Investigating the Role of the Post-transcriptional Gene Regulator MiR-24-3p in the Proliferation, Migration and Apoptosis of Human Arterial Smooth Muscle Cells in Arteriosclerosis Obliterans

Xiao-feng Zhu\textsuperscript{a} Zhen Shan\textsuperscript{a} Jie-yi Ma\textsuperscript{b} Mian Wang\textsuperscript{a} Chun-xiang Zhang\textsuperscript{a} Rui-ming Liu\textsuperscript{b,c,d} Wei-bin Wu\textsuperscript{a} Ya-wei Shi\textsuperscript{b} Wen Li\textsuperscript{b,c,d} Shen-ming Wang\textsuperscript{a,c,d}

\textsuperscript{a}Department of Vascular Surgery, First Affiliated Hospital, Sun Yat-sen University, Guangzhou
\textsuperscript{b}Laboratory of General Surgery, First Affiliated Hospital, Sun Yat-sen University, Guangzhou
\textsuperscript{c}Guangdong Engineering Laboratory for Diagnosis and Treatment of Vascular Disease, Guangzhou
\textsuperscript{d}Vascular Surgical Disease Research Center of Guangdong Province, Guangzhou, China
\textsuperscript{e}Cardiovascular Research Center, Department of Pharmacology, Rush Medical College, Rush University, Chicago, IL, USA

Key Words
Atherosclerosis • MiRNA-24-3p • Human arterial smooth muscle cells • Peripheral arterial disease • Arteriosclerosis obliterans • Platelet-derived growth factor receptor B • C-myc

Abstract
\textbf{Aims:} To explore the expression of miR-24-3p in human arteries with arteriosclerosis obliterans (ASO) as well as the role of miR-24-3p in the pathogenesis of ASO. \textbf{Methods:} We used quantitative real-time PCR (qRT-PCR) and in situ hybridization to monitor miR-24-3p expression in human arteries. To investigate the effect of miR-24-3p on human arterial smooth muscle cells (HASMCs), we applied cell counting and EdU assays to monitor proliferation and transwell and wound healing assays to investigate migration and flow cytometry to investigate apoptosis. Furthermore, we applied 3’-untranslated region (3’-UTR) luciferase assays to investigate the role of miR-24-3p in targeting platelet-derived growth factor receptor B (PDGFRB) and c-Myc. \textbf{Results:} MiR-24-3p was mainly located in the media of arteries and was downregulated in ASO arteries compared with normal arteries. Platelet-derived growth factor BB (PDGF-BB) treatment reduced the expression of miR-24-3p in primary cultured HASMCs. MiR-24-3p mimic oligos inhibited the proliferation and migration, and promotes apoptosis of HASMCs. Our 3’-UTR luciferase assays confirmed that PDGFRB and c-Myc were targets of miR-24-3p. \textbf{Conclusion:} The results suggest that miR-24-3p regulates the proliferation and migration of HASMCs by targeting PDGFRB and c-Myc. The PDGF/miR-24-3p/PDGFRB and PDGF/miR-24-3p/c-Myc pathways may play critical roles in the pathogenesis of ASO. These findings highlight the potential for new therapeutic targets for ASO.

X.-f. Zhu and Z. Shan contributed equally to this work.
Introduction

Atherosclerosis is a condition within the arterial wall in which the accumulation of cells, cholesterol and extracellular matrix causes hardening of the arterial wall [1]. Atherosclerotic lesions can grow slowly over years to eventually impede blood flow, leading to morbidity and mortality [2]. Peripheral arterial disease (PAD) is the third leading cause of atherosclerotic cardiovascular morbidity following coronary artery disease and stroke [3] and is currently estimated to affect 202 million people worldwide [4]. Those affected suffer from pain and limited mobility, which undoubtedly lead to a lower quality of life. Over 60,000 peripheral arterial disease-related amputations are performed each year in the United States [5, 6]. Furthermore, up to 45 million of the 202 million patients with peripheral artery disease will die from cardiovascular or cerebrovascular disease within 10 years [7]. Arteriosclerosis obliterans (ASO) of the lower extremities is one of the most common causes of PAD. Human arterial smooth muscle cells (HASMCs) comprise the main component of the vascular wall. It has been shown that the proliferation and migration of HASMCs out of the vascular media represent the key pathological basis for ASO.

MicroRNAs (miRNAs) are non-coding nucleotide RNAs of approximately 18-22 nucleotides in length. They are key post-translational regulators of gene expression through binding to specific, partially complementary mRNA sequences in the 3’-UTR [8–10]. MicroRNAs have been well studied since their discovery 20 years ago, although their roles in cardiovascular systems have only recently been explored [11, 12]. It has been reported that certain miRNAs (miR-21, miR-145, miR-143 and miR-221) regulate the proliferation, migration, apoptosis and cellular phenotype of HASMCs [13–16]. We previously investigated the expression profiles of miRNAs in the arteries of patients with lower extremity peripheral disease and found that the expression of miR-21, miR-140-3p, miR-24, miR-1298, miR-125b and miR-100 was significantly different in ASO arteries compared with normal arteries [13]. However, the potential implications of miRNAs in clinical practice remain unclear, and the biological functions of miRNAs in ASO are only partly understood.

MiR-24-3p is a member of the miR-24 gene cluster, which is to the miR-23 and miR-27 clusters. It functions as a tumor suppressor in colorectal cancer and laryngeal carcinoma cells [17, 18] but as an oncogene in human non-small cell lung lines [19]. In this study, we explore the role of miR-24-3p in ASO. We report that miR-24-3p is downregulated in ASO arteries. Furthermore, we show that miR-24-3p inhibits the proliferation and migration, and promotes apoptosis of primary HASMCs by directly targeting platelet derived growth factor receptor-B (PDGFRB) and c-Myc. Our results implicate miR-24-3p as a new therapeutic target for lower extremity ASO.

Materials and Methods

Sample acquisition

The research ethics committee from the first affiliated hospital of Sun Yat-sen University approved this study, and informed consent was obtained from all patients. ASO arterial specimens were collected from 6 patients (four men and two women) with an average age of 62.7 years who underwent a major amputation between January 2012 and October 2012. Six normal arterial specimens from healthy organ donors were collected from age-, sex- and ethnicity-matched controls. All artery samples were snap-frozen in liquid nitrogen immediately after arterectomy and stored at -80°C, ready for RNA extraction. Some samples were fixed in paraformaldehyde (4%) and embedded in paraffin. All samples were identified by at least three vascular surgeons.

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For the detection of miRNAs, 1000 ng RNA was subjected to reverse transcription (RT) using an miRNA RT kit (TaKaRa, Dalian, China) and used according to the manufacturer’s instructions. Real-time PCR was performed using the
cDNA as a template with the SYBR PrimeScript miRNA Real-Time PCR Kit (Takara, Dalian, China) according to the manufacturer's instructions with following primers: miR-24-3p 5’-CCCATTCAGCAGGAAACAGAAA-3’, U6 5’-AGCGAATTCTTGTGAAGGCTT-3’. For the detection of mRNA, cDNA was synthesized from 1000 ng RNA using the PrimeScript RT reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed using the cDNA as a template with the PrimeScript Real Time Master Mix (Takara, Dalian, China) on a real-time PCR machine (Bio-Rad CFX 96, Hercules, CA, USA) with the following primers: PDGFRB 5’-GGCTACATGGACATGAGCAAGG-3’ (forward), 5’-AGCTTAGACTGGAGACTCGTTGA-3’ (reverse); C-myc 5’-GCAGCTGCTTAGACGCTGGA-3’ (forward), 5’-CGCAGTAGAAATACGGCTGCAC-3’ (reverse); GAPDH 5’-GCCAGCTCAAGGCTGAGAAC-3’ (forward), 5’-TGGTGAAGAGCCAGCTGGA-3’ (reverse). Data analysis was performed by the comparative C_T method using Bio-Rad Manager 2.1 software (Hercules, CA, USA).

In situ hybridization

In situ hybridization (ISH) of miR-24-3p was performed in 5-μm-thick sections of arteries. In brief, the sections were deparaffinized with xylenes and rehydrated in graded dilutions of ethanol (2 × 100%, 75%, 50% and 25%). After washing with PBS, the sections were pre-hybridized in a hybridization solution (50% formamide, 5 × SSC, 0.5 mg/mL yeast tRNA, 1 × Denhardt’s solution) at 49.5°C for 2 hours and hybridized using a probe (LNA miRCURY probe, Exiqon, Vedbaek, Denmark) at 49.5°C overnight. The sequence of probe was as follows: 5’-Dig-CTGTTCCTGCTGAACTGAGCCA-3’ Dig. After washing, sections were blocked using Roche Block Buffer (Roche, Basel, Switzerland). Then, an anti-digoxigenin antibody (Roche, Basel, Switzerland) was added at a 1:1000 dilution and incubated at 4°C for 16 hours. After washing, the sections were incubated with 1:100 diluted NBT/BCIP staining buffer for 12 hours in the dark. After color development was terminated, the sections were washed with PBS and then dehydrated by graded dilutions of ethanol. Coverslipped, and finally visualized using an Eclipse 80i microscope (Nikon, Tokyo, Japan).

Cell culture

HASMCs were obtained from the femoral artery of a healthy organ donor. We prepared HASMCs using our established explant method [13]. HASMCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Gibco, Karlsruhe, Germany) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in an incubator humidified with 5% CO₂. All cells used in this study were from passages IV-IX.

Oligo transfection

The miR-24-3p mimic and negative control oligos were purchased from RiboBio (Guangzhou, China). The mimic and negative control oligos were transfected using RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s directions.

HASMC proliferation measurement

HASMC proliferation was measured using cell counting kit-8 (CCK-8) and EdU assays. The cells were seeded into 96-well plates. After transfection, the cells were incubated in serum-free DMEM with or without 10 ng/ml PDGF-BB (Peprotech, Rocky Hill, NJ, USA). After 24 hours, survival rates were measured using the CCK-8 kit (Dojindo, Kumamoto, Japan). Ten microliters of solution was added into each well, and the cells were incubated for 2 hours. The optical density was then measured at 450 nm.

For the EdU assay, cells were stimulated with or without PDGF-BB (10 ng/ml) for 24 hours post transfection. HASMCs were exposed to a 10 nM EdU solution (RiboBio, Guangzhou, China) for 2 hours at 37°C, fixed in 4% formaldehyde for 30 minutes and treated with 0.5% Triton X-100 for 10 minutes. After washing with PBS, the cells were treated with Apollo cocktail for 30 minutes before being stained with Hoechst for 30 minutes. Images were acquired using a fluorescence microscope (Carl Zeiss Axio Observer Z1, Jena, Germany).

HASMC migration assay

HASMC migration in vitro was assessed using transwell and wound healing assays. The transwell assay was performed using a transwell (Corning, Tewksbury, MA, USA) with a polycarbonate membrane (0.8-μm pores) inserted. After transfection, HASMCs were added to the upper chambers with 200 μl serum-free
DMEM, and the lower chambers were filled with 500 μl DMEM with or without PDGF-BB (10 ng/ml). After 12 hours, non-migrated cells on the upper surface were removed, and migrated cells on the lower surface of membrane were fixed with 4% formaldehyde and quantified by 0.1% crystal violet staining.

For the wound healing assay, HASMCs were seeded into 6-well plates (60,000 cells/well). After transfection, the nearly 100% confluence cells were wounded using a sterilized 200-μl disposable pipette tip to create a scratch wound in each well. The scratch wounds were visualized using a microscope (ZEISS Axio Observer Z1, Jena, Germany).

Cell apoptosis analysis
HASMCs apoptosis was assessed using the Annexin V-FITC apoptosis kit (Keygen, Nanjing, China) according to the manufacturer’s instructions. After transfection, the HASMCs were starved with serum free DMEM for 0 hour or 24 hours. Then, the cells were harvested, resuspended in 500 μL binding solution, and incubated with 5μL Annexin V and 5μL PI for 10 minutes in the dark. Cell apoptosis was then analyzed using flow cytometry (Beckman EPICS XL-MCL). Results were analyzed using the Kaluza software (Beckman EPICS XL-MCL).

Western blotting analysis
HASMCs were lysed in RIPA lysis buffer (Cell Signaling Technology, Boston, MA, USA) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). The total protein concentration was measured using a BCA assay kit (Keygen, Nanjing, China). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), and blotted using anti-PDGFRB, anti-C-myc, and anti-GAPDH antibodies (Cell signaling Technology, Boston, MA, USA). The bands were visualized using Luminol reagent (Thermo Pierce, Waltham, MA, USA) and imaged using a GE ImageQuant Las 4000 mini (GE, Fairfield, CT, USA).

Luciferase assay
Fragments of the PDGFRB or c-Myc mRNA 3’-UTRs containing putative or mutated miRNA binding sites for miR-24-3p were PCR amplified and cloned into a psi-CHECKTM vector (Promega, Madison, WI, USA) downstream of the Renilla luciferase coding sequence. The constructs were cotransfected with miR-24-3p mimic or control oligos into HEK 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Immunohistochemical staining
Immunohistochemistry (IHC) was performed on 4% paraformaldehyde fixed; paraffin embedded 5μm-thin artery sections according to a previously established protocol. PAGFRB antibody (Cell signaling Technology, Boston, MA, USA) or c-Myc antibody (Protein Technology, Chicago, IL, USA) was used to detect the expression of PDGFRB or c-Myc. Then, sections were treated with DAB, according to the manufacturer’s instruction, and finally visualized using Eclipse 80i microscope (Nikon, Tokyo, Japan). Integrated Optical Density (IOD) value was calculated using the Image Pro Plus 6.0 software.

Statistics
Differences between the treatment groups and control group were assessed by Student’s t test and one-way ANOVA with multiple comparisons, using the Neuman-Keuls test for the statistical analysis. We applied GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA) and SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) for our statistical analyses. The data were expressed as the mean ± SD. Statistical significance is indicated as follows: * = p < 0.05; ** = p< 0.01; *** = p < 0.001.

Results
MiR-24-3p expression was downregulated in ASO arteries
To investigate the expression of miR-24-3p within ASO arteries, six ASO and six normal artery samples were prepared. The levels of miR-24-3p were assessed using qRT-PCR. MiR-
MiR-24-3p was downregulated in ASO arteries and mainly localized within the media of arteries (Fig. 1A and B). MiR-24-3p was markedly downregulated by 3.3-fold in ASO arteries, and 3.74-fold in medium-sized arteries. No significant differences were observed in the endothelium and adventitia. ISH confirmed the abundance of miR-24-3p in the media of arteries and the downregulation of miR-24-3p in ASO arteries (Fig. 1C and D).

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**MiR-24-3p regulates the proliferation, migration and apoptosis of HASMCs**

Previous studies have shown that miR-24-3p regulates cell proliferation in many tumor cell lines. However, it remains unclear whether miR-24-3p functions on primary HASMCs of the lower extremities. We overexpressed miR-24-3p by using miR-24-3p mimic oligos (Fig. 2A) and observed the effects on HASMCs. The miR-24-3p mimic markedly inhibited the PDGF-BB-induced proliferation (Fig. 2B and C) and migration of HASMCs (Fig. 2D and E). The miR-24-3p mimic promoted the serum starvation-induced apoptosis of HASMCs (Fig. 2F).

**PDGFRB and c-Myc are direct targets of miR-24-3p in HASMCs**

Both PDGFRB and c-Myc were predicted as potential targets of miR-24-3p based on the identification of their mRNA 3'-UTR sequences being complementary to miR-24-3p, as identified using http://www.targetscan.org. To date, there are no reports showing that PDGFRB is directly affected by miR-24-3p. c-Myc is a validated target for miR-24-3p in cancer, but this has not been confirmed in HASMCs.

Fig. 1. MiR-24-3p is downregulated in ASO artery samples. (A) qRT-PCR shows that miR-24 expression is lower in ASO arteries (n=6) compared with normal arteries (n=6). (B) MiR-24-3p is highly expressed in the media of ASO arteries (n=6), compared with normal arteries (n=6), as assessed by real-time PCR. (C) and (D) Upper row, hematoxylin-eosin (HE) staining demonstrates artery structures; lower row, in situ hybridization (violet) shows that miR-24-3p is located in the artery walls. The IOD calculation for miR-24-3p staining in artery sections shows that miR-24-3p staining was significantly decreased in ASO sections (n=6) compared with normal sections (n=6). Images were taken using a 20X objective. Data are presented as the mean ± SD, *P<0.05, **P<0.01, ***P<0.001.
The upregulation of miR-24-3p decreases the expression of both PDGFRB and c-Myc protein (Fig. 3B and I); however, it does not alter the mRNA levels of PDGFRB and c-Myc.
PDGFRB and c-Myc are targets of miR-24-3p in HASMCs. (A) The putative binding sites of the PDGFRB 3'UTR. (B) PDGFRB protein levels were downregulated by the miR-24-3p mimic (50 nM/L), as measured by Western blotting. (C) The miR-24-3p mimic (50 nM/L) had no effect on PDGFRB mRNA levels, as measured by qRT-PCR. (D-G) MiR-24-3p mimic (50 nM/L) or control (50 nM/L) oligos were cotransfected with pei-CHECK-2 vector-constructed wild-type (D) or mutated PDGFRB (E-G) mRNA 3'-UTR sequences. The relative luciferase activity is reported. (H) The putative binding sites in the c-Myc 3'UTR. (I) c-Myc protein levels were downregulated by the miR-24-3p mimic (50 nM/L), as measured by Western blotting. (J) The miR-24-3p mimic (50 nM/L) had no effect on c-Myc mRNA levels, as measured by qRT-PCR. (K) and (L) MiR-24-3p mimic (50 nM/L) or control (50 nM/L) oligos were cotransfected with pei-CHECK-2 vector-constructed wild-type or mutated c-Myc mRNA 3'-UTR sequences. The relative luciferase activity is reported. Data are presented as the mean ± SD (n=3), *P<0.05, **P<0.01, ***P<0.001, experimental versus negative control.

(Fig. 3C and J). The results indicate that miR-24-3p negatively regulates PDGFRB and c-Myc expression at the post-transcriptional level.
To investigate whether miR-24-3p directly targets PDGFRB and c-Myc, we constructed luciferase reporters containing the PDGFRB-3'UTR fragment or the c-Myc-3'UTR fragment with the predicted miR-24-3p binding sites. The luciferase assay showed that the miR-24-3p mimic decreased the relative luciferase activities of Luc-PDGFRB-3'-UTR and Luc-c-Myc-3'-UTR (Fig. 3D and K). We mutated the binding sites of miR-24-3p in the PDGFRB 3'-UTR and reported that luciferase activity was abrogated only when both binding sites of miR-24-3p were mutated (Fig. 3E-G). These results suggest that miR-24-3p can bind to the two binding sites of PDGFRB 3'-UTR and downregulate the expression of PDGFRB. We also observed that the relative luciferase activity recovered upon mutating the miR-24-3p seed sequences in the c-Myc 3'-UTR (Fig. 3L), indicating that c-Myc is also a direct target of miR-24-3p.

**PDGF-BB downregulates miR-24-3p and upregulates PDGFRB and c-Myc in HASMCs**

Platelet-derived growth factors (PDGs) play a central role in atherogenesis, affecting proliferation, migration and apoptosis in vascular smooth muscle cells (VSMCs). PDGF-BB is a member of the PDGF family. It has been reported that the expression of miR-24-2 (miR-24-5p) is induced by PDGF-BB treatment in human primary pulmonary arterial smooth muscle cells (PASMCs) [20], but its function on miR-24-3p is not known. We used 20 μg/L PDGF-BB
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Cell Physiol Biochem 2015;36:1359-1370
DOI: 10.1159/000430302
Published online: July 01, 2015
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Discussion

In this study, we revealed that miR-24-3p is downregulated in ASO arteries. It has been reported that blocking miR-24 leads to preserved cardiac function and survival via the prevention of endothelial apoptosis and the enhancement of vascularity following myocardial infarction [21]. However, Qian et al. [22] demonstrated that miR-24 inhibited cardiomyocyte apoptosis, attenuated infarct size and reduced cardiac dysfunction. Recently, Di Gregoli et al. identified that microRNA-24 inhibition increased plaque size and accelerated plaque progression in ApoE-/- mice by regulating the behavior of macrophage [23]. We show that miR-24-3p is abundant in the media of arterial walls and is downregulated in ASO arteries. These findings indicate that miR-24-3p may regulate the function of HASMCs.

It is believed that the proliferation, migration and apoptosis of HASMCs represent key factors in the pathogenesis of ASO [24]. We overexpressed miR-24-3p by using an miR-24-3p mimic to investigate the effect of this miRNA in ASO. We demonstrate that miR-24-3p suppresses PDGF-BB-induced proliferation and migration in HASMCs. And we find miR-24-3p promoted the apoptosis of HASMCs. These results suggest that miR-24-3p may be involved in the pathogenesis of ASO through regulating the functions of HASMCs.

One miRNA can regulate a number of target genes, owing to flexibility in binding sites [25]. It has been reported that miR-24-3p regulates X-linked inhibitor of apoptosis protein (XIAP) [19], ALK4 [26] and E2F2 [27]. By using www.targetscan.org, we revealed PDGFRB as a potential target gene for miR-24-3p. PDGFRB is a receptor of PDGF that can bind to PDGF-B and PDGF-D [28]. Crosby et al. [29] have proven that PDGFRB is a key regulator of VSMC development. PDGFRB has a central role in atherosclerosis, and its activity remarkably enhances the migration and proliferation, and inhibits the apoptosis of VSMCs [30–32]. In this study, we revealed that PDGFRB is a direct target gene of miR-24-3p. Initially, we demonstrated that PDGFRB protein levels were downregulated using an miR-24-3p mimic, whereas PDGFRB mRNA levels were unaffected. Furthermore, we confirmed that miR-24-3p was able to bind the 3'-UTR of PDGFRB, indicating that PDGFRB is a direct target of miR-24-3p. We propose that miR-24-3p inhibits the migration, proliferation and apoptosis of HASMCs partly through regulating the expression of PDGFRB.

C-Myc is a proto-oncogene that promotes the proliferation of HASMCs after carotid artery balloon injury [33]. Biro et al. [34] found that by using antisense oligonucleotides targeting c-Myc, the proliferation and migration of VSMCs could be inhibited. In this study, we showed that miR-24-3p inhibits c-Myc protein expression in HASMCs. We also found that miR-24-3p binds to the 3'-UTR of c-Myc mRNA, suggesting that c-Myc is also a target of miR-24-3p.

We used PDGF-BB to stimulate primary HASMCs and found that PDGF-BB could inhibit the expression of miR-24-3p and induce the expression of PDGFRB and c-Myc in HASMCs. Marie-luce Vignais et al. found that PDGF regulate the activation of STAT1 [35]. And, Deepbase.sysu.edu.cn/chipbase/microrna.php predicted the STAT1 may regulate the transcription of miR-24-3p. We hypothesise the PDGF-BB regulate the expression of miR-24-3p through SATA1, it is needed further research. Ross et al. [36] first discovered PDGFs in 1974, revealing that they have a major effect on the proliferation, migration and apoptosis of VSMCs and that PDGF-B/PDGFRB is a major signaling pathway. In normal vessels, PDGFs and their receptors are expressed at low or undetectable levels, and the PDGF signal is regulated by a feedback control mechanism with stimulatory and inhibitory signals that arise in paral-
The expression of PDGFs and their receptors in eccentric atherosclerotic lesions and the mechanisms regulating their expression remain unknown. We discovered that PDGF-BB reduces the expression of miR-24-3p but induces the expression of PDGFRB. It has been reported that aggregating platelets or leukocytes result in high PDGF expression in atherosclerosis [36, 37]. We reason that the increased PDGF expression leads to miR-24-3p downregulation, relieving the inhibitory effect on PDGFRB expression. C-Myc is expressed at low levels in quiescent cells and can be induced by PDGF [38]. In this study, we found that PDGF-BB could downregulate miR-24-3p, whereas miR-24-3p negatively regulated the expression of c-Myc. Thus, we hypothesize that miR-24-3p is involved in the regulating the effects of PDGF-BB on c-Myc and mediates the function of PDGF in HASMCs.

In conclusion, this study shows that miR-24-3p is downregulated in ASO. PDGF-BB stimulates HASMCs, which could reduce the expression of miR-24-3p and thereby upregulate target genes such as PDGFRB and c-Myc and promote the pathogenesis of ASO. Our findings provide new insight into the PDGF-BB/miR-24-3p/PDGFRB and PDGF-BB/miR-24-3p/c-Myc pathways as well as a new potential target for preventing and treating ASO.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 81270378, 81070258, 81370368), by a Guangdong Department of Science & Medicine Center grant (2011A080300002), by the Guangdong Province Industry-Academia-Research Program (2011B090400117) and by the Guangzhou Science and Technology Plan Projects (2011Y2-00022).

Disclosure Statement

The authors declare that they have no conflicts of interest.

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