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The effects of miR-467b on lipoprotein lipase (LPL) expression, pro-inflammatory cytokine, lipid levels and atherosclerotic lesions in apolipoprotein E knockout mice

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ABSTRACT

Atherosclerosis is a lipid disorder disease characterized by chronic blood vessel wall inflammation driven by the subendothelial accumulation of macrophages. Studies have shown that lipoprotein lipase (LPL) participates in lipid metabolism, but it is not yet known whether post-transcriptional regulation of LPL gene expression by microRNAs (miRNAs) occurs *in vivo*. Here, we tested that miR-467b provides protection against atherosclerosis by regulating the target gene LPL which leads to reductions in LPL expression, lipid accumulation, progression of atherosclerosis and production of inflammatory cytokines in apolipoprotein E knockout (apoE$^{-/-}$) mice. Treatment of apoE$^{-/-}$ mice with intra-peritoneal injection of miR-467b agomir led to decreased blood plasma levels of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β and monocyte chemotactic protein-1 (MCP-1). Using Western blots and real time PCR, we determined that LPL expression in aorta and abdominal cavity macrophages were significantly down-regulated in the miR-467b agomir group. Furthermore, systemic treatment with miR-467b antagomir accelerated the progression of atherosclerosis in the aorta of apoE$^{-/-}$ mice. The present study showed that miR-467b protects apoE$^{-/-}$ mice from atherosclerosis by reducing lipid accumulation and inflammatory cytokine secretion via downregulation of LPL expression. Therefore, targeting miR-467b may offer a promising strategy to treat atherosclerotic vascular disease.

1. Introduction

Atherosclerosis is the major factor leading to cardiovascular complications, and macrophage-derived foam cells contribute to the initiation and progression of atherosclerosis [1]. Hypertriglyceridemia is known to be an independent risk factor for cardiovascular disease, and the prevention of high triglyceride levels is an important therapeutic goal [1,2]. Lipoprotein lipase (LPL) is a 55-kDa heparin binding glycoprotein, and it anchors to the capillary endothelium, where it hydrolyzes plasma lipoprotein triglycerides into free fatty acids and glycerol, and converts very-low-density lipoprotein (VLDL) to low-density lipoprotein (LDL) [3,4]. The relationship of LPL to the risk of atherosclerosis has long been controversial. Excess expression of systemic LPL is anti-arteriosclerotic [5]. In contrast, LPL in macrophages has been reported to be pro-arteriosclerotic, and specifically excess expression of LPL in macrophages stimulates atheroma formation [6]. One possible explanation for the pro-arteriosclerotic properties of macrophage LPL is that LPL induces foam cell formation and gene expression of inflammatory factors. It has been reported that LPL-mediated hydrolysis of VLDL plays an important role in VLDL-induced IL-1β gene expression and foam cell formation in macrophages [7].

MicroRNAs (miRNAs) are a novel class of small RNAs that negatively regulate gene expression via repression of the corresponding

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target mRNAs [8,9]. To date, several miRNAs have been found to have roles in lipid metabolism and atherosclerosis, including miR-33, miR-122, miR-378/378*, miR-758, miR-106 and miR-370 [10–18]. However, the role of miR-467b in atherosclerosis has not yet been fully elucidated, and there are no reports describing the effects of miR-467b *in vivo*.

A study by Loke and colleagues found that certain polyphenols were able to reduce atherosclerotic lesion size in apoE$^{-/-}$mice [19]. Subsequent studies by Milenkovic et al. found that hepatic miR-467b expression was upregulated in these same mice, but polyphenols enriched diets appeared to downregulate a number of miRNAs, including miR-467b [20]. Most recently, our group [21] and others (Ahn et al.) identified that miR-467b targets hepatic LPL [22]. Studies by Ahn and colleagues showed that mice fed with a high fat diet showed significantly reduction in miR-467b levels and increased hepatic levels of LPL. These studies further indicated that miR-467b regulates lipid metabolism in obese mice by affecting hepatic LPL expression [21–23]. However, whether miR-467b plays a role in the development of atherosclerosis *in vivo* via direct by targeting of LPL mRNA remains unclear.

In this study, we determined whether miR-467b expression promotes atherosclerotic lesions from apoE$^{-/-}$ mice. We hypothesize *in vivo* miR-476b over-expression inhibits LPL expression in macrophages by targeting the LPL mRNA. Accordingly, the inhibition of miR-467b promoted atherosclerotic lesion formation and increased LPL expression in lesion-associated macrophages. Taken together, these data demonstrate a crucial role for miR-476b in atherosclerosis and the inflammatory response of apoE$^{-/-}$ mice.

2. Materials and methods

2.1. Animal models

ApoE$^{-/-}$ mice on a C57BL/6 background were obtained from Beijing University of Medicine Laboratory. All experimental procedures were approved by the institutional animal care. Male 8-week-old apoE$^{-/-}$ mice were randomly divided into four groups: miR-467b agomir negative control (AG-NC), miR-467b agomir (AG), miR-467b antagomir negative control (AN-NC) and miR-467b antagomir (AN). All mice from different groups received tail vein injections of miRNA agomir/antagomir or their respective controls for 10 days after starting the high fat/high cholesterol Western diet (15% fat wt/wt, 0.25% cholesterol wt/wt). After 4 weeks in the Western diet, animals were sacrificed. Blood was collected and assayed for plasma lipids, and inflammatory cytokine expression using commercial available enzymatic methods and ELISA kits, respectively. LPL mRNA and protein expression in aorta and abdominal cavity macrophages were detected by real time (RT)-PCR and Western blot, respectively. Quantification of lesions in aortic sinus was performed by hematoxylin and eosin (HE) staining; Lipid accumulation in aorta and aortic sinus was evaluated by Oil Red O staining. All animal procedures were approved by the University of South China Medicine Animal Care and Use Committee.

2.2. Western blot analyses and RT-PCR analysis

Aortas and abdominal cavity macrophages were lysed for extraction of protein using RIPA buffer containing proteinase inhibitor cocktails (Sigma) or for purification of RNA using the RNeasy Mini Kit (QIAGEN). Protein expression of LPL was examined using Western blot and mRNA expression was examined using RT-PCR. Further details of primer pairs and Western blot technique can be found in our previously publication [21]. In brief, 20 μg of protein lysates from isolated aortic tissue or from peritoneal macrophages were used in each lane. The resulting blots were probed with primary antibodies against LPL or beta actin (1:1000, Sigma) followed by incubation with the appropriate secondary antibodies (1:2000, Sigma). Protein signals were visualized by chemiluminescence and quantified by densitometry.

2.3. Oil red O stain of lesions in aortic sinus

To quantify atherosclerosis at the aortic root, mice hearts were sectioned and stained with Oil red O. The heart was oriented so that the three valves of the aortic root were in the same plane and 12 μm sections placed onto glass slides were stained with Oil red O. Lesion area was quantified in every fourth section, and the average was reported for five measurements.

2.4. Lipid analysis

Mice were fasted for 12–14 h before blood samples were collected by retro-orbital venous plexus puncture. Plasma was separated by centrifugation and stored at −80 °C. Total plasma cholesterol and lipid were enzymatically measured with the Amplex red cholesterol assay kit (Molecular Probes, Invitrogen), according to the manufacturer's instructions. The sterol analyses were performed using an HPLC system (Model 2790, controlled with Empower Pro software; Waters Corp., Milford, MA). Analysis of cholesterol was performed after elution and detection by absorbance at 210 nm.

2.5. Cytokine ELISA

The quantitation of secreted pro-inflammatory cytokines was performed by ELISA (BioSource). IL-6, TNF-α and MCP-1 were analyzed from aliquots of conditioned medium from lesions in aortic sinus and abdominal cavity macrophages according to the manufacturer's instructions.

2.6. Statistical analysis

All data are expressed as means ± standard deviation (SD) of at least three independent experiments. Statistically significant differences among groups were analyzed by one-way analysis of variance (ANOVA) or Student's *t*-test using SPSS 18.0 software. A difference with a $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Association between miR-467b and lipid levels in apoE$^{-/-}$ mice

In our atherosclerosis animal model, we observed significant associations between miR-467b and TG, TC and LDL-C concentrations (Table 1). For example, the mean TG concentrations of

Table 1
Effects of miR-467b on the lipid level of apoE$^{-/-}$ mice ($\overline{X} + S$).

	AG-NC	AG	AN-NC	AN
BW (g)	26.92 ± 3.15	27.81 ± 2.32	28.62 ± 2.99	29.62 ± 3.78
TG (mmol/L)	2.16 ± 0.53	1.36 ± 0.32*	2.32 ± 0.39	3.29 ± 0.59#
TC (mmol/L)	15.92 ± 3.21	12.15 ± 3.89*	15.89 ± 3.52	18.99 ± 3.12#
HDL-C (mmol/L)	3.56 ± 1.12	2.25 ± 0.79*	3.52 ± 0.92	5.12 ± 0.89#
LDL-C (mmol/L)	12.10 ± 1.16	10.15 ± 2.02*	12.15 ± 1.19	14.23 ± 2.16#

$n = 10$, AG-NC: miR-467b agomir negative control, AG: miR-467b agomir, AN-NC: miR-467b antagomir negative control, AN: miR-467b antagomir, BW: body weight, TG: triglyceride, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol. Plasma from different experimental groups was measured enzymatically. The data are the means ± SEM from the indicated numbers of male apoE$^{-/-}$ mice in each group.

* $P < 0.05$ *vs.* AG-NC.

\# $P < 0.05$ *vs.* AN-NC.

Table 2
Effects of miR-467b on inflammatory cytokine production in the blood of apoE$^{-/-}$ mice ($\overline{X}+S$).

Cytokine	AG-NC	AG	AN-NC	AN
IL-6 (pg/mL)	372.8 ± 19.9	229.6 ± 23.5*	379.6 ± 29.2	476.3 ± 30.1#
IL-1β (pg/mL)	335.3 ± 37.5	203.9 ± 30.2*	328.2 ± 27.9	439.3 ± 30.6#
TNF-α (pg/mL)	479.6 ± 53.2	289.5 ± 32.6*	468.3 ± 50.1	581.6 ± 43.9#
MCP-1 (pg/mL)	229.2 ± 30.5	159.3 ± 29.2*	232.0 ± 35.2	323.5 ± 33.3#

n = 10, AG-NC: miR-467b agomir negative control, AG: miR-467b agomir, AN-NC: miR-467b antagomir negative control, AN: miR-467b antagomir, IL-6: Interleukin-6, IL-1β: Interleukin-1β, TNF-α: tumor necrosis factor alpha, MCP-1: monocyte chemotactic factor-1.
* $P < 0.05$ *vs.* AG-NC.
# $P < 0.05$ *vs.* AN-NC.

individuals with the treatment of AG were 1.36 ± 0.32 mmol/L. The corresponding mean TG concentrations were 3.29 ± 0.59 mmol/L with the treatment of AN. Therefore, these findings indicate that increased miR-467b expression is associated with lower lipid levels in apoE$^{-/-}$ mice, and conversely, lower miR-467b levels promote higher lipid levels in mice fed with high fat/high cholesterol Western diet. We then studied the impact of these correlations in atherosclerotic plaque formation (see below).

3.2. miR-467b promotes inflammatory cytokine production in the blood of apoE$^{-/-}$ mice

Early atherosclerotic lesions in apoE$^{-/-}$ mice are characterized by the increased levels of inflammatory cytokines such as TNF-α, IL-6, IL-1β and MCP-1. Since miR-467b agomir significantly reduced lipid levels, reflecting decreased inflammatory processes, we therefore hypothesized that miR-467b and its target gene could confer anti-inflammatory effects. The expression of inflammatory cytokines in plasma was greatly reduced after transfection with AG, compared with AG-NC. (Table 2). Conversely, AN increased the expression of TNF-α, IL-6, IL-1β and MCP-1 in the blood of apoE$^{-/-}$ mice (Table 2). These results indicate that miR-467b over-expression could produce anti-inflammatory effects by inhibiting the production of proinflammatory cytokines.

3.3. Effects of miR-467b on the atherosclerosis

We hypothesized that the miR-467b would promote removal of cholesterol from vessel wall foam cells, leading to plaque regression. To test this hypothesis, we first measured the surface area of atherosclerotic plaque relative to total surface area in formalin-perfused aortas. AG treated mice had a statistically significant reduction in atherosclerotic plaque formation in comparison to AG-NC, Conversely, AN increased plaque in comparison to AN-NC (Fig. 1). These results suggest that atherosclerotic plaque can be diminished by miR-467b in apoE$^{-/-}$ mice.

We then examined atherosclerotic plaque development by aortic root HE stained lesions, and found that AG mice developed significantly less than AG-NC mice (Fig. 2A and B). In contrast, as shown in (Fig. 2C and D), aortic root lesions of AN mice contained more lesion area than AN-NC mice. These results demonstrate that miR-467b reduces atherosclerosis progression in apoE$^{-/-}$ mice.

To further investigate the effects of miR-467b on atherosclerotic plaque, we characterized markers of lesion composition.

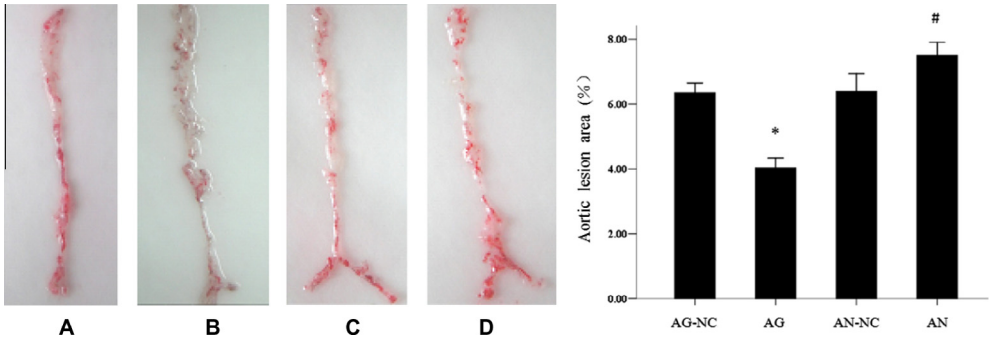

Fig. 1. Effects of miR-467b on the atherosclerotic lesion area in apoE$^{-/-}$ mice. Male 8-week-old apoE$^{-/-}$ mice were given a tail vein injection with miR-467b agomir (AG) or miR-467b antagomir (AG-NC), miR-467b antagomir (AN-NC) or miR-467b antagomir (AN) all at 80 mg/kg body wt/d. (AG-NC) Mice were injected for a total of 10 days and remained in high fat/high cholesterol Western diet until after 4 weeks. Representative Oil-red-O staining of aorta are shown for AG-NC (A), in AG (B), in AN-NC (C) and in AN (D). (n = 10 per group). Original magnification: 40×. Data are representative of three separate experiments with triplicate samples. *$P < 0.05$ *vs.* AG-NC. #$P < 0.05$ *vs.* AN-NC.

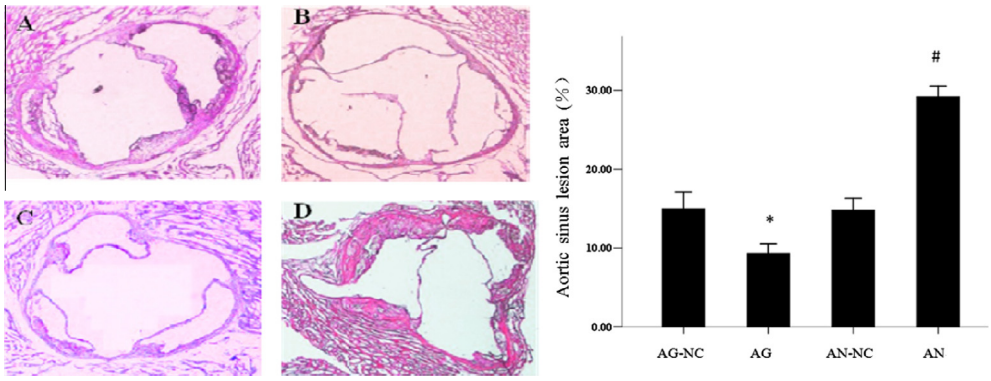

Fig. 2. Effects of miR-467b on the aortic sinus lesion area in apoE$^{-/-}$ mice. Characterization of aortic sinus atherosclerotic lesion area by HE staining in AG-NC (A), in AG (B), in AN-NC (C) and in AN (D). Images of representative sections from every group (n = 10 mice/group) are shown with the accompanying summary bar charts. Original magnification: 10×. Total HE positive area was determined using ImageJ. All of the data represent the mean ± SD from three separate experiments with triplicate samples. *$P < 0.05$ *vs.* AG-NC. #$P < 0.05$ *vs.* AN-NC.

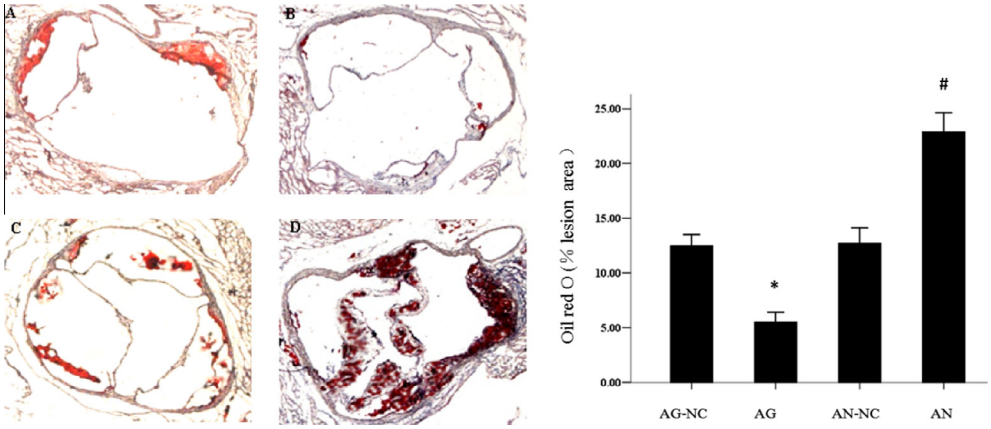

Fig. 3. Effects of miR-467b on the aortic sinus lesion composition in apoE$^{-/-}$ mice. (A) Characterization of aortic sinus atherosclerotic lesion area by Oil red O staining in miR-467b agomir negative control treated mice. (B) Characterization of aortic sinus atherosclerotic lesion area by Oil red O staining in miR-467b agomir treated mice. (C) Characterization of aortic sinus atherosclerotic lesion area by Oil red O staining in miR-467b antagomir negative control treated mice. (D) Characterization of aortic sinus atherosclerotic lesion area by Oil red O staining in miR-467b antagomir treated mice. Original magnification: 10×. Total Oil red O positive area was determined using ImageJ. All of the data represent the mean ± SD from three separate experiments with triplicate samples. $^{*}P < 0.05$ *vs.* AG-NC. $^{\#}P < 0.05$ *vs.* AN-NC.

Table 3
Effects of miR-467b on inflammatory cytokine expression in aorta of apoE$^{-/-}$ mice ($\overline{X} + S$).

Cytokine	AG-NC	AG	AN-NC	AN
IL-6 (pg/mL)	159.2 ± 25.2	93.5 ± 28.3*	162.1 ± 20.1	275.5 ± 29.2#
IL-1β (pg/mL)	122.3 ± 27.2	65.9 ± 28.5*	129.5 ± 25.3	210.1 ± 29.3#
TNF-α (pg/mL)	272.3 ± 31.5	163.9 ± 30.2*	270.1 ± 30.1	389.3 ± 35.7#
MCP-1 (pg/mL)	85.2 ± 30.3	61.2 ± 29.2*	80.9 ± 28.5	129.2 ± 30.2#

* $P < 0.05$ *vs.* AG-NC.
\# $P < 0.05$ *vs.* AN-NC.

Quantification of lipid accumulation in aortic sinus lesions by Oil red O staining demonstrated a significant decrease in lipid accumulation in AG treated mice compared with control (Fig. 3A and B). In contrast, AN treated apoE$^{-/-}$ mice had a significant increase in lesion area compared with control groups (Fig. 3C and D). Taken together, these results indicate that miR-467b agomir treatment promotes regression of atherosclerosis.

3.4. Effects of miR-467b on inflammatory cytokine expression in aorta of apoE$^{-/-}$ mice

Since the profile of plasma cytokines suggested that miR-467b inhibits pro-inflammatory cytokine production in the blood of apoE$^{-/-}$ mice, we measured aortic inflammatory cytokine expression to further explore the effects of miR-467b both on inflammation and atherosclerosis in apoE$^{-/-}$ mice. Compared to AG-NC, AG mice showed significantly decreased aortic IL-6, IL-1β, TNF-α and MCP-1 (Table 3, left panel). Furthermore, the levels of aortic inflammatory cytokine IL-6, IL-1β, TNF-α and MCP-1 were generally higher in AN mice versus control (Table 3, right panel). Therefore, miR-467b reduces expression of multiple aortic zinflammatory cytokines.

3.5. Effects of miR-467b on LPL expression in apoE$^{-/-}$ mice

To determine whether the changes in LPL expression are regulated by miR-467b *in vivo*, we examined LPL expression in isolated aorta of apoE$^{-/-}$ mice. LPL mRNA levels as well as protein expression were significantly lower in AG than AG-NC mice (Fig. 4A and B). Moreover, the data showed that AN resulted in an increase of aortic LPL mRNA expression with concomitant changes at the protein level (Fig. 4A and B). Thus, miR-467b overexpression inhibits LPL expression at the mRNA and protein levels which could account for the atherosclerotic plaque regression.

We next examined the effect of miR-467b on LPL mRNA and protein expression in apoE$^{-/-}$ mice peritoneal macrophages. Treated with AG significantly causing inhibition of LPL mRNA and protein levels (Fig. 4C and D). These results support the hypothesis that miR-467b may regulate the expression of LPL mRNA and protein levels in mouse peritoneal macrophages.

3.6. Effects of miR-467b on lipid accumulation in abdominal cavity macrophages of apoE$^{-/-}$ mice

Finally, we tested lipid levels in abdominal cavity macrophages of apoE$^{-/-}$ mice treated with intra-peritoneal injections of AG or AN. AG treatment reduced total cholesterol (TC) and cholesterol ester (CE). Notably, free cholesterol (FC) was significantly increased in abdominal cavity macrophages (Table 4). Compared with control group, AN treated mice displayed a higher level of TC and CE, but not FC, in abdominal cavity macrophages. These results provide additional evidence that miR-467b may regulate lipid accumulation in macrophages, and also suggests that other mechanisms are also involved in regulating lipid accumulation and its components.

4. Discussion

Recent studies demonstrated a causal association between elevated cholesterol and low-grade inflammation with cardiovascular disease, obesity and type 2 diabetes [24–26]. Based on this, the pursuit of novel therapies to target cardiovascular risk has focused on regulating lipid metabolism and inflammation at the same time [27]. In this study we found that miR-467b reduced lipid accumulation and inflammatory cytokine secretion by targeting the LPL gene in apoE$^{-/-}$ mice. This is the first demonstration that miR-467b can reduce atherosclerotic plaque *in vivo*.

Dysfunction of LPL leads to increased plasma cholesterol and triglyceride levels, which are closely associated with atherosclerosis and coronary artery disease [28–34]. Since the pioneering studies in *Caenorhabditis elegans*, microRNAs have emerged as critical regulators of many biological processes and diseases [35]. Recent advances in the understanding of lipid metabolism have revealed that miRNAs, including miR-33, miR-122, miR-378/378* and miR-758 play key roles in regulating cholesterol metabolism

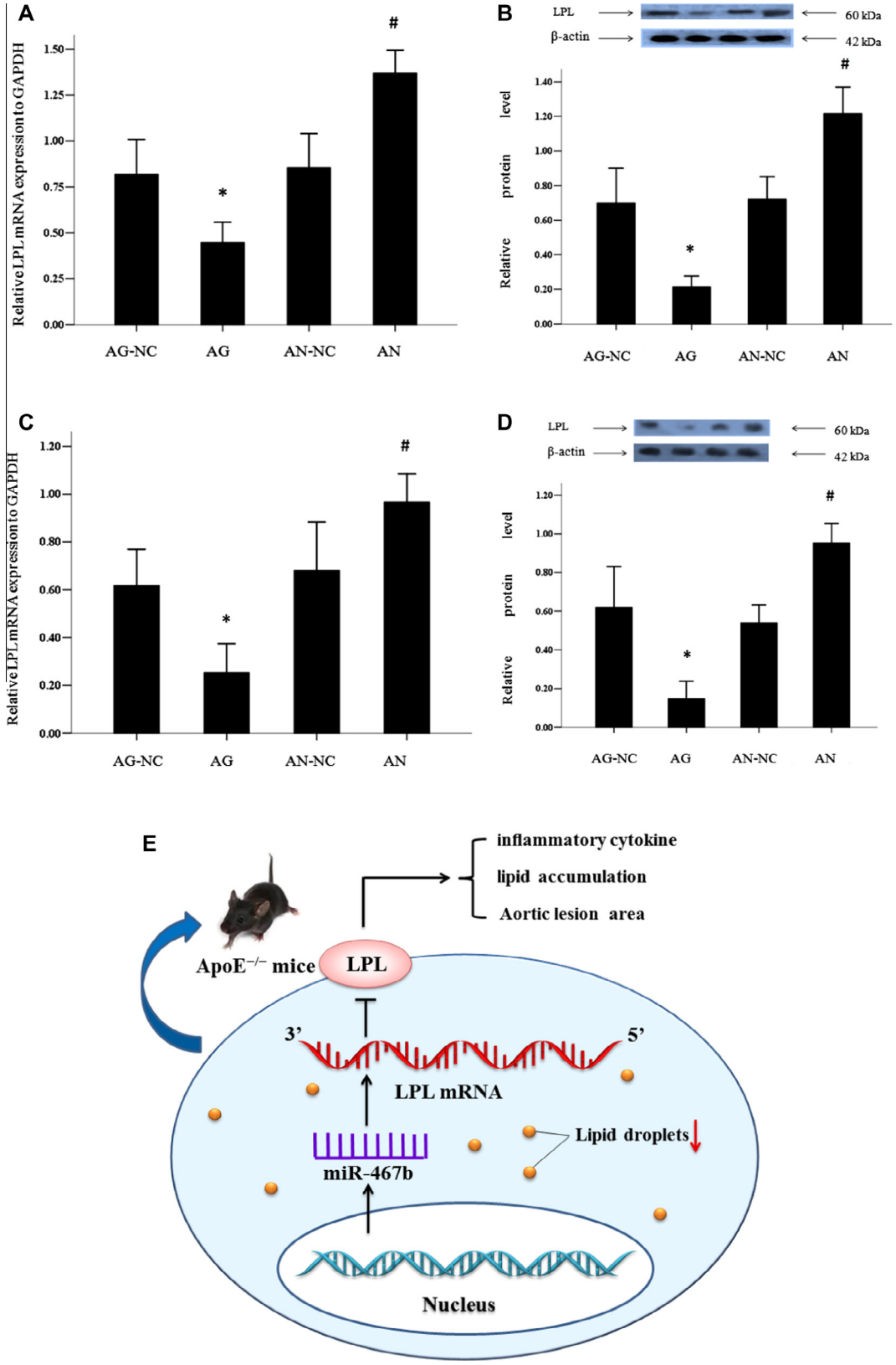

Fig. 4. Effects of miR-467b on LPL expression in aortic in apoE$^{-/-}$ mice. (A) LPL mRNA expressions in aortic tissues of apoE$^{-/-}$ mice were analyzed by qRT-PCR. (B) LPL expressions in aortic tissues of apoE$^{-/-}$ mice were analyzed by Western blotting. (C) LPL mRNA expressions in peritoneal macrophages of apoE$^{-/-}$ mice were analyzed by qRT-PCR. (D) LPL expressions in peritoneal macrophages of apoE$^{-/-}$ mice were analyzed by Western blotting. (E) Model of miR-467b regulates LPL in apoE$^{-/-}$ mice. LPL can promote lipid accumulation and proinflammatory cytokine secretion. When treated with miR-467b in apoE$^{-/-}$ mice, miR-467b can down regulate the expression of LPL by targeting the LPL 3′UTR to facilitate mRNA degradation or translational repression, and then reduce plaque formation, and lesion area in the aortic sinus, as well as decrease lipid accumulation and inflammatory cytokine secretion in apoE$^{-/-}$ mice. All the results are expressed as mean ± SD from three independent experiments, with triplicate samples. $^{*}P < 0.05$ *vs.* AG-NC. $^{\#}P < 0.05$ *vs.* AN-NC.

Table 4
Effects of miR-467b on lipid accumulation in abdominal cavity macrophages of apoE−/− mice ($\bar{X}$±S, n = 3).

	Control	AG-NC	AG	AN-NC	AN
TC	505 ± 23	503 ± 30	412 ± 39*	509 ± 35	599 ± 33#
FC	189 ± 33	192 ± 22	239 ± 22*	190 ± 35	145 ± 23#
CE	316 ± 29	311 ± 30	173 ± 32*	319 ± 37	454 ± 35#
CE/TC (%)	62.6	61.8	42.0	62.7	75.8

Unit: (mg/g protein), TC: total cholesterol, FC: free cholesterol, CE: cholesterol ester.
* $P < 0.05$ *vs.* AG-NC or control.
# $P < 0.05$ *vs.* AN-NC or control.

[13,15,16,18]. In the present study, we report a novel miRNA, miR-467b, that regulates the expression of LPL in apoE$^{-/-}$ mice. The association of miRNAs with lipid accumulation and inflammation observed in our study could be explained by regulation of its direct target LPL gene. Importantly, *in vivo* manipulation of miR-467b levels in macrophages regulates the expression of LPL, lipid and inflammatory cytokine levels in peritoneal macrophages. Since lipid metabolism and release of pro-inflammatory cytokines from peritoneal macrophages also correlate closely with coronary artery disease, miR-467b treatment may be useful to prevent atherosclerosis due to macrophage activation.

In conclusion, as shown in (Fig. 4E), our study addresses the link between miR-467b treatment and atherosclerosis by providing evidence that down-regulation of miR-467b is positively associated with the expression of LPL in apoE$^{-/-}$ mice. Our key finding was that miR-467b inhibits LPL expression in aorta and abdominal cavity macrophages of apoE$^{-/-}$ mice, suggesting that miR-467b may have therapeutic implications in the treatment of cardiovascular diseases by inhibiting macrophage LPL. The present study indicated that miR-467b directly targets LPL mRNA to inhibit LPL expression, which leads to a reduction in cellular lipid accumulation, atherosclerosis lesions and inflammatory cytokine expression in apoE$^{-/-}$ mice. We are hopeful that this knowledge will translate to novel therapeutic options for amelioration of atherosclerosis progression. More importantly, since it is well known that macrophage LPL is critical for the generation of foam cells in the atherosclerosis lesion areas of apoE$^{-/-}$ mice. It seems likely that miR-467b agomir might prove an effective therapy by decreasing lipid levels and inflammatory cytokine expression, thus improving the prognosis for patients with cardiovascular disease.

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