MicroRNA-146a promotes the proliferation of rat vascular smooth muscle cells by downregulating p53 signaling

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Abstract. The present study aimed to detect and verify gene expression profile differences for microRNA (miR)-146a and its role in the proliferation of vascular smooth muscle cells (VSMCs). Artificially synthesized miR-146a mimics, miR-146 inhibitor, scramble-miRNA or PBS was transfected into cultured primary rat VSMCs *in vitro*. Reverse transcription-quantitative polymerase chain reaction confirmed that the miR-146a expression level was significantly decreased in VSMCs treated with miR-146a inhibitor ($P<0.01$). Cell Counting Kit-8 was used to determine the proliferation ability, which demonstrated that proliferation was significantly decreased in VSMCs treated with miR-146a inhibitor ($P<0.01$). Microarray expression profiling analysis revealed that the p53 signal pathway was upregulated in VSMCs treated with the miR-146a inhibitor. Compared with untransfected VSMCs, the mRNA and protein expression levels of caspase-3 and phosphatase and tensin homolog (PTEN) in p53 signal transduction pathway did not exhibit a significant difference ($P>0.05$); however, the mRNA and protein expression levels of p53 were significantly decreased in cells transfected with miR-146a mimics and increased in miR-146a inhibitor transfected cells (both $P<0.01$). The mRNA and protein expression levels of cyclin D1 significantly increased in miR-146a mimics transfected cells and decreased in cells transfected with the miR-146a inhibitor (both $P<0.05$). The present data indicated that miR-146a may promote the proliferation of rat VSMCs by downregulating p53 and upregulating cyclin D1 expression.

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Introduction

Vascular neointimal remodeling is a crucial pathological process in proliferative cardiovascular diseases, such as atherosclerosis and restenosis following angioplasty (1). Vascular smooth muscle cells (VSMCs) are not terminally differentiated; their phenotypic transition from the differentiated state to the dedifferentiated state induces alterations in vascular biology and subsequently leads to vascular neointimal remodeling (2). However, the molecular mechanisms behind this pathological process have not been fully determined.

Endogenous, non-coding microRNAs (miRNAs) negatively regulate the expression of greater than one-third of human genes at the post-transcriptional level by degrading or inhibiting the translation of their specific target genes (3). Certain miRNAs determine cell fate and tissue homeostasis, including VSMCs biology and vascular remodeling. Identification of these specific miRNAs may aid in the discovery of novel targets in the prevention and treatment of proliferative cardiovascular diseases (4,5).

miRNA (miR)-146a was the first small RNA to be discovered that was demonstrated to be involved in the regulation of the immune system, mainly associated with the development of rheumatoid arthritis, cancer and sepsis (6). A previous study demonstrated that the level of miR-146a expression was significantly higher in the peripheral blood mononuclear cells of patients with acute coronary syndrome (7). miR-146a may promote differentiation and functions of type 1 T helper (Th1) cells, and may be involved in immune regulation in patients with coronary heart disease (7). The level of miR-146a expression was reported to be significantly upregulated in the carotid artery following carotid artery balloon injury, which suggested that miR-146a may be involve in the pathophysiology of restenosis following balloon injury (8). A recent study also revealed that miR-146a was involved in atherosclerosis in apolipoprotein E-deficient ($ApoE^{-/-}$) rats (9). Our previous study demonstrated that the level of miR-146a was significantly increased during VSMC proliferation, and that miR-146a promoted the proliferation and migration of VSMCs and inhibited their apoptosis, but its mechanisms remains unclear (10).

The present study aimed to explore the target genes of miR-146a and the signaling pathways that may be involve in promoting VSMC proliferation to gain a better understanding of its role in cardiovascular diseases.

Materials and methods

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethics Committee of the Shenzhen People's Hospital (Shenzhen, China); all efforts were made to minimize suffering.

Rat VSMC culture and miR-146a interference. Male Sprague-Dawley rats (8 weeks-old; 150±20 g; n=3) were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China) and housed in specific pathogen-free animal room on 12-h light/dark cycle at 40-70% humidity and 22±2˚C with access to food and water *ad libitum*. Primary VSMCs were obtained and isolated from the thoracic aortic media of SD rats (10). The VSMCs were divided into four groups: i) miR-146a mimics group; ii) miR-146a inhibitor group; iii) miR-Scramble negative control group; and iv) untransfected VSMC control group. miR-146a inhibitors were labeled with 6-carboxyfluorescein. Cells ($1x10^5$ cells/ml) in miR-146a mimics group, miR-146a inhibitor group and negative control group were transfected with miR-146a mimics (50 nmol/l; Shanghai GenePharma Co., Ltd., Shanghai, China), miR-146a inhibitor (siRNA) (50 nmol/l; Shanghai GenePharma Co., Ltd.) or miR-Scramble negative control (50 nmol/l; Shanghai GenePharma Co., Ltd.), respectively, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator containing 5% CO_2 at 37˚C for 5 h. PBS (Gibco; Thermo Fisher Scientific, Inc.) was added at the same equivalent dosage to normal VSMC group. The medium was replaced with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) 5 h post-transfection, and transfection efficiency was observed under a fluorescence microscope. VSMCs were subsequently incubated for 48 h in the incubator containing 5% CO_2 at 37˚C prior to further experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following incubation at 37˚C for 48 h post-transfection, total RNA of VSMCs ($3x10^5$ cells/ml) was extracted using the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The cDNA was synthesized using a PrimeScript Reagent Kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR was accomplished using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR Premix Ex Taq Kit (Takara Bio, Inc.). Primer sequences were as follows: miR-146a, forward 5'-TGAGAACTGAATTCCATGGGTT-3', reverse 5'-GGCCAACCGCGAGAAGATGTTTTTTTTT-3'; caspase-3, forward 5'-GCTGGACTGCGGTATTGAGA-3', reverse 5'-CCATGACCCGTCCCTTGA-3'; phosphatase and tensin homology (PTEN), forward 5'-AGACCATAACCCACCACAGC-3', reverse 5'-TTACACCAGTCCGTCCTTTCC-3'; p53, forward 5'-AGATTGGGGAATGGGTTGG-3', reverse 5'-GATAGAATCTTACAGGCGGTGG-3'; cyclin D1, forward 5'-GCGTACCCTGACACCAATCTC-3', reverse 5'-CTCTTCGCACTTCTGCTCCTC-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-ACGCTTCACGAATTTGCGT-3'. PCR conditions were as follows: One cycle at 95˚C for 30 sec, 40 cycles at 95˚C for 5 sec and

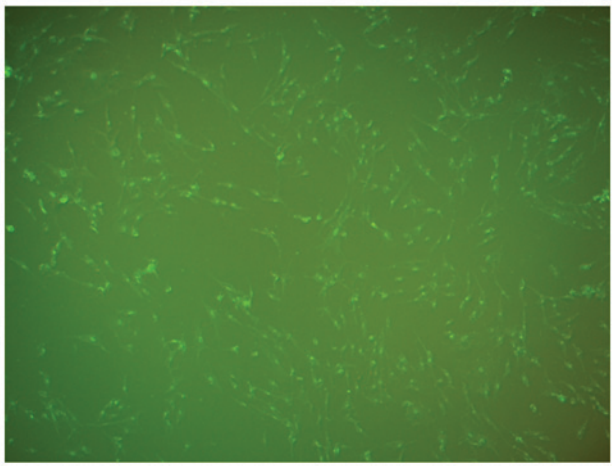

Figure 1. microRNA-146a inhibitors labeled with 6-carboxyfluorescein fluorescence in vascular smooth muscle cells were detected under fluorescence microscopy.

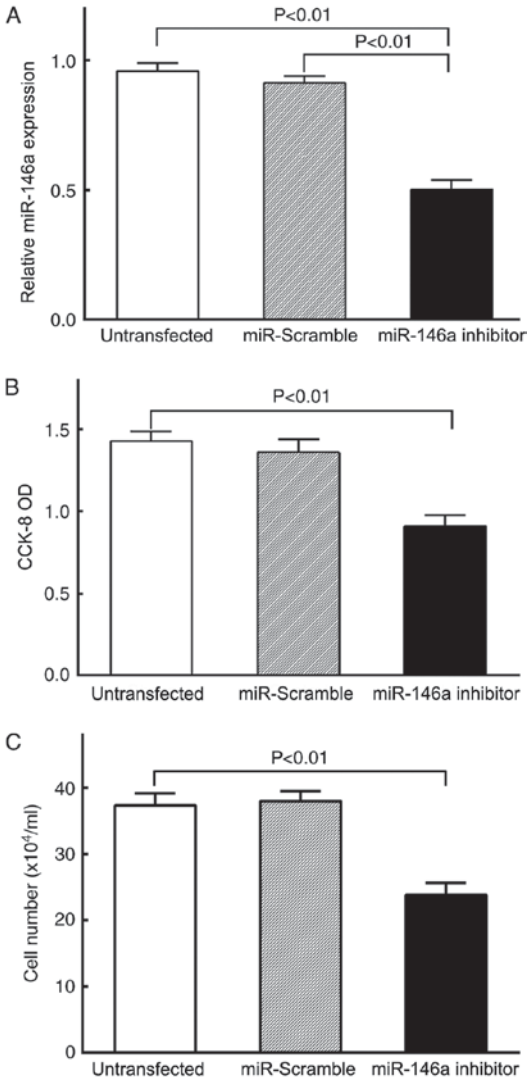

Figure 2. miR-146a promotes proliferation of VSMCs. (A) Reverse transcription-quantitative polymerase chain reaction demonstrated that the expression of miR-146a in VSMCs was knockdown by transfection with miR-146a inhibitors. (B), Cell Counting Kit-8 analysis and (C) total cell counting by hemocytometer indicated that the proliferation of VSMCs was reduced in cells transfected with the miR-146a inhibitor. miR, microRNA; VSMCs, vascular smooth muscle cells.

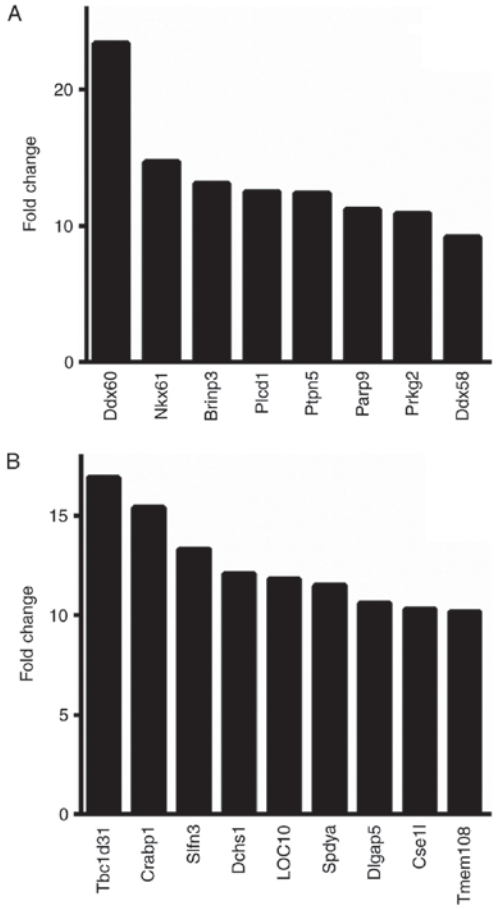

Figure 3. Top genes identified as being regulated by microRNA-146a. (A) The top eight upregulated genes with fold change >10. (B) The top nine downregulated genes with fold change >10.

one cycle at 60˚C for 30 sec. Relative expression levels of miR-146a, caspase-3, PTEN, p53, cyclin D1 and U6 (internal control) were calculated by using SDS 2.1.1 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All Experiments were performed according to the manufacturer's protocol.

VSMC proliferation. VSMCs seeded ($1x10^4$ cells/well) in the 96-well plate were cultured in the incubator containing 5% CO_2 at 37˚C for 24 h. The cells were transfected with miR-146a inhibitor (50 nM), miR-Scramble control (50 nM) or PBS for 5 h at 37˚C. The proliferative properties of the VSMCs were measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and cell counting was performed 48 h after VSMC transfection. The absorbance was measured at 450 nm with a reference wavelength at 650 nm using microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The number of VSMCs was counted using a hemocytometer under an IX51 light microscope (Olympus Corporation, Tokyo, Japan). All experiments were performed six times.

Microarray expression profiling analysis. VSMCs ($1x10^5$ cells/ml) cultured in an incubator containing 5% CO_2 at 37˚C were divided into miR-146a inhibitor group and miR-146a scramble group (n=3 samples/group). VSMCs were transfected

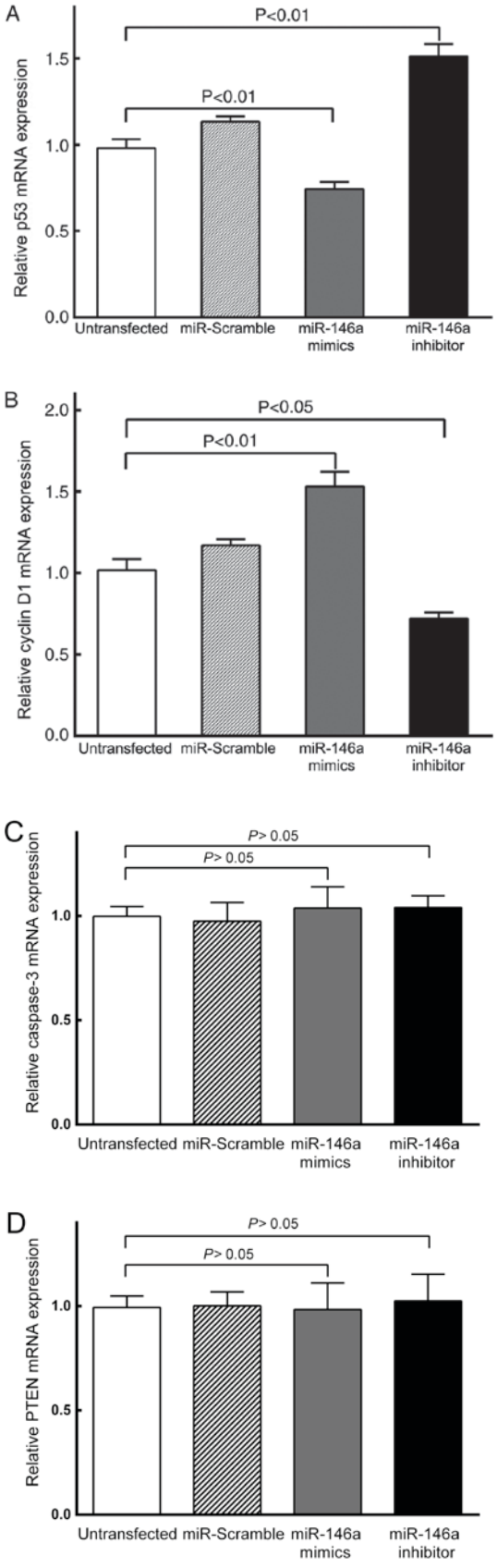

Figure 4. Effect of miR-146a knockdown on mRNA expression levels of p53 and cyclin D1 in transfected VSMCs. (A) RT-qPCR demonstrated that p53 mRNA expression was downregulated by miR-146a. (B) RT-qPCR demonstrated that cyclin D1 mRNA expression was upregulated by miR-146a. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VSMCs, vascular smooth muscle cells. There were no significant differences observed in mRNA levels of (C) caspase-3 or (D) PTEN between the four experimental groups.

with miR-146a inhibitor (50 nmol/l) or miR-146a scramble (50 nmol/l) in 5% CO_2 at 37˚C for 5 h at room temperature prior to further incubation in 5% CO_2 at 37˚C for 48 h. Total RNA was isolated from VSMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Gene profile was scanned using an Agilent DNA Microarray Scanner (Agilent Technologies, Inc., Santa Clara, CA, USA) and data were collected using Agilent Feature Extraction software (v11.0.1.1; Agilent Technologies, Inc.). Gene Ontology (www.geneontology.org) analysis was carried out to detect different genes and Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/KEGG) analysis was carried out to detect different pathways. Enrichment scores were calculated to rank distinct pathways according to the P-values of genes detected in these pathways.

Western blot analysis. Following incubated at 5% CO_2, 37˚C for 48 h post-transfection, cultured cells were lysed on ice for 30 min in 1x RIPA Lysis Buffer (Sangon Biotech Co., Ltd.) supplemented with a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Protein concentrations of the cell lysates were measured with the BCA Protein Assay kit (Sangon Biotech Co., Ltd.). Denatured proteins (20 μg/lane) were electrophoresed on 10% SDS-polyacrylamide gels and were electrotransferred from the gel to PDVF membranes (EDM Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk in a Tris buffered saline with 0.05% Tween-20 for 2 h at room temperature, membranes were incubated overnight at 4°C with polyclonal rabbit anti-rat Caspase-3 (1:1,000; ab217; Abcam, Cambridge, MA, USA), polyclonal rabbit anti-rat PTEN (1:1,000; ab32199; Abcam), polyclonal rabbit anti-rat P53 (1:200; ab1431; Abcam), monoclonal rabbit anti-rat cyclinD1 (1:1,000; ab134175; Abcam) and GAPDH (1:1,000; ab8245; Abcam). The blots were subsequently incubated in 1:6,000 dilutions of goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. ECL kit (Thermo Fisher Scientific Inc.) developing solution (100 μl) was added to each sample to detect the protein signal with a Quant RT ECL cold CCD imaging system (GE Healthcare Life Sciences, Little Chalfont, UK). GAPDH was used as the internal control.

Statistical analysis. Data are presented as the mean ± standard deviation and were analyzed using SPSS 12.0 statistical software (SPSS, Inc., Chicago, IL, USA), one-way analysis of variance was used to analyze the data, with a Student-Newman-Keuls-q test used for pairwise comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-146a promotes VSMC proliferation. Following transfection with the miR-146a inhibitor for 5 h, a large number of fluorescent granules were visible in the cytoplasm of VSMCs under fluorescence microscopy, which indicated that miR-146a inhibitor was successfully transfected into the VSMCs (Fig. 1). The level of miR-146a expression was detected by RT-qPCR. Compared with cells transfected with the miR-Scramble negative control and the untransfected VSMCs, the relative expression level of miR-146a in the miR-146a inhibitor group was significantly decreased (P<0.01; Fig. 2A). No significant differences were identified between the miR-Scramble and the untransfected control cells (P>0.05; Fig. 2A). VSMC proliferation was detected 48 h post-transfection by CCK-8 analysis and cell counting. No significant differences were identified for the OD values and the cell numbers between the miR-Scramble negative control group and the untransfected VSMC group (P>0.05; Fig. 2B and C); however, the OD values and the cell numbers were significantly decreased in cells transfected with the miR-146a inhibitor (P<0.01; Fig. 2B and C).

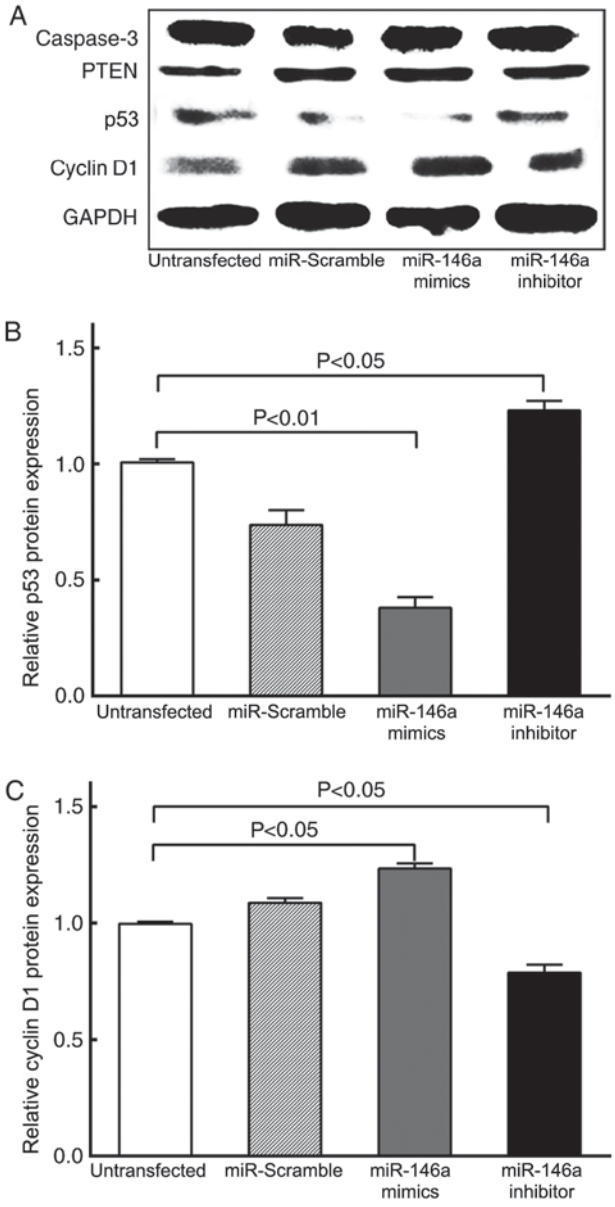

Figure 5. Effects of miR-146a inhibitor transfection on protein expression levels in VSMCs. (A) Protein expression levels of caspase-3, PTEN, p53 and cyclin D1 were investigated by western blot analysis; GAPDH was used as an internal control. (B) Relative quantification of p53 protein expression levels from (A); p53 expression was significantly reduced by miR-146a mimics transfection. (C) Cyclin D1 protein expression was significantly increased by miR-146a mimics transfection. miR, microRNA; PTEN, phosphatase and tensin homolog; VSMCs, vascular smooth muscle cells.

p53 signaling pathway is a miR-146a target. Microarray expression profiling analysis revealed that, among the total 16,802 target genes identified, 8,547 (50.9%) genes were upregulated

in cells treated with the miR-146a inhibitor and 8,255 (49.1%) genes were downregulated. A fold-change cut-off value >2.0 was used to select for differential expression, which identified 806 genes as upregulated in cells treated with the miR-146a inhibitor, and of these, 8 genes exhibited a fold change >10.0 (Fig. 3A). Among the 1,026 genes that were identified as downregulated in cells treated with the miR-146a inhibitor, >2.0-fold change, 9 genes exhibited a >10.0-fold change (Fig. 3B). Kyoto Encyclopedia of Genes and Genome analysis identified that the p53 signaling pathway was upregulated by miR-146a inhibitor (P=0.019; enrichment score, 1.72).

miR-146a downregulates p53 expression. The key molecules in the p53 signaling pathway, including p53, cyclin D1, caspase-3 and PTEN, were detected by RT-qPCR and Western blot analysis (Figs. 4 and 5). The results demonstrated that there were no significant differences in mRNA and protein expression levels of caspase-3 and PTEN between the four experimental groups (P>0.05; Figs. 4C, D and 5A). However, p53 mRNA and protein expression levels were decreased in cells transfected with the miR-146a mimics compared with the untransfected VSMC group (P<0.01; Figs. 4A, 5A and B); p53 mRNA and protein expression levels were increased in cells transfected with the miR-146a inhibitor compared with untransfected VSMC cells (P<0.01). Compared with the untransfected VSMC group, cyclin D1 mRNA and protein expression levels were increased in miR-146a mimics transfected cells (P<0.01; Figs. 4B, 5A and C), and were decreased in the miR-146a inhibitor group (P<0.05).

Discussion

miRNAs are important and widespread gene regulators, and serve important roles in physiological and pathological processes, including cell growth, development, differentiation, signal transduction, disease and death (3). Previous studies reported that miRNAs may not only be used as diagnostic biomarkers of disease, but may also be used as therapeutic targets for treating diseases (11,12).

miR-146a was reported as one of the major miRNAs involved in the regulation of immune functions (13). It was also previously confirmed that stimulation of monocytes by lipopolysaccharide caused an increase in the expression of miR-146a. Tumor necrosis factor (TNF-α) and interleukin (IL)-1β have been reported to promote miR-146a expression through nuclear factor (NF)-κB-dependent pathway (14). miR-146a was revealed to negatively regulate inflammation and the immune response by downregulating the expression levels of TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1, to avoid excessive inflammatory response (15). miR-146a was also reported to serve an important role in the occurrence and development of rheumatoid arthritis, cancer and sepsis, and may be used as a biomarker of the diseases (16-18). A previous study reported that the level of miR-146a in the peripheral blood mononuclear cells was significantly increased in patients with acute coronary syndrome (7), miR-146a expression upregulated the function of Th1 cells, promoted Th1 cell differentiation and NF-κB p65 synthesis, which indicated that miR-146a may be associated with immune function regulation in patients with coronary heart disease. The expression level of miR-146a was revealed to be significantly upregulated in animal models of restenosis following the balloon injury (8). ApoE serves an important role in lipid metabolism and negatively regulates atherosclerosis (19). ApoE proteins were revealed to inhibit NF-κB activation, inflammation and atherosclerosis by promoting miR-146a expression in monocytes and macrophages (9). miR-146a may aid in predicting ventricular remodeling in patients with acute ST-elevation myocardial infarction (20). Results from these previous studies suggested that there may be a close correlation between miR-146a expression and the development of atherosclerosis, coronary heart disease and stenosis. However, these findings are not consistent, as Guo *et al* (7) reported that miR-146a was able to induce Th1 cells to synthesize NF-κB, whereas Li *et al* (9) demonstrated that in monocytes and macrophages, miR-146a inhibited NF-κB expression.

Our previous study indicated that the levels of miR-146a expression were significantly increased during VSMC proliferation (10); following miR-146a expression inhibition by RNA interference, VSMC proliferation and migration were significantly reduced, and the apoptosis was increased, but the underlying mechanism is unclear. The dual-luciferase reporter assay is the classical method for studying miRNA target genes; however, systematized study of signaling pathways by this method is still lacking. p53 is a classic tumor suppressor gene and a pro-apoptotic gene, p53 may not only serve an important role in tumor growth and invasion, but is also closely related to atherosclerosis and ventricular remodeling (19). Induction of p53 expression may inhibit VSMC proliferation (21) and p53-knockout may lead to atherosclerosis (22). p53 may directly or indirectly inhibit the expression of miR-146a in R6/2 rats (23). The cyclins and cyclin-dependent kinase (cyclin/CDK) complex serves an important role in the regulation of the G1/S transition phase; cyclin D1/CDK4 is an important regulatory protein (24). Cyclin D1 activation is closely associated with VSMC proliferation and intima reconstruction, and is an important target to promote VSMC proliferation (25). The present study demonstrated that miR-146a expression may be able to induce VSMC proliferation by downregulating the expression of the key tumor suppressor gene p53, and by upregulating the expression of cyclin D1.

In summary, miR-146a may promote the proliferation of rat VSMCs by downregulating p53 and upregulating cyclin D1 expression, which is related to vascular neointimal remodeling and proliferative cardiovascular diseases. Further work is required to better understand the role of miR-146a in the pathophysiological processes of cardiovascular diseases and its possible use as therapeutic agent.

Acknowledgements

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