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MicroRNA-21 deficiency attenuated atherogenesis and decreased macrophage infiltration by targeting *Dusp-8*

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HIGHLIGHTS

- miR-21$^{-/-}$ attenuates atherosclerotic lesions, with reduced presence of macrophages, SMCs and collagen content in mice.
- miR-21$^{-/-}$ impairs macrophage migration and weakens macrophage-endothelium adhesion.
- *Dusp-8* - p38/JNK signaling is the downstream target of miR-21 in regulating the activation of macrophage.

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ABSTRACT

Background and aims: Atherosclerosis is a chronic inflammatory disorder mediated by macrophage activation. MicroRNA-21 (miR-21) is a key regulator in the macrophage inflammatory response. However, the functional role of miR-21 in atherogenesis is far from clear.

Methods and results: Here, we report that miR-21 is significantly upregulated in mouse atherosclerotic plaques and peripheral monocytes from patients with coronary artery disease. Compared with *miR-21*$^{+/+}$*apoE*$^{-/-}$ mice (*apoE*$^{-/-}$ mice), *miR-21*$^{-/-}$ *apoE*$^{-/-}$ (double knockout, DKO) mice showed less atherosclerotic lesions, reduced presence of macrophages, decreased smooth muscle cells(SMC) and collagen content in the aorta. We further explored the role of miR-21 in macrophage activation *in vitro*. Bone marrow-derived macrophages (BMDMs) from DKO mice not only exhibit impaired function of migration induced by chemokine (C–C motif) ligand 2 (CCL2) but also a weakened macrophage-endothelium interaction activated by tumor necrosis factor-α (TNF-α). However, atherogenic inflammatory cytokine secretion was not affected by miR-21 *in vitro* or *in vivo*. Additionally, miR-21 knockdown in BMDMs directly derepressed the expression of dual specificity protein phosphatase 8 (*Dusp-8*), a previously validated miR-21 target in cardiac fibroblasts, which negatively regulates mitogen-activated protein kinase (MAPK) signaling, particularly the p38-and c-Jun N-terminal kinase (JNK)-related signaling pathways.

Conclusions: These data demonstrate that inhibition of miR-21 may restrict the formation of atherosclerotic plaques partly by regulating macrophage migration and adhesion, while, reduced SMCs and collagen content in plaques may lead to a less stable phenotype with the progression of atherosclerosis. Thus, the absence of miR-21 reduces atherosclerotic lesions but may not represent all benefit in atherosclerosis development.

1. Introduction

Atherosclerosis is a chronic inflammatory process characterized by lipid accumulation and foam cell formation beneath the intimal space [1–3]. It is generally accepted that monocytes/macrophages play a key role in the process by maintaining vessel wall lipid homeostasis and regulating inflammatory responses. Monocytes adhere to the endothelium and transmigrate into the subendothelial space and then differentiate into macrophages and induce a vascular inflammatory response with lipid overload [4,5]. However, the complex signaling

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mechanisms that control these processes are far from clear.

MicroRNAs (miRNAs), endogenous, highly conserved noncoding RNAs, regulate target gene expression post-transcriptionally by repressing translation or promoting mRNA degradation [6]. Increasing evidence has proven that miRNAs exert essential functions in various physiological and pathological processes related to cardiovascular diseases, including endothelial cell activation, macrophage inflammation, cholesterol efflux and vascular remodeling [7,8]. The identification of these miRNAs and their respective targets may offer new therapeutic strategies for the treatment of cardiovascular diseases. In our previous study, we analyzed the expression profiles of miRNAs in oxidized low-density lipoprotein (ox-LDL)-stimulated human peripheral blood monocytes and validated several significantly upregulated miRNAs, including the multifunctional miR-21 [9].

miR-21 has been well-investigated in tumors during the past several decades. miR-21 has been identified as an onco-mir because of its oncogenic activity [10]. Recent studies indicate that miR-21 plays a role in modulating the processes of cardiovascular diseases, including changes of functions of endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and macrophages/foam cells [11]. A previous study indicated that shear stress-induced miR-21 expression regulated endothelial cell function by decreasing apoptosis and increasing endothelial nitric oxide synthase phosphorylation by targeting phosphatase and tensin homolog (PTEN) [12]. Recently, Tang et al. [13] pointed out that miR-21 alleviates ox-LDL-induced endothelial cell injuries by enhancing autophagic flux. In vascular smooth muscle cells (VSMCs), several reports have noted the proliferative and anti-apoptotic effects of miR-21 in mice [11,14,15]. miR-21 has also been indicated as a key mediator in the pathogenesis of macrophages [16,17]. miR-21 blocks NF-kappa B activity and decreases pro-inflammatory cytokine secretion by targeting programmed cell death 4 (PDCD4) [18,19]. However, other reports are inconsistent with these “anti-inflammatory” results. In models of in-stent restenosis, miR-21 promoted vascular inflammation by inducing pro-inflammatory mediator secretion [20]. miR-21 is also considered a regulator of macrophage polarization; however, whether miR-21 promotes the M1 or M2 phenotype in macrophages is still controversial [20–22].

Considering the importance of macrophages in atherogenesis and the lack of a complete understanding of the effects of miR-21 on atherogenic monocyte/macrophage activation, we used both *in vivo* and *in vitro* models to elucidate the association between miR-21 and atherogenesis. We compared atherosclerosis development in *apoE*$^{-/-}$ mice and DKO mice, and showed that miR-21 knockout attenuated atherogenesis with reduced macrophage infiltration and SMCs content.*In vitro*, we demonstrated the inhibitory role of miR-21 deficiency in macrophage migration and adhesion, which was due, at least in part, by targeting the *Dusp-8* gene.

2. Materials and methods

2.1. Human primary peripheral blood monocyte isolation

Peripheral human blood was obtained from patients undergoing coronary angiography at the Department of Cardiology, Shanghai Ninth People's Hospital. Patients were divided into the non-CAD, SAP and ACS groups according to the diagnostic criteria mentioned in our previous study [23]. Mononuclear cells were isolated for analysis of miRNA expression. The clinical study was approved by the hospital ethnics review board of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

2.2. Mice and model of atherosclerosis

miR-21$^{-/-}$ mice (Jackson Laboratory) were crossed with *apoE*$^{-/-}$ mice (Jackson Laboratory) to generate DKO mice. For atherosclerotic models, *apoE* $^{-/-}$ mice (8–10 weeks old, male) and DKO (8–10 weeks old, male) mice were fed a high-fat diet (16.9% fat, 1.3% cholesterol, 21.1% crude protein, and 46.5% carbohydrate) for 3 months. Mice were euthanized by cervical dislocation and aortas were harvested for the evaluation of atherosclerotic lesions. All animal procedures were approved by the Committee on the Ethics of Animal Experiments of the Shanghai JiaoTong University School of Medicine.

2.3. Quantification of atherosclerosis

At the time of sacrifice, the mice were weighted and plasma was separated for lipid analysis. Atherosclerosis was quantified by staining aortic plaque with Oil Red O as previously described [24]. Atherosclerotic lesion sizes were assessed using commercial software (Image Pro Plus 6.0, Cybernetics, Bethesda, MD, USA) and are expressed as the percentage of plaque area relative to total intimal area. The aortic root was also sliced for Oil Red O staining. Plaque lesion size was assessed in the same manner and is expressed as the percentage of plaque area relative to cross-sectional luminal area.

2.4. Histological examination and immunostaining

The harvested aortic roots with attached left ventricles were embedded and sliced for Masson's trichrome staining to display collagen components in the plaque, which were quantified by measuring the percentage of Masson's trichrome stained area relative to aortic root area.

Quantitative immunostaining was performed for macrophages and smooth muscle cells in frozen slides of the aortic root using anti-CD68 antibody (Abcam, USA) and anti-α-SMA antibody(Biolegend, San Diego, CA). Macrophage staining was expressed as the percentage of CD68-positive area relative to plaque area. Smooth muscle cells staining was expressed as the percentage of α-SMA-positive area relative to cross-sectional luminal area of aortic root.

2.5. Cell culture

Bone marrow-derived macrophages (BMDMs) were isolated from the bone marrow of 4–6-week-old *apoE* $^{-/-}$ mice and DKO mice as described previously [25]. Briefly, bone marrow cells taken from tibiae and femurs were incubated in RPMI-1640 medium containing 10% (v/v) FBS, 1% penicillin/streptomycin and 50 ng/mL M-CSF. Non-adherent cells were removed and adherent cells were incubated for an additional 3–4 days. After reaching full confluence, the BMDMs were harvested and confirmed with F4/80 and CD11b expression by flow cytometry.

2.6. Enzyme-linked immunosorbent assay (ELISA)

BMDMs were pretreated with ox-LDL (50 μg/ml) for 24 h and cell supernatants were collected for further detection. TNF-α, IL-1β, IL-6, MMP-2, and MMP-9 protein levels were assayed in both collected cell supernatants and in mouse plasma using commercially available ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

2.7. Macrophage migration assay

The chemotaxis of BMDMs was assessed *in vitro* using a two-chamber migration assay. Unstimulated BMDMs (1×10^5 cells) were plated onto a transwell upper chamber (8 μm pore size, Millipore) and allowed to migrate across the porous filter for 6 h at 37 °C towards CCL2 (50 ng/ml, PeproTech, Inc.). Migrated BMDMs on the bottom of the filter were stained with crystal and counted using a fluorescence microscope. The number of cells was determined in five random fields (magnification × 200) for each experiment.

2.8. Macrophage-endothelial adhesion assay

Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Bank of Chinese Academy of Sciences. For adhesion experiments, HUVECs were pretreated with TNF-α (10 ng/ml) for 12 h after reaching confluence. Next, the BMDMs from *apoE* $^{-/-}$ mice and DKO mice were stained with fluorescent probes (CellTracker Green CMFDA, Invitrogen) and then added to activated HUVECs and incubated for 30 min. After incubation, the suspended BMDMs were removed and the adherent BMDMs were visualized using an inverted epifluorescence microscope. The number of macrophages that adhered to endothelial cells was determined in five random fields (magnification × 200) for each experiment.

2.9. In vivo influx to the peritoneum

apoE $^{-/-}$ mice and DKO mice were injected intraperitoneally with 2 mL of 3% Brewer's thioglycollate (Sigma, St Louis, USA). Three days after injection, mice were sacrificed, and cells were collected from peritoneal lavage with 10 ml RPMI medium. Cells were identified with F4/80 and CD11b expression to confirm the monocyte-macrophage phenotype and then countered on a cell counter (Beckman, CA, USA).

2.10. RNA isolation, miRNA expression and microarray analysis

Total RNA from BMDMs or human peripheral blood monocytes was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was quantified on a NanoDrop ND-2000 (Thermo Fisher Scientific, Wilmington, USA) and RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). The primer sequences used for qPCR have been listed in supplementary material 1.

For the analysis of miRNA expression, cDNA was synthesized with reverse transcriptase (TaKaRa Biotechnology, Otsu, Japan) and real-time PCR was carried out with a SYBR Ex Tag kit on an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA). The miRNA primers used are commercially available (catalog number: BK1010; Biotend, Shanghai, China).The relative gene expression level was calculated with the comparative $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression.

Genes of BMDMs from *apoE* $^{-/-}$ mice and DKO mice were detected with an Agilent Mouse Gene Expression microarray (8*60 K, Design ID: 028005) according to the manufacturer's standard protocols. The differentially expressed genes were identified through a fold change ≥2.0 and a p value ≤ 0.05 (each experiment was repeated at least 3 times).

2.11. RNA interference

The siRNA targeting *Dusp-8* (si-*Dusp-8*) was purchased from Santa Cruz (USA). siRNA duplexes with nonspecific scramble sequences were used as the negative control. Raw 264.7 cells were seeded into 6-cm culture dishes in antibiotic-free medium before transfection. The mmu-miR-21 inhibitor together with si-*Dusp-8* or nontargeting control siRNA (siNTC) were transfected into Raw 264.7 cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were then harvested 48 h later for real-time PCR or Western blot analysis.

2.12. Western blot analysis

Cells were lysed in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 2 mM EDTA supplemented with protease inhibitors (complete protease inhibitor cocktail, Roche). Total cell lysates were boiled for 10 min in 1 × loading buffer in the presence of 2-mercaptoethanol. Samples were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. After blocking with 5% nonfat dry milk in TBS-T, the membranes were incubated with primary antibodies at 4 °C overnight followed by appropriate secondary antibodies. Anti-DUSP8 antibody (Abcam, Cambridge, USA), MAPK and Phospho-MAPK Family Antibody (Cell Signal Technology, Danvers, MA, USA)were used to detect the protein expression levels of *Dusp-8, p-p38, p38, p-Jnk, Jnk, p-Erk, Erk*. Protein bands were visualized by Odyssey V3.0 image scanning (Li-COR. Inc., Lincoln, NE, USA). The intensity of each band was analyzed using Image J software.

2.13. Statistics

Data are presented as the mean ± SEM. Statistical analyses were performed with GraphPad Prism software 6.0 (GraphPad Software, Inc., La Jolla, CA). The significance of differences between two groups was determined by t-tests. The significance of differences among three or more groups was evaluated by ANOVA with Tukey's post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. miR-21 is upregulated in mouse aortic atherosclerotic lesions and circulating monocytes from patients with coronary heart disease

We previously demonstrated that miR-21 expression is upregulated in ox-LDL-stimulated human peripheral blood monocytes. To further assess the relevance of miR-21 in atherosclerosis, we extracted total RNAs from the aortic segments of a diet-induced atherosclerosis model for qRT-PCR analysis. The expression of miR-21 in *apoE*$^{-/-}$ mice was significantly increased after 12 weeks of high-fat diet (HFD) feeding compared with the normal diet (ND) (Fig. 1A). We further examined the expression of miR-21 in circulating monocytes from patients with coronary artery disease (CAD). miR-21 expression in circulating monocytes was significantly the highest in patients with acute coronary syndrome (ACS), followed by patients with stable angina pectoris (SAP) and non-CAD patients (Fig. 1B).

3.2. Effect of miR-21 deficiency on atherogenesis in vivo

To investigate the functions of miR-21 in atherogenesis, 8-week-old *apoE*$^{-/-}$ and DKO mice were treated with an atherogenic diet to induce atherosclerosis. After 12 weeks, the two groups showed equivalent

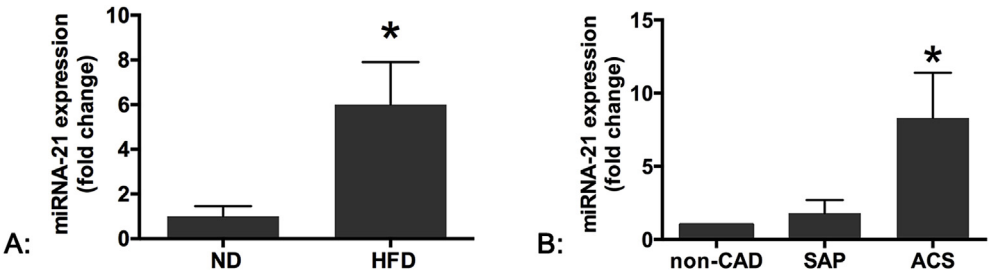

Fig. 1. miR-21 expression is upregulated in mouse aortic atherosclerotic lesions and circulating monocytes from patients with ACS. (A) miR-21 expression in the mouse aorta was analyzed by qRT-PCR. miR-21 expression in aortic segments from *apoE*$^{-/-}$ mice on HFD is significantly increased compared to *apoE*$^{-/-}$ mice on ND. *$p < 0.05$, *vs.* ND(n = 5). (B) Circulating monocytes from patients with non-CAD, SAP or ACS were analyzed for miR-21 expression. *$p < 0.05$, *vs.* non-CAD (n = 8–10).

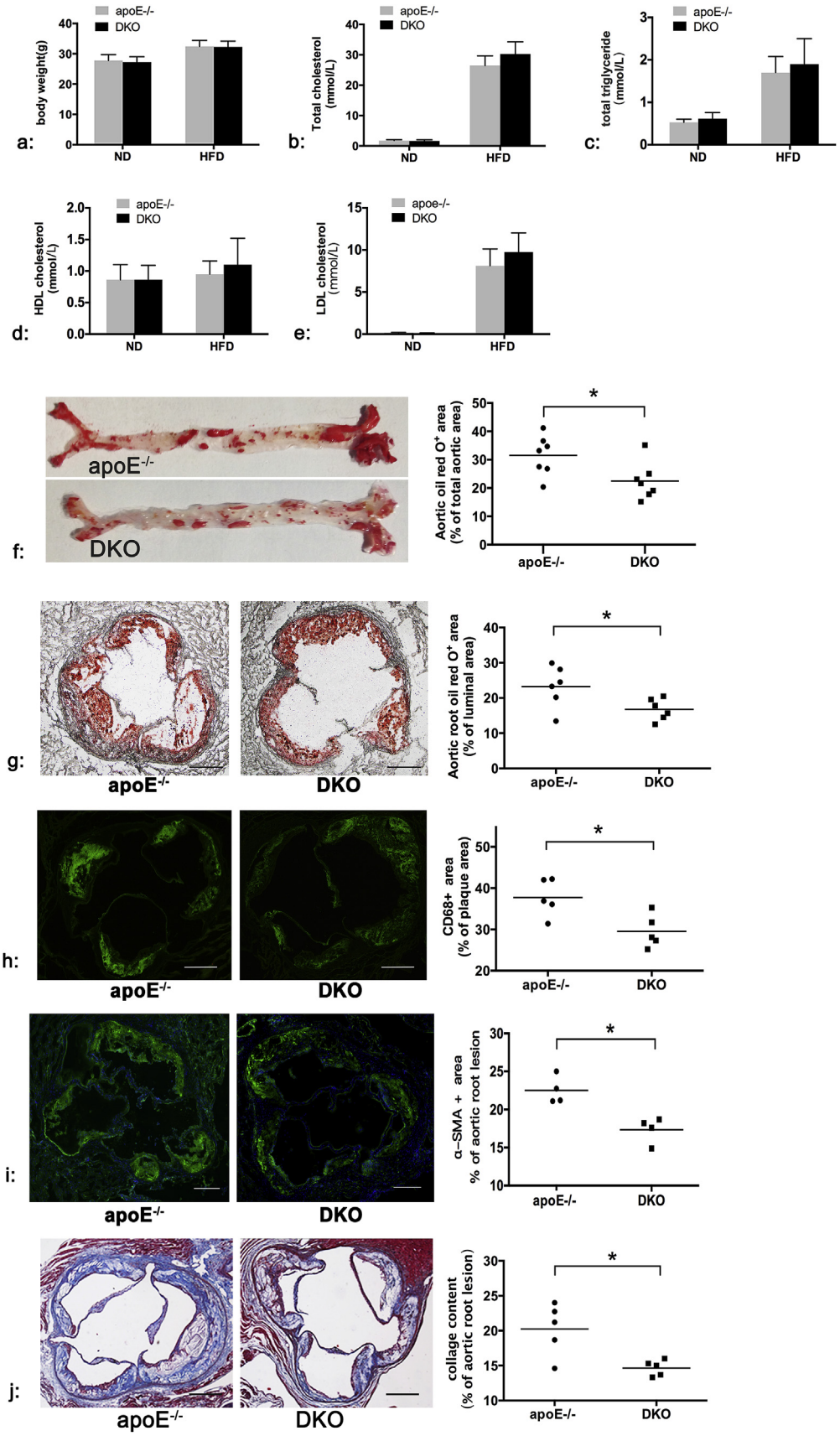

Fig. 2. miR-21 deficiency attenuated atherosclerosis and reduced macrophage accumulation in atherosclerotic lesions. (A–E) Body weights and plasma levels of total cholesterol, triglyceride, HDL and LDL cholesterol were determined in *apoE*$^{-/-}$ and DKO mice before and after 12 weeks of HFD feeding. *$p < 0.05$ *vs.* the *apoE*$^{-/-}$ group(n = 12–15). (F and G) Atherosclerotic lesions were analyzed in *apoE*$^{-/-}$ and DKO mice after HFD feeding for 12 weeks. Atherosclerotic lesion areas in *en face* aortas and aortic roots were assessed by Oil Red O staining. *$p < 0.05$ *vs.* *apoE*$^{-/-}$ mice(n = 6–7). (H and I) Macrophage and SMC content in atherosclerotic lesions were determined by immunohistochemical staining for CD68 or α-SMA,respectively. *$p < 0.05$ *vs.* *apoE*$^{-/-}$ mice(n = 5). (J) Collagen fibrous content in aortic roots was observed by Masson's trichrome staining. *$p < 0.05$ *vs.* *apoE*$^{-/-}$ mice(n = 5. The quantitative analysis and representative images are shown. Scale bar = 250 μm.

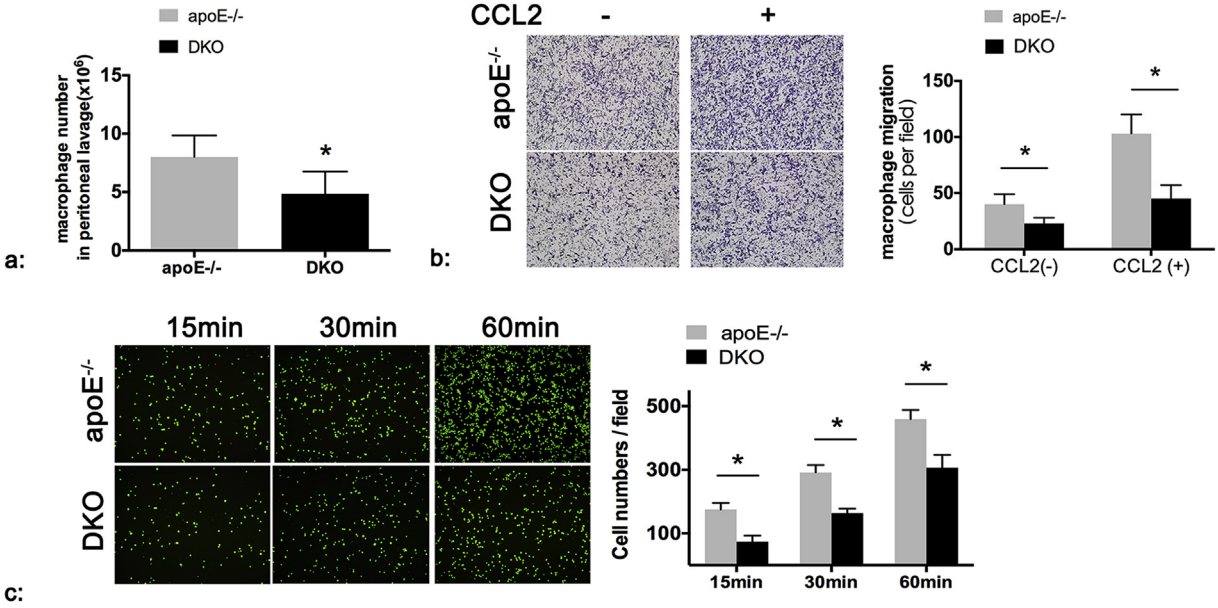

Fig. 3. miR-21 deficiency impaired macrophage migration and the macrophage-endothelial interaction. (A) Fewer macrophages were found in the peritoneal cavity in DKO mice in a thioglycollate-induced peritonitis model. *$p < 0.05$ *vs.* the $apoE^{-/-}$ group(n = 5). (B) Migration towards medium lacking or containing CCL2 (50 ng/mL) was significantly reduced in BMDMs from DKO mice, as determined by transwell assay. *$p < 0.05$ *vs.* the $apoE^{-/-}$ group(n = 5). (C) Fewer interacting BMDMs were observed on the tumor necrosis factor-α (Tnf-α)-activated endothelial cell surface in DKO mice. *$p < 0.05$ *vs.* the $apoE^{-/-}$ group(n = 5. Data represent the mean values ± SEM. Magnification × 40.

weights (Fig. 2A) and plasma lipid levels (Fig. 2B–E). Atherosclerotic lesion areas were evaluated in en face aortas by Oil Red O staining. Compared with $apoE^{-/-}$ mice, the area fraction of atherogenic lesions in DKO mice was significantly decreased (Fig. 2F). We next stained the aortic roots and found smaller atherosclerotic lesion areas in DKO mice (Fig. 2G). Importantly, DKO mice exhibited a smaller macrophage area and fewer SMC area relative to $apoE^{-/-}$ mice (Fig. 2H and I). Moreover, the collagen fiber content, which is closely related to the vulnerability of atherosclerotic plaque, tended to decrease in DKO mice (Fig. 2J). We further detected the mRNA expression of MMP-9 and TIMP-1 in aorta tissue,and the ratio of MMP9 mRNA/TIPM1 mRNA tends to be higher in DKO mice with HFD, but has no statistical difference compared with $apoE^{-/-}$ group (Supplementary material 2). These results indicate that miR-21 does not affect hypercholesterolemia induced by a high-fat diet. miR-21 deficiency attenuates atherosclerosis likely by reducing the recruitment of macrophages. However, decreased SMC area and collagen may lead to less stabilization.

3.3. Role of miR-21 deficiency in macrophage migration and the monocyte-endothelial interaction

Circulating monocytes attach to the damaged endothelium and initiate the progression of atherosclerosis. To investigate the underlying mechanisms involved, we first performed macrophage chemotaxis in DKO mice in a model of thioglycollate-induced peritonitis. Macrophage influx into the peritoneal cavity in DKO mice was markedly impaired compared to that of $apoE^{-/-}$ controls (Fig. 3A). Next, we verified the results *in vitro* using an CCL2-induced transwell assay. BMDMs from DKO mice showed significantly lower migration capacity compared with $apoE^{-/-}$ mice (Fig. 3B). Finally, we explored BMDM cell adhesion to endothelial cells. An approximate 50% reduction of adhesion was observed on the endothelial cell surface in DKO mice (Fig. 3C). Taken together, we conclude that an impaired capability of migration and adhesion to the endothelium in DKO mice may, at least in part, account for the decreased macrophage influx in atherosclerotic plaques.

3.4. Role of miR-21 in atherosclerotic inflammatory responses

Due to the previously reported role of miR-21 in inflammation, we first compared inflammatory cytokine expression in plasma from $apoE^{-/-}$ and DKO mice. As a result, DKO mice secreted lower levels of plasma MMP-2 at baseline. However, after 3 months of HFD feeding, the difference was diminished. Surprisingly, the expression of IL-1β, IL-6, TNF-α, and MMP-9 in plasma did not differ substantially between $apoE^{-/-}$ and DKO mice (Supplementary material 4A-E). We also analyzed the concentrations of these inflammatory cytokines in BMDMs from $apoE^{-/-}$ and DKO mice. After pretreatment with ox-LDL for 24 h, BMDM culture supernatant was collected for ELISA. ELISA showed that the expression of IL-1β, IL-6, TNF-α, MMP-2, and MMP-9 did not change significantly in miR-21-deficient BMDMs either before or after ox-LDL treatment (Supplementary material 4F-J) compared with BMDMs from $apoE^{-/-}$ mice. These *in vivo* and *in vitro* results suggest that miR-21 does not participate in the regulation of the ox-LDL-induced macrophage inflammatory response.

3.5. Mechanism of miR-21's effect on macrophage activation

To explore the potential mechanism of miR-21 on the regulation of monocyte/macrophage migration and adherence, we used gene microarrays to evaluate the pattern of gene expression in $miR\text{-}21^{+/+}$ and $miR\text{-}21^{-/-}$ BMDMs. Compared with $miR\text{-}21^{+/+}$ BMDMs, 90 genes were upregulated in miR-21-deficient BMDMs (fold change ≥2.0, $p \leq 0.05$, n = 3 per group; Supplementary material 5). In reference to the computational prediction of miR-21 targets by TargetScan, 21 genes were chosen as candidates,and 11 genes among them were further verified by quantitative RT-PCR (Fig. 4A). Among these candidates, we identified the previously validated miR-21 target [36], *Dusp-8* (a negative regulator of mitogen-activated protein kinase (MAPK) signaling) as our target gene involved in the regulation of miR-21 on macrophage migration and adhesion.

Next, to clarify the downstream target of *Dusp-8*, we assayed the phosphorylation of MAP kinases, which are related to macrophage activation. Remarkably, miR-21 deficiency significantly downregulated the phosphorylation of p38 and JNK at baseline and after ox-LDL

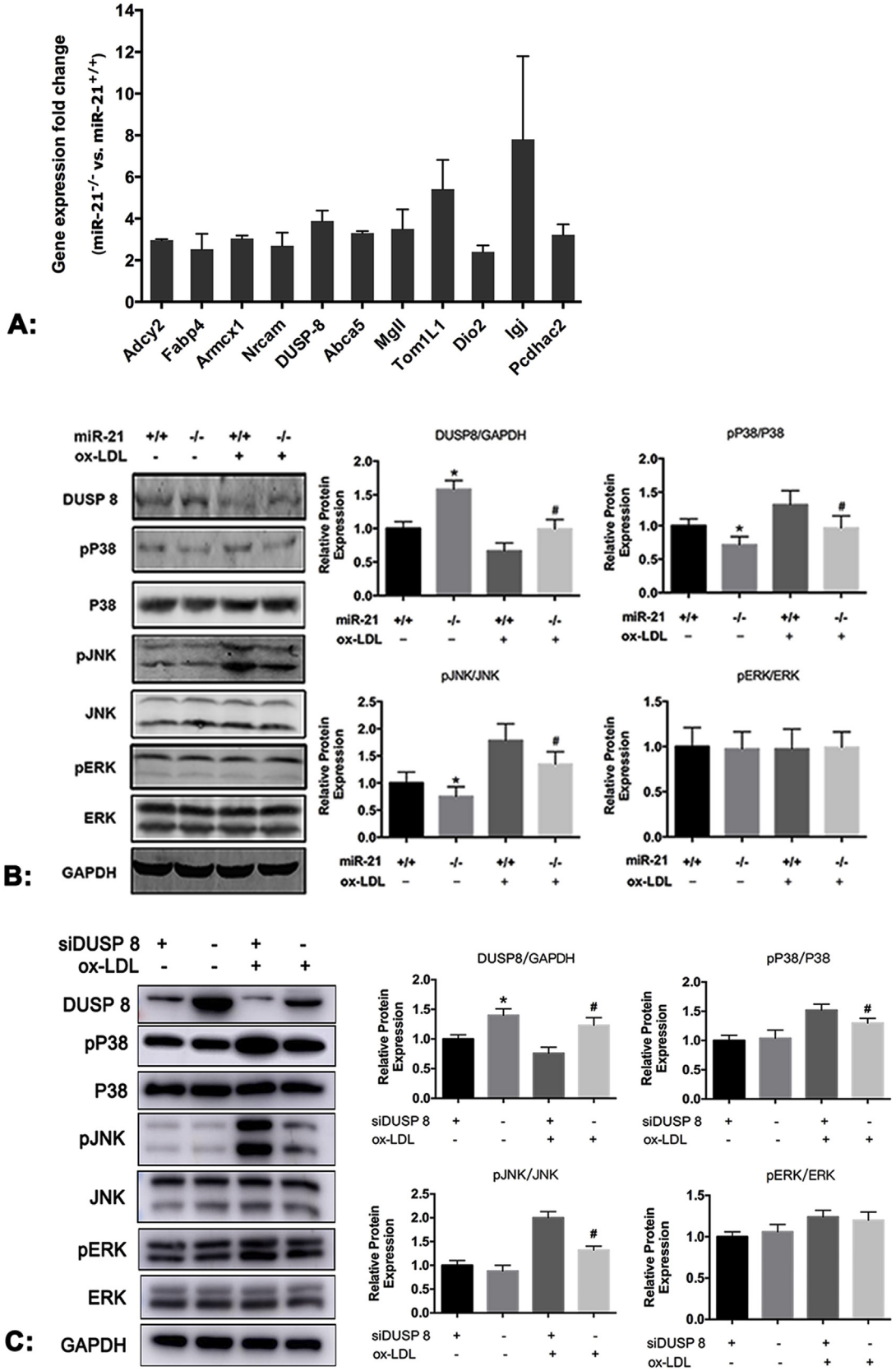

Fig. 4. miR-21 deficiency upregulated the expression of target *Dusp-8* and subsequently suppressed p38 and JNK signaling. (A) Effects of miR-21 deficiency on the expression of potential miR-21 targets in ox-LDL-stimulated BMDMs by quantitative RT-PCR(n = 3, fold change ≥2.0 and $p \leq 0.05$. (B) Effects of miR-21 deficiency on *Dusp-8* expression and MAPK pathway activation in unstimulated and ox-LDL-stimulated BMDMs. The level of *Dusp-8* was significantly increased at baseline and following ox-LDL stimulation in miR-21-deficient BMDMs compared with WT. (C)The phosphorylation state of p38/JNK/ERK after si-Dusp-8 tansfection in Raw 264.7 cells. Phosphorylation of p38 and JNK significantly increased in ox-LDL activated macrophages after si-Dusp-8 tansfection. Western blot was used to detect protein expression levels of Dusp-8, p-p38, p38, p-Jnk, Jnk, p-Erk, Erk, and Gapdh. The average ratios of Dusp-8, p-p38/p38, p-Erk/Erk, and p-Jnk/Jnk were calculated based on the gray intensity analysis. *$p < 0.05$ *vs.* the unstimulated WT group, #$p < 0.05$ *vs.* the ox-LDL stimulated WT group(n = 3).

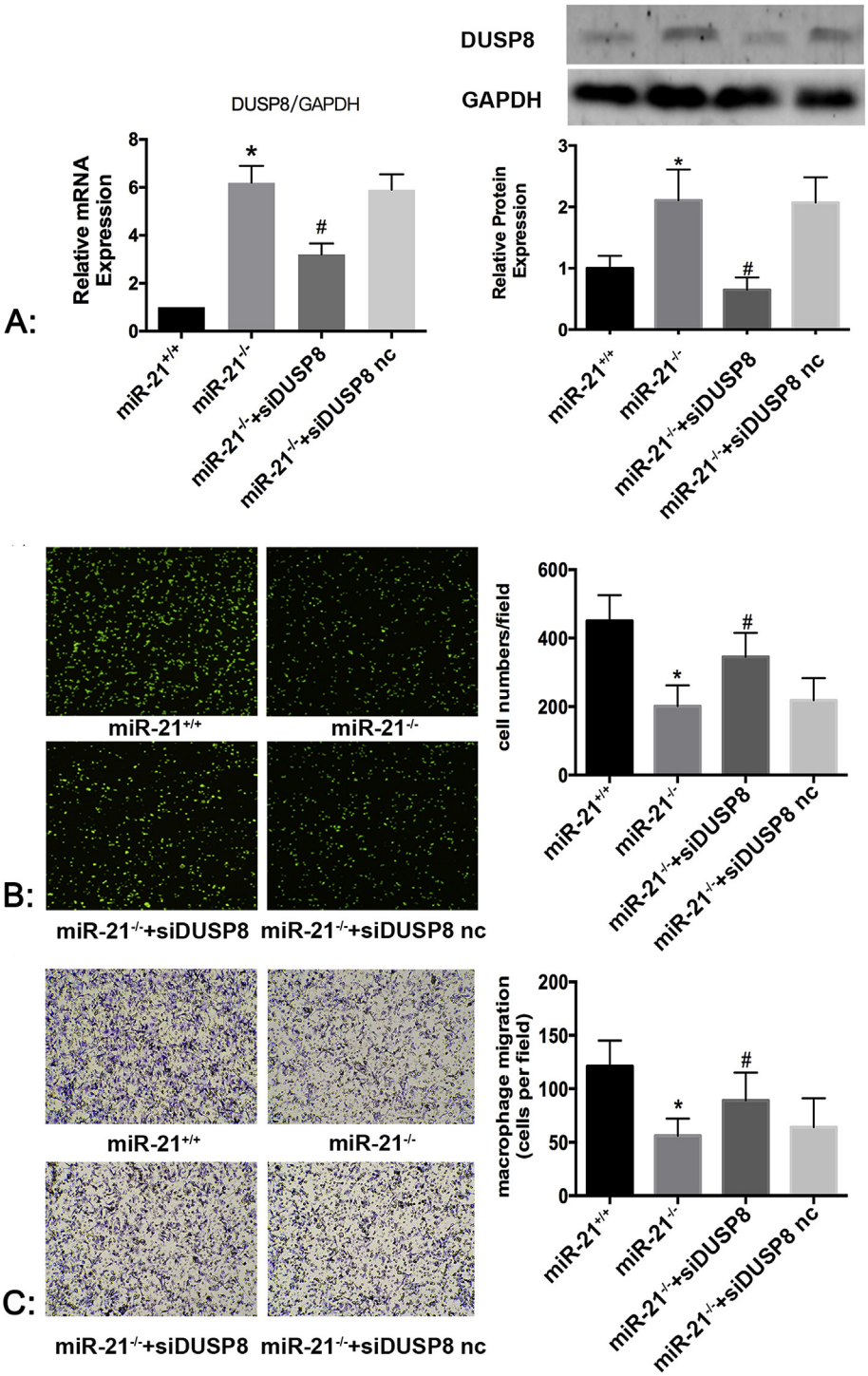

Fig. 5. Targeting *Dusp-8* partly restored the impaired migration and adhesion mediated by miR-21 deficiency in BMDMs. (A) The mRNA and protein level of Dusp-8 was assayed. Dusp-8 expression decreased in *miR-21*$^{-/-}$ BMDMs after silencing by a specific small interfering RNA (siRNA) compared with the non-targeting control siRNA (siNTC). *$p < 0.05$ *vs*. the WT group, #$p < 0.05$ *vs.* the miR-21 KO group (n = 3). (B–C) Effect of *Dusp-8* siRNA treatment on macrophage-endothelial interaction (B) and macrophage migration (C) in *miR-21*$^{-/-}$ BMDMs. BMDMs were transfected with either *siDusp-8* or siNTC. Macrophage adhesion and migration were analyzed as previously described. Data represent the mean values ± SEM. Magnification × 40. *$p < 0.05$ *vs.* the WT group, #$p < 0.05$ *vs*. the miR-21 KO group (n = 3).

stimulation in macrophages. Extracellular signal-regulated kinase (ERK) signaling was not obviously affected by miR-21 (Fig. 4B). To further prove that p38/JNK is the downstream target of Dusp-8, we detected the phosphorylation state of p38/JNK/ERK after si-Dusp-8 tansfection in Raw 264.7 cells. After si-Dusp-8 tansfection,the phosphorylation of p38 and JNK significantly increased in ox-LDL activated macrophages.

Besides, a rescue study was performed to prove that *Dusp-8* can serve as an effective target gene of miR-21. *Dusp-8* expression was knocked down by a small-interfering RNA strategy in miR-21-deficient BMDMs, and cell migration and adhesion were examined. Silencing of *Dusp-8* resulted in an obvious enhancement of macrophage migration and the macrophage-endothelial interaction (Fig. 5).

Taken together, these results indicate that reduced macrophage migration and adhesion in miR-21-deficient BMDMs is attributable to the derepression of *Dusp-8* expression.

4. Discussion

In this study, we present the effects of miR-21 on the migration and adhesion of macrophages and on atherogenesis *in vitro* and *in vivo*. miR-21 deficiency decreased macrophage infiltration, thereby restricted the development of atherosclerotic plaques, however, the reduced SMC and collagen content may lead to an instable plaque phenotype. *Dusp-8-*

JNK/p38 signaling is, at least in part, the downstream target of miR-21 and is involved in the regulation of macrophage function and the pathophysiological progression of atherosclerosis.

Atherosclerosis is a chronic inflammatory response. Monocytes/macrophages play a major role in its initiation, propagation and progression. We previously found that miR-21 was upregulated in ox-LDL-stimulated human peripheral blood monocytes [9]. To further demonstrate the relationship between miR-21 and activating monocytes, we performed additional *in vivo* and *in vitro* studies. Our data revealed that the expression of miR-21 is significantly upregulated in both mouse aortic atherosclerotic lesions and circulating monocytes from patients with coronary disease. These findings are consistent with our earlier report and other studies. Raitoharju et al. reported that miR-21 is upregulated in human atherosclerotic plaques compared to non-atherosclerotic arteries [26]. Based on these results, we hypothesize that miR-21 may be an important regulator in the pathological process of atherosclerosis.

To prove our hypothesis, we utilized *miR-21*$^{-/-}$ *apoE*$^{-/-}$ mice and *apoE*$^{-/-}$ mice to develop a model of atherosclerosis. miR-21 knockout clearly decreased atherogenesis in the aorta. Theoretically, this phenomenon may be attributable to the following two causes: (1) the role of miR-21 in the regulation of macrophage function, including inflammation, infiltration and migration, lipid influx and efflux, and (2) other unknown effects on other tissues or cells, such as endothelial cells, smooth muscle cells, and fibroblasts. Considering the decreased macrophage infiltration in DKO mice and the importance of macrophages in atherosclerosis, we focused on the effect of miR-21 on macrophages.

Previous studies have indicated that miR-21 may serve as a key switch in the transition of inflammation. Several studies considered miR-21 as a negative regulator in the NF-κB signaling pathway and reported that it exerts an anti-inflammatory effect by targeting PDCD4 or MyD88; the suppression of microRNA-21 expression increased tumor necrosis factor-α (TNF-α) and lL-6 and decreased IL-10 levels after LPS stimulation [18,19,27]. Moreover, miR-21 upregulates Arg1 expression, which indicates a relationship with the M2 phenotype [20]. However, other studies have reported opposing results [21,22,28,29]. In models of in-stent restenosis, miR-21 promoted vascular inflammation by inducing the secretion of pro-inflammatory mediators, such as IL-1α, IL-1β, IL-6 and macrophage inflammatory protein-1α in macrophages. miR-21-knockout macrophages are prone to exert an M2 phenotype [21]. Wang Z et al. also revealed that miR-21 inhibition enhances the PGE2-mediated expression of M2 genes by targeting $STAT_3$ [22]. In our study, we analyzed the impact of miR-21 deficiency on BMDMs polarization. It shows that the mRNA expression of iNOS、TNF-α and IL-6,which indicate M1 macrophage polarization, has no statistical difference between WT group and miR-21 KO group (supplementary material 3). The pro-inflammatory cytokines remained unchanged in the absence of miR-21 both *in vitro* and *in vivo*. The reason for this discrepancy is still unclear but may be due to the different cell and animal models used or different inflammatory stimuli. On the other hand, our results showed that miR-21 deficiency decreased the leukocyte-endothelial interaction and macrophage migration, which represent the impairment of atherosclerosis initiation. This finding is consistent with several previous papers. Shi et al. reported that miR-21 knockout improved the survival rate in a fatal colitis model by reducing $CD68^+$ and $CD3^+$ cell infiltration [30]. Other studies have also demonstrated that miR-21 enhances the migration and invasion of tumor cells [31–34].

Lipid metabolism is a crucial factor in the development of atherosclerosis. Miśkowiec et al. reported that free circulating miR-21 levels inversely correlate with TC, LDL-C, and non-HDL-C in patients with acute coronary syndromes without persistent ST-segment elevation [35]. However, in our study, the cholesterol, triglyceride, LDL and HDL levels were all comparable between DKO mice and *apoE*$^{-/-}$ mice. We further evaluated the effect of miR-21 on lipid accumulation in macrophages; it appears that miR-21 has no effect on macrophage uptake of ox-LDL *in vitro* (Supplementary material 6). One explanation for this discrepancy may be attributable to the different research objects.

It is worth noting that decreased SMCs and collagens content were also prominent in DKO mice. Collagens constitute a major portion of the extracellular matrix in the atherosclerotic plaque, dysregulated collagen metabolism lead to plaque destabilization [36]. The decreased collagen content may attribute to decreased collagen synthesis and/or increased collagen degradation. SMC are thought to be the primary source of collagen within the fibrous cap [37], plenty of studies have identified the proliferative and anti-apoptotic effects of miR-21 [11,14,15], which may partly account for decreased SMC content in DKO mice and thus cause impaired collagen synthesis. On the other hand, MMPs/TIMPs imbalance disrupt collagen homeostasis [38]. So we detected the mRNA expression of MMP-9 and TIMP-1 in aorta tissue by qRT-PCR,and the ratio of MMP9 mRNA/TIPM1 mRNA tends to be higher in DKO mice with HFD, but has no statistical difference compared with *apoE*$^{-/-}$ group (Supplementary material 2). Taken together, we proved that miR-21 deficiency in mice alleviates atherogenesis partly through impaired leukocyte-endothelial interaction and macrophage migration, while deceased collagen content in plaque may mean less stable with the progress of atherosclerosis. Chipont et al. considered miR-21 deficiency reduced early-stage atherosclerosis but increased plaque instability at later stages of atherosclerosis [39]. It is mostly consistent with our findings. However, a recent study reported conflicting results. Hong et al. reported that miR-21−/−apoE−/− mice presented with more atherothrombotic events and substantially higher levels of arterial macrophage infiltration[44].In their study, the carotid tandem stenosis model was used to induce instability plaques. It is reported that wall shear stress influences miR-21 expression and causes endothelial dysfunction and vascular remodeling[12,45,46], which may have led to this discrepancy.

The next question then is how miR-21 regulates monocyte migration and infiltration. To address this question, we used gene microarrays and computational prediction to identify the target mRNAs of miR-21. We found that dual-specificity phosphatase 8 (*Dusp-8*) may serve as a potential downstream mediator. *Dusp-8* is a member of the dual specificity phosphatase subfamily and negatively regulates members of the mitogen-activated protein (MAP) kinase superfamily (MAPK/ERK, SAPK/JNK, and p38), which determine the type of physiological response. Actually, Liu SL et al. used a luciferase reporter assay and reported that miR-21 binds to the 3′UTR of *Dusp-8* and promotes high-glucose-induced cardiac fibrosis [40]. In the present study, we also verified that *Dusp-8* is a downstream target of miR-21 in BMDMs. The expression of *Dusp-8* was significantly increased in BMDMs from DKO mice compared with *apoE*$^{-/-}$ mice. Our rescue study further demonstrated the role of *Dusp-8* in serving as an effective target of miR-21. Knockdown of *Dusp-8* in *miR-21*$^{-/-}$ BMDMs reversed the effect of miR-21 deficiency on macrophage activation. *Dusp-8* prefers p38 and JNK as dephosphorylated substrates, but can also affect ERK1/2 [41–43]. In this study, the upregulated *Dusp-8* subsequently decreased the phosphorylation of p38 and JNK but did not alter the activation of ERK. These results indicate that *Dusp-8*-p38/JNK signaling is the downstream target of miR-21 and is involved in macrophage activation.

Lastly, we should address some limitations to the current study. First, the DKO mice we used are a whole system. Thus, the effect of miR-21 on atherogenesis could also be the subsequent effect of other issues besides macrophages. Canfrán-Duque A. et al. demonstrated that miR-21 expression increased in the aorta during atherosclerosis and the absence of miR-21 in macrophages accelerated the progression of atherosclerosis due to enhanced macrophage apoptosis and vascular inflammation [47]. Thus, the pathophysiological role of miR-21 on vascular smooth muscle cells and endothelial cells during atherogenesis, requires further investigation. Second, the regulation of macrophage activation by miR-21 was mostly examined *in vitro*. Therefore, further *in vivo* experiments would be more convincing.

4.1. Conclusions

In summary, our results indicate that the inhibition of miR-21 expression in mice reduce atherosclerotic plaque but may has some disadvantageous effects on plaque stability with the progress of atherosclerosis. miR-21 is an important regulator of macrophages in atherogenic programming, partly by regulating *Dusp-8* and p38/JNK MAPK signaling.

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Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.10.003.

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