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Identification of miRNAs as atherosclerosis biomarkers and functional role of miR-126 in atherosclerosis progression through MAPK signalling pathway

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Abstract. – OBJECTIVE: Mounting evidence suggests the role of microRNAs (miRNAs) in regulating inflammatory responses in various vascular diseases. Inflammation is the key mechanism leading to atherosclerosis (AS) and various miRNAs are aberrantly expressed in response to AS pathophysiology. However, there are very limited studies that serve to elucidate the role of specific miRNA in in vivo or in vitro AS models.

MATERIALS AND METHODS: Microarray analysis of blood plasma of apolipoprotein deficient (apoE-/-) mice was performed followed by the confirmation using qPCR. Bone marrow mononuclear cells (BMMCs), plasma, and vessel tissue were obtained from apoE-/- mice that were induced with miR-126 mimic or inhibitor. Ox-LDL-induced THP-1 macrophages served as in vitro AS model. The release of inflammatory cytokines was detected using ELISA. The regulatory effect of miR-126 on MAP3K10 was confirmed by luciferase reporter activity and immunohistochemical analyses.

RESULTS: The results showed that the miR-126 exhibited a greater fold change of expression in AS mice. Further, the functional role of miR-126 in atherosclerosis pathophysiology was demonstrated both *in vivo* and *in vitro*. miR-126 reduced the cytokine release and also decreased the AS progression. miR-126 was also found to be involved in mitogen-associated protein kinase (MAPK) signaling pathway. MAP3K10 was identified to be a direct target.

CONCLUSIONS: miR-126 might serve as a biomarker of AS and its over-expression might prevent the AS progression and development.

Key Words:
Atherosclerosis, miR-126, MAP3K10, Biomarker.

Introduction

Atherosclerosis (AS) is associated with chronic inflammation characterized by cholesterol accumulation in large arteries. It contributes to coronary artery disease (CAD) and presents a wide array of complex pathophysiological processes including endothelial cell dysfunction, oxidative stress, hemodynamic changes, inflammatory responses and so on[1]. Atherogenesis occurs largely due to inflammation mediated by macrophages, dendritic cells, T-lymphocytes and various proinflammatory cytokines[2]. There have been limited studies to determine the mechanistic data regarding the regulation of inflammatory responses in AS. MicroRNAs, non-coding class of small RNAs, are differentially expressed in manifold cardiovascular diseases and are significantly involved in atherosclerosis[3-6].

miRNAs that regulate immune cell functions control inflammation in vascular diseases. miR-126 has been previously reported to be involved in regulating the inflammatory responses and promoting angiogenesis[7,8]. Fatty acid oxidation is reported to be controlled by miR-370 and miR-122 with implications in vascular diseases[9]. miR-181a expression is also associated with the inflammatory stimuli along with hemostatic responses[10]. However, the role of miR-126 in atherosclerosis in response to inflammation still needs to be explored. Interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are important proinflammatory cytokines that have a pivotal role in atherosclerotic plaque progression[11]. Experimental evidence has proved that TNF-a deficient apolipoprotein E ($apoE$)$^{-/-}$ mice had smaller lesion size[12]. Similarly, IL-6 is also known to increase lesion development and is considered to be a pro-atherogenic cytokine[13]. Regulating the release of these cytokines might prove beneficial in controlling the AS plaque progression[14].

Inflammatory stimuli activate a number of intracellular signalling pathways that promote the release of inflammatory cytokines and media-

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tors. Mitogen-activated protein kinase (MAPK) is required for the activation of nuclear factor-κB (NF-κB), which in turn activates TNF-α. There are three distinct groups of MAP kinases namely, p38, JNK, and ERK1/2. All these kinases share similarities in being involved in cell growth, differentiation, inflammation and apoptosis[15]. This work was designed to determine the expression level of different miRNAs involved in atherosclerosis that might serve as potential biomarkers. Further, the role of miR-126 has been elaborately studied in *in vitro* as well as an *in vivo* model for atherosclerosis along with its implications in regulating MAPK signalling pathway.

Materials and Methods

Animals

To develop an atherosclerosis model, 20 male C57BL/6 (B6) mice (n=10 for apoE$^{-/-}$ mice and n=10 for healthy) were obtained from SLAC company, Songjiang District, Shanghai, China. The research involving animals was conducted according to the relevant guidelines and was approved by the local Ethical Committee. They were fed for 8 months with standard food which comprised of fat (6.5%), protein (19.5%) and no cholesterol. They were kept under optimum conditions with 12 hours light/dark cycle, the temperature of 22±0.5°C and humidity of 50±10%.

THP-1 Cell Culture

The THP-1 cell line was obtained from ATCC, Manassas, VA, USA and was cultured with PMA (100 nM) to differentiate to macrophage.

Sampling of Blood

Intraperitoneal injection of sodium pentobarbital (75 mg kg^{-1}) (Sigma-Aldrich, Huangpu, Shanghai, China) was done to anesthetize mice. A tube was rotated, inserted into the eyeball of mice through the sinus membrane. The blood was collected avoiding contamination followed by the immediate addition of EDTA to avoid coagulation. The sample was stored no more than 2h at RT and was then centrifuged at 4°C for 10 min at 1200 g. The supernatant was separated and then centrifuged to remove cellular debris (1200 g at 4°C for 10 min). Trizol LS reagent (Thermo Fisher Scientific, Pudong, Shanghai, China) was added to the purified plasma and stored at -80°C. Each mice was subjected to plasma pooling for analysis.

Microarray analysis of miRNA

The miRCURY Hy3/Hy5 Power Labeling Kit (Exiqon, Changping District Beijing, China) was used to separately label 1 μg plasma RNA from apoE$^{-/-}$ and control mice respectively. It was then hybridized using Exiqon miRCURY LNA Array v.16.0 followed by washing and scanning of slides using microarray scanner (Axon GenePix, Sunnyvale, CA, USA), where the images were analyzed using the GenePix Pro 6.0. The normalization factor was calculated based on the intensities of miRNAs (> 50) in all the samples. Following the normalization of expression data, the upregulation and downregulation of each miRNA were defined by a threshold value that was significant with a fold change greater than 3.0.

qRT-PCR to Determine the Expression of Selected miRNAs

Trizol LS RNA isolation kit (Thermo Fisher Scientific, Pudong, Beijing, China) was used to isolate total RNA according to the protocol described by the manufacturer. NanoDrop 1000 system (Thermo Fisher Scientific, Pudong, Beijing, China) was used to detect the total RNA purity and then stored at -80 °C immediately. The expression level of five miRNAs (miR-17-5p, miR-126, miR-370a, miR-122, and miR-181a) in apoE$^{-/-}$ and healthy B6 mice was quantified by using TaqMan MicroRNA assays (Thermo Fisher Scientific, Pudong, Beijing, China). qPCR was performed (Applied Biosystems, Chaoyang District, Beijing, China), where U6 RNA served as an internal control to normalize the mean Ct values.

In vivo Assay

The miR-126 agomiR was modified chemically and conjugated with cholesterol. A scrambled miR-126 agomiR acted as a control. 10 nmol of miR-126 agomir was conjugated with cholesterol and scrambled miR-126 agomir in 0.1 ml saline buffer was injected into the tail vein of mice once every 3 days for 2 weeks. Further, flushing bone marrow with the phosphate buffered saline following the removal of femurs and tibia produced bone marrow-derived dendritic cells. Incubation with NH_4Cl lysing buffer removed the red blood cells (RBC).

Transfection with Small RNA

THP-1 macrophage-induced with PMA was transfected with miR-126 agomir or inhibitor according to the instructions for Hiperfect transfection reagent from Qiagen (Valencia, CA, USA)

with a final concentration of 50 nM. Incubation of cells with oxLDL (50 ug/ml) was done for 24 hours followed by transfection for 24 hours with miRNA mimic/inhibitor. The efficiency of transfection was confirmed by using Cy3-labeled negative control.

miRNA and mRNA RT-qPCR

Total RNA was isolated using the miRVana miRNA isolation kit (Ambion, Thermo Fisher Scientific, Pudong, Beijing, China) according to the protocol described by manufacturers. cDNA was generated with TaqMan® MicroRNA Reverse Transcription Kit, and miRVana quantitative RT-PCR primer sets for miRNAs (Ambion, Thermo Fisher Scientific, Pudong, Beijing, China). ABI PRISM 7000 (Applied Biosystems, Chaoyang District, Beijing, China) was used to monitor PCR reaction and endogenous control was U6 RNA. SYBR-GREEN reagent kits (Thermo Fisher Scientific, Pudong, Beijing, China) were used to analyze mRNA levels. GAPDH was used as an internal control.

Enzyme-linked Immunosorbent Assay (ELISA) of TNF-a and IL-6

Enzyme Immunoassay kits (Enzo Life Sciences, Chang Shou, Shanghai China) were used to determine the presence of inflammatory markers TNF-α and IL-6.

Western blot Analysis

20 μg of protein was separated and by subjecting the denatured and solubilized proteins to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%). The blot was blocked using 5% blocking solution (non-fat dry milk in phosphate buffered saline – PBS) and then incubated with primary antibody to rabbit anti-human-MAP3K10, P38, JNK, ERK1/2 (1:1000), or rabbit anti-actin (1:5000) (Abcam, Xuhui District, Shanghai, China). The blots were then washed thrice with PBS/0.1% Tween followed by the incubation of blot in the secondary antibody goat anti-rat IgG diluted to 1:5000 (Abcam, Xuhui District, Shanghai, China) that was labeled with Alexa Fluor 680 dye. The blots were washed and the proteins were detected using a chemiluminescence system. The Odyssey system (Li-cor, Lincoln, NE, USA) was used to detect signals and densitometric analysis was performed.

Luciferase Reporter Assay

p-MAP3K10 UTR and mut-MAP3K10 UTR miRNA luciferase reporter vector, as well as a miR-126 inhibitor, cotransfected the THP1 cells using Lipofectamine 2000 (Invitrogen, Pudong, Beijing, China). To monitor the transfection efficiency, PGL3-control vector transformed the cells. A non-homologous miRNA, anti-miR negative served as the control. pRL-TK (Promega, Pudong, Shanghai, China) transfection in cells served as a normalization control. Dual-luciferase reporter assay system was used to determine the firefly luciferase activity, after 24 h. Normalization to renilla control provided the relative reporter activity by normalization.

Immunohistochemical Analysis of Atherosclerotic Plaque

Following two weeks, the rats were injected with ketamine (60 mg/kg) and xylazine (6 mg/kg) intraperitoneally to anesthetize them and then killed immediately by cervical. Cholesterol kit (Sigma-Aldrich, Huangpu, Shanghai, China) was used to measure plasma cholesterol. Aortic sinus and thoracic aorta were subjected to morphometric and immunohistochemical studies. The entire aortic valve was divided into serial sections and stained with hematoxylin and eosin. The plaque size was obtaining images of 3 sections (Olympus, Shinjuku-ku Tokyo, Japan). Image-Pro Plus v 6.0 software was used to obtain lesion areas. The extent of atherosclerosis for each animal was estimated by obtaining the mean value of 3 cross-sectional plaque areas. For immunohistochemical analysis, primary antibodies anti-mouse CD68 (Sigma-Aldrich, Huangpu, Shanghai, China), anti-MAP3K10 (Santa Cruz Biotechnology, Chai Wan Hongkong, China), anti-IL-6 antibody (Sigma-Aldrich, Huangpu, Shanghai, China) and anti-TNF-a (Sigma-Aldrich, Huangpu, Shanghai, China) were used. Immunohistochemical analysis performed according to the previously described protocol[16].

Statistical Analysis

The values are represented as the means±SD and ANOVA or Student's *t*-test was used for statistical analyses with $p<0.05$ as statistical significance. The differential expression of miRNAs was considered significant if the change in expression was at least 3 fold. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Altered miRNA Expression in the apoE$^{-/-}$ mice Plasma

Ten miRNAs were found to have altered expressed in the plasma of apoE deficient mice

Table I. Altered expression levels of circulating microRNAs in apoE deficient mice compared with control wild type mice, where the value of threshold is >3.

Accession Number	miRNA	Fold Change
MI0000139	miR-1	+3.34
MI0000256	miR-122	+3.63
MI0000153	miR-126	-4.17
MIMAT0000649	miR-17-5p	+3.43
MIMAT0000210	miR-181a	-3.79
MI0000143	miR-29b	+3.21
MI0000623	miR-340	+2.93
MI0001165	miR-370	-3.65
MI0004676	miR-499	+3.12
MI0005521	miR-92b	-3.13

when compared to the controls. Out of these 10 miRNAs, 6 were upregulated and 4 were downregulated. The fold change of each miRNA is shown in Table I. The fold change was obtained following the normalization of average value of each miRNA. The differential expression of these miRNAs in CAD was in congruence with the previously published reports.

Differential Expression of miRNAs in CAD Confirmed by qRT-PCR

The microarray results were validated by qRT-PCR which determined the expression levels of miRNAs in plasma. To confirm the differential expression of miRNAs in plasma of apoE$^{-/-}$ mice, miRNAs with a greater fold change and those reported to have a high significance in CAD (miR-17-5p, miR-126, miR-370, miR-122, miR-181a) were subjected to qPCR analysis. The results were consistent with the microarray analysis and are shown in Figure 1. miR-126 showed a greater alteration in expression and hence it was interesting to study its further effect on atherosclerosis *in vitro* and *in vivo* models.

TNF-a and IL-6 secretion by miR-126

The introduction of miR-126 mimic reduced the secretion of inflammatory cytokines, IL-6 and TNF-α, in macrophages induced with oxLDL, whereas the miR-126 inhibitor elevated the secretions at protein and mRNA levels. This indicates that miR-126 regulates the inflammatory response of oxLDL-treated macrophages (Figure

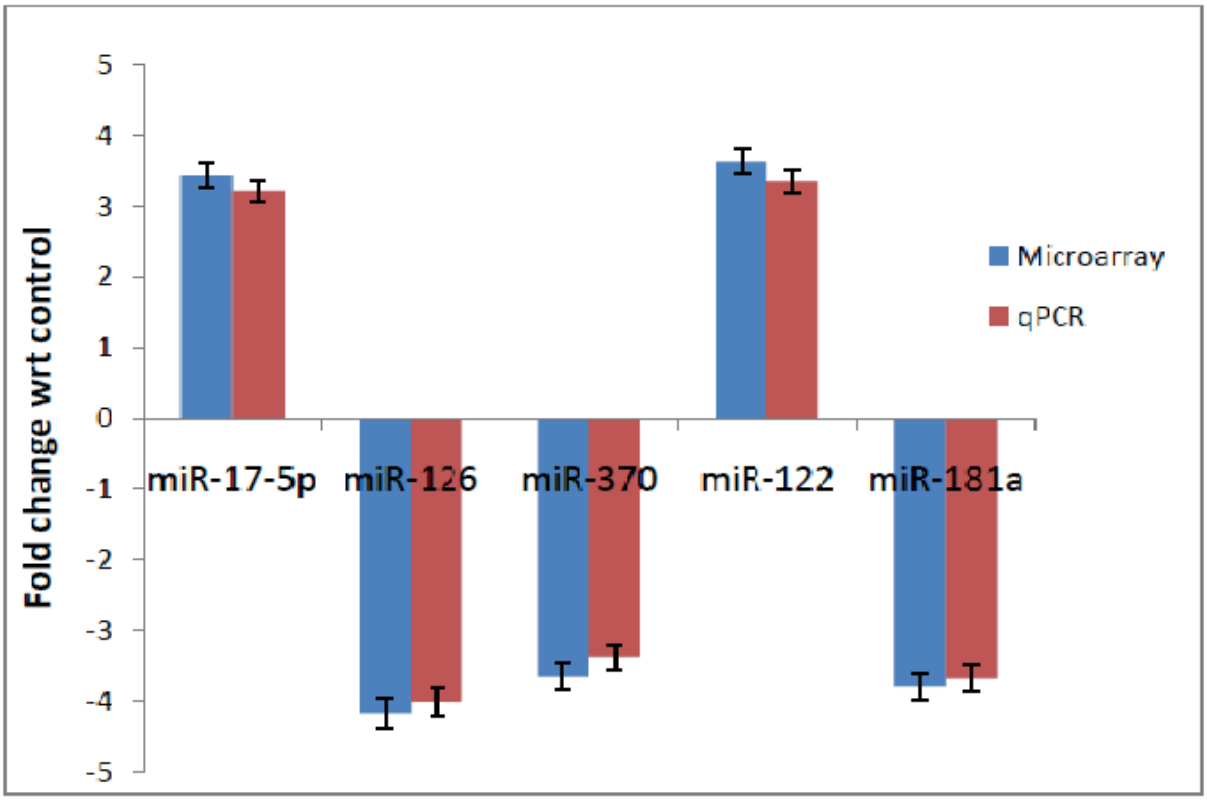

Figure 1. qRT-PCR confirmed the microarray results in five selected miRNAs with greater altered expression in the plasma of apoE$^{-/-}$ mice. The results are expressed in terms of fold change with respect to the controls and compared with the microarray results.

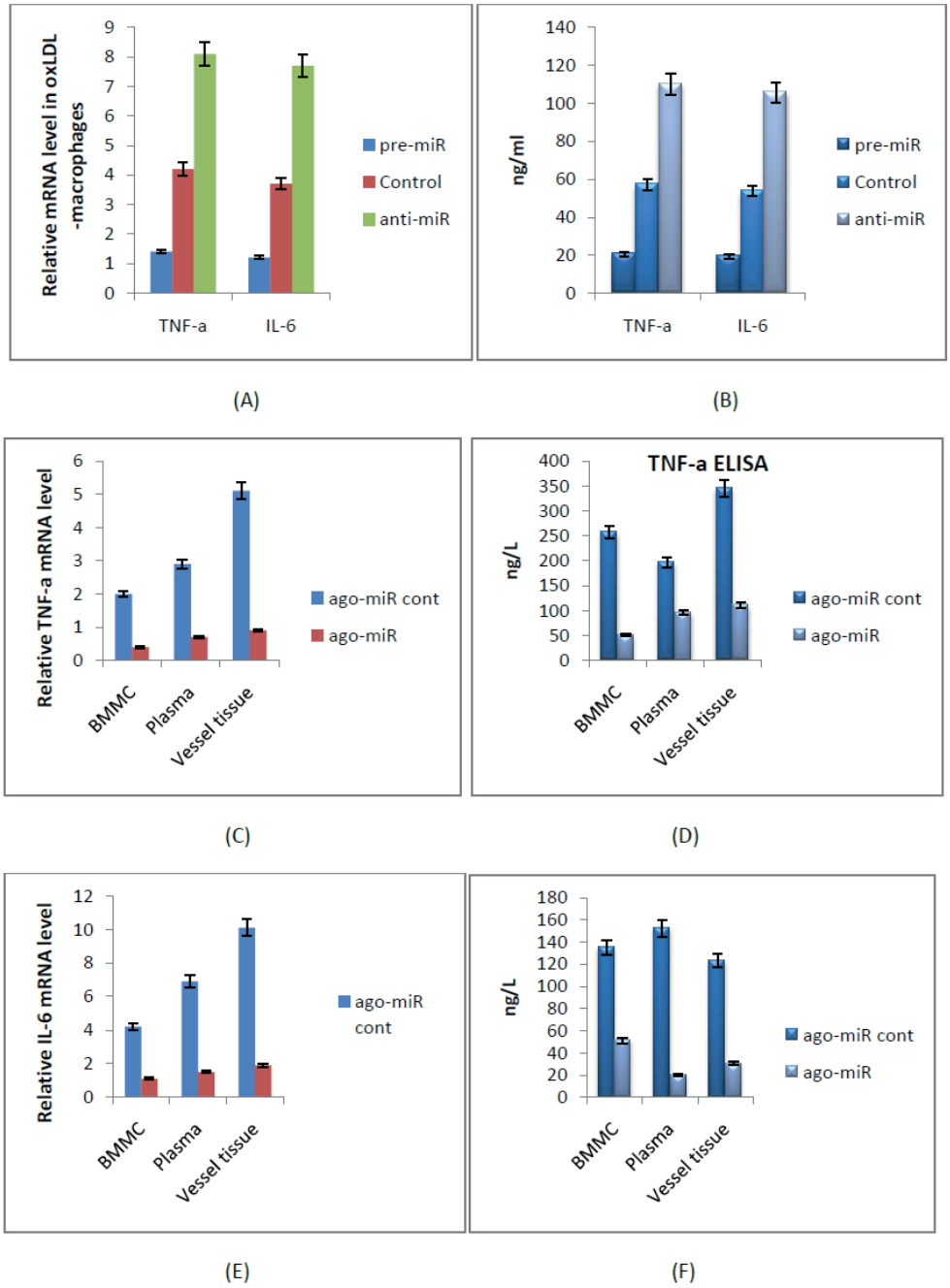

Figure 2. miR-126 regulation of IL-6 and TNF-α secretion. miR-126 transfected THP-1 cells were detected the expression levels of TNF-α and IL-6. ***(A)*** TNF-α and IL-6 relative mRNA levels in oxLDL treated THP-1 cells, where miR-126 was overexpressed. ***(B)*** ELISA to detect protein level of TNF-α and IL-6 ***(C)*** and ***(E)*** TNF-α and IL-6 relative mRNA levels in BMMCs, plasma, and vascular tissues. ***(D)*** and ***(F)*** TNF-α and Il-6 protein detection in BMMCs, plasma and vascular tissues of apoE$^{-/-}$ mice. Values are represented as mean±SD, $p<0.05$ versus the control group.

2A, 2B). The *in vivo* effect of miR-126 was studied by inducing agomiR-126 by single tail vein injection which led to a decreased protein levels of IL-6 and TNF-α. The expression of miR-126 and the effect on TNF-α, IL-6 secretion in BMMCs, plasma and vascular tissues of apoE$^{-/-}$ mice was determined by RT-PCR analyses (Figure 2D, 2F). Further, the decrease in cytokine secretion (TNF-α, IL-6) was confirmed by ELISA (Figure 2C and 2E).

miR-126 Overexpression Prevents in vivo Atherosclerosis Development and Progression

The image analysis following the staining of the thoracic aorta with hemolysin and eosin revealed that there is a very slight decrease in the lesions in thoracic aorta treated with miR-126 agomiR. CD68, TNF-α, and IL-6 stained regions showed a significant reduction in the plaque area of mice group induced with miR-126 agomir (Figure 3). These results demonstrate that miR-126 might prove to be effective in reducing the plaque progression in atherosclerosis due to its anti-inflammatory effect.

MAPK Pathway Inhibition by miR-126

In oxLDL induced macrophages, miR-126 inhibitor significantly upregulated the phosphorylation pathways of p38, JNK, and ERK1/2, whereas the mimic had an opposite effect and downregulated the phosphorylation pathways. Also, overexpression of miR-126 resulted in the inhibition of MAPK pathway proteins (p38, ERK1/2, and JNK) (Figure 4).

Determining MAP3K10 as a Putative Target of miR-126

One of the putative targets of miR-126 could be MAP3K10 identified with bioinformatics tools in different databases (picTar, miRwalk, miRanda). However, the effect of miR-126 in an anti-inflammatory response associated with atherosclerosis has not been reported. MAP3K10 expression regulation by miR-126 was determined to perform luciferase reporter assay. The activity of the cells cotransfected with miR-126 was increased and decreased when compared to the plasmid co-transfected cells (Figure 5A). The endogenous MAP3K10 mRNA levels following miR-126 transfection were measured using qPCR. MAP3K10 expression was increased with miR-126 inhibition, whereas decreased with the miR-126 mimic compared to the control cells (Figure 5B). The *in vivo* results were also consistent in plasma, BMMCs, and tissues (Figure 5C). MAP3K10 protein levels also showed a similar change (Figure 5D). Further, immunohistochemical analysis confirmed the expression of MAP3K10 in miR-126 induced mice (Figure 5E).

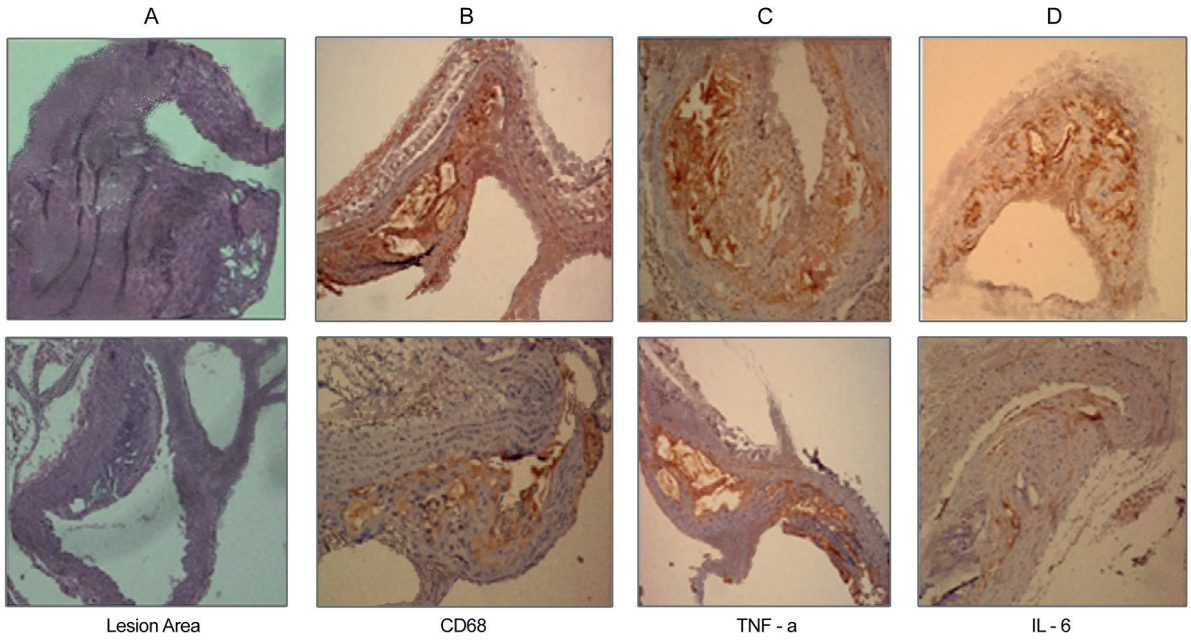

Figure 3. Inducing miR-126 has a direct effect on suppressing atherosclerotic plaque progression, reducing the accumulation of macrophages and also drops the IL-6 and TNF-α levels in apoE$^{-/-}$ mice. ***(A)*** Cross-sections stained with HE from apoE$^{-/-}$ mice infused with agomiR control or agomiR-126-infused. ***(B)*** Reduction in macrophage accumulation represented by CD68 marker expression. ***(C)***, ***(D)*** Reduced expression of TNF-α and IL-6 in miR-126 induced mice compared to ago-miR control infused group.

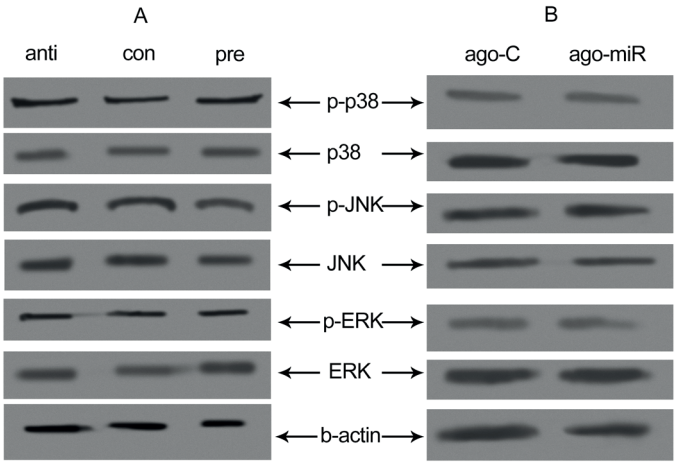

Figure 4. Effect of miR-126 on MAPK signalling pathway confirmed in oxLDL-macrophages as well as in apoE$^{-/-}$ mice. Western blot assays to determine the phosphorylation of p38, JNK, and ERK1/2 pathways. ***(A)***. Protein levels in PMA induced THP-1 cells infused with anti-miR-126, control cells, and pre-miR-126. ***(B)*** Western blot assay in thoracic aorta of apoE$^{-/-}$ mice.

Discussion

Atherosclerotic plaque progression is driven by inflammation leading to lesion growth through the action of macrophages and T-lymphocytes releasing cytokines[1,2]. miRNAs have become an integral part in determining the mechanisms associated with atherosclerosis that is aberrantly expressed and also act as biomarkers[6]. The present study detected the expression of 10 miRNAs in apoE knockdown mice and qRT-PCR confirmed the differential expression of five selected miRNAs (miR-17-5p, miR-126, miR-370, miR-122, miR-181a) (Table I, Figure 1). miR-126 showed the greatest fold change. Hence, it was interesting to explore the novel mechanisms behind the activity of miR-126 in atherosclerosis. A greater wealth of evidence suggests the involvement of miR-126 in association with the inflammatory responses[7,8]. However, its activity with respect to MAPK signalling pathway in atherosclerosis has not been studied.

Recent studies have indicated the multifunctional role of miR-126 in inflammation, immunity and also in cardiovascular diseases[8]. In the current study, we have demonstrated the anti-atherogenic effect of miR-126 *in vitro* and *in vivo*. AgomiR used in the study was cholesterol conjugated as it is directly involved in the human disease cause and pathway enabling easier dose control and rendering effective overexpression of target miRNA *in vivo* conditions[17,18]. miR-126 overexpression poses beneficial effects by decreasing the proinflammatory cytokine expression (Figure 2) and reducing the accumulation of macrophages in atherosclerotic lesions. To further elucidate the effect of miR-126 on the molecular mechanism of atherosclerosis, MAP3K10 was found to be a potential target of miR-126 verified through luciferase reporter assay (Figure 5A). MAP3K10 expression at mRNA and protein level in THP-1 macrophages and apoE$^{-/-}$ mice was also found to be under the regulation of miR-126 by inducing miRNA mimics and inhibitors.

MAP3K10 is associated with a mixed lineage of kinases that function with JNK signalling pathway and is involved in the phosphorylation of several proteins that are important in inflammatory and immune responses[19]. MAP3K10 also regulates endocytic functions[20]; hence, taken together, MAP3K10 has a significant role in atherosclerotic processes. Effect of miR-126 and MAP3K10 on inflammatory response was further investigated by determining the release of TNF-α and IL-6 *in vitro* and *in vivo*. miR-126-5p has been previously reported to limit atherosclerosis by increasing the endothelial cell proliferation and suppressing Notch1 inhibitor delta-like 1 homolog (Dlk1)[7]. Studies have revealed the anti-inflammatory effect of miR-126 by promoting angiogenesis by increased endothelial progenitor cell proliferation by regulating the expression of VCAM-1, but its effect on inflammatory cytokines has not been clear[21]. However, the current study focuses on the effect of miR-126 on inflammatory cytokines (TNF-α and IL-6) and the consequent effect on atherosclerosis.

MAPK plays a significant role in the signal transduction and regulation of genes involved in inflammation leading to atherosclerosis. miR-126 has been found to regulate the differentiation of mesenchymal stem cells to endothelial cells through MAPK/ERK pathway activation and also promotes ischemic angiogenesis[22,23]. The direct relation between MAPK and miR-126 in atherosclerosis was not studied and hence, we suggested

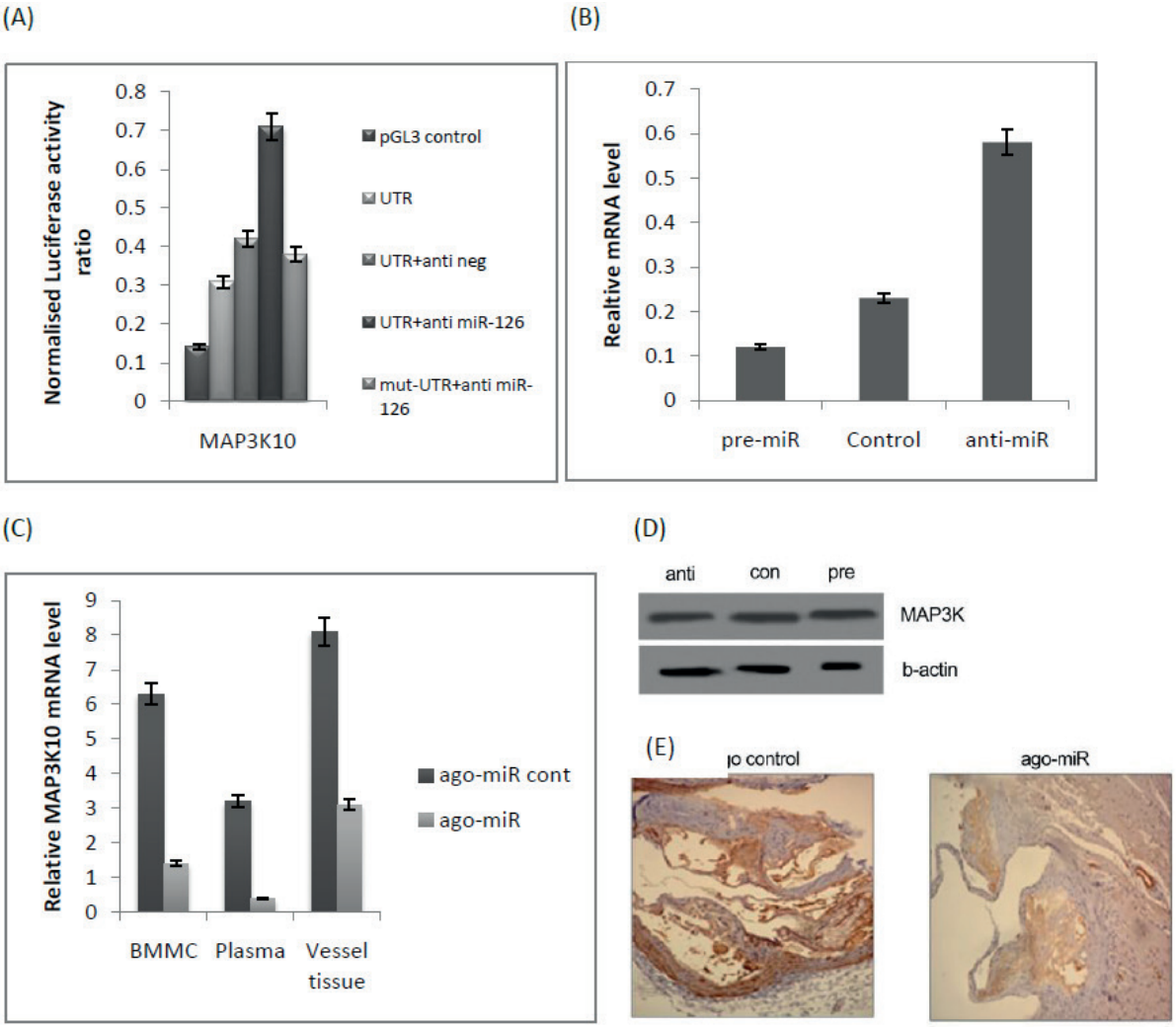

Figure 5. Determining the target of miR-126 along with its expression ***(A)*** Luciferase reporter activity of THP-1 cells cotransfected with anti-negative miRNA and anti-miR-126 as well as with pGL-3 control, p-mutant-MAP3K10-UTR, and p-MAP3K10-UTR. Renilla control's luciferase activity was used to normalize luciferase values. Luminometer evaluated the ratio of each construct's luciferase activity. $p<0.01$ *vs.* control plasmids group. ***(B)*** Effect of miR-126 on MAP3K10 mRNA in TH-P cells ***(C)*** the relative mRNA expression of MAP3K10 in BMMCs, plasma and vessel tissues of $ApoE^{-/-}$ mice following miR-126 treatment ***(D)*** Western blot of MAP3K10 expression in THP-1 induced with anti-miR-126, pre-miR-126 and controls. ***(E)*** Immunohistochemical analyses to determine MAP3K10 expression in aortic lesion following miR-126 treatment in $apoE^{-/-}$ mice.

that MAP3K10 might be a direct target of miR-126 in suppressing inflammation in atherosclerosis. Subsequently, it is proved that p38, JNK, and ERK1/2, three major classes of MAPKs, were activated in anti-miR-126 induced macrophages with reverse effects in cells induced with miR-126 agomir (Figure 4). All the results were confirmed *in vivo* and therefore, corroborates the protective role of miR-126 in atherosclerosis by limiting the excessive production of inflammatory cytokines. These results also strongly reflect one of the possible mechanisms underlying the effect of mir-126 on atherosclerosis by targeting MAP3K10.

Conclusions

This study found the differential expression of a few selected miRNAs in atherosclerosis that might serve to biomarkers. The role of miR-126 in AS through the MAPK signalling pathway was confirmed that has further given mechanistic insights into the atherosclerotic plaque progression.

Conflicts of interest

The authors declare no conflicts of interest.

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