Attenuated macrophage activation mediated by microRNA-183 knockdown through targeting NR4A2

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Abstract. Atherosclerosis is considered a chronic inflammatory disease, and macrophages function as important mediators in the development of atherogenesis. MicroRNA (miR)-183 is a small non-coding RNA that acts as a novel tumor suppressor and has recently been proposed to affect cardiac hypertrophy. However, the exact role and underlying mechanism of miR-183 in macrophage activation remain unknown. In the present study, miR-183 showed upregulated expression in atheromatous plaques and in bone marrow-derived macrophages (BMDMs) subjected to stimulation with oxidized low-density lipoproteins. Using a miR-183 loss-of-function strategy, it was demonstrated that miR-183 knockdown significantly increased resolving M2 macrophage marker expression but decreased proinflammatory M1 macrophage marker expression, as well as attenuated NF-κB activation. Moreover, decreased foam-cell formation accompanied by upregulation of genes involved in cholesterol efflux and downregulation of genes implicated in cholesterol influx was found in BMDMs transfected with a miR-183 inhibitor. Mechanistically, macrophage activation mediated by miR-183 silencing was partially attributed to direct upregulation of NR4A2 expression in BMDMs. Thus, the present study suggests that neutralizing miR-183 may be a potential therapeutic strategy for the treatment of atherosclerosis.

Introduction

Atherosclerosis is well recognized as a chronic inflammatory disease (1) that can cause various cardiovascular pathologies, including myocardial infarction, sudden cardiac death and stroke (2). During the development of atherosclerosis, infiltrated macrophages triggered by endothelial dysfunction play an important role (3). Macrophage polarization refers to an estimate of macrophage activation at a given point in space and time. Currently, the macrophage field has arrived at a partial consensus to describe the broad grouping of macrophage activation phenotypes and the underlying mechanisms (4). The classical inflammatory macrophage phenotype has been termed the M1 phenotype and is usually induced by a combination of interferon-γ and the Toll-like receptor 4 ligand lipopolysaccharide. By contrast, the alternatively activated macrophage populations are termed M2 populations and can be induced by incubation of macrophages with interleukin (IL)-4 and IL-13. Upon exposure to multiple environmental stimuli, macrophages exhibit considerable plasticity, which is responsible for the inflammatory response underlying atherogenesis through a switch between classically activated M1 macrophages, which produce a wide variety of proinflammatory mediators, and alternatively activated M2 macrophages that alleviate inflammation (5-7). Additionally, lesion macrophages can give rise to lipid-laden foam-cell formation following excess engulfment of oxidized low-density lipoprotein (Ox-LDL) (8,9). Importantly, previous studies have also suggested that M1 or M2 macrophages may be the main foam cell precursors through the regulation of cholesterol transport by a liver-X-receptor-dependent mechanism (10,11). Macrophage activation constituted by the aforementioned macrophage polarization shift and foam-cell formation leads to the enlargement of necrotic cores and formation of vulnerable plaques (12). Thus, exploring the key regulators and potential mechanisms of atherosclerosis-associated macrophage activation may provide clinicians with an effective strategy for preventing atherosclerosis.

MicroRNAs (miRNAs/miRs) are an evolutionarily conserved class of small non-coding RNAs with 20-23 nucleotides (13) and exert important regulatory effects on various biological processes, including cell development, cell differentiation, cell proliferation, apoptosis and immune inflammation, through regulation of gene expression by directly binding to the 3’-untranslated regions (3’-UTRs) of their target mRNAs (14-16). Accumulating evidence has demonstrated that miR-183 is strongly implicated and functions in various aspects of tumorigenesis. miR-183 is reported to be a potent prognostic marker for lung cancer through...
negative regulation of the metastatic potential of lung cancer but acts as a tumor suppressor in human non-small cell lung cancer by downregulating MTA1 (17). miR-183 inhibits osteosarcoma cell growth and invasion by regulating the LRP6-Wnt/β-catenin signaling pathway (18), and promotes proliferation and invasion in esophageal squamous cell carcinoma by targeting programmed cell death 4 (19). Recently, miR-183 has emerged as an important player in cardiovascular disease and showed downregulated expression in hypertrophic cardiac tissues subjected to transverse aortic constriction surgery (20). Additionally, inhibition of miR-183 expression in HUVECs with Ox-LDL-induced injury can enhance cell activity, decrease inflammation level, and thus prevent cell injury by increasing insulin receptor substrate 1 expression (21). miR-183 alleviates early injury, including decreasing brain edema, neurobehavioural defects, inflammation, oxidative stress and iron deposition, and by inhibiting heme oxygenase-1 expression after intracerebral hemorrhage. A miR-183 inhibitor was also found to suppress anaphylaxis and atopic dermatitis by decreasing NF-κB activation (22). However, the potential role and precise underlying mechanism of miR-183 in macrophage activation have not been elucidated.

The present study demonstrated that significantly upregulated miR-183 expression was found in atherosclerotic plaques of ApoE−/− mice fed a high-fat diet (HFD) and in BMDMs upon Ox-LDL stimulation. miR-183 knockdown dramatically accelerated the activation of resolving M2-polarized macrophages but attenuated the activation of classical M1-polarized macrophages, as well as inhibited NF-κB activation. Additionally, miR-183 silencing attenuated foam-cell formation accompanied by increased upregulation of genes involved in cholesterol efflux, and downregulation of genes implicated in cholesterol influx. Mechanistically, it was verified that NR4A2 was the direct target for the attenuating effect on macrophage activation regulated by miR-183 knockdown. Overall, the present study results demonstrated that blockade of the miR-183-NR4A2 axis might be a promising strategy for the regulation of macrophage activation implicated in atherogenesis.

Materials and methods

Animals and morphological analysis. The animal study procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Tongren municipal people’s Hospital. A total of 25 male ApoE deficient mice (ApoE−/−; age, 8-12 weeks; weight, 21-28 g; Beijing Zhongke Biotechnology Co., Ltd.) were fed a high-fat diet (HFD; 15.8% fat and 1.25% cholesterol) or normal chow (NC) purchased from Beijing Huaufukang Bioscience Co Inc., respectively, for up to 12 weeks. Animals were maintained under a 12 h light/dark cycle with free access to food and water at a controlled temperature of 22-26°C and relative humidity (65±10%). Hearts from mice subjected to NC or HFD treatment for 12 weeks were excised after anesthesia via the intraperitoneal injection of pentobarbital sodium (50 mg/kg). The tissues were subsequently dehydrated at room temperature in 85% alcohol for 15 min, 95% alcohol twice for 10 min each and in 100% alcohol twice for 15 min and embedded in paraffin for histological analysis. Consecutive 5-µm sections of the atrioventricular valve region of each heart were collected and stained with hematoxylin and eosin (H&E) for 5 min for morphological analysis at a temperature of 26°C. Samples were observed under a light microscope at a x40 magnification.

Cell culture and small interfering (si)RNA transfection. Approximately 5x10³ nucleated BMDMs isolated from bone marrows and tibias of ApoE-mice were cultured in 10 ml of RPMI with 10% fetal bovine serum and Macrophage Colony-Stimulating Factor (MCSF; 50 ng/ml; cat. no. M9170, Sigma-Aldrich; Merck KGaA) (23-25). Cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck KGaA) containing 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 1% penicillin-streptomycin. The synthetic miR-183 antagonist was synthesized by GenePharma. BMDMs were transfected with miR-183 antagonist and the corresponding controls (NC) at a concentration of 50 nM using a riboFEC CP Transfection kit according to the manufacturer’s protocol. The infected BMDMs stimulated with 15 ng/ml Ox-LDL, for 24 h after serum starvation for one day, were utilized for the in vitro functional study.

Reverse transcription-quantitative (RT-q)PCR and western blotting. Total mRNA was extracted by trichloromethane, dissolved in DEPC-water, and then reverse transcribed into cDNA with a Transcriptor First Strand cDNA Synthesis kit (F. Hoffmann-La Roche AG) as previously described (26). The expression levels of the target genes were quantified by real-time PCR using LightCycler 480 SYBR Green 1 Master mix and a LightCycler 480 QPCR System (F. Hoffmann-La Roche AG). The thermocycling conditions were as follows: 95°C for 10 min followed by 50 cycles of 95°C for 10 sec, 55°C for 10 sec, 72°C for 5 sec; 99°C for 1 sec; 59°C for 15 sec; 95°C for 1 sec; and cooling at 40°C. The relative transcription levels of the target genes were normalized against GAPDH, while the level of miR-375 was normalized to U6 level. The 2ΔΔCq method was used (27). The primers are listed in the Table I.

Protein concentrations were determined by Bicinchoninic Acid Kit assay (cat. no. BCA1; Sigma-Aldrich; Merck KGaA). The proteins (5 µg) were separated by 7.5% SDS-PAGE (Invitrogen; Thermo Fisher Scientific, Inc.) and then transferred to polyvinylidene fluoride membranes, which were blocked in Tris-buffered saline (TBS) and Tween-20 containing 5% non-fat milk 1 h at room temperate. Subsequently, the membranes were incubated with the appropriate primary antibodies overnight at 4°C.

The membranes were then incubated with a secondary IRDye® 800CW-conjugated antibody (1:5,000; cat. no. ab216773; Abcam) for 1 h at 37°C, and treated with enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) before being visualized with Molecular Imager ChemiDoc™ XRS (Bio-Rad Laboratories, Inc.) using Image Lab™ Software 5.1 (Bio-Legend Scientific Co., Ltd). The expression levels of specific proteins were normalized against GAPDH expression. The antibodies used are listed in the Table II.

Foam-cell formation. BMDMs serum-starved for 24 h and stimulated with 15 ng/ml Ox-LDL for 24 h were collected. To
miR-183 expression is upregulated in atheromatous plaques and macrophages. To investigate the involvement of miR-183 in atherogenesis, atherosclerotic plaques from ApoE–/– mice induced by HFD feeding were first examined to determine whether miR-183 expression was altered. Hematoxylin and eosin staining demonstrated the typical pathological changes of atherosclerotic plaques in ApoE–/– mice fed a HFD for 12 weeks, characterized by the thin fibrous cap and large necrotic core (Fig. 1A). RT-Qpcr analysis revealed that miR-183 Mrna expression levels were markedly upregulated in arteries of HFD-fed ApoE–/– mice compared with those of NC-treated mice (Fig. 1A). Furthermore, the expression of miR-183 in BMDMs subjected to Ox-LDL stimulation was also evaluated. It was found that miR-183 expression was gradually upregulated in BMDMs following Ox-LDL stimulation (Fig. 1B). These findings suggest that miR-183 is involved in the development of atherosclerosis, partially through functioning in macrophages.

miR-183 silencing ameliorates the inflammatory response. Given the significant change in miR-183 expression in atheromatous plaques and macrophages treated with Ox-LDL, miR-183 loss-of-function experiments were performed in macrophages. It was observed that miR-183 expression was notably decreased in BMDMs following miR-183 inhibitor transfection (Fig. 2A). Furthermore, the Mrna levels of genes associated with proinflammatory M1-polarized macrophages, including tumor necrosis factor-α, inducible nitric oxide synthase, cyclooxygenase 2 and IL-6, were dramatically downregulated in BMDMs transfected with the miR-183 inhibitor upon Ox-LDL treatment compared with the control group (Fig. 2B). In contrast, the Mrna expression levels of genes associated with resolving M2-polarized macrophages, including Arginase-1 (Arg-1), transforming growth factor-β, mediator of replication checkpoint protein 1 and peroxisome proliferator-activated receptors γ were significantly upregulated in miR-183 inhibitor-transfected BMDMs with Ox-LDL treatment compared with control cells (Fig. 2C). Moreover,
miR-183 promotes macrophage activation. IL-6 and Arg-1 protein levels determined by western blotting analysis were consistent with the mRNA expression levels (Fig. 2D). Furthermore, phosphorylation of p65, an important event in the activation of the inflammatory mediator nuclear factor-kappa B (NF-κB), was decreased in BMDMs transfected with the miR-183 inhibitor (Fig. 2E).

miR-183 knockdown attenuates foam-cell formation. After excess engulfment of Ox-LDL, macrophages form foam cells, which play an important role in atherogenesis. A significant decrease in foam-cell formation in BMDMs transfected with the miR-183 inhibitor was observed upon Ox-LDL stimulation, as evaluated by neutral lipid staining with oil red O, compared with the control group (Fig. 3A). Subsequently, the markers associated with cholesterol uptake and efflux that contribute to foam-cell formation were evaluated. BMDMs transfected with miR-183 inhibitor presented with decreased Mrna levels of genes (SR-A and CD36) involved in cholesterol influx, whereas the expression levels of markers implicated in cholesterol efflux (ABCA1 and ABCG1) were increased (Fig. 3B). Moreover, the CD36 and ABCA1 protein levels determined by western blot analysis were consistent with the mRNA expression levels (Fig. 3C).

Knockdown of miR-183 promotes NR4A2 expression. Next, bioinformatic analysis using the program TargetScan was performed to elucidate the mechanism by which miR-183 affects macrophage activation. The results indicated that NR4A2 contained a putative binding site for miR-183 in its 3'UTR (Fig. 4A). Moreover, luciferase reporter assays demonstrated that miR-183 inhibitor transfection promoted NR4A2 luciferase activity, whereas the above effect was abolished when the predicted binding sites within the NR4A2 3'UTR were mutated (Fig. 4B). As expected, NR4A2 showed significantly upregulated Mrna and protein expression in BMDMs transfected with the miR-183 inhibitor (Fig. 4C and D). Collectively, these findings suggested that the effects of miR-183 knockdown on macrophage activation were partially mediated by the upregulated expression of its molecular target, NR4A2.

### Table II. Antibodies for immunoblot analyses.

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Figure 1. miR-183 expression in atherosclerotic plaques and macrophages. (A) Left: Aortic roots from ApoE<sup>-/-</sup> mice treated with normal chow or a HFD for 12 weeks were stained with hematoxylin and eosin. Scale bar, 500 µm. Right: Qpcr analysis of miR-183 Mrna expression in the entire aorta in ApoE<sup>-/-</sup> mice treated with NC for 12 weeks or with HFD for 12 weeks. n=3. *P<0.05 vs. ApoE<sup>-/-</sup> with NC-12W treatment group. (B) RT-PCR analysis of miR-183 Mrna expression in bone marrow-derived macrophages stimulated with PBS and Ox-LDL for 12 or 24 h. *P<0.05 vs. PBS-treated group. *P<0.05 vs. Ox-LDL treated for 12 h group. miR, microRNA; RT-PCR, reverse transcription-quantitative PCR; HFD, high-fat diet; Ox-LDL, oxidized low-density lipoprotein; NC, negative control.
Figure 2. Pro-inflammatory macrophage response regulated by miR-183 knockdown. (A) miR-183 expression in BMDMs infected with miR-183 inhibitor or NC. Mrna expression of M1 marker genes (B) and M2 marker genes (C) in BMDMs transfected with miR-183 inhibitor or NC examined by reverse transcription-quantitative PCR analysis. (D and E) The protein level of IL-6 and Arg-1, as well as phosphorylated and total p65 in BMDMs infected with miR-183 inhibitor or NC tested by western blot analysis. *P<0.05 vs. control group. miR, microRNA; BMDMs, bone marrow-derived macrophages; NC, negative control; TNFα, tumor necrosis factor α; Inos, inducible nitric oxide synthase; COX, cyclooxygenase; IL, interleukin; Ox-LDL, oxidized low-density lipoprotein; Arg, arginase; TGF, transforming growth factor; Mrc, mediator of replication checkpoint protein 1; PPAR, peroxisome proliferator-activated receptors.

Figure 3. miR-183 silencing ameliorates foam-cell formation. (A) Foam-cell formation in macrophages infected with miR-183 inhibitor or NC induced by Ox-LDL. Scale bar, 50 µm. (B) The Mrna expression of markers associated with cholesterol uptake ability (CD36 and SR-A) and cholesterol efflux (ABCA1 and ABCG1) in BMDMs infected with miR-183 inhibitor or NC induced by Ox-LDL. (C) The protein expression levels of CD36 and ABCA1 in BMDMs infected with miR-183 inhibitor or NC. *P<0.05 vs. control group. miR, microRNA; BMDMs, bone marrow-derived macrophages; Ox-LDL, oxidized low-density lipoprotein; NC, negative control.
Discussion

The present study first demonstrated that miR-183 is a novel and independent indicator of macrophage activation implicated in atherogenesis, which exhibited an upregulated expression in atherosclerotic plaques of ApoE-/- mice fed with HFD and BMDMs with Ox-LDL stimulation. The loss-of-function strategy demonstrated that miR-183 knockdown promoted M2 phenotypic macrophage but attenuated M1 macrophage switching. Meanwhile, miR-183 knockdown significantly ameliorated foam cell forming by increased cholesterol efflux but decreased cholesterol uptake. Mechanistically, NR4A2 was the direct target of miR-183 and the silencing of miR-183 dramatically promoted NR4A2 expression. On the basis of the results from the present study, an unexplored role of miR-183 in regulation of macrophage activation was identified, which is at least partially through regulation of NR4A2 expression.

Over the past decade, multiple studies have demonstrated that various miRNAs are widely involved in numerous human disorders and are especially implicated in multiple cardio-metabolic diseases, such as obesity, diabetes, heart failure and atherosclerosis; the collective evidence implies their potential as therapeutic targets and provides a new direction for the study of these diseases (15,28-34). Atherosclerosis is recognized as a complex multifactorial pathological process (35), and microRNAs have emerged as important regulators of atherosclerosis development through the regulation of the inflammatory response via the switch between classical proinflammatory M1 macrophage activation and alternative inflammation-resolving M2 macrophage activation (36-39). In the present study, it was observed that the expression of miR-183 was gradually upregulated in atherosclerotic plaques of ApoE-/- mice and in BMDMs following Ox-LDL stimulation. miR-183 silencing attenuated the Mrna expression of prototypical genes associated with proinflammatory M1-polarized macrophages but increased the Mrna expression levels of genes associated with anti-inflammatory M2-polarized macrophages. The predominance of M2-polarized macrophages in bladder cancer affects angiogenesis, the tumor grade and invasiveness. Macrophage polarization has been well recognized to exert an important role in the regulation of tumorigenesis and angiogenesis (40), and has emerged as the main precursor of foam-cell formation mediated by macrophages, which together play important roles in the development of atherosclerosis. The present study demonstrated that a miR-183 inhibitor attenuated the formation of foam cell contaminants, with decreased Mrna levels of genes involved in cholesterol influx but increased expression levels of markers implicated in cholesterol efflux.

miRNAs are capable of regulating target gene expression by binding to the 3'UTRs of their target mRNAs, and miRNAs are involved in negatively regulating gene expression at the posttranscriptional level (13). Bioinformatic analysis was performed using the program TargetScan, which found that NR4A2 contained a putative binding site for miR-183 in its 3'UTR. Moreover, it was demonstrated that miR-183 inhibitor transfection increased NR4A2 luciferase activity, as evaluated by a luciferase reporter assay, whereas the aforementioned effect was abolished when the predicted binding sites within the NR4A2 3'UTR were mutated. Importantly, NR4A2 Mrna and protein expression were also significantly increased in BMDMs transfected with the miR-183 inhibitor. A previous study demonstrated that members of the NR4A subfamily (NR4A1/Nur77, NR4A2/Nurr1 and NR4A3/Nor1) of orphan NRs (nuclear receptors) act as ligand-independent and constitutively active receptors, which are tightly controlled at the levels of expression, posttranslational modification and subcellular localization (41). NR4A subfamily members have emerged as key transcriptional regulators of cytokine and growth factor
action in various diseases. Recently, accumulating evidence has suggested that NR4A nuclear receptors play pivotal roles in immunity and atherosclerosis (41), while all three NR4A family members are expressed within atherosclerotic lesions. The orphan nuclear receptor NR4A1 attenuates atherosclerosis by downregulating inflammatory gene expression in macrophages (42,43). NR4A3 deficiency has been shown to inhibit vascular injury and atherosclerosis in mice (44,45). NR4A2 has been shown to act as a repressor of NF-kB activation in microglia and astrocytes and to attenuate atherosclerosis in mice (46). Exogenous expression of NR4A2 in macrophages leads to polarization toward their alternative phenotype, with induction of genes that are prototypical M2 markers (47). Moreover, NR4A2 transcriptionally activates Arg1 expression by directly binding to its promoter (7). Thus, it is speculated that miR-183 knockdown attenuated macrophage activation partially by upregulating NR4A2 expression, which may play an important protective role against atherogenesis.

In conclusion, the present study demonstrated that miR-183 attenuated macrophage activation contributed by decreasing the classic pro-inflammatory M1 macrophage but increasing alternative inflammation resolving M2 macrophage activation, accompanied by attenuation of foam-cell formation, along with enhanced genes associated with cholesterol efflux compared to uptake. This study highlights the important role of neutralizing miR-183 as a promising therapeutic strategy for atherosclerosis management.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
FHG and LL designed and conducted in vitro experiments, analyzed the data and drafted the paper. YSY and DHS performed the biomolecule experiment. YSZ, XSW and XPZ performed the pathological study and analyzed the data. QXQ designed all of the experiments, supervised and funded the study, and contributed to the data analysis and to the writing of the paper. All authors read and approved the final manuscript and FHG and QXQ confirm the authenticity of all the raw data.

Ethics approval and consent to participate
The animal study procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Tongren municipal people’s Hospital (approval no. 20190609).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


