Original Paper

Mir-22-3p Inhibits Arterial Smooth Muscle Cell Proliferation and Migration and Neointimal Hyperplasia by Targeting HMGB1 in Arteriosclerosis Obliterans

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Key Words

Mirna-22-3p • Arteriosclerosis • Human arterial smooth muscle cell • Proliferation • Migration • Neointimal hyperplasia • HMGB1

Abstract

Background: Aberrant vascular smooth muscle cell (VSMC) proliferation and migration contribute to the development of vascular pathologies, such as atherosclerosis and post-angioplasty restenosis. The aim of this study was to determine whether miR-22-3p plays a role in regulating human artery vascular smooth muscle cell (HASMC) function and neointima formation. **Methods:** Quantitative real-time PCR (qRT-PCR) and fluorescence in situ hybridization (FISH) were used to detect miR-22-3p expression in human arteries. Cell Counting Kit-8 (CCK-8) and EdU assays were performed to assess cell proliferation, and transwell and wound closure assays were performed to assess cell migration. Moreover, luciferase reporter assays were performed to identify the target genes of miR-22-3p. Finally, a rat carotid artery balloon-injury model was used to determine the role of miR-22-3p in neointima formation. **Results:** MiR-22-3p expression was downregulated in arteriosclerosis obliterans (ASO) arteries compared with normal arteries, as well as in platelet-derived growth factor-BB (PDGF-BB)-stimulated HASMCs compared with control cells. MiR-22-3p overexpression had anti-proliferative and anti-migratory effects and dual-luciferase assay showed that high mobility group box-1 (HMGB1) is a direct target of miR-22-3p in HASMCs. Furthermore, miR-22-3p expression was negatively correlated with HMGB1 expression in ASO tissue specimens. Finally, LV-miR-22-3p-mediated miR-22-3p upregulation significantly suppressed neointimal hyperplasia specifically by reducing HMGB1 expression *in vivo*. **Conclusions:** Our results indicate that miR-22-3p is a S. Huang and M. Wang are contributed equally to this work.

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key molecule in regulating HASMC proliferation and migration by targeting HMGB1 and that miR-22-3p and HMGB1 may be therapeutic targets in the treatment of human ASO.

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Introduction

Arteriosclerosis obliterans (ASO) of the lower extremities significantly affects 17% of humans between 55 and 75 years worldwide and is characterized by lower extremity ischemia causing intermittent claudication, rest pain and gangrene [1-3]. Endovascular procedures, including balloon angioplasty procedures and stent implantations, yield immediate positive results and have thus become the main approaches to treat ASO [4]. However, approximately 30%~50% patients develop post-angioplasty restenosis within 1 year after the procedure, and almost 12% suffer from severe ischemia and eventually require limb amputation [5]. Vascular smooth muscle cells (VSMCs) are the main component of blood vessel walls, and excessive VSMC proliferation and migration are believed to be critical events in the pathogenesis of arteriosclerosis and post-angioplasty restenosis [6, 7]. Thus, regulate aberrant VSMC proliferation and migration has become a major method of treating ASO. Great progress has been made with respect to the understanding of VSMC biology in recent years, however, the molecular mechanisms through VSMC contribute to the development of ASO and restenosis have not been fully elucidated.

MicroRNAs (miRNAs) negatively regulate the expression of their target genes at the post-transcriptional level by binding the 3'-untranslated regions (UTRs) of the genes, and participate in a series of physiological and pathological process, such as embryogenesis, cell differentiation, angiogenesis, wound repair and tumorigenesis [8-10]. Ji et al. [11] first reported the miRNA expression profile of the rat carotid artery in 2007 and they noted that some miRNAs played important roles in regulating various processes in VSMCs. Moreover, recent studies have shown that some miRNAs, such as miR-21, miR-143, miR-145, miR-221, miR-222 are involved in regulating VSMC proliferation, migration and apoptosis [12-16]. We previously explored the miRNA expression profiles of human arteries affected by ASO and found that certain miRNAs, including miR-21, miR-22, miR-24 and miR-1298 were significantly differentially expression between those arteries and normal arteries [12].

It has been reported that miR-22-3p functions as a tumor suppressor gene and displays decreased expression in multiple carcinomas [17-20]. However, the role of miR-22-3p in the regulation of human artery vascular smooth muscle cell (HASMC) proliferation and migration in ASO remains unknown. Here, we studied the potential roles of miR-22-3p in HASMC function *in vivo* and *in vitro*, as well as the molecular mechanisms through which those roles are fulfilled.

Materials and Methods

Artery specimen acquisition

This study was approved by the research ethics committee of the First Affiliated Hospital, Sun Yat-sen University, and the patients or donors from whom the samples were obtained provided informed consent before participating in the study. ASO arterial specimens were acquired from 12 patients with ASO who had undergone lower limb amputations, and normal arterial specimens were obtained from 6 healthy organ donors. Some specimens were snap-frozen in liquid nitrogen after arterectomy for RNA extraction, while other specimens were fixed with 4% paraformaldehyde and embedded in paraffin for further analysis.

Cell culture and transfection

HASMCs were collected from the walls of femoral arterial specimens obtained from healthy organ donors. The cells were subsequently prepared in our lab as described previously and identified via SM- α -actin antibody staining [21]. The cells were maintained in Dulbecco's modified Eagle' medium (DMEM; Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Gibco, Karlsruhe, USA) at 37°C

in a humidified incubator with 5% CO₂. Cells from Passages IV to IX were utilized in this study. MiR-22-3p mimics, miR-22-3p inhibitors and negative control oligo nucleotides (NCs) (RiboBio, Guangzhou, China) were transfected into the cells using Lipo RNA iMax (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

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

Total RNA was isolated from the above mentioned arterial specimens or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was then reverse-transcribed into cDNA before being amplified with a real time-PCR miRNA (or mRNA) detection kit (Takara, Dalian, China) on a Roche LightCycler 480 Real-Time PCR System. The reaction comprised the following steps: one cycle of 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 20 sec. The following primers were used for the experiment: miR-22-3p, 5'-CGAAGCTGCCAGTTGAAGAA-3'; and high mobility group box-1 (HMGB1), 5'-GATCCCAATGCACCCAAGAG-3' (forward) and 5'-TCGCAACATCACC AATGGAC-3' (reverse). U6 and GAPDH served as reference genes for the detection of miR-22-3p and HMGB1, respectively. Relative gene expression levels were calculated by the 2^{-ΔΔCt} method.

Fluorescence in situ hybridization (FISH), immunofluorescence (IF) and immunohistochemistry (IHC)

FISH of miR-22-3p was performed using a 5'-DIG-and 3'-DIG-labeled miRCURY LNA Detection Probe (Exiqon, Vedbaek, Denmark) and 4-μm tissue sections. The sequence of the miR-22-3p probe was as follow: 5' Dig-ACAGTTCTTCAACTGGCAGCTT-Dig 3'. The procedure comprised the following steps: 1. The tissue sections were deparaffinized with xylene and then rehydrated in graded ethanol solutions (100%, 100%, 95%, 75% and 60%). 2. The sections were subsequently digested with proteinase K (40 μg/ml) for 10 min at 37°C, washed in glycine/PBS (0.2%) for 5 min, fixed with paraformaldehyde (4%) for 10 min, and then acetylated for 10 min. 3. The sections were then prehybridized in hybridization buffer (50×Denhardt's solution, 20×SSC, 60% formamide, 300-μg/ml yeast tRNA and 1 M DTT) for 30 min at 49°C. 4. The sections were hybridized with the above mentioned probe (1:500 dilution) overnight at 49.5°C; 5. The sections were washed in 2×SSC twice for 5 min at 49.5°C and then blocked for 1 h at room temperature using Roche blocking buffer. 6. The sections were subsequently treated with anti-digoxigenin antibody (1:200 dilution; Roche, Basel, Switzerland) before being incubated with the antibody for 2.5 h at 37°C. 7. The sections were then incubated with TSA buffer (1:50 dilution) for 20 min at room temperature in the dark; 8. The sections were subsequently washed thrice with PBS before being mounted with cover slips. IF and IHC were performed to detect SM-α-actin, HMGB1 and ki-67 (Abcam, MA, USA) localization as previously described [21]. The intensity of the staining in the acquired images was determined by measuring integrated optical density (IOD) values, and the images were analyzed by Image Pro Plus 6.0.

Western blot analysis

The cells were lysed in lysis buffer supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland), after which the proteins were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), which was blocked with 5% non-fat milk in TBS and incubated with a rabbit anti-HMGB1 monoclonal antibody (1:1000 dilution; Abcam, MA, USA) or GAPDH monoclonal antibody (1:1000 dilution; Cell Signaling). The PVDF membrane was subsequently incubated with chemiluminescence detection reagent (Thermo Pierce, Waltham, MA, USA), and the resultant bands were imaged with a GE Image Quant Las 4000 mini (GE, Fairfield, CT, USA). The densities of the bands were normalized to those of GAPDH.

Measurement of HASMC proliferation

Cell Counting Kit-8 (CCK-8) and EdU assays were performed to assess cell proliferation. The cells (2~3×10³ /well) were plated in 96-well plates and then incubated in serum-free DMEM with or without 20 ng/ml platelet-derived growth factor-BB (PDGF-BB; R&D, Minneapolis, MN, USA) for 24 h after transfection.

For the CCK-8 assay, the cells were incubated with 10 μl /well CCK-8 solution (Dojindo, Kumamoto, Japan) for 2~3 h, after which the optical density was measured at an absorbance of 450 nm.

For the EdU assay, the cells were incubated in 50 nmol/L EdU solution (RiboBio, Guangzhou, China) for 2 h and then visualized, according to the manufacturer's protocol. Images were acquired by an inverted fluorescence microscope (Zeiss Axio Observer Z1, Jena, Germany) and analyzed by Image Pro Plus 6.0 software.

Measurement of HASMC migration

Transwell and wound closure assays were performed to detect cell migration.

For the transwell assay (Corning, Tewksbury, NY, USA), after transfection, the cells (5×10^5 cells/ml) were resuspended in 200 μ l of serum-free DMEM before being added to the upper chambers of the transwell apparatus. The lower chamber of the apparatus was filled with 500 μ l of serum-free DMEM with or without PDGF-BB (20 ng/ml). After 16 h, the migrated cells, i.e., the cells on the bottom surface of the chamber membrane, were fixed with 4% formaldehyde and stained with 0.1% crystal violet.

For the wound closure assay, after transfection, the HASMCs were seeded in 12-well plates (10,000 cells/well), and a straight scratch-wound was created in the cells layer, which had reached almost 100% confluence, using a sterilized 200- μ l disposable pipette tip. The scratch wounds were subsequently visualized and photographed using a microscope (Zeiss Axio Observer Z1, Jena, Germany).

Luciferase reporter assays

Fragments of the 3'-UTR of the HMGB1 mRNA sequence containing the putative (wild-type) or mutated binding sites for miR-22-3p were amplified and cloned into luciferase reporter constructs (RiboBio, Guangzhou, China). HEK 293 cells were subsequently cotransfected with these constructs and miR-22-3p mimics or NCs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours of transfection, the cells were treated with Dual-luciferase Reporter Assay Reagent (Promega, Madison, WI, USA) to determine luciferase activity according to the manufacturer's instructions.

Rat carotid artery balloon-injury model

The rat carotid artery balloon-injury model was used to evaluate the ability of miR-22-3p to inhibit neointima formation. Briefly, male SD rats (300~350 g) were anesthetized with phenobarbital sodium (50 mg/kg), after which a 2F Fogarty catheter (Baxter Edwards Healthcare, CA, USA) was used to injure the intima of the right common carotid artery. The procedure was monitored with a dissecting microscope. After balloon-injury induction, a solution containing lentiviral (LV)-miR-22-3p (5×10^9 pfu/ml) or LV-control (5×10^9 pfu/ml) was infused into the temporarily ligated segment of the vessel for 30 min, after which blood flow within the right common carotid artery was restored. The animals were sacrificed 14 days later, and the carotid arteries were fixed in formalin. This study was reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Sun Yat-sen University.

Statistical analysis

All data are presented as the mean $\pm$ SD. Statistical analysis was performed using student's *t* test and one-way ANOVA followed by the Newman-Keuls test. All data analysis were performed with SPSS17.0 software. The following P values were indicative of statistical significance: * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$.

Results

MiR-22-3p expression levels were decreased in ASO arteries

First, miR-22-3p expression levels were detected in ASO arterial walls samples and normal arterial walls samples from the lower extremities of patients with ASO and healthy control subjects, respectively, by qRT-PCR which we demonstrated that miR-22-3p expression levels were downregulated in ASO arteries compared with normal arteries (Fig. 1 A). Second, qRT-PCR was performed to determine the distribution of miR-22-3p in the three layers of the ASO arterial wall. The results of the analysis suggested that miR-22-3p expression level in the media were higher than those in the adventitia and intima (Fig. 1 B). Third, FISH and IF were performed to determine the localization of miR-22-3p and SM- α -actin and confirmed that miR-22-3p and SM- α -actin were primarily located in the media and neointima of the ASO arterial wall (Fig. 1 C). The colocalization of miR-22-3p and SM- α -actin indicated that miR-22-3p is expressed mainly in VSMCs. Finally, the IOD value for miR-22-3p showed that its expression was significantly downregulated in ASO arteries compared

Fig. 1. The characteristics of miR-22-3p expression in ASO tissue samples. (A) miR-22-3p was downregulated in the ASO arteries compared with normal arteries, as demonstrated by qRT-PCR (n=6). (B) miR-22-3p expression levels in the three layers of ASO arteries walls were determined by qRT-PCR (n=6). (C) HE staining demonstrating arterial structures. Co-staining of miR-22-3p (red) and SM- α -actin (green) in artery sections. The FISH and IF results

showed that miR-22-3p localized mainly in the media and neointima, indicating that it is expressed in HASMCs (n=6). (D) The IOD values for miR-22-3p staining in the arterial sections showed that miR-22-3p staining was significantly decreased in the ASO sections (n=6) compared with the normal sections (n=6). Original magnification: 100 $\times$. *P<0.05, **P<0.01, ***P<0.001.

with normal arteries (Fig. 1 D). This result confirmed that miR-22-3p expression levels are decreased in ASO HASMCs compared with normal HASMCs.

MiR-22-3p inhibited proliferation and migration in PDGF-BB-stimulated HASMCs

The expression levels of miR-22-3p in proliferating HASMCs were analyzed by qRT-PCR. HASMCs were starved in serum-free DMEM for 24 h to return to a quiescent state. Then, the cells were stimulated with PDGF-BB (20 ng/ml) to trigger proliferation. As shown in Fig. 2 A, miR-22-3p expression levels were significantly decreased in proliferating HASMCs, especially at 12 h after PDGF-BB stimulation, compared with quiescent cells. These findings suggested that miR-22-3p may be indispensable for PDGF-BB-stimulated HASMC proliferation and migration.

To investigate the role of miR-22-3p in PDGF-BB-induced HASMC proliferation and migration, we transfected miR-22-3p mimics or NCs into HASMCs and then performed CCK-8 and EdU assays to measure cell proliferation and transwell and wound closure assays to measure cell migration. MiR-22-3p mimic-induced miR-22-3p upregulation significantly reduced HASMC proliferation in mimic-transfected cells compared with control cells, as determined by CCK-8 (Fig. 2 B) and EdU (Fig. 2 C and D) assay. Regarding cell migration, the miR-22-3p mimics significantly inhibited cell migration in mimic-transfected cells compared with control cells, as demonstrated by transwell (Fig. 2 E and F) and wound closure assay (Fig. 2 G and H). These results demonstrate that miR-22-3p inhibited HASMC proliferation and migration *in vitro*.

Identification of HMGB1 as a direct target of miR-22-3p in HASMCs

HMGB1 was identified as a potential miR-22-3p target. The putative seed sequences for miR-22-3p within the 3'-UTR of HMGB1 were highly conserved (Fig. 3 A) (predicted by the miRwalk website).

Fig. 2. miR-22-3p suppressed HASMC proliferation and migration in vitro. (A) miR-22-3p expression levels were significantly decreased in proliferating HASMCs, especially at 12 h after PDGF-BB stimulation, compared with quiescent cells, as demonstrated by qRT-PCR (n=6). HASMCs were transfected with miR-22-3p mimics (50 nmol/L) and NCs (50 nmol/L). The miR-22-3p mimic significantly inhibited PDGF-BB-induced (20 ng/ml) HASMC proliferation, as demonstrated by the CCK-8 (B) assay and EdU assay (C and D). (E and F) The miR-22-3p mimic significantly decreased PDGF-BB-induced (20 ng/ml) HASMC migration, as determined by the transwell assay. (G and H) The miR-22-3p mimic significantly suppressed PDGF-BB-induced (20 ng/ml) HASMC migration, as determined by wound closure assay. Original magnification: 100× (EdU and transwell); 50× (wound closure). *P<0.05, **P<0.01, ***P<0.001.

We first transfected HASMCs with miR-22-3p mimics, inhibitors and NCs and detected HMGB1 expression by western blot and qRT-PCR. As shown in Fig. 3 B and C, HMGB1 protein expression levels were downregulated by the miR-22-3p mimic but were significantly upregulated by the miR-22-3p inhibitor. However, neither the miR-22-3p mimic nor the inhibitor altered HMGB1 mRNA expression levels (Fig. 3 D). These data indicate that miR-22-3p negatively regulates HMGB1 expression at the post-transcriptional level in HASMCs. We then performed dual-luciferase reporter assay to determine whether HMGB1 is a direct target of miR-22-3p. As shown in Fig. 3 E, miR-22-3p mimics significantly decreased relative luciferase activity levels in the wild-type (putative) HMGB1 3'-UTR group compared with the negative-control group. However, the inhibitory effect of miR-22-3p on relative luciferase activity was abolished in the mutant type HMGB1 3'-UTR group compared with the negative-

Fig. 3. HMGB1 was the target of miR-22-3p in HASMCs and associated with miR-22-3p-induced cellular effects. (A) There are two miR-22-3p binding sites in the 3'-UTR of HMGB1. (B and C) Western blot was used to determine HMGB1 protein expression levels in HASMCs transfected with miR-22-3p mimics (50 nmol/L), inhibitors (100 nmol/L) and NCs (50 nmol/L). (D) Neither miR-22-3p mimics nor inhibitors altered HMGB1 mRNA expression levels, as determined by qRT-PCR (n=6). (E) Luciferase reporter assay was performed to determine whether HMGB1 was a direct target of miR-22-3p. The miR-22-3p mimic significantly reduced luciferase activity in wild-type HMGB1 compared with the negative control. However, the miR-22-3p mimic did not affect luciferase activity in mutant HMGB1 compared with the negative control. (F) HMGB1 overexpression in HASMCs was achieved with a lentiviral vector (LV-HMGB1). Overexpressing HMGB1 promoted HASMC proliferation and significantly attenuated the antiproliferative effects of miR-22-3p on HASMCs, as determined by CCK8 (G) and Edu assay (H and I) (n=6). Overexpressing HMGB1 promoted HASMC migration and significantly attenuated the antimigratory effects of miR-22-3p on HASMCs, as determined by transwell (J and K) and wound closure (L and M) assays (n=6). Original magnification: 100× (Edu and transwell); 50× (wound closure). *P<0.05, **P<0.01, ***P<0.001.

control group. These results indicated that miR-22-3p directly binds to the 3'-UTR of HMGB1.

To further investigate whether HMGB1 is a functional target gene of miR-22-3p in HASMCs, we used a lentiviral vector (LV-HMGB1) to overexpress HMGB1 in HASMCs. Successful HMGB1 overexpression was confirmed by western blot (Fig. 3 F). As expected, overexpressing HMGB1 promoted HASMC proliferation and migration and significantly attenuated the suppressive effects of miR-22-3p on HASMC proliferation and migration (Fig. 3 G~M). These data indicated that HMGB1 was a functional and downstream target of miR-22-3p and was involved in miR-22-3p-treated HASMC proliferation and migration.

Mir-22-3p expression was negatively correlated with HMGB1 expression in ASO

To illustrate the relationship between HMGB1 and miR-22-3p in human ASO, we performed IHC, western blot and IF assays to detect HMGB1 expression and FISH to detect

Fig. 4. A negative correlation between miR-22-3p and HMGB1 expression levels in ASO. (A and B) HMGB1 expression levels were much higher in ASO arteries than in normal arteries, as demonstrated by IHC staining for HMGB1 (brown) (n=12). Original magnification:100× (left row); 200× (right row). (C) HMGB1 protein expression was more abundant in ASO arteries than in normal arteries, as determined by western blot (n=6). (D) HE staining demonstrating arteries structures. Co-staining of miR-22-3p (red) and HMGB1 (green) in consecutive arteries sections. The IF results showed that HMGB1 expression levels were much higher in ASO arteries than in normal arteries. HMGB1 was mainly localized in the media and neointima, indicating that HMGB1 was expression in HASMCs. Similar findings were noted regarding miR-22-3p localization (n=6). Original magnification:100×. (E) Comparison of HMGB1 IOD values in normal (n=6) and ASO samples (n=6). (F) Calculating HMGB1 and miR-22-3p IOD values in consecutive ASO sections revealed the existence of a negative correlation between miR-22-3p and HMGB1 expression levels (n=12). *P<0.05, **P<0.01, ***P<0.001.

miR-22-3p expression. The IHC (Fig. 4 A and B), western blot (Fig. 4 C) and IF results (Fig. 4 D and E) showed that HMGB1 protein expression levels were increased in ASO arteries compared with normal arteries. Moreover, the FISH and IF data (Fig. 4 D) demonstrated that HMGB1 was expressed at significantly higher levels in the media and neointima of ASO arteries than in the adventitia of ASO arteries, findings consistent with those pertaining to miR-22-3p expression. We calculated the IOD values for HMGB1 and miR-22-3p in 12 pairs of consecutive ASO aretrial sections. Interestingly, we noted that a negative correlation exists between HMGB1 and miR-22-3p expression in human ASO specimens (Fig. 4 F). These findings indicated that miR-22-3p upregulation may suppress HMGB1 expression at the post-transcriptional level in ASO.

Fig. 5. miR-22-3p attenuated neointimal hyperplasia in balloon-injured rat carotid arteries. (A) The sequence of miR-22-3p is conserved among many species and miR-22-3p has similar binding sites in the 3'-UTR of HMGB1 in different species. (B) Large amounts of ASMCs were noted throughout the media and neointima after vascular injury, as demonstrated by IHC (n=6). Original magnification: 50× (upper row); 100× (lower row). (C and D) miR-22-3p expression levels were decreased in balloon-injured arteries compared with normal arteries, as determined by FISH; (C~E) LV-miR-22-3p-mediated miR-22-3p re-overexpress in injured vascular walls, as determined by FISH and qRT-PCR (n=6). (F and G) HMGB1 expression levels were significantly increased in balloon-injured arteries compared with normal arteries, as determined by IHC (n=6). (H and I) No neointima formation was observed in the normal control group, and significant neointimal formation was observed in the LV-control group; however, a thin neointima was observed in the LV-miR-22-3p group (n=6). Original magnification: 50× (upper row); 100× (lower row). *P<0.05, **P<0.01, ***P<0.001.

Mir-22-3p regulated HMGB1 expression and suppressed neointimal hyperplasia in balloon-injured rat carotid arteries in vivo

Mir-22-3p and its target, the 3'-UTR of HMGB1, are highly conserved among different species, including humans, rats and mice, according to the miRwalk and NCBI databases (Fig. 5 A). To determine the role of miR-22-3p in HASMC proliferation and neointimal formation *in vivo*, we used a well established and reproducible rat carotid balloon-injury model, which has been described in detail elsewhere [21]. Large amounts of ASMCs appeared throughout the media and neointima after balloon injury, as detected by IHC and as shown in Fig. 5 B.

We next used LV-miR-22-3p to re-overexpress miR-22-3p in the injured vascular wall of the indicated cells. Cells treated with an LV-control vector, which contained no miRNA, served as control group. MiR-22-3p expression levels in the vascular wall of the balloon-injury group were significantly downregulated compared with those in the vascular wall of the normal group, as determined by FISH (Fig. 5 C and D). Successfully miR-22-3p overexpression was confirmed by FISH and qRT-PCR (Fig. 5 C and E). In contrast, HMGB1 expression levels in the vascular wall of the balloon-injury group were significantly upregulated compared with those in the vascular wall of the normal group, as determined by IHC (Fig. 5 F and G). As shown in Fig. 5 H and I, no neointimal formation were observed in the normal control group, and significant increases in neointimal formation were observed in the LV-control group; however, a thin neointima was observed in the LV-miR-22-3p group. These results confirmed that miR-22-3p overexpression significantly inhibited neointima formation in rat carotid arteries after balloon injury.

Furthermore, after balloon injury, the expression levels of the cell proliferation marker Ki67 were significantly decreased in the LV-miR-22-3p group compared with the LV-control group, indicating that LV-miR-22-3p significantly suppressed ASMC proliferation *in vivo* (Fig. 6 A and B). Importantly, as expected, HMGB1 expression levels were also significantly decreased when miR-22-3p expression upregulated by lentiviral transfection in the LV-miR-22-3p group (Fig. 6 A and C).

Finally, the FISH and IF results showed that miR-22-3p and HMGB1 displayed similar distributions in the media and neointima. Moreover, these results, including qRT-PCR and western blot analysis showed that miR-22-3p expression was upregulated, while HMGB1 expression was downregulated in the LV-miR-22-3p group; however, contrasting results were observed in the LV-control group (Fig. 6 D~F). We determined the IOD values of HMGB1 and miR-22-3p in 12 pairs of consecutive balloon-injured rat carotid arteries sections and also noted the existence of a negative correlation between HMGB1 and miR-22-3p expression levels (Fig. 6 G). Taken together, these findings demonstrated that miR-22-3p inhibited neointima formation mainly by targeting HMGB1 *in vivo*.

Discussion

Great progress has been made with respect to the understanding the role of miRNAs in VSMC biology; however, the molecular mechanisms through which HASMC proliferation, migration and phenotypic switching contribution to ASO have not been fully elucidated. Here, we confirmed that miR-22-3p functions as a novel modulator of HASMC proliferation and migration. We also demonstrated that miR-22-3p expression levels were significantly downregulated in both human ASO arterial tissue samples and proliferating HASMCs. In addition, miR-22-3p suppressed proliferation and migration in PDGF-BB-stimulated HASMCs, most likely by targeting HMGB1 expression. Furthermore, miR-22-3p expression levels were negatively correlated with HMGB1 expression levels in human ASO arterial tissue samples. Finally, miR-22-3p regulated HMGB1 expression and alleviated neointimal hyperplasia in balloon-injured rat carotid arteries *in vivo*.

It is well established that tissue-specificity is an important feature of miRNA expression [22]. For example, a previous study showed that miR-1 was abundantly expressed in skeletal and cardiac muscle tissue but was poorly expressed in vascular smooth muscle tissue [23].

Fig. 6. A negative correlation between miR-22-3p and HMGB1 expression levels in balloon-injured rat carotid arteries. (A and B) Ki67 expression levels were significantly decreased in the LV-miR-22-3p group compared with the LV-control group (n=6). (A and C) HMGB1 expression levels were significantly decreased in the LV-miR-22-3p group compared with the LV-control group (n=6). Original magnification: 100×. (D) miR-22-3p expression levels were significantly increased in the LV-miR-22-3p group compared with the LV-control group as detected by qRT-PCR (n=8). (E) HMGB1 protein expression were significantly decreased in the LV-miR-22-3p group compared with the LV-control group as detected by western blot (n=8). (F) miR-22-3p (red) and HMGB1 (green) displayed similar distributions in the medial and neointimal layers of the rat artery sections. miR-22-3p expression levels were upregulated, while HMGB1 expression levels were downregulated in the LV-miR-22-3p group; however, contrasting results were observed in the LV-control group (n=6). (G) The IOD values for miR-22-3p and HMGB1 in consecutive rat artery sections revealed the existence of a negative correlation between miR-22-3p and HMGB1 expression (n=12). Original magnification: 200×. *P<0.05, **P<0.01, ***P<0.001.

In this study, we found that miR-22-3p expression levels were significantly downregulated in ASO arterial walls compared with normal arterial walls, findings supported by our qRT-PCR and FISH results. We dissected ASO arterial walls into their three constituent layers to detect miR-22-3p expression by qRT-PCR. Interestingly, we found that miR-22-3p was located mainly in the media, which contains only ASMCs, rather than in the intima and adventitia. Furthermore, miR-22-3p expression was decreased in proliferating HASMCs stimulated with PDGF-BB, compared with quiescent cells. All of these findings indicate that HASMCs may be the main effector cells of miR-22-3p in the pathogenesis of ASO. However, little is known about the biological function of miR-22-3p in HASMCs.

MiRNAs function as important mediators in multiple physiological and pathological processes mainly, by modulating target gene expression at the post-transcriptional level [24]. Our previous studies revealed that miR-21 regulates VSMCs proliferation and migration and promotes ASO development and progression via the HIF-1 α /miR-21/TPM1 pathway by regulating the TPM1 gene expression at the post-transcriptional level [12]. Another study showed that miR-24 inhibits HASMC proliferation and migration and promotes apoptosis by targeting PDGFRB and c-Myc *in vitro* [25]. Accumulating amounts of evidence suggest that miR-22-3p participates mainly in cell growth and differentiation. Previous studies have shown that miR-22-3p can promote CLL-B cell and cardiomyocytes proliferation [26, 27]; however, in most carcinomas, including breast cancer [28], gastric carcinoma [29], medulloblastomas [30] and liver cancer [31], miR-22-3p suppressed cancer cell proliferation. Thus the biological functions of miR-22-3p in HASMCs require further study. It is believed that HASMC proliferation and migration play crucial roles in the pathogenesis of atherosclerosis and post-angioplasty restenosis [32, 33]. To assess the effects of miR-22-3p in HASMCs in ASO, we performed CCK-8 and EdU assays to assess cell proliferation, and transwell and wound closure assays to assess cell migration. We found for the first time that miR-22-3p plays a significant role inhibiting proliferation and migration in PDGF-BB-stimulated HASMCs. These findings confirmed that miR-22-3p participates in the aberrant HASMC proliferation and migration that contribute to ASO and restenosis development.

In this study, we determined that HMGB1 is an important target of miR-22-3p in HASMCs. HMGB1 was initially identified as a potential miR-22-3p target by miRwalk. Moreover, a previous study have demonstrated HMGB1 is a target of miR-22-3p in osteosarcoma cells [34], this had not been verified in vascular cells. Thus, we investigated whether HMGB1 is a target of miR-22-3p via the following process: first, using both gain-of-function and loss-of-function approaches, we determine that miR-22-3p mimics and miR-22-3p inhibitors altered HMGB1 protein expression but did not affect HMGB1 mRNA expression. These data suggested that miR-22-3p negatively regulates HMGB1 expression at the post-transcriptional level in HASMCs. Then, we performed luciferase assay to confirm that HMGB1 was a direct target of miR-22-3p. We also found that overexpressing HMGB1 via LV-HMGB1 alleviates the antiproliferative and antimigratory effects of miR-22-3p in HASMCs. Finally, we noted that a negative correlation exists between the expression of HMGB1 and that of its endogenous inhibitor, miR-22-3p, in ASO specimens, which indicates that HMGB1 protein expression upregulation in ASO was related to miR-22-3p downregulation. These findings indicated that HMGB1 was a functional and downstream target of miR-22-3p and was involved in miR-22-3p-mediated regulation of HASMCs proliferation and migration.

HMGB1, which is known as a DNA-binding protein and cytokine, is highly expressed in human atherosclerotic lesions. The protein was first identified by Kalinina et al. [35, 36], who suggested that HMGB1 may be involved in the process of arteriosclerosis. It has been reported that VSMCs are the source of HMGB1 in advanced atherosclerotic plaques [36, 37]. In this study, we found that HMGB1 protein expression was significantly higher in ASO arteries than in normal arteries, findings supported by the outcomes of our IHC and western blot analyses, whose results were consistent with the those of previous studies [36, 37]. Notably, our IHC and IF results confirmed that HMGB1 is expressed mainly in the media and neointima of ASO arteries, indicating that proliferating ASMCs are the major cells in which HMGB1 is localized. Moreover, previous studies have shown that HMGB1 not only promoted

human pulmonary ASMC and primary arterial endothelial cell proliferation [37, 38], but also enhanced rat ASMC migration *in vivo* [39]. In the current study, we used LV-HMGB1 to overexpress HMGB1 in HASMCs and found that HMGB1 overexpression significantly promoted HASMC proliferation and migration and that LV-HMGB1 attenuated the effects of miR-22-3p on HASMCs proliferation and migration. These results showed that HMGB1 participates miR-22-3p-mediated regulation of HASMC proliferation and migration.

Finally, we used a rat carotid balloon-injury model to study the role of miR-22-3p in ASMC proliferation and neointima formation *in vivo*. We found that miR-22-3p expression levels were significantly decreased, while HMGB1 expression levels were increased in rat carotid arteries after balloon injury. It has been reported that HMGB1 protein expression accelerated neointima formation in vascular injury mice [40], whereas HMGB1 inhibition suppressed post-vascular injury neointimal hyperplasia in mice and rats, there by reducing VSMC migration and proliferation [41, 42]. In this study, LV-miR-22-3p-induced miR-22-3p overexpressing in rat arteries led to reductions in HMGB1 expression and suppression of ASMC proliferation and neointimal hyperplasia. These findings showed that HMGB1 is a functional target of miR-22-3p *in vivo* and suggest that exogenous miR-22-3p may be administered to suppress ASO and restenosis.

Conclusion

In conclusion, the present study demonstrated that miR-22-3p is downregulated in ASO samples and that miR-22-3p inhibits HASMC proliferation and migration and alleviates neointimal hyperplasia at least in part by directly targeting HMGB1. These results indicate that miR-22-3p and HMGB1 may represent new therapeutic targets in the prevention and treatment of ASO and restenosis.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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