Abstract. MicroRNA (miR)-31 serves a key role in various biological processes, including tumor development, angiogenesis and inflammation. Whether miR-31 is involved in the pathological processes of arteriosclerosis obliterans (ASO) remains to be elucidated, as does the mechanism of miR-31 regulation of arterial smooth muscle cells (ASMCs). In the present study, miR-31 expression was detected by reverse transcription-quantitative polymerase chain reaction and in situ hybridization, and was significantly upregulated in human ASO arterial walls compared with normal arterial walls (P<0.001). In addition, miR-31 proliferation was detected by Cell Counting Kit-8 and EdU assays; proliferation was significantly promoted in platelet-derived growth factor (PDGF)-BB-induced human ASMCs (HASMCs) (P<0.001). miR-31 migration was detected by transwell and wound closure assays, and was revealed to be promoted in PDGF-BB-induced HASMCs (P<0.001). Lastly, HASMCs were transfected with miR-31 mimics and inhibitors, and negative controls. A dual-luciferase reporter assay was performed to verify that mitofusin-2 (MFN2) was a direct target of miR-31 and that MFN2 expression was significantly downregulated by miR-31 at a post-transcriptional level in HASMCs (P<0.01). These findings suggest that miR-31 is able to promote the proliferation and migration of HASMCs, at least in part, by targeting MFN2. The results of the present study provide novel insight into the underlying mechanisms and roles of miR-31/MFN2 in the pathology of ASO, which may offer a potential therapeutic target for the treatment of ASO.

Introduction

Atherosclerosis is a chronic, progressive systemic disease affecting arteries, particularly those in the brain, heart, and lower extremities. Arteriosclerosis obliterans (ASO) of the lower extremities is one of the most significant health-related issues worldwide and may have devastating consequences, including intermittent claudication, resting pain and gangrene (1-3). Notable progress has been made in endovascular surgical techniques to treat ASO; however, restenosis following angioplasty remains a primary limiting factor for the long-term success of artery reconstruction (4). Vascular smooth muscle cells (VSMCs) comprise the main component of blood vessel walls, and excessive proliferation and migration of VSMCs is a critical event in the pathogenesis of atherosclerosis and vascular stenosis (5,6). It is unclear whether the molecular mechanism underlying the proliferation and migration of human arterial smooth muscle cells (HASMCs) is associated with ASO.

MicroRNAs (miRNAs or miRs) have previously been shown to serve important roles in cardiovascular systems (7-9). miRNAs comprise a class of non-coding, short (18-22 nucleotides), endogenous RNAs that negatively regulate their targets through binding of the 3' untranslated region (3'-UTR) at a post-transcriptional level. Aberrant miRNA expression serves a key role in the development of ASO (10). There have been notable breakthroughs in studies of gene expression regulation in recent decades, and so miRNAs have been investigated for their functions in VSMC differentiation, proliferation, migration and apoptosis (11,12). A previous study by the present authors demonstrated that miR-21 promoted HASMC proliferation and migration, and inhibited apoptosis in ASO.
of the lower extremities (11). Another study revealed that miR-663 regulated the human VSMC phenotypic switch and vascular neoointimal formation by targeting JunB/myosin light chain 9 expression, which suggests that targeting miR-663 or its targets may provide a promising approach for the treatment of proliferative vascular diseases (12).

Although miR-31 is involved in various biological processes, including tumor development (13-16), angiogenesis (17,18) and inflammation (19), the role of miR-31 in ASO, particularly the regulatory mechanism, remains elusive. The present study aimed to elucidate the potential roles of miR-31 in HASMCs within ASO processes, and to identify the underlying molecular mechanism.

**Materials and methods**

**Source of artery specimens.** ASO artery specimens were obtained from 6 patients with ASO (4 males and 2 females) with an average age of 65.3 years who had undergone major amputations, and normal artery specimens were acquired from 6 healthy organ donors (3 males and 3 females) with an average age of 55.1 years. Specimens were obtained between June 2013 and December 2014. ASO artery specimens were used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses, in situ hybridization (ISH), immunofluorescence, and 4,6-diamidino-2-phenylindole (DAPI) and haematoxylin and eosin (H&E) staining. Artery specimens were harvested in a sterile state and some specimens were snap-frozen in liquid nitrogen immediately after arteriectomy and stored at -80˚C for RNA extraction, while other specimens were fixed with paraformaldehyde (4%) at 4˚C overnight and embedded in paraffin for further analysis. The present study was approved by the research ethics committee of the First Affiliated Hospital, Sun Yat-Sen University (Guangzhou, China) and prior informed consent was provided by the patients.

**RT-qPCR analysis.** The separation of three layers of artery samples was performed using a light microscope with anatomical lens (magnification, x5), micro scissors and micro forceps (both Shanghai Medical Instruments (Group) Ltd., Corp. Surgical Instruments Factory, Shanghai, China). The normal tissues or untreated cells were used as control. Total RNA was extracted from arterial specimens or cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., USA), according to the manufacturer's protocol. RNA was reverse transcribed -quantitative polymerase chain reaction using a miRNA RT kit (Invitrogen; Thermo Fisher Scientific, Inc.) at 95˚C for 5 sec and 60˚C for 20 sec. U6 was used as a reference gene. Relative expression was analyzed using the 2^(-∆∆Cq) method (20). All experiments were independent and performed at least in triplicate.

**ISH, immunofluorescence, and DAPI and H&E staining.** ISH was performed using a S-Digoxigenin (DIG)- and 3’-DIG-labeled miRCURY LNA Detection Probe (Exiqon A/S, Vedbaek, Denmark) to human miR-31 (5′-AGCTATGGCC AGCATCTGGCT-3′) in 4-µm-thick sections of tissues. The tissues were fixed with 4% paraformaldehyde at 4˚C overnight, and embedded in paraffin then stored at room temperature for further analysis. Briefly, the sections were deparaffinized and rehydrated in graded dilutions of ethanol (100, 95, 75 and 60%), digested with 40 µg/ml proteinase K (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 10 min, rinsed in 0.2% glycine/PBS for 5 min, fixed with 4% paraformaldehyde at room temperature for 10 min, and acetylated for 10 min. Sections were subsequently prehybridized in a hybridization buffer [50X Denhardt's solution, 60% formamide, 300 µg/ml transfer RNA, 20X Saline Sodium Citrate (SSC) Buffer and 1 M dithiothreitol (Invitrogen; Thermo Fisher Scientific, Inc.)] at 49˚C for 30 min and then hybridized with the miR-31 probe (1:500) at 49.5˚C overnight. Sections were subsequently washed with 2X SSC for 5 min at 49.5˚C and then blocked for 1 h at room temperature using 2% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and incubated with anti-DIG antibodies (1:200; cat. no. 11093274910; Roche Diagnostics) at 37˚C for 2.5 h. Sections were incubated with tyramide signal amplification buffer (1:50; Focofish, Inc., Guangzhou, China) at room temperature for 20 min in the dark, washed with PBS and covered with a cover slip. Immunofluorescence was performed to determine SM-α-actin localization using anti-SM-α-actin primary antibodies (1:500; cat. no. ab5694; Abcam, Cambridge, MA, USA) at 37˚C for 2 h (11) and Alexa Fluor® 488-conjugated secondary antibodies (1:1,000; cat. no. A12379; Thermo Fisher Scientific, Inc.) at room temperature for 30 min.

DAPI staining was used as a nuclear counterstain in fluorescence microscope. Following the fluorescence staining of SM-α-actin, DAPI staining was performed using a SlowFade™ Diamond Antifade Mountant with DAPI reagent (cat. no. 36968; Invitrogen; Thermo Fisher Scientific, Inc.) in of tissue sections at room temperature overnight in the dark. Images of the sections were captured by an inverted fluorescence microscope (magnification, x100; Axio Observer Z1, Carl Zeiss AG, Oberkochen, Germany) and analyzed by calculating the integrated optical density (IOD) value using Image Pro Plus software (version 6.0, Media Cybernetics, Inc., Rockville, MD, USA).

H&E staining was used to demonstrate arterial structures. The tissue sections were deparaffinized and rehydrated in graded dilutions of ethanol (100, 100, 95 and 75%) for 5 min each. The slides were the incubated with hematoxylin solution for 20 sec, then washed the slides with H2O for 1 min. The slides were then stained with 1% eosin solution for 3 min and dehydrated with graded dilutions of ethanol (75, 95, 100 and 100%) for 1 min each. Alcohol was extracted with two changes of xylene and covered with a cover slip. All of the above steps were performed at room temperature. Images of H&E sections were observed by a light microscope (magnification, x100).
Western blotting. The HASMCs harvested from the femoral arterial walls of healthy organ donors were lysed at 0°C for 15 min in a lysis buffer (cat. no. 9806S; Cell Signaling Technology, Inc., Danvers, MA, USA) supplemented with a protease inhibitor cocktail (cat. no. 04693132001; Roche Diagnostics) and centrifuged in 12,500 x g at 4°C for 15 min. The proteins (20 µg/lane) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBS at 37°C for 2 h, incubated with a rabbit anti-mitofusin-2 (MFN2) monoclonal antibody (1:1,000; cat. no. ab50843; Abcam) at 4°C overnight and washed with TBST at room temperature, three times for 10 min. The signals were detected using Luminol reagent (Pierce; Thermo Fisher Scientific, Inc.), imaged using a GE ImageQuant Las 4000 mini phosphorimager (GE Healthcare Life Sciences, Chalfont, UK) with ImageJ (version 1.48; National Institutes of Health, Bethesda, MD, USA) and presented as the density ratio vs. GAPDH (1:1,000; cat. no. 2118S; Cell Signaling Technology, Inc.). Densitometric analyses were taken as an average of three experiments.

Cell culture. The primary HASMCs were obtained from the femoral arterial walls of amputation from patients who suffered serious trauma, and the cells were prepared by the explant method, previously established in our lab (11). HASMCs from healthy donors were used in all subsequent experiments and the ASO cell model was established by platelet-derived growth factor-BB (PDGF-BB) treatment. The HASMCs were identified via staining with anti-SM-α-actin antibodies at 37°C for 2 h (1:1,000; cat. no. ab5694; Abcam), and passages from 4 to 9 were utilized in the present study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂, then the cells were added to a cell cryopreservation solution (10% dimethylsulfoxide and 90% FBS) and stored in liquid nitrogen.

HASMC transfection. Cells (2-3x10⁵ cells/well) were seeded into wells and transfection was performed using Lipo RNA iMax (Invitrogen; Thermo Fisher Scientific, Inc.) 24 h later. For miR-31 function analysis, miR-31 mimics (50 nmol/l), miR-31 inhibitor (100 nmol/l) and negative control oligos (50 nmol/l; all Guangzhou RiboBio Co., Ltd., Guangzhou, China) were transfected into the cells, following the manufacturer's protocol.

Measurement of HASMC proliferation. Cell Counting Kit-8 (CCK-8) and EdU assays were performed to measure
cell proliferation. HASMCs were seeded into 96-well plates (2-3x10^3 cells/well). Cells were incubated at 37°C in serum-free DMEM with or without 20 ng/ml PDGF-BB (R&D Systems, Inc., Minneapolis, MN, USA) for 24 h following transfection. For the CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay, 10 µl CCK-8 solution was added to each well, and the absorbance was measured at 450 nm following 3 h of incubation at 37°C.

For the EdU (Guangzhou RiboBio Co., Ltd.) assay, the cells were incubated at 37°C in EdU solution (50 nmol/l) for 2 h, and fixed at room temperature in 4% formaldehyde for 30 min. The cells were subsequently exposed to 1,000 µl Cell-Light™ EdU Apollo®488 In Vitro Imaging kit (100T) (938 µl dH2O, 50 µl reaction buffer, 10 µl catalyst solution, 3 µl fluorescent dye solution, 9 µg buffer additive) for 30 min, followed by nuclear staining at room temperature with Hoechst 33,342 for 30 min (all Guangzhou RiboBio Co., Ltd.). An inverted fluorescence microscope was used to capture and analyze the images (magnification, x100; Axio Observer Z1).

Measurement of HASMC migration. Transwell and wound closure assays were used to assess cell migration. For the Transwell assay (Corning Incorporated, Corning, NY, USA), cells were resuspended following transfection and added to the upper chambers with 200 µl serum-free DMEM (5x10^5 cells/ml), and the lower chamber was filled with 500 µl serum-free DMEM with or without PDGF-BB (20 ng/ml). After 16 h, the migrated cells on the lower face of the chamber membrane were fixed with 4% formaldehyde and stained with 0.1% crystal violet.

For the wound closure assay, the HASMCs were seeded into 12-well plates following transfection (10,000 cells/well), and a straight scratch wound was created using a sterilized 200 µl disposable pipette tip in each well. The scratch wounds were visualized every hour at 37°C using a live cell imaging system (magnification, x100; Axio Observer Z1). Image Pro Plus software (version 6.0) was used to measure the widths of the scratch wounds.

Luciferase reporter assay. The MFN2 mRNA 3’-UTR, containing putative or mutated binding sites for miR-31,
were amplified by PCR using the aforementioned protocol and cloned into the pmiR-RB-Report vector (Guangzhou RiboBio Co., Ltd.). The HEK 293T cells (American Type Culture Collection, Manassas, VA, USA) were co-transfected with miR-31 mimics (50 nmol/l) or negative control oligos (50 nmol/l) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase values were determined 48 h later using the Dual-Luciferase Reporter Assay System kit (Promega Corporation, Madison, WI, USA), according to the manufacturer’s protocol.

Statistical analysis. The data are presented as the mean ± standard deviation. Student’s t-test and one-way analysis of variance, with multiple comparisons using the Newman-Keuls test, were used for the statistical analysis. The data analysis was performed with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-31 is upregulated in ASO arteries of the lower extremities. The miR-31 expression levels were analyzed in ASO and normal arterial walls from the lower extremities via RT-qPCR. miR-31 expression was significantly upregulated in the ASO arteries compared with the normal arteries (P<0.001; Fig. 1A). The expression level of miR-31 in the media was significantly higher compared with the adventitia and intima in ASO arteries (P<0.001; Fig. 1B). Furthermore, the ISH and immunofluorescence results demonstrated that miR-31 was primarily located in the artery media and neointima of ASO (Fig. 1C). The colocalization of miR-31 and SM-α-actin indicates that miR-31 is primarily expressed in ASO HASMCs. The IOD value also indicated that miR-31 expression was significantly increased in the ASO arteries compared with the normal arteries (P<0.001; Fig. 1D). These results suggest that miR-31 is upregulated in ASO HASMCs.
Mir-31 promotes proliferation in PDGF-BB-induced HASMCs. To investigate the role of miR-31 in HASMC proliferation induced by PDGF-BB, miR-31 mimics and negative control oligos were transfected into HASMCs and proliferation was assessed via an EdU and a CCK-8 assay. These assays demonstrated that upregulation of miR-31 significantly promoted HASMC proliferation compared with the control group (P<0.001; Fig. 2). These results suggest that miR-31 overexpression promotes HASMC proliferation in vitro.

Mir-31 promotes migration in HASMCs following PDGF-BB treatment. To explore whether miR-31 promoted HASMC migration induced by PDGF-BB, miR-31 mimics and negative control oligos were transfected into HASMCs to overexpress miR-31. Cell migration was measured via Transwell assay. As shown in Fig. 3A and B, the miR-31 mimics significantly enhanced PDGF BB-induced HASMC migration compared with the control group (P<0.001). Similar results were obtained in the wound closure assay (P<0.001; Fig. 3C and D). These results demonstrate that miR-31 overexpression promotes HASMC migration in vitro.

MiR-31 targets the 3'-UTR of MFN2 and downregulates its expression. TargetScan online software was utilized for the bioinformatics prediction and MFN2 was identified as a potential target of miR-31. The putative seed sequences for miR-31 at the 3'-UTR of MFN2 were conserved (Fig. 4A). A dual-luciferase reporter assay was performed to verify whether MFN2 was a direct target gene of miR-31. As shown in Fig. 4B, in the wild-type MFN2 3'UTR group, the miR-31 mimics significantly reduced Renilla/firefly luciferase activity compared with the results in the negative control group (P<0.01). However, in the mutant type MFN2 3'UTR group, there was no significant difference between miR-31 and negative control transfection in the mutant MFN2. (C and D) Western blotting was performed to determine the levels of MFN2 protein in HASMCs transfected with miR-31 mimics (50 nmol/l), inhibitor (100 nmol/l) and negative control oligos (50 nmol/l). "P<0.01 vs. negative control. MFN2, mitofusin-2; miR, microRNA; HASMC, human arterial smooth muscle cell; UTR, untranslated region.

MFN2 expression is negatively regulated by miR-31 at a transcriptional level in HASMCs. To further investigate whether MFN2 is a functional target gene of miR-31 in HASMCs, HASMCs were transfected with miR-31 negative control oligos, mimics and an inhibitor, and the MFN2 expression levels were determined via western blotting. As shown in Fig. 4C and D, the protein level of MFN2 was significantly downregulated following miR-31 overexpression (P<0.01). However, MFN2 was significantly upregulated in the HASMCs transfected with a miR-31 inhibitor (P<0.01; Fig. 4C and D). These observations demonstrate that miR-31 negatively regulates the expression of MFN2 at a post-transcriptional level in HASMCs.

Discussion

It has been well established that miRNAs function as key mediators in multiple physiological and pathological processes, predominantly via modulating the expression of target genes at a post-transcriptional level (21). In the present study, miR-31 expression was markedly upregulated in human ASO arterial walls compared with normal arterial walls. Furthermore, an ASO cell model was established to investigate whether miR-31 overexpression had an effect on HASMCs. To the best of our knowledge, the present study demonstrated for the
first time that miR-31 promotes proliferation and migration in PDGF-BB-induced HASMCs, most likely by regulating the expression of MFN2.

ASO of the lower extremities is one of the most painful vascular diseases, with high morbidity and mortality; ASO seriously affects survival and quality of life (22). It is well established that aberrant proliferation and migration of VSMCs serves an important role in arteriosclerosis and vascular stenosis (23). Despite great progress in the understanding of VSMC biology (24), the molecular mechanisms underlying the contribution of HASMCs to the pathogenesis of ASO has yet to be elucidated. miRNAs have previously been reported to serve important roles in cardiovascular systems (7-9).

miR-31 functions as an oncogene in the majority of cancers, including colorectal cancer (13), lung cancer (14), and esophageal cancer (15); however, it has been demonstrated to act as a tumor suppressor in glioblastoma and liver cancer (16,25). In the present study, miR-31 was significantly upregulated in ASO arterial walls compared with normal arterial walls as determined by RT-qPCR and ISH. miR-31 was located primarily in the media and neointima, which indicates that HASMCs may be the main effector cells of miR-31 in the ASO pathological process. The characteristics of miR-31 distribution in the present study are consistent with the observations of a previous study, which was performed in rat carotid arteries with neointimal lesions (26).

The biological functions of miR-31 in the HASMC physiological and pathological processes require further study. To investigate the detailed roles of miR-31 in HASMCs in ASO, CCK-8 and EdU assays were performed to investigate cell proliferation, wound closure and Transwell assays were used to detect cell migration. In the present study, miR-31 was demonstrated to promote proliferation and migration in PDGF-BB-induced HASMCs, which is consistent with the observation of a previous study by Liu et al (27), who reported that VSMC proliferation in rats was significantly inhibited by miR-31 knockdown. Cell proliferation was increased by the overexpression of Rattus norvegicus-miR-31, which demonstrates that miR-31 has a pro-proliferative effect on VSMCs in rats. Wang et al (28) previously reported that miR-31 was involved in the regulation of a human thoracic ASMC phenotype switch by targeting the cellular repressor of E1A-stimulated genes.

MFN2, also known as a hyperplasia suppressor gene, has a crucial role in regulating cell proliferation, migration, apoptosis and differentiation, which are involved in the pathophysiological processes of severe cardiovascular diseases, including arteriosclerosis, restenosis after angioplasty, hypertension and myocardial infarction (29-31). A previous study demonstrated that MFN2 acted as an anti-proliferation gene, by preventing VSMC proliferation in cultured cells and a rat carotid artery balloon-injury model (31). Another study revealed that overexpression of MFN2 reduced VSMC proliferation and increased apoptosis in human and experimental pulmonary arterial hypertension model (32). In the present study, a dual-luciferase reporter assay was applied to confirm that MFN2 was a direct target gene of miR-31. In addition, via gain-of-function and loss-of-function approaches, transfection with miR-31 mimics reduced the protein expression of MFN2 in HASMCs; however, transfection with an miR-31 inhibitor resulted in increased MFN2 expression. These previous observations and the results of the present study suggest that miR-31 has a role in promoting proliferation and migration in HASMCs, which is, at least in part, dependent on directly targeting MFN2.

In conclusion, miR-31 was significantly increased in arterial walls of patients with ASO. Furthermore, miR-31 promoted the proliferation and migration of HASMCs, at least partially by directly targeting the expression of MFN2. The results of the present study may provide a novel insight into the mechanisms and significant role of miR-31/MFN2 in the pathological processes of ASO and offers a potential therapeutic target for the treatment of ASO.

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