Blockade of 146b-5p promotes inflammation in atherosclerosis-associated foam cell formation by targeting TRAF6

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Abstract. Atherosclerosis (AS) is a chronic inflammation in response to lipid accumulation. Increasing evidence has demonstrated that numerous microRNAs (miRs) have critical roles in inflammatory responses. A previous study suggested that miR-146b-5p is possibly associated with AS; however, its exact role has remained largely elusive. The present study aimed to investigate the potential role of miR-146b-5p in AS and to explore the underlying mechanism. Fist, the levels of miR-146b-5p were determined in foam cells and clinical specimens from patients with AS by reverse-transcription quantitative PCR. The role of miR-146b-5p in AS was then investigated by using miR-146b-5p inhibitor. The results demonstrated that the expression levels of miR-146b-5p were elevated in the lesions of patients with AS. In addition, the levels of miR-146b-5p in THP-1 cells stimulated with phorbol 12-myristate 13-acetate (100 nM) to induce their differentiation into macrophages were dose- and time-dependently elevated by oxidized low-density lipoprotein treatment applied for inducing foam cell formation. miR-146b-5p was also revealed to directly target tumor necrosis factor receptor-associated factor 6 (TRAF6), which functions as a signal transducer in the nuclear factor-κB (NF-κB) pathway. Furthermore, the present study reported for the first time that miR-146b-5p inhibition promotes the inflammatory response and enhances lipid uptake during foam cell formation. In conclusion, miR-146b-5p inhibition promoted chronic inflammation and had a detrimental role during AS-associated foam cell formation by targeting TRAF6.

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Introduction

Atherosclerosis (AS), one of the most common cardiovascular diseases, is a major cause of morbidity and mortality worldwide (1). Hyperlipidemia, monocyte recruitment, differentiation into macrophages, foam cell formation and induced inflammation are the key cellular events of AS (2). Chronic inflammation has a key role in the occurrence and development of AS (3), and macrophages with the ability to stimulate the vascular inflammatory reaction are the major effector cells throughout the pathological process of AS (4). Therefore, genes and cytokines involved in immune system control are essential in regulating AS. Recent studies have indicated the potential roles of microRNAs (miRNAs) in the regulation of AS-associated processes (5,6).

miRNAs are small (~22 nucleotides in length), endogenous, non-coding, single-stranded RNAs, which have important roles in gene expression regulation by binding to the 3'-untranslated region (UTR) of target mRNAs (7,8). A growing body of evidence suggested that miRNAs have different roles in the development of AS. Li *et al* (9) reported that increased miR-155 relieves chronic inflammation in AS. Xu *et al* (10) suggested that miR-135b-5p and miR-499a-3p promoted cell proliferation and migration in AS. Zhang *et al* (11) demonstrated that miR-26a prevented endothelial cell apoptosis under AS conditions. Ouimet *et al* (12) found that miR-33 antagonism exerted atheroprotective roles via regulating macrophage-associated inflammation.

A previous study demonstrated upregulation of miR-146b-5p in oxidized low-density lipoprotein (oxLDL)-stimulated monocytes (13); however, to the best of our knowledge, no further study on the role of miR-146b-5p in AS has been performed. Thus, the present study investigated the potential role of miR-146b-5p in AS and explored the underlying molecular mechanisms.

Materials and methods

Specimens. A total of 10 pairs of atherosclerotic lesion tissues and normal veins were identified and collected during biopsies from 10 patients (gender ratio: 1:1; age, ranging from 42 to 59 years old) with AS who were diagnosed by clinical symptoms and angiography at the Affiliated Hospital of Qingdao

University (Qingdao, China) between August 2015 and August 2016. The exclusion criteria of the patients were as previously described (9). Informed consent was obtained from each patient. The present study was approved by the Affiliated Hospital of Qingdao University (Qingdao, China).

Cell culture and foam cell model construction. The THP-1 human monocytic and the HEK293T human embryonic kidney cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). THP-1 cells were grown in RPMI-1640 medium, supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA USA), and 1% streptomycin and penicillin mixed solution (Thermo Fisher Scientific, Inc.). HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.). All cells were incubated in a humidified atmosphere with 5% CO_2 at 37˚C. A foam cell model was established as previously described (14-16). In brief, THP-1 cells were first seeded in culture plates at $1x10^6$ cells/ml with 100 nM phorbol 12-myristate 13-acetate (PMA; Sangon Biotech Co., Ltd., Shanghai, China) for 12 h to differentiate them into macrophages. Subsequently, the cells were stimulated with different concentrations of oxLDL (10, 50 or 100 μg/ml; Sangon Biotech Co., Ltd.) for specific durations (0, 6 or 12 h) in order to induce foam cell formation; control cells were treated with PBS.

Oil Red O staining. Macrophages derived from THP-1 cells were transfected with a miR-146b-5p inhibitor or its negative control (GenScript Biotech Corporation; Piscataway, NJ, USA) using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection with miR-146b-5p inhibitor or control, cells were treated with oxidized low-density lipoprotein (50 μg/ml) for 24 h. The cells were then washed with PBS, fixed with 4% paraformaldehyde, stained with Oil Red O (Sangon Biotech Co., Ltd.) at room temperature for 20 min, and then de-stained with 60% isopropanol for 1 min. The foam cells were then imaged by using a microscope (Olympus Corporation,, Tokyo, Japan) at a magnification of x40.

RNA isolation and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). miR-146b-5p was extracted from atherosclerotic lesion tissues and normal veins by using the mirVana PARIS kit (cat no. AM1556; Ambion; Thermo Fisher Scientific, Inc.) in line with the manufacturer's instructions. The TaqMan MicroRNA Reverse Transcription kit (cat no. 4366596; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for RT of miRNA, and the complementary (c)DNA was amplified by PCR using TaqMan Fast Advanced Master Mix (cat no. 4444556; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The amplification conditions were as follows: 37 cycles of denaturation at 95˚C for 10 sec, followed by annealing and extension at 58˚C for 60 sec. U6 was used as an endogenous control of miRNA expression. The primer sequences were as follows: miR-146b-5p forward, 5'-TGA CCCATCCTGGGCCTCAA-3' and reverse, 5'-CCAGTGGGC AAGATGTGGGCC-3'; and U6 forward, 5'GCTTCGGCA GCACATATACTAAAAT3' and reverse, 5'CGCTTCACG AATTTGCGTGTCAT3'.

For tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) mRNA expression analysis, total RNA from THP-1-derived macrophages was isolated by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was obtained by using a RT-PCR detection kit (cat no. 18091050; Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Real-time PCR was performed to amplify the synthesized cDNA by using the Fast SYBR Green Master Mix (cat no. 4385610; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Amplification conditions were 95˚C for 10 min, followed by 38 cycles at 95˚C for 15 sec, and 72˚C for 30 sec. GAPDH acted as an internal control. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression (17). The primer sequences were as follows: TRAF6 forward, 5'-GAGTTTGAC CCACCTCTGGA-3' and reverse, 5'-TTTCATTGTCAACTG GGCACT-3'; GAPDH forward, 5'-CTTTGGTATCGTGGA AGGACTC-3' and reverse, 5'-GTAGAGGCAGGGATGATG TTCT-3'.

Western blot analysis. Cells were dissolved by using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and a bicinchoninic acid protein assay kit (cat no. 23225; Thermo Fisher Scientific, Inc.) was used for determining the protein concentration. Protein samples (25 μg per lane) were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blocked with 5% skimmed milk powder. The membrane was incubated with a primary antibody against GAPDH (cat no. 5174), nuclear factor (NF)-κB (p65; cat no. 8214) or TRAF6 (cat no. 8028) (dilution for all, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C, and then incubated with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (cat no. 7074; 1:5,000 dilution; Cell Signaling Technology, Inc.) at room temperature for 2 h. The protein levels were detected by enhanced chemiluminescence using the Chemiluminescent ECL reagent (EMD Millipore, Billerica, MA, USA).

ELISA. Pro-inflammatory cytokines [interleukin (IL)-6, cat no. E-EL-H0102c; Elabscience Biotechnology, Inc., Wuhan, China), IL-2 (cat no. 8629), TNF-α (cat no. 8668) (both from Cell Signaling Technology, Inc.) and transforming growth factor (TGF)-β (cat no. BMS249-4FIVE; Invitrogen; Thermo Fisher Scientific, Inc.) secreted from THP-1-derived macrophages transfected with miR-146b-5p inhibitor or control, followed by treatment with oxidized low-density lipoprotein (50 μg/ml) for 24 h, were detected by using respective ELISA kits according to the manufacturer's instructions.

Luciferase reporter assay. To predict the potential targets of miR-146b-5p, TargetScan (http://www.targetscan.org/vert_71/) was performed in the present study. To confirm our prediction, miR-146b-5p recognition sequences from the wild-type (WT) and mutant (MUT) 3'-UTR of TRAF6 (forward, 5'-GCGATCGCTATATGTAATATATTAAAAGTG AAA-3' and reverse, 5'-GGAGCTCAAATAATTAAGGTT ATATTTAGG-3') were amplified and then cloned into the

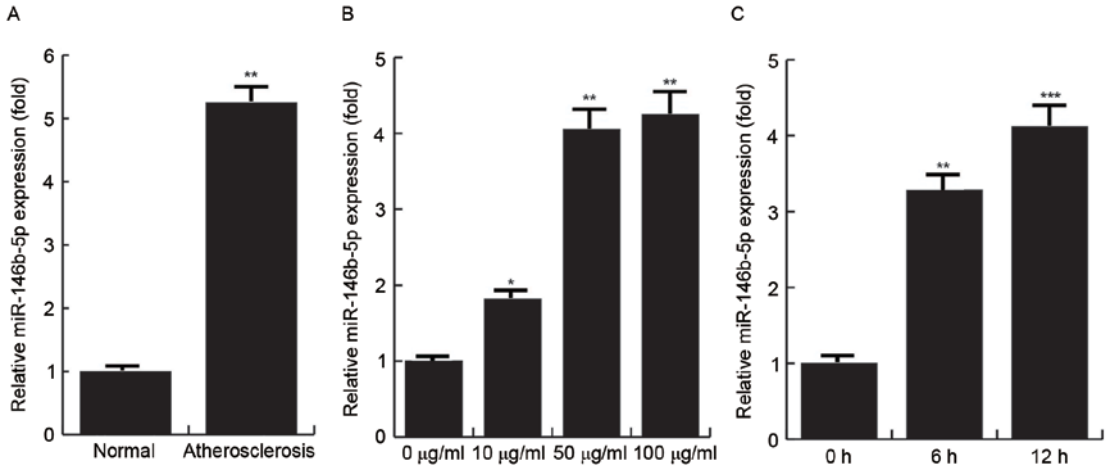

Figure 1. Expression of miR-146b-5p is increased in human macrophages in clinical specimens and is induced by oxLDL. (A) Levels of miR-146b-5p in atherosclerotic lesions (n=10) and normal veins (n=10) from the same patients with AS. (B) Analysis of miR-146b-5p expression in THP-1 cells, which were first stimulated with PMA (100 nM) to induce them to differentiate into macrophages and then treated with oxLDL at the indicated doses (0, 10, 50 or 100 μg/ml). (C) miR-146b-5p expression in THP-1 cells, which were first stimulated with PMA (100 nM) to induce them to differentiate into macrophages and then treated with oxLDL (50 μg/ml) for the indicated times (0, 6 or 12 h). miR-146b-5p was determined by reverse-transcription quantitative polymerase chain reaction analysis. *P<0.05, **P<0.01, ***P<0.001 vs. control. miR, microRNA; oxLDL, oxidized low-density lipoprotein; PMA, phorbol 12-myristate 13-acetate.

psiCHECK-2 reporter vector (Promega Corporation, Madison, WI, USA). The resulting miR-146b-5p-TRAF6-3'UTR-WT or miR-146b-5p-TRAF6-3'UTR-MUT vectors were respectively co-transfected with miR-146b-5p or control miR into HEK293T cells using Lipofectamine 2000 transfection reagent in line with the manufacturer's protocol. At 48 h after transfection, the Dual-Luciferase Reporter Assay system (Promega Corporation) was used for luciferase activity detection. The luciferase activity was then normalized, expressed and Renilla luciferase activity was used as the internal control.

Statistical analysis. Data are presented as the mean ± standard deviation. Student's t-test was performed for comparison between two groups. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. All tests were independently performed at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-146b-5p is overexpressed in the atherosclerotic lesions of patients with AS and is induced by oxLDL in human macrophages. The relative expression levels of miR146b-5p in atherosclerotic lesions and normal veins from the same AS patients were detected by RT-qPCR, and the results demonstrated that the miR146b-5p expression levels were significantly increased in the atherosclerotic lesions compared with those in the normal veins. To determine whether overexpression of miR-146b-5p is induced by oxLDL, THP-1 cells were first treated with 100 nM PMA to stimulate their differentiation into macrophages and then stimulated with various concentrations of oxLDL (0, 10, 50 and 100 μg/ml) for 24 h or with 50 μg/ml oxLDL for specific durations (0, 6 or 12 h) in order to induce foam cell formation (18). The findings demonstrated that the expression of miR-146b-5p was significantly increased by oxLDL stimulation, and the effect was dose- and time-dependent (Fig. 1).

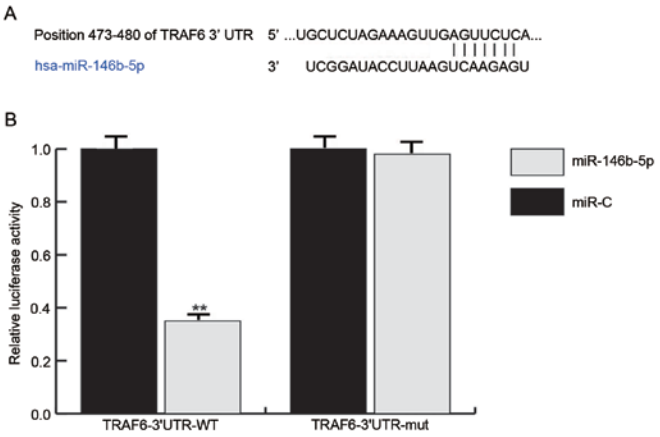

Figure 2. miR-146b-5p directly targets TRAF6. (A) Interaction between miR-146b-5p and the 3'UTR of TRAF6 was predicted using TargetScan and MiRanda databases. (B) Luciferase activity of cells transfected with a reporter vector containing a WT or mutant TRAF6 3'UTR together with miR-146b-5p or miR-C are presented in the bar graph. **P<0.01 vs. control. TRAF6 3'UTR-MUT, TRAF6 3'UTR with a mutation in the miR-146b-5p binding site; UTR, untranslated region; WT, wild-type; TRAF6, tumor necrosis factor receptor-associated factor 6; miR, microRNA; miR-C, control miR; hsa, *Homo sapiens*.

TRAF6 is a functional target of miR-146b-5p. To explore the mechanisms by which miR-146b-5p affects macrophage-derived foam cell formation and the inflammatory response, TargetScan and miRanda database searches were performed to predict the direct mRNA targets of miR-146b-5p. To confirm the prediction of TRAF6 mRNA being a target, a luciferase reporter assay was performed. The results revealed that the luciferase activity was significantly decreased in the HEK293 cells co-transfected with miR-146b-5p and miR-146b-5p-TRAF6-WT, while co-transfection of miR-146b-5p with miR-146b-5p-TRAF6-MUT did not (Fig. 2). This result indicated that TRAF6 is a direct target of miR-146b-5p.

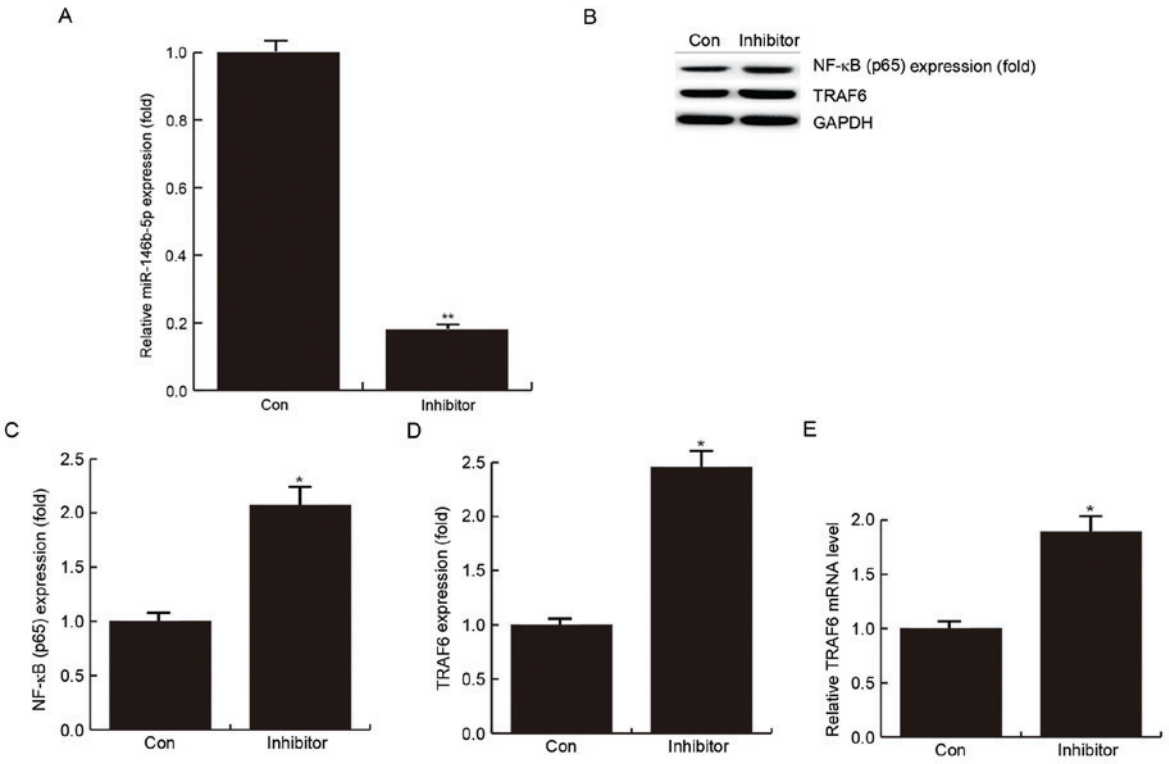

Figure 3. Confirmation of miR-146-5p inhibition and its effect on the protein levels of TRAF6 and NF-κB (p65) as well as TRAF6 mRNA expression. (A) Relative miR-146-5p level; (B) protein level of TRAF6 and NF-κB (p65); protein expression levels of (C) NF-κB (p65) and (D) TRAF6 were expressed as fold changes compared with the control group; and (E) Relative TRAF6 mRNA expression. THP-1 cells, which had been treated with 100 nM phorbol 12-myristate 13-acetate for 12 h to differentiate them into macrophages, were transfected with miR-146b-5p inhibitor or its negative control for 24 h, and then treated with 50 μg/ml oxidized low-density lipoprotein for 24 h. RT-qPCR and western blot analysis were then used for miRNA/mRNA and protein detection. *P<0.05, **P<0.01 vs. control. TRAF6, tumor necrosis factor receptor-associated factor 6; miR, microRNA; Con, control; NF, nuclear factor; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

miR-146b-5p inhibition during foam cell formation increases TRAF6. To investigate the role of miR-146b-5p in the pathological process of AS, miR-146b-5p was inhibited using miR-146b-5p inhibitor. THP-1 cells, which had been treated with 100 nM PMA for 12 h to induce their differentiation into macrophages, were transfected with miR-146b-5p inhibitor or its negative control for 24 h, and then treated with 50 μg/ml oxLDL for 24 hto induce foam cell formation. Efficient inhibition of miR-146b-5p was confirmed by RT-qPCR (Fig. 3A).

In addition, western blot analysis demonstrated that after transfection with miR-146b-5p inhibitor for 24 h, the protein levels of NF-κB (p65) (P<0.05; Fig. 3B and C) and TRAF6 were significantly enhanced (P<0.05; Fig. 3B and D). The results also indicated that miR-146b-5p inhibition may promote the inflammatory response, possibly by promoting the expression of NF-κB (p65) in foam cell formation. At the mRNA level, TRAF6 was significantly enhanced in the group transfected with miR-146b-5p inhibitor (Fig. 3E). This further supported the result that TRAF6 is a direct target of miR-146b-5p.

miR-146b-5p inhibition promotes the inflammatory response during foam cell formation. The effect of miR-146b-5p inhibition on inflammatory factor secretion in macrophages treated with oxidized low-density lipoprotein (50 μg/ml) for 24 h was examined by ELISA. The results demonstrated that in the group transfected with miR-146b-5p inhibitor, the secretion of IL-6, IL-2, TNF-α and TGF-β was significantly increased (Fig. 4).

miR-146b-5p inhibition enhances lipid uptake during foam cell formation. The effect of miR-146b-5p inhibition on foam cell formation was analyzed by Oil Red O staining. The results indicated that in the group transfected with miR-146b-5p, lipid uptake was notably enhanced (Fig. 5).

Discussion

Increasing evidence suggested that chronic inflammation contributes to the formation of atherosclerotic lesions. Foam cell formation may be induced by exposure of macrophages to oxLDL (19). Exploration of the potential molecular mechanisms of inflammatory processes and foam cell formation will provide novel strategies for the treatment of AS.

Various studies have demonstrated that miRNAs have an important role in the pathogenesis of AS. Zernecke *et al* (20) demonstrated that miR-126 prevents atherosclerotic lesion formation through regulating angiogenesis and vascular inflammation, and an anti-AS function of miR-126-5p has been reported recently (21). Consistent with these results, Tabet *et al* (22) suggested that the anti-inflammatory function of high-density lipoprotein is conferred through miR-223. By contrast, Zhang *et al* (23) reported that miR-150 promotes

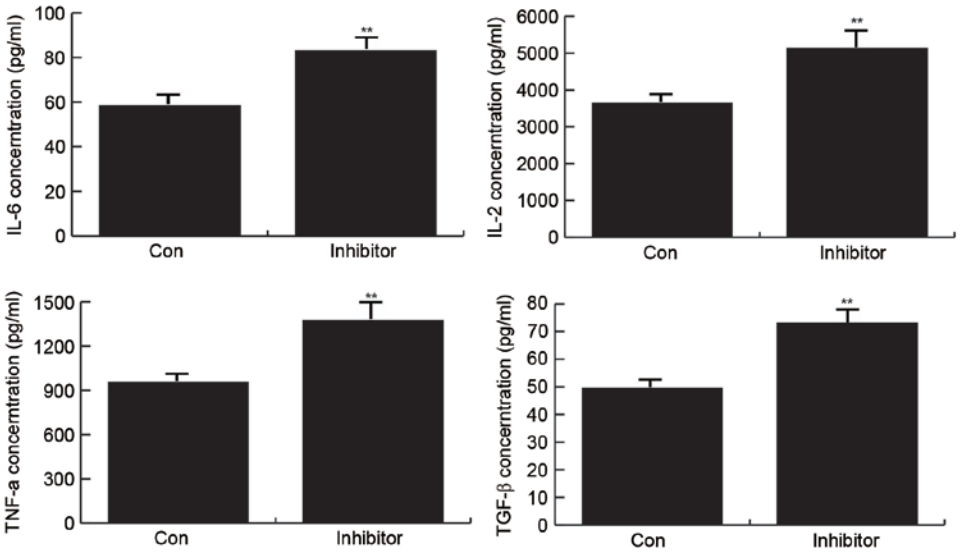

Figure 4. miR-146b-5p inhibition promotes inflammation. After transfection of miR-146-5p inhibitor or its negative control into macrophages, the cells were treated with oxidized low-density lipoprotein (50 μg/ml) for 24 h, and the secreted protein concentration of IL-6, IL-2, TNF-α and TGF-β was determined by ELISA. Values are expressed as the mean ± standard deviation of 3 independent experiments as determined by Student's t-test. **P<0.001 vs. Con. IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; miR, microRNA; Con, control.

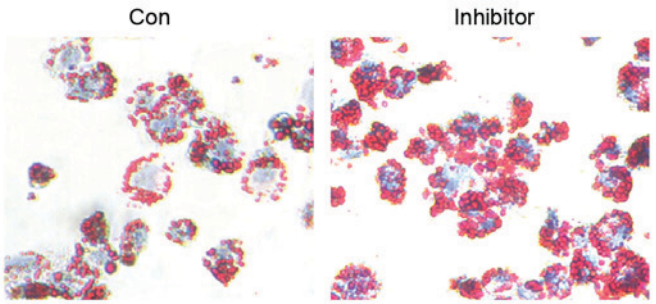

Figure 5. miR-146b-5p inhibition enhances lipid uptake. Lipid uptake was measured by Oil Red O staining after transfection of miR-146b-5p inhibitor or its negative control into macrophages, followed by treatment with oxidized low-density lipoprotein (50 μg/ml) for 24 h (magnification, x40). miR, microRNA; Con, control.

AS via enhancing endothelial cell migration. The present study investigated the role of miR-146b-5p in AS-associated processes to identify novel therapeutic strategies for the treatment of AS and other vascular diseases.

First, the expression levels of miR-146b-5p were determined in atherosclerotic lesions of patients with AS, and the results indicated that miR-146b-5p was highly expressed in the atherosclerotic lesions compared with that in normal veins from the same patient. The present study also found that miR-146b-5p may be induced by oxLDL stimulation in a time- and dose-dependent manner, which was consistent with the results of a previous study (13).

Next, the present study confirmed TRAF6 as a direct target of miR-146b-5p. A statistically significant inverse association was identified between miR-146b-5p and TRAF6 expression in oxLDL-stimulated macrophages, suggesting a significant biological function of the TRAF6-miR-146b-5p complex in AS. TRAF6 functions as a signal transducer in the NF-κB pathway, and the activation of NF-κB was reported to be elevated in patients with acute coronary syndrome and in oxLDL-induced mast cells (24,25). Various molecules involved in the immune response and early inflammation are modulated by the NF-κB pathway. Thus, it was hypothesized that TRAF6-miR-146b-5p is involved in the regulation of AS-associated inflammation. The results of the present study suggested that inhibition of miR146b-5p increases TRAF6 and NF-κB (p65) expression, as well as the secretion of pro-inflammatory cytokines (IL-6, IL-2, TNF-α and TGF-β) in oxLDL-simulated macrophages. These results confirmed the prediction that miR-146b-5p acts as a promoter of inflammation in oxLDL-stimulated macrophages, partly via targeting TRAF6. Furthermore, the present study investigated the effect of miR-146b-5p on foam cell formation via Oil Red O staining. The results indicated that miR-146b-5p inhibition significantly enhanced the lipid uptake by oxLDL-stimulated macrophages.

In conclusion, the present study found that miR146b-5p is overexpressed in the atherosclerotic lesions of patients with AS, and it may be induced by oxLDL in human macrophages. Blockade of miR146b-5p promoted inflammation and foam cell formation by increasing TRAF6-mediated activation of NF-κB expression, indicating the anti-AS function of miR146b-5p.

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