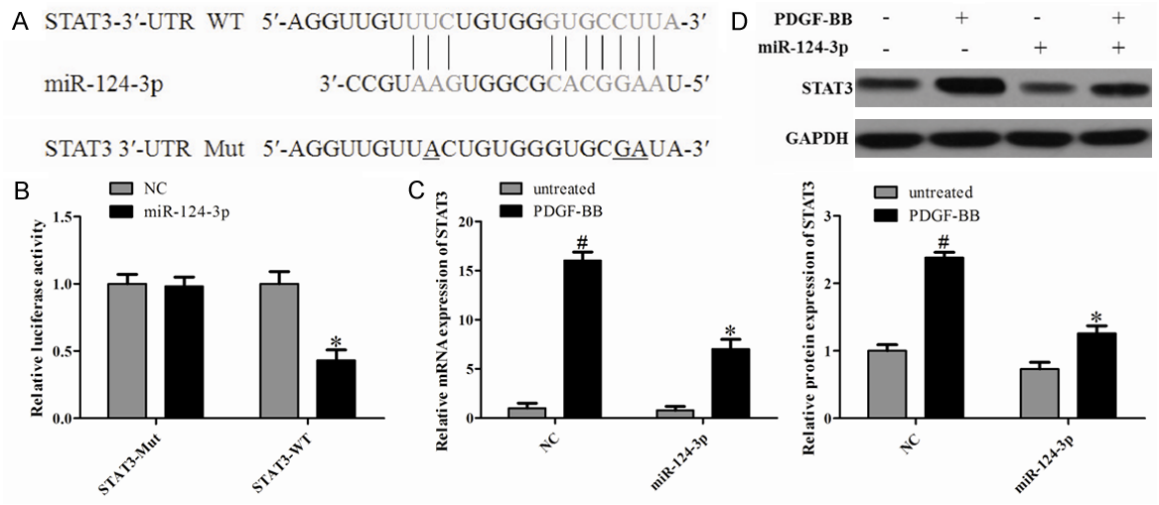

Figure 3. Identification of STAT3 as a target of miR-124-3p in human VSMCs. A. Schematic of the miR-124-3p putative binding site in human STAT3 3'-UTR and alignment of wild-type (WT) miR-124-3p and mutated (Mut) STAT3 3'-UTR binding site of miR-124-3p. B. HEK293 cells were co-transfected with the luciferase reporter carrying WT-STAT3 or Mut STAT3, together with plasmid bearing miR-124-3p or negative control. Forty-eight hours after transfection, renilla luciferase activities were measured. C. VSMCs were transfected with miR-124-3p or negative control for 24 h and then serum-deprived for 48 h, STAT3 mRNA was measured by qRT-PCR in VSMCs 3 h after stimulation with or without PDGF-BB (20 ng/mL). D. Effects of miR-124-3p overexpression on STAT3 protein expression 6 h after PDGF-BB stimulation. #*P*<0.05 *vs.* untreated; **P*<0.05 *vs.* control and NC group.

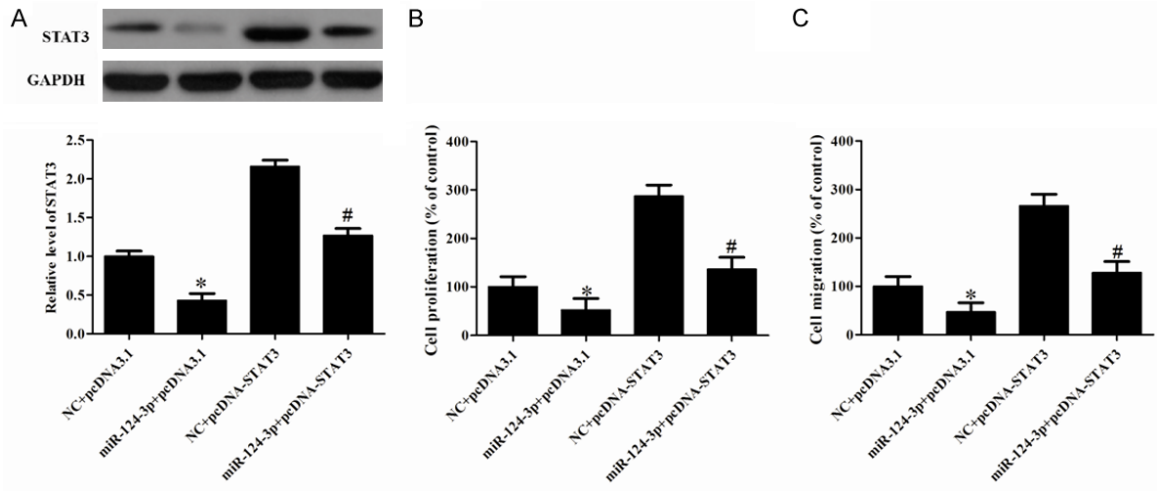

Figure 4. STAT3 involved in the effect of miR-124-3p in PDGF-BB-induced VSMCs. A. The protein expression of STAT3 was measured by Western blot. B. CCK-8 assays were used to detect to explore the effects of miR-124-3p/STAT3 interaction on cell proliferation of VSMCs. C. Overexpression of STAT3 promoted miR-124-3p-induced inhibition of VSMCs migration. Data are presented as means ± SD. **P*<0.05 *vs.* NC+pcDNA3.1, #*P*<0.05 *vs.* NC+pcDNA-STAT3.

from the media to intima in injured vessels [11]. To further examine the functional role of miR-124-3p in VSMCs, we sought to determine whether restoration of miR-124-3p could affect PDGF-BB induced SMCCs proliferation using the CCK-8 assay. To this end, miR-124-3p was transfected into VSMCs, which caused miR-124-3p expression levels to be significantly increased (**Figure 2A**). In addition, PDGF-BB treatment significantly increased the proliferation of VSMCs compared to the non-stimulated group, and overexpression of miR-124-3p significantly suppressed PDGF-BB-induced cell proliferation (**Figure 2B**). The proliferating cell nuclear antigen (PCNA) that plays a crucial role in the life and death decisions of cells was also

investigated. Representative expression of PCNA was significantly decreased in miR-124-3p-overexpressed VSMCs compared to cells treated with PDGF-BB (**Figure 2C**). To confirm the inhibitory effect of miR-124-3p on VSMCs migration, a Transwell assay was performed. As the **Figure 2D** shown, the number of migratory cells was suppressed in miR-124-3p-overexpressed VSMCs compared to cells treated with PDGF-BB.

STAT3is a direct target of miR-124-3p

By using the TargetScan and PicTar algorithms, we found that STAT3 is a putative target of miR-124-3p (**Figure 3A**). To determine whether miR-124-3p directly binds to the 3'-UTR sequence of STAT3 mRNA and affects its expression, the 3'-UTR sequence of STAT3 containing the putative binding site for miR-124-3p was cloned into a luciferase reporter vector. The construct vector was then co-transfected with either NC or a miR-124-3p into VSMCs. The luciferase assay was inhibited in cells transfected with miR-124-3p compared to that with NC, but that does not bind to the 3'-UTR of STAT3 (**Figure 3B**). To further verify that STAT3 is a functional target gene of miR-124-3p in VSMCs, we determined the levels of STAT3 mRNA by RT-PCR. Interestingly, PDGF-BB increased mRNA levels of STAT3, but miR-124-3p effectively downregulated the expression of STAT3 induced with PDGF-BB (**Figure 3C**). As determined by western blot analysis, overexpression of miR-124-3p downregulated both basal and PDGF-BB-induced STAT3 expression in VSMCs (**Figure 3D**).

STAT3 involved in the effect of miR-124-3p in VSMCs

The data described above showed that miR-124-3p inhibited STAT3 mRNA and protein expression in VSMCs. The remaining question was whether miR-124-3p inhibits cell proliferation and migration by down-regulation of STAT3. The results showed that the plasmid of STAT3 promoted the STAT3 protein expression detected using Western blot (**Figure 4A**). CCK8 analysis demonstrated that overexpression of STAT3 promoted miR-124-3p-induced inhibition of PDGF-BB-induced VSMCs proliferation (**Figure 4B**). Moreover, ectopic expression of STAT3 promoted miR-124-3p-induced inhibition migration in PDGF-BB-induced VSMCs (**Figure 4C**).

Discussion

Proliferation of VSMCs plays an essential role in the progression of atherosclerosis lesions. Inhibition of VSMCs proliferation is an important therapeutic strategy for atherosclerosis related diseases. Abnormal proliferation of PDGF-BB-stimulated VSMCs increases the risk of vascular disorders, and it is critical for the progression of restenosis and atherosclerosis [12]. In the present study, we first detected the miRNA expression in PDGF-BB-stimulated human VSMCs and found that seven miRNAs were up-regulated and six miRNAs were down-regulated in response to PDGF-BB stimulation.

MiRNAs are small noncoding RNA molecules that post-transcriptionally or translationally regulate biological processes including the modulation of stem cell self-renewal, differentiation, apoptosis, and proliferation by binding to the 3'-UTR of target genes [13]. However, the mechanism by which miRNAs exert these effects remains under investigation. Recent studies have suggested important regulatory roles for miRNAs such as miR-21, miR-216, miR-217, miR-181b, miR-31b and miR-34a, which were confirmed to be upregulated in senescing human umbilical vascular endothelial cells (HUVECs) [14]. These miRNAs are related to cardiovascular diseases [15]. In the present study, to the best of our knowledge, we demonstrate for the first time that miR-124-3p is significantly down-regulated in human proliferative VSMCs and restoration of miR-124-3p markedly inhibits the PDGF-BB-induced VSMCs proliferation and the expression of PCNA. In addition, miR-124-3p also suppresses the PDGF-BB-induced VSMCs migration.

Signal transducer and activator of transcription-3 (STAT3) is a critical member of the STAT transcription factor family. STAT3 has been reported to participate in a variety of physiological progresses, such as cell proliferation, apoptosis and so on [16]. Previous studies, both *in vitro* and *in vivo* (in neointimal cells), have shown that phosphorylation of STAT3 induces trans-activation of cyclin D1 and surviving in SMCs, which therefore promotes proliferation and migration of SMCs as well as reducing apoptotic cell death [17]. The interplay between miRNAs and STAT3 signaling pathway has been widely studied [18]. STAT3 has been reported to be the target gene of miR-124 in

endometrial cancer cells, and be involved in the miR-124-mediated suppressive effects on endometrial cancer cells [19]. In the current study, STAT3 was identified as a direct target of miR-124-3p and down-regulates the mRNA and protein expression of STAT3 in VSMCs. Furthermore, overexpression of STAT3 abolished the suppressive effects of miR-124-3p on the proliferation and migration in response to PDGF-BB stimulation. Collectively, these findings demonstrated that miR-124-3p inhibits the PDGF-BB-induced proliferation and migration of VSMCs through STAT3. Thus, our study provides cognate evidence implicating miR-124-3p as a novel key regulator in human VSMCs biology.

Taken together, our results for the first time establish a functional link between miR-124-3p and STAT3 expression in VSMCs, demonstrating that STAT3 is directly repressed by miR-124-3p, which subsequently inhibits its downstream signaling pathway. miR-124-3p expression was substantially down-regulated in proliferative human VSMCs and restoration of its expression markedly inhibited both VSMCs proliferation and migration in response to PDGF stimulation through downregulation of STAT3. In this regard, our study provides significant novel insight into the molecular mechanisms associated with VSMCs proliferation and migration, and suggests a potential therapeutic target for preventing and treating human vascular diseases, such as atherosclerosis and restenosis.

Disclosure of conflict of interest

None.

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