

## Original Article

# Downregulation of miR-145 alleviates ox-LDL-induced inflammation by targeting QKI in macrophages

Xueqiang Feng<sup>1</sup>, Fuwang Yao<sup>2</sup>, Yuqin Lang<sup>3</sup>, Zhibin Cao<sup>4</sup>

<sup>1</sup>Department of Vascular Intervention, Affiliated Hospital of Weifang Medical University, Weifang, Shandong Province, China; <sup>2</sup>Department of Anesthesiology, Affiliated Hospital of Weifang Medical University, Weifang, Shandong Province, China; <sup>3</sup>Department of Endoscopic Outpatient Operating Room, Affiliated Hospital of Weifang Medical University, Weifang, Shandong Province, China; <sup>4</sup>Department of Endocrinology, Affiliated Hospital of Weifang Medical University, Weifang, Shandong Province, China

Received February 20, 2022; Accepted April 11, 2022; Epub June 15, 2022; Published June 30, 2022

**Abstract:** Objective: Atherosclerosis (AS) is a chronic inflammatory disease with high mortality. miRNAs perform a vital role in its development. This study aimed to discuss the effect of miR-145 in AS occurrence and development. Methods: The effects of miR-145 mimics and inhibitors on IL-6, IL-1 $\beta$  and TNF- $\alpha$  expressions were assessed by qRT-PCR and ELISA. CCK-8 was applied to examine the influence of miR-145 on macrophage proliferation. The influence of miR-145 on the QKI transcriptional activity was analyzed using luciferase reporter gene assay. Results: Overexpression of miR-145 could enhance the expression of IL-6, IL-1 $\beta$ , and TNF- $\alpha$ . Down-regulation of miR-145 could inhibit the proliferation of macrophages and the expression level of inflammatory cytokines. The effect of miR-145 inhibitor on the expression of inflammatory factors was partially reversed by interfering with the transcription of QKI with siRNA. Conclusion: miR-145 regulates the inflammatory response induced by macrophage activation through targeting QKI. It provides a means for AS targeted therapy.

**Keywords:** miR-145, inflammatory reaction, Quaking (QKI), atherosclerosis

## Introduction

Atherosclerosis (AS) is a chronic multifactorial disease with high morbidity and mortality worldwide [1, 2]. The etiology of AS includes monocyte recruitment, hyperlipidemia, foam cell formation, macrophage differentiation, and activation of inflammatory factors [3]. Inflammation and lipid metabolism have become the focus of atherosclerosis research [4]. It has been proven that there is a relation between many pathogenic factors and the AS occurrence and development. ox-LDL (oxidized low-density lipoprotein) induces macrophages to express various inflammatory cytokines, especially pro-inflammatory cytokine, which can accelerate the occurrence of atherosclerosis [5]. AS is known as a chronic inflammatory disease. Evidence suggested that inflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are pivotal in AS development and progression [6]. Prevention of ox-LDL-induced in-

flammation in macrophages has become a therapeutic target for cardiovascular diseases.

As a set of highly conserved non-coding RNAs, MicroRNAs (miRNAs) bring an important regulatory effect on cell development and metabolism, immune inflammation, and tumorigenesis. They are widely found in eukaryotes [7]. miRNAs realize their biological functions by regulating target mRNAs, and becoming new biomarkers for the diagnosis of the occurrence and development of various diseases [8]. miRNAs have been confirmed to have a crucial effect on the pathological process and molecular signaling pathway of AS. It has been found that miR-146a expression is significantly down-regulated and the release of inflammatory factors is reduced in THP-1 cells stimulated by ox-LDL, affecting the development of AS [9]. The increase of miR-125a-5 expression in THP-1 cells stimulated by ox-LDL may protect AS by regulation of scavenger receptors expression [10]. miR-155 promotes an inflammatory mediator

**Table 1.** Primer sequence for RT-PCR

Gene	Primer sequence
miR-145	Forward: 5'-CTGGCTGGCC GGG ACCTGACA-3' Reverse: 5'-ACCGCTCGTTGCCAATAGTGATGA-3'
QKI	Forward: 5'-CTGGACGAAGAAATTAGCAGAGT-3' Reverse: 5'-ACTGCCATTTAACGTGTCATTGT-3'
TNF- $\alpha$	Forward: 5'-GACGTGGAAGTGGCAGAAGAG-3' Reverse: 5'-TTGGTGGTTTGAGTGTGAG-3'
IL-1 $\beta$	Forward: 5'-GAAATGCCACCTTTTGACAGTG-3' Reverse: 5'-TGGATGCTCTCATCAGGACAG-3'
IL-6	Forward: 5'-TGGATGCTCTCATCAGGACAG-3' Reverse: 5'-GAATTGCCATTGCACAACCTCTTT-3'
GAPDH	Forward: 5'-GAAGGTGAAGTCCGGAGTC-3' Reverse: 5'-GAAGATGGTGATGGGATTC-3'

expression by targeting the inhibition of B-cell leukemia/lymphoma 6 (Bcl6) and cytokine transduction signal 1 (SOCS1). It plays an important role in aggravating AS through a macrophage-driven aberrant proinflammatory response [11]. These findings indicate that miRNAs shed a vital effect on the development of atherosclerosis by regulating inflammatory responses.

It has been proven that miR-145 is a key factor in AS development [12, 13]. It is crucial in the differentiation of vascular smooth muscle cells which are key cells in the development of atherosclerosis and inhibition of its proliferation [14]. miR-145 was significantly decreased in M1 cells induced by interferon  $\gamma$  and bacterial lipopolysaccharides [15], and significantly increased in M2-type macrophages induced by monocytes stimulated by interleukin-4 (IL-4). MiR-145 also plays a regulatory role in cytokine secretion and macrophage infiltration [16]. miR-145 accelerates inflammatory responses through activation of NF- $\kappa$ B signaling in ox-LDL-stimulated THP-1 cells [17]. miR-145's function in AS and its molecular mechanism have not been fully elucidated.

In this paper, it was found that miR-145 over-expression induced the expression of a variety of pro-inflammatory factors, and inhibition of miR-145 inhibited macrophage proliferation and expression of pro-inflammatory factors. Studies revealed that miR-145 may regulate inflammatory factors by targeting Quaking I (QKI). Interference with QKI could partially reverse the inhibition of miR-145 inhibitors on inflammatory factors. This may be a targeted therapy for AS, laying the foundation for the

occurrence and development of molecular network of AS.

## Materials and methods

### Cell culture

This study was approved by the Affiliated Hospital of Weifang Medical University (approval No. 2021-153) and was in accordance with the rules and regulations of the laboratory. Mouse Raw264.7 macrophages (American Type Culture Collection, USA) were cultured in Minimum Essential Medium- $\alpha$  (MEM- $\alpha$ ) containing 10% fetal bovine serum and kept in the incubator with the condition of 5% carbon dioxide at 37°C. Before the experiment, the cells were cultured in MEM- $\alpha$  containing 0.5% FBS for 12 h. After 12 h, the cells were transfected with si-QKI or treated with miR-145 mimics or inhibitor for 24 h. Then treated with 100  $\mu$ g/ml ox-LDL for 24 h.

### Cell proliferation assay

CCK-8 (Cell counting kit, Dojindo Laboratories, Japan, CK04) was used to test proliferation of Raw264.7 cells. Raw264.7 cells were inoculated into 96-well plates at a density of 8  $\times 10^3$  cells/well. The optical density for each well was tested at 450 nm with a microplate reader.

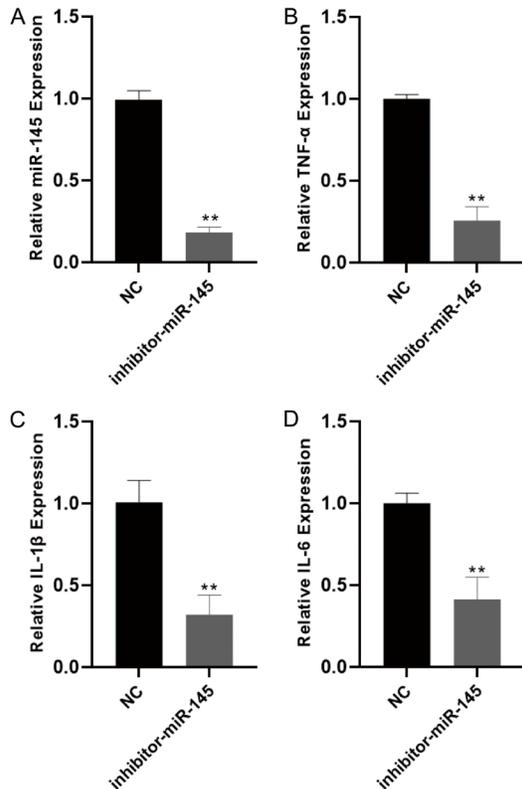
### qRT-PCR analysis

The RNA of RAW264.7 cells was extracted from each group after treatment using the Trizol method (Invitrogen). The reverse transcription kit was used to synthesize the first strand cDNA (Takara, RR420a) in accordance with the instructions of the manufacturer. The mRNA was quantitatively analyzed by TB SYBR Premix Ex Taq (Takara Bio, RR820A). Gene expression was analyzed by  $2^{-\Delta\Delta CT}$  method. Primers applied for qRT-PCR amplification are detailed in **Table 1**.

### siRNA transfection

The design and construction of miR-145 inhibitor (5'-AGGGAUUCCUGGGA AACUGGAC-3'), miR-145 mimics (Sense: 5'-GUCCAGUUUCCCCAGGAAUCCCU-3', Antisense: 5'-GGAUUCCUGG-GAAAACUGGACUU-3'), and siRNA QKI (Sense: 5'-GGACUUACAGCUAACAACCTT-3', Antisense: 5'-GUUGUUUAGCUGUAAGUCCTC-3') were conducted by Guangzhou Geneseeed Biotech Co., Ltd, China. RAW264.7 cells were transfected

## The role of miR-145 in inflammation



**Figure 1.** The effect of miR-145 downregulation on mRNA expression of inflammatory cytokines in RAW264.7 macrophage. A: The miR-145 mRNA expression level after transfection with miR-145 inhibitor was analyzed by RT-PCR. B-D: mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in RAW264.7 macrophages treated with miR-145 inhibitor for 24 h were analyzed by RT-PCR. Compared with NC, \*\* $P < 0.01$ .

with miR-145 mimics, miR-145 inhibitor, and siRNA QKI by liposome 2000 (Invitrogen) following the instructions of the manufacturer and then used for further experiments.

### Luciferase reporter assay

Using the online tool Targscan, QKI was predicted to be a putative target gene of miR-145. For the purpose of verifying the relationship between miR-145 and QKI, the recombinant pMIR-reporter luciferase vector was used for luciferase assay. Lipofectamine 2000 was performed to co-transfect miR-145 mimic or miR-145 inhibitor with pMIR-reporter luciferase vector containing the 3'-untranslated region (3'-UTR) of wild or mutant QKI into RAW264.7 cells. Dual-luciferase reporter gene assay (Promega Corp.) was used to detect the luciferase activity at 48 h of incubation. The luciferase reporter gene activity was normalized.

### Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells were cultured and transfected with miR-145 mimics or miR-145 inhibitor. Supernatant was collected, centrifuged at 3000 rpm for 10 min, and the contents of IL-1 $\beta$  (R&D systems, USA, MLB00C), IL-6 (R&D systems, USA, M6000B), and TNF- $\alpha$  (R&D systems, USA, MTA00B) were analyzed using corresponding ELISA Kits in accordance with the instructions of the manufacturer.

### Western blot

The protein lysis buffer RIPA was added to extract the total protein. The BCA method was used to detect the protein concentration. The protein samples were transferred to the PVDF membrane following SDS-PAGE gel electrophoresis. The membrane was kept in the TBST of 5% non-fat milk powder and blocked for 30 min at room temperature. The primary antibody (QKI, dilution: 1:500, ab126742, Abcam, USA) was incubated overnight under the conditions of 4°C. After washing, the PVDF membrane was incubated with the second antibody (Dilution: 1:800, ab6721, Abcam, USA). ECL reagent was added to develop the color and the integrated optical density of the target bands was detected using the Bio-Rad image software. GAPDH served as the internal reference.

### Statistics and methods

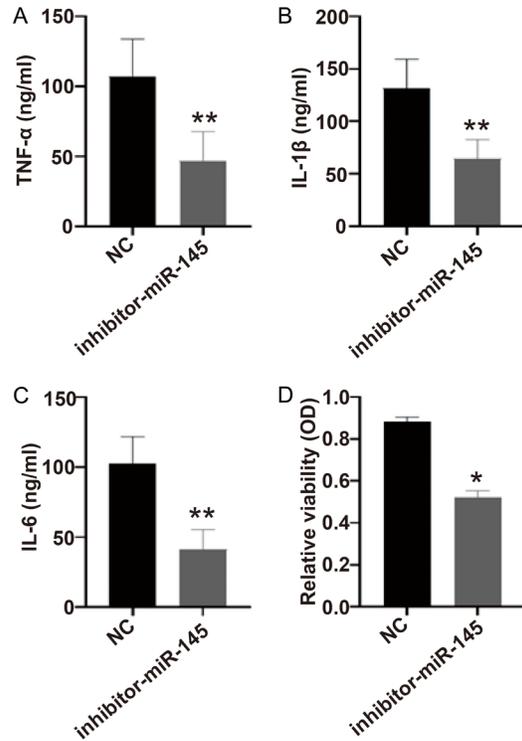
All statistical data was analyzed by SPSS 23.0 software. The measured data was represented by mean  $\pm$  standard deviation. The independent sample t-test was used for the comparison between the two groups.  $P < 0.05$  was considered as the statistical significance.

## Result

### Down-regulation of miR-145 inhibited the proliferation and inflammation in RAW264.7 macrophages

To study miR-145 effect in macrophages, we down-regulated the miR-145 expression in RAW264.7 macrophages with miR-145 inhibitors. **Figure 1A** shows the efficiency after transfection (**Figure 1A**). After 24 h of transfection, RAW264.7 cells were stimulated with ox-LDL for 24 h to induce an inflammatory response. Compared with the NC group, the IL-1 $\beta$ , IL-6, and TNF- $\alpha$  mRNA levels in miR-145 inhibitor group decreased significantly (**Figure 1B-D**).

## The role of miR-145 in inflammation

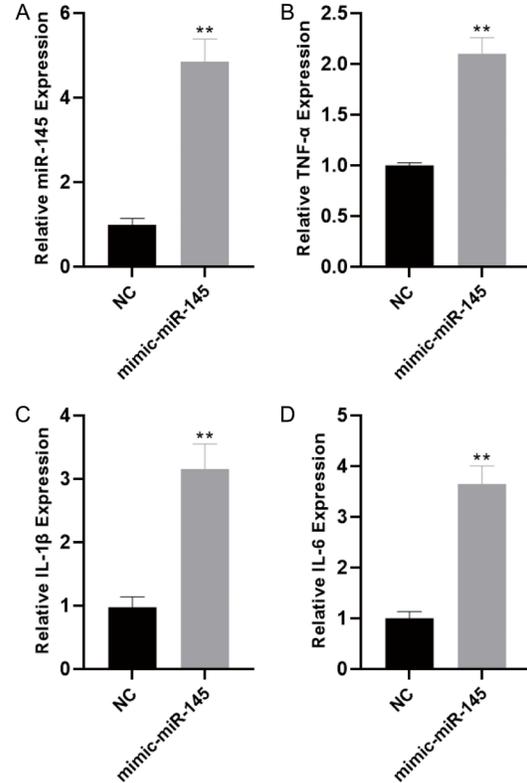


**Figure 2.** Effects of down-regulated miR-145 on RAW264.7 macrophage proliferation and inflammatory cytokines concentration. A-C: The concentration of IL-6 IL-1β and TNF-α in RAW264.7 macrophage was detected by ELISA assay. D: Proliferation ability of macrophages after transfection with miR-145 was tested by CCK-8. Compared with NC, \*P<0.05, \*\*P<0.01.

ELISA results confirmed reduced IL-6, IL-1β, and TNF-α levels in cell supernatant (**Figure 2A-C**). The results of CCK-8 suggested that the proliferation capacity of cells in the miR-145 inhibitor group was lower than that of the control group (**Figure 2D**). These findings indicated that inhibition of miR-145 reduces the inflammatory response and proliferation capacity of RAW264.7 macrophages, which may have a supplementary effect in the progression of AS.

### *miR-145 mimics aggravated the inflammatory reaction of RAW264.7*

miR-145 mimic was adopted to up-regulate miR-145 expression in RAW264.7 macrophages (**Figure 3A**). The findings of qRT-PCR indicated that miR-145 mimics raised the mRNA levels of TNF-α, IL-6, and IL-1β (**Figure 3B-D**). ELISA results confirmed the raised IL-6, IL-1β, and TNF-α levels in cell supernatant (**Figure 4A-C**). CCK-8 findings indicated that there was



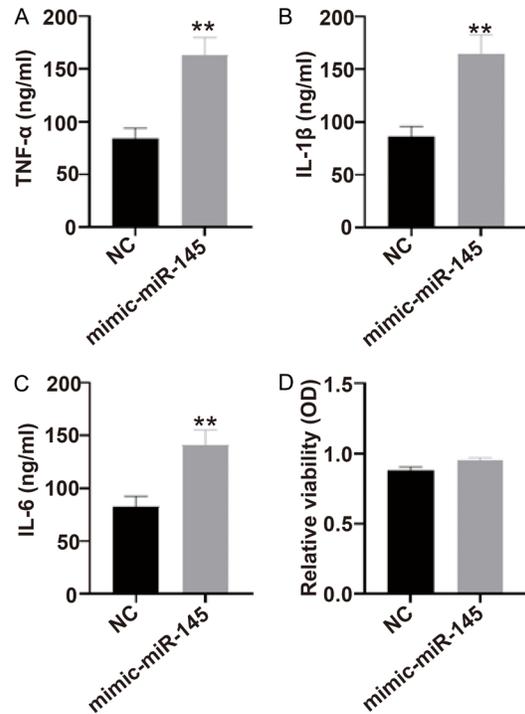
**Figure 3.** The effect of miR-145 mimics on mRNA expression of inflammatory cytokines in RAW264.7 macrophage. A: miR-145 mRNA expression level after mimic miR-145 transfection was analyzed with qRT-PCR. B-D: mRNA levels of TNF-α, IL-1β, and IL-6 in RAW264.7 macrophages treated with inhibitor miR-145 for 24 h were analyzed by RT-PCR. Compared with NC, \*\*P<0.01.

no significant difference in cell proliferation between miR-145 mimic group and the control group (**Figure 4D**). These findings suggested that miR-145 expedited the inflammatory response of RAW264.7 macrophages.

### *QKI was predicted to be a direct target of miR-145*

To illustrate the mechanism by which miR-145 affects macrophage activation, bioinformatics analysis was conducted by means of the TargetScan program. It showed that QKI contained a presumed binding site with miR-145 in 3'UTR (**Figure 5A**). qRT-PCR and Western blot indicated that miR-145 mimics suppressed QKI expression, and miR-145 inhibitor induced QKI expression (**Figures 5C and 6**). By using luciferase reporter gene analysis, the reporter vector was constructed by inserting the wild or mutant

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**Figure 4.** Effects of miR-145 over-expression on cell proliferation and concentration of inflammatory cytokines in RAW264.7 macrophage. A-C: ELISA assay was applied to measure the concentration of Raw264.7 IL-6, IL-1 $\beta$ , and TNF- $\alpha$ . D: Proliferation ability of macrophages after transfection with miR-145 mimics was tested by CCK-8. Compared with NC, \*\*P<0.01.

3'-UTR sequence of QKI mRNA into the plasmid. The results proved that miR-145 inhibitor transfection enhanced the luciferase activity of QKI-wild. The influence was interdicted when the predicted binding site in QKI 3'UTR was mutated (**Figure 5B**). It suggested that QKI might be a direct target of miR-145.

### *QKI mediated the inflammatory response regulated by miR-145 in macrophages*

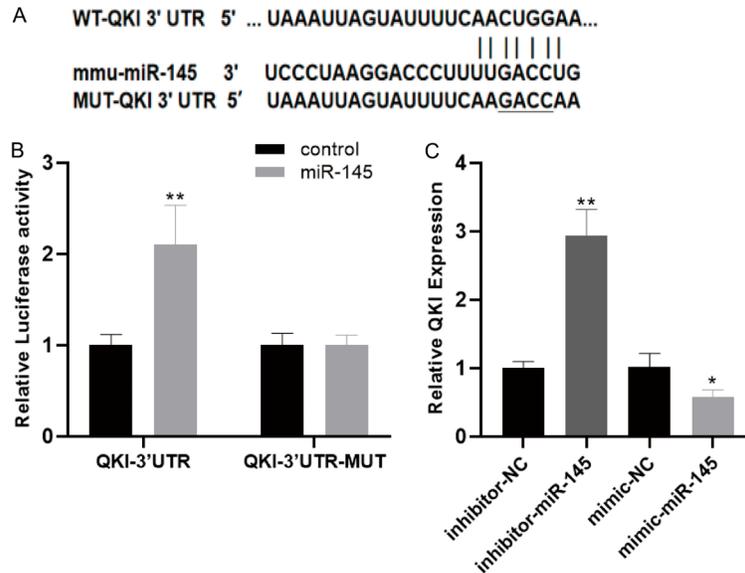
To confirm whether regulation of miR-145 was mediated by QKI, we used si-RNA to down-regulate QKI expression in macrophages. qRT-PCR assay indicated that after transfection of si-RNA-QKI, the expression of QKI was significantly decreased (**Figure 7A**). To evaluate whether inhibition of miR-145 reduces inflammatory cytokines due to QKI, especially in macrophages, we used siRNA to down-regulate QKI expression. The inhibitory effect of miR-145 inhibitor on IL-1 $\beta$ , IL-6, and TNF- $\alpha$  expression was par-

tially reversed by si-QKI transfection (**Figure 7B-D**). Similar results were found in detecting the levels of inflammatory factors in Raw264.7 cell supernatants with ELISA (**Figure 8A-C**). These results confirmed that miR-145 targets QKI may have an important effect on its regulation of macrophage-mediated inflammation.

## Discussion

This study demonstrated for the first time that miR-145-targeted QKI is a pathway for regulating AS inflammation *in vitro*. We used ox-LDL-treated mouse macrophages RAW264.7 as a cellular model of AS and investigated its effects on ox-LDL-mediated inflammation using miR-145 inhibitors and mimics. Our data support that miR-145 inhibitor reduces inflammatory responses by regulating QKI. The mechanism of miR-145 in atherosclerotic mice still needs to be investigated in depth in future experiments.

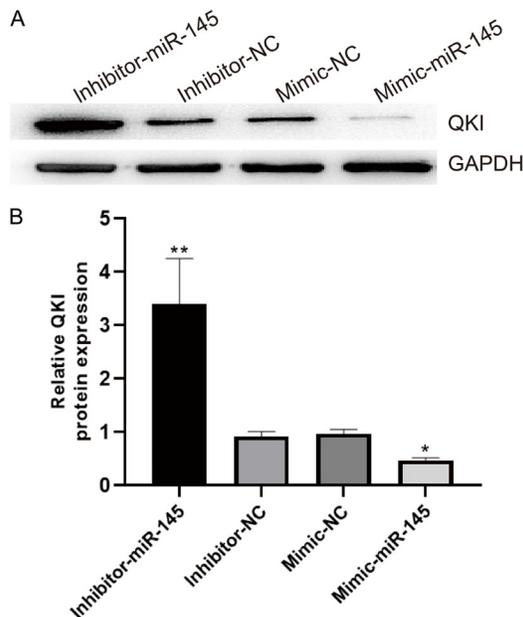
AS is the pathological basis of cardiovascular and cerebrovascular diseases. The inflammatory response of macrophages is also one of the research hotspots in this field. miRNA has been considered to play an important role in cardiovascular and cerebrovascular diseases [18, 19]. miRNA in serum and plasma has gradually become biomarkers for disease detection. Studies have proven that detection of miRNA expression in serum of patients has an important guiding role in disease diagnosis and treatment. More evidence indicates that miR-145 has an important effect on vascular smooth muscle cells and macrophages. It has also been proven to be a crucial factor in AS occurrence and development [20-22]. Guo et al. found that miR-145 could inhibit VSMC proliferation by regulating CD40 [23]. Li et al. found that miR-145 inhibited the secretion of LPS-mediated inflammatory factors (IL-1 $\beta$ , TNF- $\alpha$ , and IL-6) through regulation of Arf6 [24]. The researcher also found that miR-145 inhibitor significantly suppressed IL-1 $\beta$ , TNF- $\alpha$ , CCL-2, and CCL-4 expressions in ox-LDL-treated THP-1. It increased the number of VSMC, reduced the number of macrophages, and attenuated aortic sinus lesions [25]. This study shed light on the effect of miR-145 in Raw264.7 cells treated by ox-LDL, providing a preliminary evaluation of the roles of miR-145 in atherosclerosis progression. We demonstrated that inhibition of miR-145 expression reduces the prolif-



**Figure 5.** Up-regulation of QKI expression caused by loss of miR-145. A: TargetScan predicted the binding sites of miR-145 in the 3'UTR of QKI mRNA. B: The QKI 3'UTR construct was transfected into RAW264.7 cells with control or miR-145 inhibitors, followed by dual-luciferase report analysis. C: RT-PCR was applied to test the influence of miR-145 inhibitor or mimics on QKI mRNA expression level. Compared with control, \*\*P<0.01; Compared with NC inhibitor, \*\*P<0.01; Compared with NC mimic, \*P<0.05.

ed by ox-LDL. miR-145 mimics enhanced the secretion of inflammatory factors. miR-145 inhibitor has been shown to exert anti-inflammatory and anti-proliferative effects by regulating QKI expression, which puts forward a new strategy for prevention and treatment of AS.

As a member of the RNA-binding protein activator (STAR) family, RNA-binding protein Quaking (QKI) is involved in the physiological activities such as myocardial ischemia [26], gastric cancer [27], oligodendrocytes [28], gastrointestinal epithelial cells [29], and myelin formation of Schwann cells [30]. Its expression is down-regulated in the differentiation of monocytes into macrophages [31]. Wang et al. found that lack of QKI in macrophages exacerbated DSS-

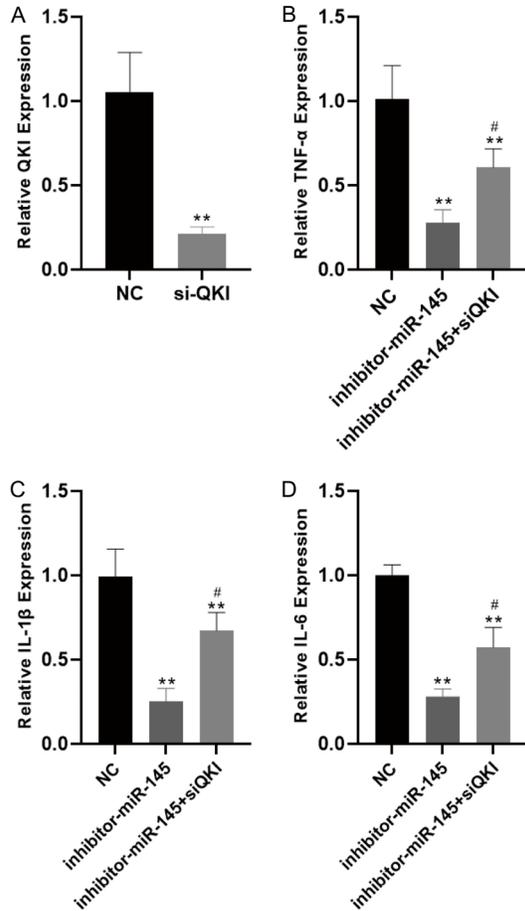


**Figure 6.** The effect of miR-145 inhibitor or mimics on QKI protein expression level. A: Western Blot. B: Quantitative analysis of QKI protein expression. Compared with inhibitor-NC, \*\*P<0.01; Compared with mimic-NC, \*P<0.05.

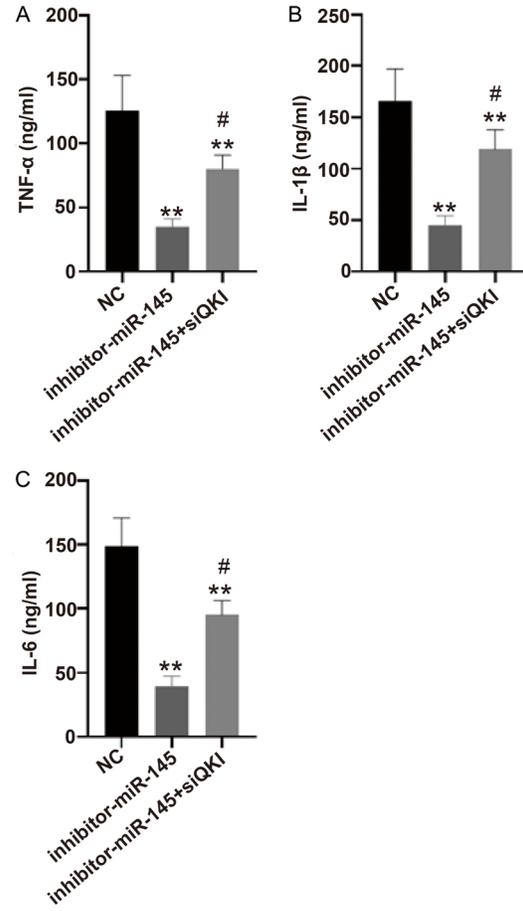
induced enterocolitis in mice [32]. De Bruin et al. found abundant QKI in macrophages of advanced atherosclerotic plaques. Adhesion of monocyte, migration, differentiation into macrophages, and formation of foam cells were impaired by depletion of QKI [33]. It has been found that QKI deficiency exacerbates LPS-induced macrophage polarization, increases iNOS, TNF- $\alpha$ , and IL-6 expressions and amplifies inflammation in experimental endotoxemia through the Ahr/STAT1-NF- $\kappa$ B pathway. This was consistent with our findings [34]. Our study found that miR-145 inhibitor suppressed TNF- $\alpha$ , IL-6, and IL-1 $\beta$  expressions in mice. The increased expression of inflammatory factors was found by interfering with the transcript of QKI with siRNA, indicating that the anti-inflammatory effect of miR-145 inhibitor was partially reversed. We suggest that miR-145 may directly target QKI to regulate macrophage inflammatory responses.

This study has some limitations that should be noted. Only cell experiments were conducted without animal or human experiments. The mechanisms of signal transduction system for QKI regulation were not evaluated. Conducting a multicenter, randomized, controlled, and lar-

eration and inflammatory cytokines (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ) secretion of macrophage treat-



**Figure 7.** miR-145 could directly bind to QKI to regulate the inflammatory cytokines in macrophages. A: mRNA expression was tested after si-QKI transfection with qRT-PCR. B-D: mRNA expression levels of inflammatory factors TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were tested with qRT-PCR after inhibition of miR-145 and co-transfection of miR-145+Si-QKI for 24 h. Compared with NC, \*\*P<0.01; Compared with miR-145 inhibitor, # P<0.05.



**Figure 8.** miR-145 could directly bind to QKI to regulate the concentration of inflammatory cytokines in macrophages. A-C: The concentration of Raw264.7 IL-6, IL-1 $\beta$ , and TNF- $\alpha$  were tested with ELISA assay. Compared with NC, \*\*P<0.01; Compared with miR145 Inhibitor, #P<0.05.

ge-sample study to investigate the roles of miR-145 in ox-LDL-induced inflammation in development of atherosclerosis is warranted to guide the clinical practices in prevention and treatment of atherosclerosis.

In summary, we have found that miR-145 mimics increases the production of inflammatory factors, and inhibition of miR-145 expression reduces the inflammatory reaction and proliferation of macrophages induced by ox-LDL by targeting QKI. These findings shed light on the molecular mechanisms by which miR-145 inhibitor attenuates inflammation. miR-145 can be used as an effective target for preventing and detecting arteriosclerosis.

**Disclosure of conflict of interest**

None.

**Address correspondence to:** Zhibin Cao, Department of Endocrinology, Affiliated Hospital of Weifang Medical University, No. 2428 Yuhe Road, Kuiwen District, Weifang 261041, Shandong Province, China. Tel: +86-0536-3081278; Fax: +86-0536-3081278; E-mail: wfcaozb@126.com

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