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Long Noncoding RNA XR007793 Regulates Proliferation and Migration of Vascular Smooth Muscle Cell via Suppressing miR-23b

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Funds Collection G

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Background: Long noncoding RNAs (lncRNAs) were identified as potential regulatory factor in vascular disease. However, the role of XR007793 in the regulation of neointima formation after vascular injury remains largely unknown.

Material/Methods: LncRNA expression levels were detected using real-time polymerase chain reaction (RT-PCR). *In vivo* and *in vitro* assay were performed in Sprague-Dawley rats and VSMCs. Cell Counting Kit-8 (CCK-8) assay, Transwell assay, and scratch wound healing assay were performed to detect cell proliferation and migration. Western blotting was used to detect protein expression.

Results: The results of qRT-PCR indicated that XR007793 expression was significantly increased in the injured carotid artery of Sprague-Dawley rats and platelet-derived growth factor-BB induced rat aortic smooth muscle cells. Knockdown of XR007793 repressed the proliferation and migration of VSMC *in vitro*. The expression level of miR-23b was reduced in mouse carotid injured tissues and cell line. Bioinformatics analysis and luciferase reporter assay revealed that XR007793 directly bonds to miR-23b. Pearson correlation analysis showed that XR007793a and miR-23b were negatively correlated in carotid samples. Furthermore, bioinformatics analysis and luciferase assay indicated that miR-23b targeted the Forkhead box O 4 (FOXO4) 3'-UTR to inhibit FOXO4 expression. After transfecting miR-23b inhibitor, the expression both of XR007793 and FOXO4 was increased. The effects on expression were reversed after transfected with miR-23b mimics. Rescue experiments results indicated that miR-23b inhibitor reduced the expression of VSMC marker and promoted proliferation and migration of VSMC.

Conclusions: This study shows that XR007793 aggravates the loss of function of VSMCs by negatively regulating miR-23b. It does so by targeting FOXO4, which could serve as a novel therapeutic target in post-angioplasty restenosis.

MeSH Keywords: Cell Migration Assays • Cell Proliferation • MicroRNAs • Muscle, Smooth, Vascular • RNA, Long Noncoding

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Background

Percutaneous transluminal coronary angioplasty (PTCA) is widely used in the clinical setting. It is an important means of diagnosis and treatment of coronary artery disease, but the occurrence of in-stent restenosis (ISR) affects the efficiency of coronary intervention and the patient's quality of life. Although the use of drug-eluting stents (DETs) reduces the incidence of restenosis (RS), its occurrence is still as high as 10% to 15%, especially if patients are comorbid with diabetes and multiple lesions [1]. The main pathogenesis of luminal stenosis after balloon injury is proliferation of neointima, which is because of the VSMCs phenotypic switch [2,3]. Under physiological and pathological conditions, VSMCs have significant phenotypic plasticity [4]. They regulate the synthesis of contractile related proteins to maintain vascular tension, regulate blood pressure, and play a crucial part in vascular disease, like atherosclerosis and restenosis after PTCA [5]. Current research is focused on the VSMCs function that is impaired in atherosclerosis, particular after bare metal stent (BMS) deployment.

Previous studies have shown that lncRNA can be used as a competitive endogenous RNA (ceRNA) and interacts with miRNAs, participates in the regulation of expression of target genes, and plays an important role in the progression of cardiovascular disease [6]. Zhou et al. demonstrated XIST promoted myocardial infraction by targeting miR-130a-3p [7]. knockdown of lncRNA MIAT was identified to inhibit angiotensin II (Ang-II)-induced cardiac hypertrophy by targeting miR-93/TLR4 axis [8]. LncRNA XR007793 was first identified as a novel regulator of VSMCs proliferation and migration in vascular remodeling of hypertension [9]. However, the role of XR007793 in formation and proliferation of neointima after vascular injury remains unclear.

MiR-23b belongs to the miR-23~27~24 clusters, is a highly conserved sequence on human chromosome 9, which is expressed abnormally in various types of tumors, such as gastric cancer, colorectal cancer, and endothelial cells, and is involved in the regulation of cell proliferation, migration, and apoptosis [10–12]. Most importantly, a study showed miR-23b as a novel regulator of VSMCs phenotypic switch *in vitro* and *in vivo* [13]. The study confirmed that Forkhead box O 4 (FOXO4) can promote vascular smooth muscle migration and dedifferentiation, thereby aggravating the progress of atherosclerosis. A growing number of miRNAs have been shown negatively correlated to FOXO4, which means that FOXO transcripts are tightly regulated by the miRNA network [14].

In this study, we focus on the interaction effects between lncRNA XR007793 and miR-23b in VSMCs proliferation and migration after vascular injury. Finally, we revealed that lncRNA XR007793 serve as a "sponge" to decrease miR-23b and finally increased FOXO4 expression at post-transcriptional level.

Material and Methods

Cell culture, transfection, and reagents

Rat aortic smooth muscle cell (RA-SMC) line was purchased from ScienCell Research Laboratories and cultured (at 37°C, 5% CO_2) in Dulbecco's modified Eagle medium (GIBCO, LifeTechnologies, USA), containing 10% FBS (GIBCO, LifeTechnologies, USA) with 1% of penicillin-streptomycin. Cells were treated with different concentrations of platelet-derived growth factor-BB (PDGF-BB) for different lengths of time. XR007793 siRNA, miR-23b mimic, and miR-23b inhibitor and were purchased from Genepharma Company (Shanghai, China). Cells at 70% confluence were transfected with siRNA or miRNA mimics/inhibitor by Lipofectamine 2000 reagent (Life Technologies, USA). Antibodies against FOXO4 antibody (ab128908, 1: 5000), SMα-actin antibody (ab5694, 1: 5000), SM22 antibody (ab10135, 1: 5000) were purchased from Abcam (Cambridge, MA, USA), secondary antibody (SA00001-2, 1: 2000) was purchased from Proteintech (Wuhan, China)

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, Calif, USA). Then reverse transcription (RT) and real-time SYBR Green quantitative PCR were performed according to the kit instructions (Takara, Dalian, China). The primers used to amplify rat genes were synthesized by Sangon Biotech (Shanghai, China) and the specific sequence was as follows: XR007793: forward, 5'-CATAACCCAAGCGTCAAAGG-3', reverse, 5'-CATGAAGGCAGGTAAGAAAACAC-3'. MiR-23b: forward, 5'-GAGCATCACATTGCCAGGG-3', reverse, 5'-GTG CAGGGTCCGAGGT-3'. FOXO4: forward, 5'-CTTTCTGAAGACTGGCA GGAATGTG-3', reverse, 5'-GATCTAGGTCTATGATCGCGGCAG-3', U6: forward, 5'-CGCTAGCACATATCGGCTA-3', reverse, 5'-TTCTGCGACGAATTTGTCAT-3'. U6 acted as the internal reference. The thermal cycling conditions were carried out as follows: at 94°C for 10 min, followed by 40 cycles (94°C for 15 sec; 60°C for 30 sec; and 72°C for 60 sec). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative fold changes.

Cell proliferation assay

The VSMC (2×10^4/well) were seeded in 96-well plates per well in 100 µL culture medium. After incubation for 24 hours, the cells were stimulated with PDGF-BB (20 ng/mL) for another 24 hours, then Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) solution (10 µL/well) were added and incubated 2 hours and the absorbance was measured at 450 nm (Tecan Sunrise, Männedorf, Switzerland).

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Cell migration assay

Cell migration was assessed using the scratch wound healing assay and Transwell assay. After starving in FBS-free medium for 12 hours, a linear wound was gently introduced to the center of the cell monolayer using a 200 μL tip. The culture was subjected to stimulation with or without PDGF-BB (Peprotech, Anaheim, CA, USA) at a final concentration of 40 ng/mL, cells were incubated 24 hours at 37°C, and images were captured and counted using a microscope (Olympus, Tokyo, Japan). Similarly, cells were transferred to the upper chamber of a Transwell (1×10^5/mL cells per chamber) coated with fibronectin after starvation. Then, 500 μL of PDGF-BB (20 ng/mL) was added to the bottom chamber medium, and cells were incubated 12 hours at 37°C. After pass through the polycarbonate film, the non-migrated cells were scraped off using swab. The migrated cells were counted after crystal violet staining, and 3 to 5 randomly selected fields were chosen to take pictures and count using a microscope.

Luciferase reporter assay

The 3'-UTR of miR-23b and XR007793 was amplified using PCR. Mutant sequence was structured according to the miRNA complementary sites. Sequences were subcloned into the luciferase reporter vector pGL3 vectors (Promega, Madison, WI, USA) with 3'-UTR. VSMc cells were co-transfected with luciferase reporter plasmids and miRNA mimics as well as the internal control pRSV-β-galactosidase vector by using the Lipofectamine™ 2000 transfection reagent. Then, 48 hours post-transfection, VSMc cells activity was measured using a dual-luciferase reporter gene assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase expression was analyzed by GloMax-Multi-function detector (Promega, Madison, WI, USA). The expression of Renilla luciferase (pRL-TK) divided by the expression of firefly luciferase equaled the relative luciferase expression.

Western blot analysis

The cells were lysed by protein lysis buffer (Beyotime, Shanghai, China) and centrifuged at 4°C 2000 g for 5 min, the supernatant was transferred to a 1.5 mL tube and the loading buffer was added. SDS-PAGE was conducted to separate the cellular proteins. The candidate proteins were blotted onto the polyvinylidene difluoride (PVDF) membrane and incubated overnight at 4°C with primary antibodies. Next, the membrane was washed and incubated with a second antibody (anti-rabbit/horseradish peroxidase) at room temperature for 1.5 hours. The protein brand was detected by the standard chemiluminescence method and quantified using the gel image analysis system (SynGene, USA).

Rat carotid balloon-injured model

All animal procedures were conducted in accordance with the Animals Care and Use Committee of Huaihe Hospital of Henan University. Male Sprague-Dawley rats (n=24, 350–450 g) obtained from the SLAC of China (Shanghai, China), were anesthetized with 5% chloral hydrate (1 mL/100 g, intraperitoneally), the neck was depilated and disinfected after being fixed on the anatomy plate. The left common artery was isolated and the 2F balloon catheter (Sigma, St. Louis, MO, USA) was inserted through the external carotid arteriotomy incision. Next, the balloon was inflated at a pressure of 1.3 kg/cm^2 and pulled back 3 times to cause common carotid artery endothelial injury. Next, the catheter was removed and the external carotid artery was ligated. The rats in the sham group underwent the same procedures, except the balloon was not inserted. The rats were sacrificed on day 14 and the tissues were harvested for next experiments.

Pathological change

The carotid artery sections were dissected, fixed in 4% paraformaldehyde and embedded in paraffin. The slices were cut to 5-μm thickness during the routine follow-up procedure and stained with hematoxylin and eosin (H&E). The pictures were taken using a light microscope (Leica, Heidelberg, Germany) at 200× and 400×, respectively.

Statistical analysis

All data are presented as the means ±SEM. Statistical evaluation of the data was performed using the 2-tailed unpaired Student's *t*-tests and ANOVA analysis using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA: IBM Corp.) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Spearman Rho correlation coefficient was used to assess relation between miR-23b and XR007793. Values of $P<0.05$ were considered a statistically significant.

Results

Expression of XR007793 in VSMC *in vitro* and *in vivo*

To investigate the biological role of XR007793 in vascular disease, we first compared the pathological changes of carotid intima injury in both groups. The results suggested that the area of intimal hyperplasia significantly increased and the artery lumen showed remarkable stenosis in 14 days (Figure 1A). After that, we measured the expression levels of XR007793 in injured rat carotid artery using qRT-PCR. As shown in Figure 1B, XR007793 expression levels were significantly increased in

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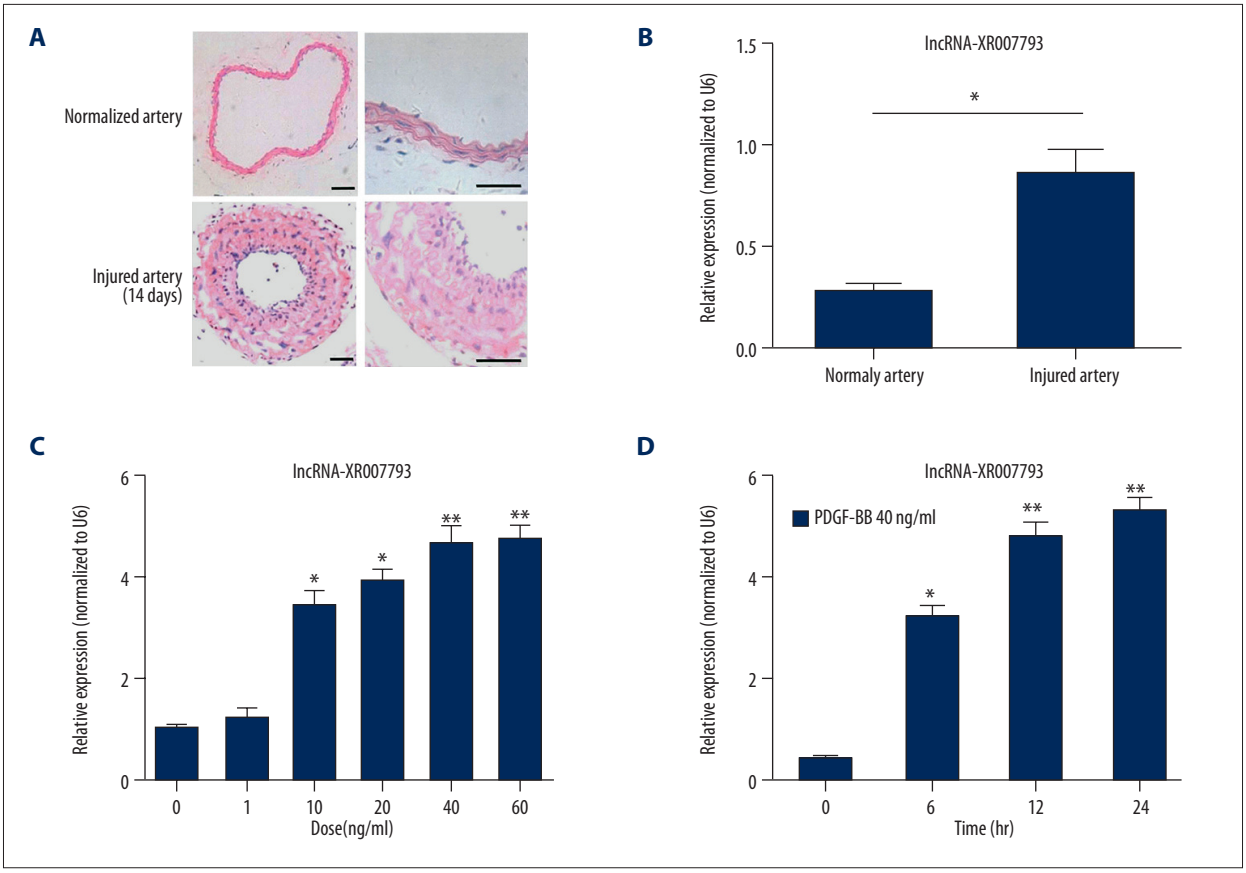

Figure 1. XR007793 was upregulated in carotid tissues of rats and cell lines. (**A**) Carotid artery sections with or without injury shown by hematoxylin and eosin (H&E) staining. Scale bars, 100 μm and 50 μm. (**B**) The expression of XR007793 in injured carotid tissues was higher than normal tissues. (**C**) After stimulated by different concentration of PDGF-BB and incubated 24 hours, the expression of XR007793 in RA-SMCs was tested by qRT-PCR. (**D**) Cells were incubated with 40 ng/mL PDGF-BB and the expression of XR007793 was gradually increased in different time periods. Data were represented as mean ±SD, * $P<0.05$, ** $P<0.01$, compared to normal group or control group.

balloon injured vascular tissue compared with the sham group tissue. We examined the expression levels of XR007793 in cultured RA-SMC (Figure 1C, 1D), which were gradually upregulated after induction with different concentrations of PDGF-BB. Dramatically, 40 ng/mL and 60 ng/mL PDGF-BB stimulated RA-SMC induced an increase in XR007793. Hence, we focused on 40 ng/mL PDGF-BB in our further experiments. When stimulated with 40 ng/mL PDGF, the level of XR007793 gradually increased with time, suggesting that the increase of XR007793 was time-dependent. Taken together, these results manifested that XR007793 genuinely overexpressed in balloon injured carotid artery and cell lines, which suggests that it might be involved with VSMCs in neointima.

XR007793 knockdown inhibited VSMC proliferation and migration

Based on the aforementioned results, we investigated the function of XR007793 on cell proliferation and migration. RA-SMC siRNA were transfected into the VSMC line to inhibit the expression of lncRNA-XR007793. As show in Figure 2A, the efficiency of siRNA transfection was tested and confirmed using qRT-PCR. CCK-8 assay indicated that XR007793 knockdown significantly suppressed the proliferation over time (Figure 2B). The Transwell and scratch wound assay showed that XR007793 knockdown markedly decreased the migration ability of RA-SMCs (Figure 2C, 2D). Together, these data indicate that XR007793 knockdown inhibits the proliferation and migration of VSMCs, this underscores the potential neointima promoting effect.

XR007793 regulated miR-23b via direct targeting

Increasing evidence suggests that lncRNA acts as a miRNA sponge to regulate physiological functions. Studies have shown that overexpression of miR-23b inhibits VSMC proliferation and migration. Therefore, we focused on miR-23b as our research target to probe the complementary bindings. As

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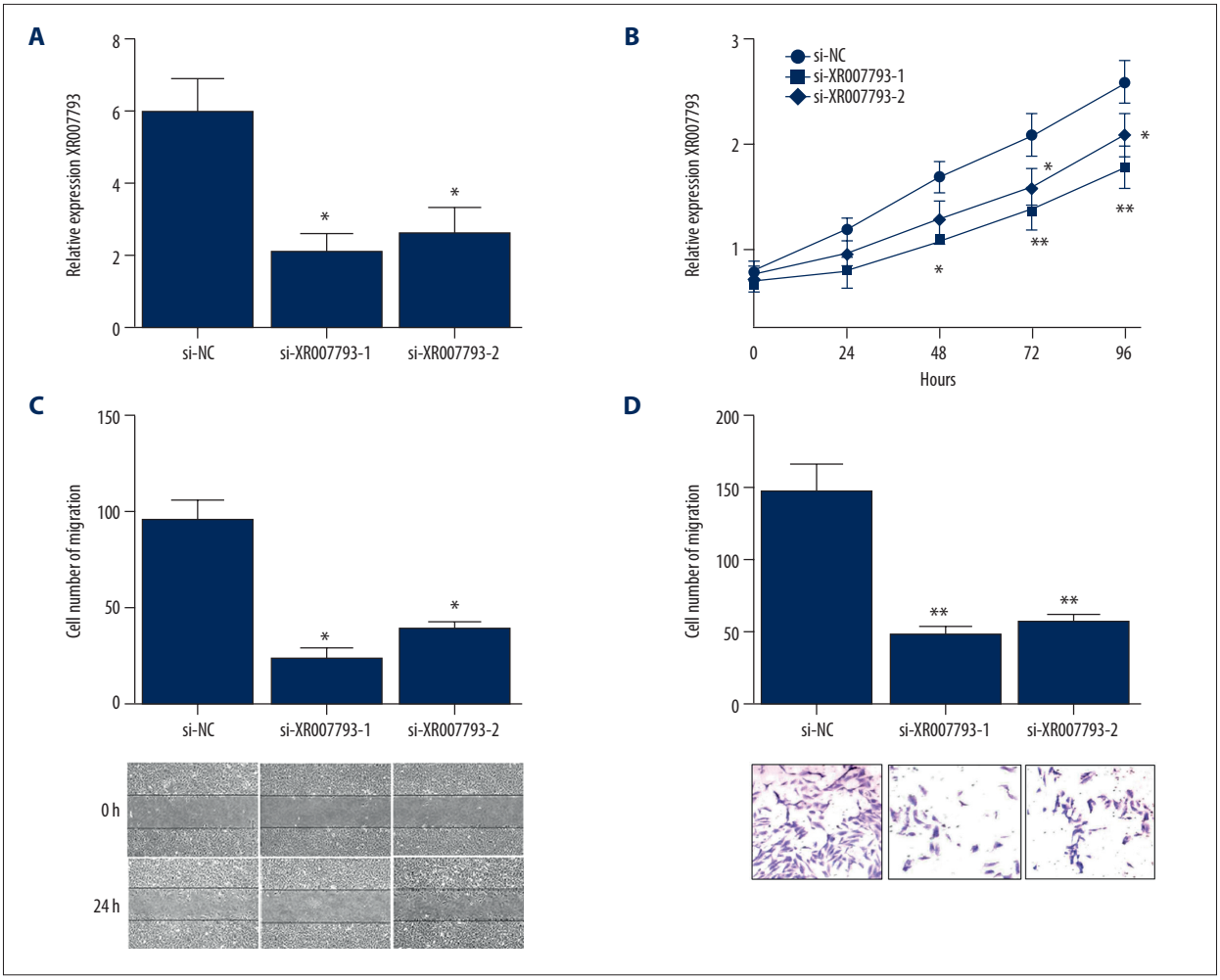

Figure 2. Knockdown of XR007793 suppressed proliferation and migration of RA-SMCs. (**A**) 10 nM of si-XR007793 was transfected into RA-SMC and incubated 24 hours, the expression of XR007793 was tested by qRT-PCR. (**B**) Cell viability was measured at special time point after seeding in 96-well plates using the CCK-8 assay. (**C**) The migration ability of RA-SMC was detected by plate scribing after transfected with si-XR007793. (**D**) Representative images of vertical migration after transfected with si-XR007793 and randomly selected fields are shown. Data were represented as mean ±SD, * $P<0.05$, ** $P<0.01$, compared to normal group.

shown in Figure 3A, alignment of complementary regions between miR-23b and XR007793 3'-UTR manifested in a presumed miR-23b target site in the XR007793 gene. The luciferase reporter construct linked to the 3'UTR of miR-23b was repressed by co-transfection of si-XR007793 (Figure 3B). The expression levels of miR-23b in injured artery tissue and RA-SMCs were obviously decreased compared to the control group (Figure 3C, 3D). Pearson's correlation analysis showed that the expression level of miR-23b in injured artery tissue was negatively related to the expression of XR007793 (Figure 3E). These results suggested that XR007793 function as a miR-23b sponge.

XR007793 promotes VSMCs proliferation and migration by suppressing miR-23b via FOXO4 targeting

After detecting the interaction of XR007793 and miR-23b, the possible binding sites between miR-23b and the downstream target gene FOXO4 were predicting using bioinformatics analysis (TargetScan Human 7.1). The results are shown in Figure 4A and verified by luciferase reporter assay (Figure 4B). After that, the miR-23b inhibitor and miR-23b mimics were individually transfected into cells. The expression of XR007793 and FOXO4 were measured by qRT-PCR. The results demonstrated that miR-23b inhibitor evidently increased the expression of XR007793 and FOXO4, while miR-23b mimics showed the opposite effects (Figure 4C, 4D). Moreover, co-transfection with si-XR007793 and miR-23b inhibitor inhibited the expression

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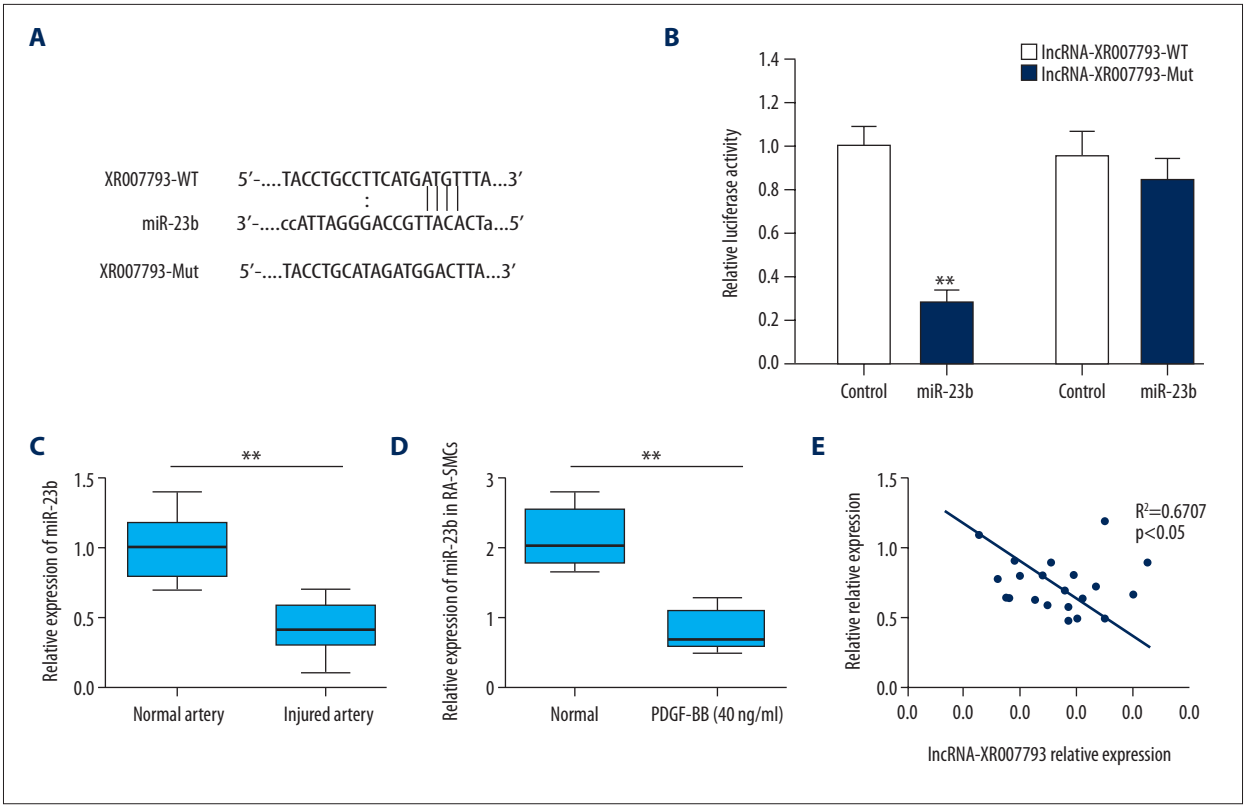

Figure 3. Validation of XR007793 as a direct target of miR-23b. (**A**) Putative complementary sites within miR-23b and 3′-UTR of XR007793 predicted by starBase. (**B**) Dual-luciferase reporter assay verified the complementary sites within miR-23b with the 3′-UTR of XR007793. (**C**) The expression of miR-23b in carotid tissues. (**D**) MiR-23b relative expression was assessed by qRT-PCR in cells. (**E**) The correlations between XR007793 and miR-23b expression was showed by Pearson's correlation (R^2=0.6707, P<0.05). All data were expressed as mean ±SD. ** P<0.01 *vs.* control group.

of FOXO4 and smooth muscle-specific markers smooth muscle α-actin and smooth muscle protein 22-α (SMα-actin and SM22α), however, was promoted by XR007793 knockdown (Figure 4G–4I). Finally, both the cell viability and migration number were significantly repressed by si-XR007793, and the miR-23b inhibitor rescued the suppression (Figure 4J–4L). In conclusion, these results indicate that XR007793 aggravated the loss of function of VSMCs by negatively regulating miR-23b via FOXO4 targeting.

Discussion

The proliferation and migration of VSMCs are thought to be key events in the development of atherosclerotic lesions and restenosis of blood vessels. Under physiological and pathological conditions, VSMCs have significant phenotypic plasticity. When a variety of causes stimulate vascular endothelial injury and release of inflammatory cytokines, VSMCs will dedifferentiate and regain the ability to proliferate, migrate, and synthesize extracellular matrix, as well as reduced expression of differentiation markers (i.e., SMα-actin and SM22α), which also becomes the pathological basis for atherosclerosis and restenosis after angioplasty.

Accumulating evidence have identified the potential function of lncRNAs in SMC' proliferation and migration [15]. LncRNA-21 was shown to repress VSMC proliferation and induce apoptosis to delay the progression of atherosclerotic plaque damage [16]. PVT1 was upregulated in asthmatic patients with severe corticosteroid-insensitive asthma. Targeting PVT1 might be helpful for alleviating airway remodeling [17]. Upregulated XR007793 has been shown to exacerbate vascular remodeling of hypertension by promoting Ang-II induced VSMC proliferation and migration [8], but its role in vascular intimal hyperplasia after stent injury and downstream miRNA-mRNA regulatory networks is unclear. In the present study, we identified XR007793 as a novel regulator of VSMCs proliferation and migration, which reveals that the expression of XR007793 is closely related to differentiation of VSMC and neointima caused by stent implantation.

As competing endogenous RNAs, lncRNA regulate miRNA levels by modulating mRNA stability and translation by homologous

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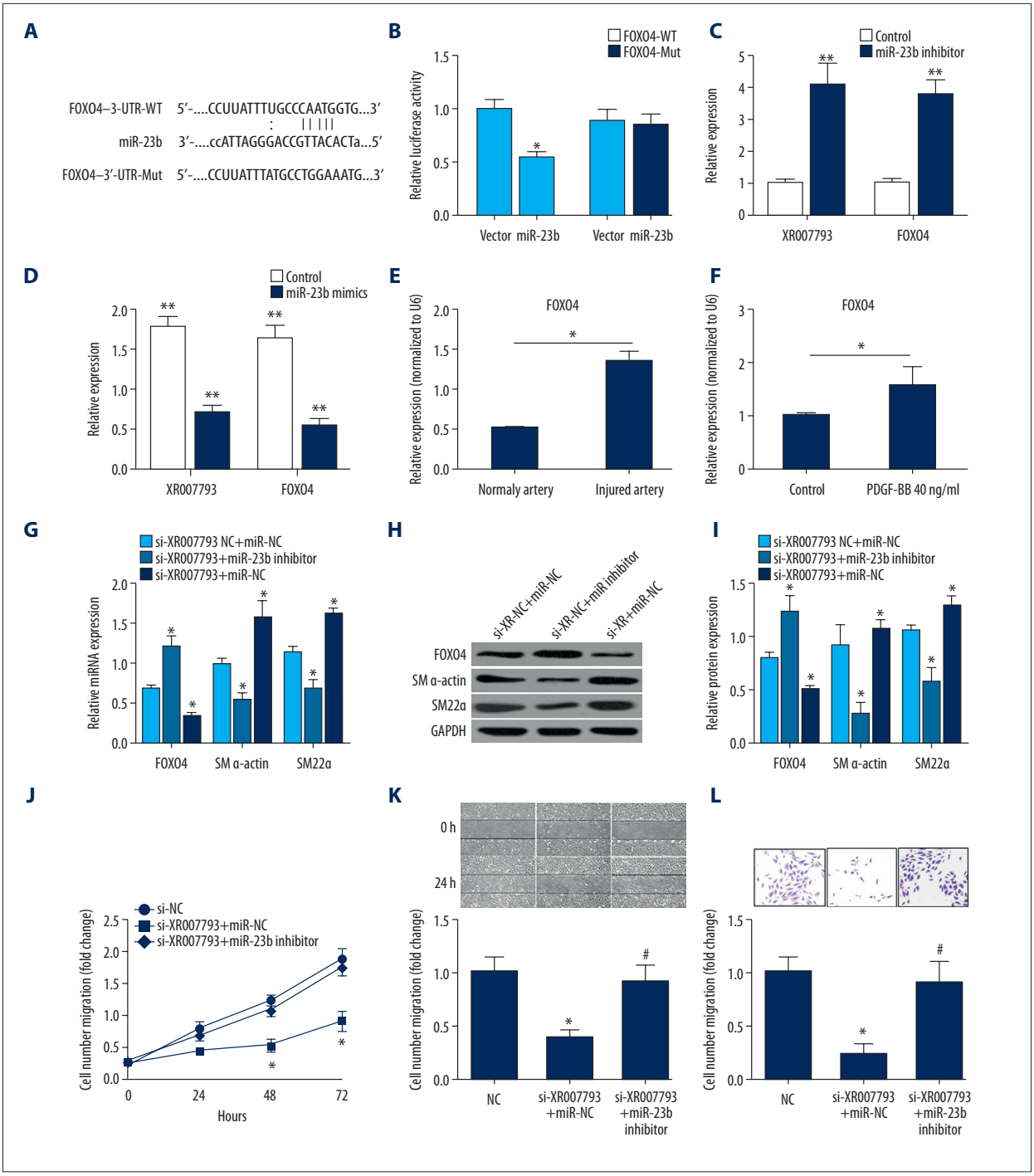

Figure 4. XR007793 is a target of miR-23b and regulates the expression of FOXO4. (**A**) A putative complementary sequence alignment between miR-23b and FOXO4 3'-UTR. (**B**) The miR-23b mimics and WT/Mut FOXO4 vectors were co-transfected into RA-SMCs and the probability of binding of miR-23b with the 3'-UTR of FOXO4 was assessed by luciferase reporter assay. (**C**) After transfected with miR-23b inhibitor, the expression of XR007793 and FOXO4 were significantly increased. (**D**) The expression levels of XR007793 and FOXO4 in RA-SMCs transfected with miR-23b control or miR-23b mimics. (**E**) Expression of FOXO4 in tissues. (**F**) Expression of FOXO4 in cells. (**G**) FOXO4, SMα-actin, and SM22α mRNA level were analyzed by qRT-PCR. (**H**) FOXO4, SMα-actin, and SM22α proteins in RA-SMCs transfected with si-NC +miR-NC, si-XR007793 +miR-23b inhibitor or si-XR007793 +miR-23b-NC detected by western blot. (**I**) Quantitative expression of FOXO4, SMα-actin, and SM22α. (**J**) RA-SMCs proliferation viability detected cell using CCK-8. (**K, L**) The migratory behavior of RA-SMCs was evaluated by scratch test and Transwell assay. Data were expressed as mean ±SD. * $P<0.05$, ** $P<0.01$ *vs.* si-NC group, # *vs.* si-XR007793+miR-23b inhibitor group.

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base pairing [18]. Recently, cancer-related triplets of lncRNA-miRNA-mRNA were analyzed by an integrative network to illustrate the intricate transcriptome, in breast cancer [19] and uterine corpus endometrial carcinoma [20]. LncRNA H19 and ANRIL have been shown to play roles in the pathogenesis of atherosclerosis. Leung et al. found that lnc-Ang362 knockdown decreased the proliferation of SMCs by targeting miR-221 and miR-222 [21]. The main contribution of our study lies in the discovery of XR007793 may act as endogenous sponge RNAs to interact with miR-23b and influence the expression of FOXO4. This work provides new insights into the regulation of VSMCs events by lncRNAs. It is also an interesting scientific topic that we will continue to focus on in future research.

Forkhead box O (FOXO) proteins use the forkhead box domain to bind as monomers to the consensus sequence and regulate gene expression [22]. As a member of the transcription factor FOXO family, FOXO4 is a promoter of smooth muscle dedifferentiation genes and as an activator of VSMCs migration [23]. Li et al. [24] reported that FOXO4 regulates tumor necrosis factor alpha (TNF-α)-induced SMC migration against proliferative arterial diseases. Emerging evidence shows that miRNAs such as miR-499-5p [25] and miR-150 [26] target FOXO4. MiR-23b was identified as a regulator of the VSMCs phenotypic switch by targeting FOXO4 [13]. In this study, we provide experimental evidence that miR-23b negatively regulates the expression of FOXO4 through the FOXO4 3'-UTR, and that it effects the expression of SM-αactin and SM22α in SMCs. These results are particular interesting, since they might explain our finding that the miR-23b modulates the expression levels of several key partakers of VSMC phenotypic modulators, such as SMα-actin and SM22α.

Conclusions

Our study results reveal the critical interaction between lncRNA XR007793 and miR-23b and their effect on the regulation of the VSMCs phenotypic switch in a rat carotid balloon-injury model. LncRNA XR007793 aggravates the proliferation and migration of RA-SMCs by sponging miR-23b and the post-transcriptional regulation of FOXO4. These findings may lead to progress in lncRNA therapies, other than XR007793. This may lead to novel preventative strategies in ISR and atherosclerosis.

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