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Regression of atherosclerosis in apolipoprotein E-deficient mice by lentivirus-mediated gene silencing of lipoprotein-associated phospholipase A_2

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ABSTRACT

Overexpression of lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is implicated in atherosclerosis. We tested the hypothesis that lentivirus-mediated *Lp-PLA_2* silencing could inhibit atherosclerosis in apolipoprotein E-deficient mice. Sixty eight apolipoprotein E-deficient mice were fed a high-fat diet and a constrictive collar was placed around the left carotid artery to induce plaque formation. The mice were randomly divided into control, negative control (NC) and RNA interference (RNAi) groups. *Lp-PLA_2* RNAi or scrambled NC lentivirus viral suspensions were constructed and transfected into the carotid plaques 8 weeks after surgery; the control group was administered saline. The carotid plaques were assessed 7 weeks later using hematoxylin and eosin, Masson's trichrome and oil red O staining; plasma and lesion inflammatory gene expression were examined using ELISAs and real-time PCR. Seven weeks after transfection, the serum concentration and plaque mRNA expression of Lp-PLA_2 was significantly lower in the RNAi group, and lead to reduced local and systemic inflammatory gene expression. *Lp-PLA_2* RNAi also ameliorated plaque progression, reduced the plaque lipid content and increased the plaque collagen content. The effects of *Lp-PLA_2* RNAi were independent of serum lipoprotein levels, as the triglyceride and total cholesterol levels of the control, NC and RNAi groups were not significantly different. These findings support the hypothesis that lentivirus-mediated *Lp-PLA_2* gene silencing has therapeutic potential to inhibit atherosclerosis and increase plaque stability, without altering the plasma lipoprotein profile.

1. Introduction

Despite intensive management of the conventional atherosclerosis risk factors, many patients continue to experience the onset and recurrence of coronary events. Atherosclerosis is not only a lipid disease, but a complex process intertwined with inflammatory disease [1], which may explain why atherosclerosis still presents a significant risk of morbidity and mortality, despite state of the art therapies such as statins [2].

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2, also termed platelet-activating factor acetylhydrolase, PAFAH) hydrolyzes platelet activating factor (PAF) and oxidizes phospholipids, generating the bioactive lipid mediators lysophosphatidylcholine (LPC) and oxidized non-esterified fatty acid (oxNEFA), which both play a key role in atherosclerosis [3,4]. Experimental and epidemiological studies have demonstrated that Lp-PLA_2 is a useful independent cardiovascular risk marker, which can be used in addition to traditional risk factors [5,6]; therefore, Lp-PLA_2 provides an attractive target for intervention to reduce atherosclerosis.

RNA interference (RNAi) can effectively silence target genes in both dividing and non-dividing cells [7]. However, no research has yet investigated the effects of *Lp-PLA_2* RNAi on the progression of atherosclerosis in an in vivo mouse model. This research is important, as it has been suggested that high-density lipoprotein (HDL)-associated Lp-PLA_2, the predominant form in mice, may be antiatherogenic; whereas low-density lipoprotein (LDL)-associated Lp-PLA_2, which predominates in humans, may be atherogenic. However, other research has suggested an antiatherogenic role for Lp-PLA_2 in mice, as adenovirus-mediated gene transfer of human *Lp-PLA_2* prevented injury-induced neointima formation and spontaneous atherosclerosis in apolipoprotein E-deficient mice [8]. Therefore, the biological role of Lp-PLA_2 in atherosclerosis is controversial; however, most evidence suggests a proatherogenic role for Lp-PLA_2 [2].

To further investigate the role of Lp-PLA_2 in atherosclerosis, we constructed lentivirus vectors (LV) encoding short hairpin RNAs (shRNAs) against mouse Lp-PLA_2, and evaluated the effects of Lp-PLA_2 RNAi in atherosclerosis-prone apolipoprotein E-deficient mice.

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2. Materials and methods

2.1. RNAi Lp-PLA$_2$ lentiviral vectors

Four different sites of the mouse *Lp-PLA$_2$* gene were selected as RNAi targets: 5′-GCAAGCTGGAATTCTCCTTTG-3′ (A), 5′-GGGTCTTAGTATATTTCTTGG-3′ (B), 5′-GGCTCTCAGTGCGATTCTTGA-3′ (C) and 5′-GCAACACTCTCCAGGATCACA-3′ (D). The scrambled shRNA sequence 5′-TTCTCCGAACGTGTCACGT-3′ served as a negative control (NC). The shRNAs were subcloned under control of the human U6 promoter into LV-1-U6-GFP which expresses green fluorescent protein (GFP), as previously described [9]. Third-generation HIV-1-based LVs were prepared using a standard calcium phosphate transfection method in 293T cells. Replication-incompetent LV were produced by co-transfection of 293T cells with shRNA expression vectors and ViraPower packaging mix (genepharma, shanghai, China). Viral supernatant was harvested 48 h after transfection and the titers averaged 1×10^9 TU (transduction units)/ml.

2.2. Cell culture

RAW264.7 mouse macrophage cells were cultured in DMEM containing 10% FBS, penicillin and streptomycin (100 U/ml) at 37 °C in a 5% CO_2 and 95% air humidified atmosphere. Previous studies demonstrated that unstimulated macrophage cells failed to produce detectable levels of Lp-PLA$_2$, while oxidized (ox)-LDL upregulated the expression of Lp-PLA$_2$ in a concentration- and time-dependent manner. In our preliminary cell experiments, the expression of Lp-PLA$_2$ reached the platform stage after 60 μg/ml of oxLDL stimulation. Therefore, we pretreated the cells with 60 μg/ml oxLDL.

2.3. RNAi target screening

After 60 μg/ml of oxLDL pretreatment, the expression of Lp-PLA$_2$ increased sharply. Mouse RAW264.7 cells (90% confluent) were then untransfected or transfected with NC LV or *Lp-PLA$_2$* RNAi LV (multiplicity of infection = 50) to determine their silencing efficiency. It is universally acknowledged that the transfection efficiency of lentivirus reached the platform stage 3–4 days after transfection. GFP provides an efficient, convenient method to monitor transfection efficiency. In our study, GFP fluorescence in the cells was very weak 1 day after transfection, and more fluorescence was detected 2 days after transfection. The most GFP fluorescence was visualized 4 days after transfection, indicating peak transfection efficiency. So mRNA and protein expression of Lp-PLA$_2$ was analyzed by real-time PCR and Western blotting 4 days after transfection.

2.4. Animal protocol

Sixty eight apolipoprotein E-deficient mice received a high-fat diet (0.25% cholesterol and 15% cocoa butter) throughout the experiment. After anesthesia (40 mg/kg pentobarbital sodium *i.p.*) a constrictive Silastic collar (0.30 mm) was placed on the left common carotid artery following the technique of von der Thüsen et al. [10]. The mice were randomly allocated to the control group ($n = 18$), NC group ($n = 32$) or RNAi group ($n = 18$). At the end of week 8, the carotid collars were removed and LV suspension (5×10^7 TU) or PBS (control group) was instilled around the left common carotid artery, incubated in situ for 30 min, and then drawn off before closure of the incision. To check the plaque transfection efficiency, two mice from the NC group were killed every week after the second operation. Cryosections were prepared and viewed by fluorescence microscopy to visualize GFP expression. The remaining 54 animals were sacrificed at week 15. The experimental protocol complied with the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001) and was approved by the Ethics Committee of Zhengzhou University (Zhengzhou, China).

2.5. Histological and morphometric analyses

Mice were perfused with PBS followed by 4% formaldehyde at physiological pressure via the left ventricle, the left common carotid artery segments were dissected, fixed in 4% formaldehyde overnight at 4 °C, embedded in O.C.T. compound and stored at −20 °C. The entire length of the common carotid artery was cross-sectioned (6 μm sections at 50 μm intervals) and stained with hematoxylin and eosin (HE); plaque collagen and lipid deposition were identified using Masson's trichrome and oil red O (ORO) staining, respectively. The site of maximal plaque size was selected for morphological analysis. The images were digitized using an OLYMPUS microscope and an automated image analysis system (Image-Pro Plus 5.0; MediaCybernetics, Silver Spring, MD, USA) was used for quantitative measurements. The positively-stained lipid and collagen areas were quantified by computer assisted color-gated measurement; the positively-stained:intimal area ratio was calculated.

2.6. Plasma lipid and biological analysis

Blood samples were obtained from the retro-orbital plexus, plasma was separated by centrifugation at 1500*g* for 30 min at 4 °C and stored at −80 °C. Lp-PLA$_2$, PAF, LPC, matrix metalloproteinase-2 (MMP-2), total cholesterol (TC) and triglyceride (TG) plasma levels were measured using commercial kits (CoWin-Bioscience, Beijing, China).

2.7. RNA extraction and real-time PCR

Total RNA was extracted from the left common carotid arteries using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The relative levels of *Lp-PLA$_2$*, monocyte chemotactic protein-1 (*MCP-1*), *MMP-2* and *MMP-9* mRNA were quantified using SYBR green on the ABI Prism 7300 Sequence Detection System (Applied Biosystems, CA, USA) by the $2^{-\triangle\triangle CT}$ method using β-actin as an internal control. The forward and reverse primers were: *MCP-1*, 5′-GCTCAGCCAGATGCAGTTAACG-3′ and 5′-TCTTGGGGTCAGCACAGACCTC-3′; *MMP-9*, 5′-CTGAATCATAGAGGAAGCCCAT-3′ and 5′-GTCCAGAGAAGAAGAAAACCC-3′; *Lp-PLA$_2$*, 5′-CCAGAGATTCAGATGTGGAGTT-3′ and 5′-TGGCAGAGTTGATAAAGAGGAG-3′; *MMP-2*, 5′-CTGGGAGCATGGAGATGGATAC-3′ and 5′-GACCGTTGAACAGGAAGGGGAA-3′; β-actin, 5′-GCTATGCTCTCCCTCACGCCAT-3′ and 5′-TCACGCACGATTTCCCTCTCAG-3′.

2.8. Western blot analysis

Equal amounts of protein from RAW264.7 cells were separated on sodium dodecyl sulfate (SDS)–14% polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% nonfat milk, washed with PBS containing 0.1% Tween 20 (TBST), incubated with antibodies against β-actin or Lp-PLA$_2$ at 4 °C overnight, then incubated with secondary antibody conjugated to horseradish peroxidase (1:1000). The blots were visualized using enhanced chemiluminescence; quantitative analysis was performed using ImageJ.

2.9. Data analysis

Data are presented as mean values ± standard deviation (SD). Data were compared among groups using one-way analysis of

variance (ANOVA) followed by the Student–Newman–Keuls (SNK) test for post-hoc comparisons. All statistical analyses were performed using SPSS Version 16.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of lentiviral transfection in vitro and in vivo

RAW264.7 cells had a low expression level of Lp-PLA$_2$ before oxLDL stimulation. After pretreatment with 60 μg/ml oxLDL, Lp-PLA$_2$ expression increased sharply. Gene-silencing analysis demonstrated that the site A lentivirus most effectively blocked Lp-PLA$_2$ mRNA and protein expression (Fig. 1). Throughout the experiments, the mice remained in good health and gene transfer was tolerated very well. GFP provides an efficient, convenient method to monitor transfection efficiency [11]. GFP fluorescence was examined in the carotid artery plaques every week after transfection. Moderate GFP fluorescence was observed one week after transfection, and faint fluorescence was still visible 7 weeks after transfection. The strongest GFP fluorescence was observed 2 weeks after transfection (Fig. 2). Taken together, these results demonstrated that the in vivo transfection was effective.

3.2. Body weight and serum lipid profiles

We found no significant difference in the body weight of the control (27.81 ± 3.62 g), NC (27.19 ± 3.76 g) and RNAi (27.56 ± 3.70 g) groups, demonstrating that RNA interference was safein these animals. Likewise, serum TC and TG levels in the control group (30.25 ± 2.25 and 2.97 ± 0.95 mmol/l, respectively) and NC group (29.23 ± 2.86 and 2.92 ± 0.94 mmol/l) did not differ significantly from the RNAi group (29.58 ± 3.08 and 3.02 ± 0.92 mmol/l), suggesting that the therapeutic effects of gene transfer were independent of the serum lipid profile.

3.3. Effect of RNAi on the plasma concentration of Lp-PLA$_2$ and other inflammatory markers

The RNAi group had lower plasma levels of Lp-PLA$_2$, LPC and MMP-2 than the control and NC groups, whereas the PAF levels were similar in all three groups. The serum levels of Lp-PLA$_2$, LPC

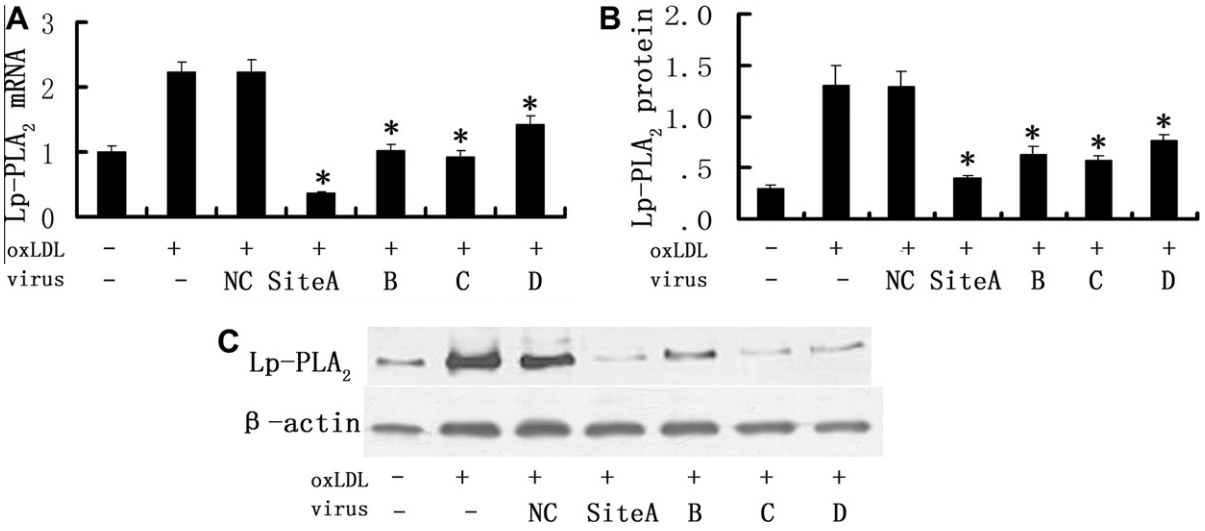

Fig. 1. Silencing of Lp-PLA$_2$ in RAW264.7 cells by LV-Lp-PLA$_2$ shRNAs. Cells were pretreated with 60 μg/ml of oxLDL. Cells were then transduced with MOI 50 of each vector to determine their silencing efficiency. (A) Real-time PCR quantification of Lp-PLA$_2$ mRNA expression in the control, NC and four RNAi groups pretreated with or without oxLDL; after oxLDL pretreatment, the expression of Lp-PLA$_2$ increases sharply. Compared with cells treated with oxLDL alone, cells treated with virus suspension demonstrated significant inhibition of Lp-PLA$_2$ expression. Site A lentiviruses is the most effective. (B and C) Western blotting and result analysis of Lp-PLA$_2$ protein expression. Symbols − and + indicate the absence and presence of oxLDL or virus suspension. $^*P < 0.05$ versus control and NC groups, by one-way ANOVA ($n = 6$).

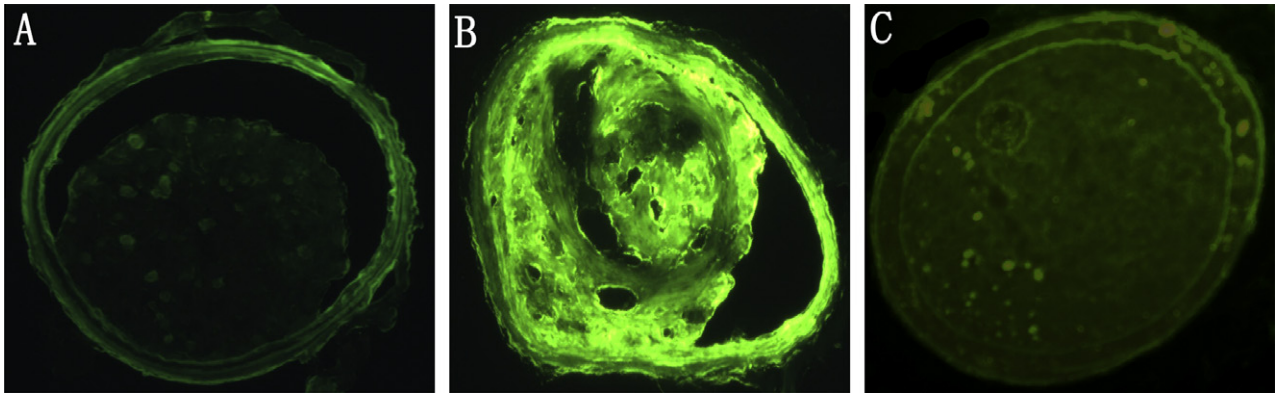

Fig. 2. Efficiency of transfection in carotid plaques. (A)–(C) were fluorescence images of plaques obtained 1 week, 2 weeks and 7 weeks after transfection, respectively. The strongest GFP fluorescence was displayed 2 weeks after transfection. GFP was still visible 7 weeks after transfection. Magnification 200×.

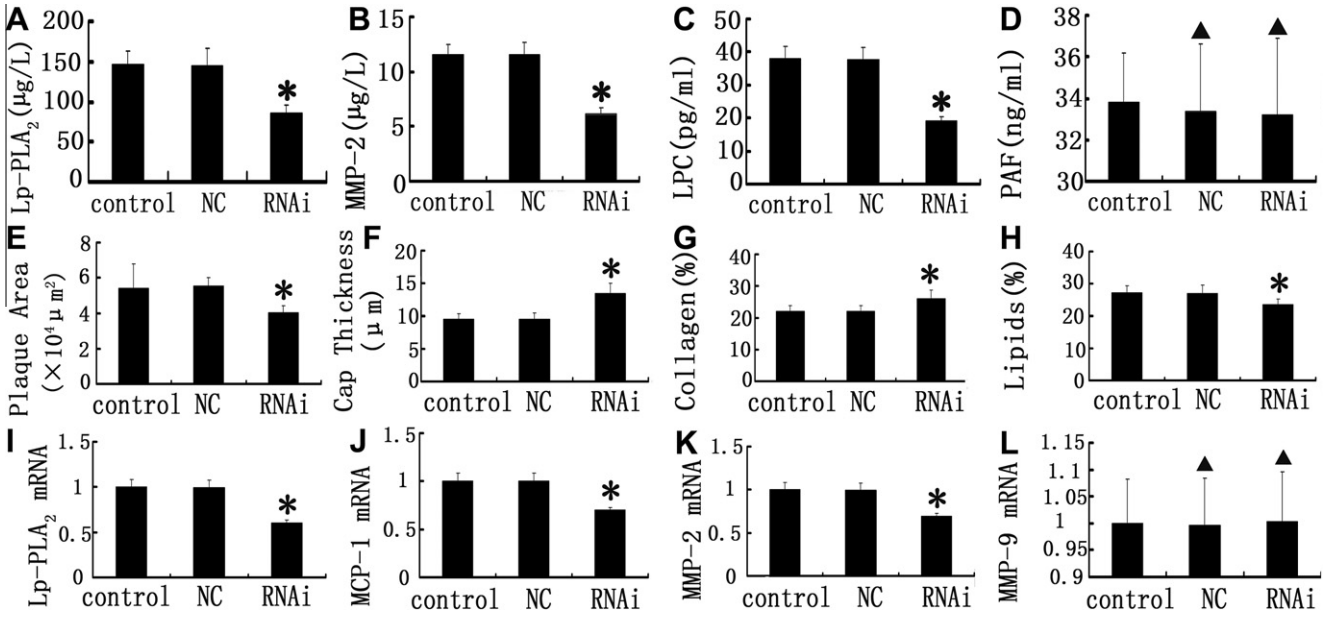

Fig. 3. (A)–(D) are plasma concentration of Lp-PLA_2 and inflammation markers in the control, NC, and RNAi groups. (A) Lp-PLA_2; (B) MMP-2; (C) LPC; (D) PAF. Data were expressed as the mean ± SD (n = 18). (E)–(H) are comparison of plaque area (E) fibrous cap thickness (F) relative collagen content (G) and relative lipid content (H) in the control, NC and RNAi groups. (I)–(L) are carotid plaque mRNA expression of Lp-PLA_2 (I), MCP-1 (J) MMP-2 (K) and MMP-9 (L) in the control, NC and RNAi groups. No significant differences were found between control and NC groups. *P < 0.05 versus control and NC groups; (▲)P > 0.05 versus control group (n = 18). NC: negative control group; RNAi: RNA interference group; Lp-PLA_2; lipoprotein-associated phospholipase A_2; MMP: matrix metalloproteinase; LPC: lysophosphatidylcholine; PAF: platelet activating factor; MCP-1: monocyte chemotactic protein-1.

and MMP-2 did not differ in the control group and NC group (Fig. 3).

3.4. Effect of RNAi on the formation and composition of atherosclerotic plaques

The cross-sectional plaque area was significantly lower in the RNAi group than the control and NC groups (P < 0.01). Fibrous cap thickness was significantly higher in the RNAi group (13.42 ± 1.61 μm) than the control and NC groups (9.53 ± 0.77 and 9.52 ± 0.94 μm, respectively; P < 0.01). No significant difference was observed between the plaque area and fibrous cap thickness of the control and NC groups (Figs. 3 and 4).

The plaque lipid content was significantly lower in the RNAi group (23.46%) than the control and NC groups (27.08% and 26.92%, respectively; P < 0.01). The relative collagen content of the plaques in RNAi group (26.04%) was significantly higher than the control and NC groups (22.06% and 21.26%, respectively; P < 0.01). Plaque area and composition among all groups were not statistically significant before transfection (week 8). This data suggested that LV-mediated silencing of *Lp-PLA_2* effectively attenuated atherosclerotic plaque formation and decreased plaque vulnerability in apolipoprotein E-deficient mice.

3.5. Effect of RNAi on expression of Lp-PLA_2 and other inflammatory genes in plaques

The RNAi group had lower levels of *Lp-PLA_2*, *MCP-1* and *MMP-2* mRNA expression than the control and NC groups (all P < 0.01); *MMP-9* expression was similar in all three groups. Expression of *Lp-PLA_2* mRNA did not differ significantly in the control group and NC groups (Fig. 3).

4. Discussion

We investigated the effect of LV-mediated *Lp-PLA_2* silencing on the progression of atherosclerosis following collar-induced atherosclerosis in apolipoprotein E deficient mice. We demonstrate that *Lp-PLA_2* RNAi attenuated the expression of Lp-PLA_2, ameliorated atherosclerosis and reduced *MCP-1* and *MMP-2* expression, suggesting that *Lp-PLA_2* silencing can exert a potentially therapeutic effect.

Lp-PLA_2 is responsible for the metabolism of oxidized phospholipids to the proatherogenic mediators LPC and oxNEFA [3–4], which contribute to inflammation and render plaques vulnerable to rupture [4,12]. Previous studies demonstrated that darapladib, a selective Lp-PLA_2 inhibitor, ameliorated inflammation and necrotic core formation in animal models of atherosclerosis. However, chronic administration of darapladib did not reduce the primary end point of coronary plaque deformability, nor alter the plasma hs-CRP concentration in a phase II clinical study [12,13]. In summary, experimental and epidemiological evidence remains equivocal about the effects of darapladib. RNAi is a clinically feasible method to down-regulate the expression of target genes efficiently and selectively. However, no research in vivo investigated the effects of Lp-PLA_2 silencing on the progression of atherosclerosis. So we choose to blunt Lp-PLA_2 expression at the mRNA, protein, and functional levels by LV-mediated RNAi. In this study, the efficacy of LV-mediated gene silencing was confirmed by reduced Lp-PLA_2 expression and visualization of GFP fluorescence in the plaques. Additionally, the plaques of the RNAi group had a higher collagen content, lower lipid content, reduced inflammatory gene expression, and thicker fibrous caps than the control and NC groups. Furthermore, the plaque area, inflammatory gene expression and plaque vulnerability were not significantly different in the control and NC groups, excluding the possibility that the effects observed in the RNAi group were due to non-specific immune stimulation induced by transfection. Thus, LV-mediated *Lp-PLA_2* silencing provides a safe, efficient, specific approach which could potentially be used to treat atherosclerosis.

Several lines of evidence suggest that mice have a different lipoprotein profile to humans and are inadequate for studying the role of Lp-PLA_2 in atherosclerosis, as the role of Lp-PLA_2 may depend on its plasma lipoprotein carrier [13]. Lp-PLA_2 is mainly carried by LDL

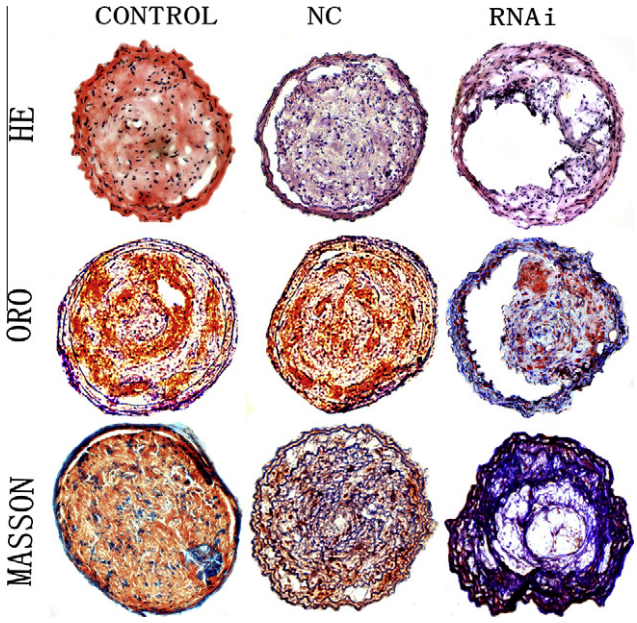

Fig. 4. Histopathologic staining shows plaque composition in the control, NC, and RNAi groups. Cross-sections of carotid plaques from the various groups were stained with HE, masson's trichrome and ORO respectively. Magnification 200×.

in humans, whereas HDL is the preferred carrier in mice. HDL-associated Lp-PLA_2 may contribute to reduce atherosclerosis, whereas LDL-associated Lp-PLA_2 may stimulate atherosclerosis [5]. Controversy exists regarding this process [14,15]. Lp-PLA_2 is thought to exert an antiatherogenic and anti-inflammatory effect by hydrolyzing and inactivating PAF, a typical pro-inflammatory factor which contributes to inflammation and atherosclerosis [16]. However, individuals with reduced levels of Lp-PLA_2 activity do not display a rampant inflammatory response due to uncontrolled PAF accumulation [13], nor an acute bronchoconstriction response to PAF inhalation [13,17,18]. Moreover, there is no evidence that Lp-PLA_2 hydrolyzes PAF in vivo. In a recent study by Hu et al., Lp-PLA_2 inhibition using darapladib did not alter serum PAF levels [16]. Similarly, intravenous administration of human recombinant Lp-PLA_2 failed to alter PAF-mediated responses or mortality in patients with asthma or septic shock [17,19]. Additionally, Liu et al. [13] indicated that circulating PAF is primarily cleared by PAF receptor-independent transport in vivo, rather than intravascular Lp-PLA_2-mediated hydrolysis [5,18]. From the traditional point of view, Lp-PLA_2 may be antiatherogenic if it degrades PAF [14]. Therefore, we hypothesize that the development of atherosclerotic plaques is determined not only by the total amount of PAF, but also by the degradation products of oxidized phospholipids such as LPC. Consequently we investigated the concentration of PAF and LPC in mice. We observed no difference in the plasma levels of PAF amongst the three experimental groups. However, the RNAi group had lower serum levels of LPC, despite the fact that the PAF levels were similar in all groups; suggesting that the equilibrium between the degradation and generation of proatherogenic phospholipids may favor a proatherogenic role for Lp-PLA_2. Indeed, most research favors a proinflammatory and proatherogenic role for Lp-PLA_2 [14,15].

In the present study, *Lp-PLA_2* RNAi reduced inflammation by attenuating expression of *MCP-1*, *LPC* and *MMP-2*. Inflammation is a major component of atherosclerosis [3]. LPC stimulates macrophage proliferation, upregulates tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, implying a complex interaction between Lp-PLA_2 and other inflammatory mediators [20]. MCP-1 is responsible for the recruitment of monocytes to inflammatory lesions and MMP-2 is expressed in plaque macrophage-rich areas, especially the cap shoulder region, which promotes weakening of the fibrous cap [21,22]. Our results are in line with the work of Kuzuya et al. which indicated that MMP-2 deficiency reduces atherosclerosis in mice [23]. However, we did not observe different *MMP-9* expression in the RNAi, NC and control groups, and further research is needed to explain this phenomenon.

Gene silencing of *Lp-PLA_2* ameliorated the progression of atherosclerosis in apolipoprotein E-deficient mice, in accordance with previous studies which associated increased plasma Lp-PLA_2 levels with inflammation and atherosclerosis [14,24]. However, our results differ from Quarck et al. who reported that adenovirus-mediated *Lp-PLA_2* gene transfer prevented injury-induced neointima formation and reduced spontaneous atherosclerosis in apolipoprotein E-deficient mice [8,25]. These contradictions may be due to the varied penetrance of pro-atherogenic stimuli in different strains of mice. Similar site-specific effects for pro-atherogenic stimuli are not uncommon in LDL receptor-knockout ($LDLR^{-/-}$) mice [26], which probably reflect distinct mechanisms operating at different arterial sites. For example, local hemodynamic forces at different arterial sites exert distinct effects on endothelial cell proatherogenic and vasoprotective factor gene expression [27]. In addition, strain-specific differences in the susceptibility of mice to injury-induced neointima formation and diet-induced atherosclerosis vary: some strains susceptible to atherosclerosis are resistant to neointima formation, and vice versa [28]. Furthermore, distinct temporal roles for Lp-PLA_2 during lesion pathogenesis cannot be ruled out; further studies are needed to clarify these issues.

The limitations of this study should be considered. Collar-induced carotid atherosclerosis in apolipoprotein E-deficient mice does not fully resemble the process of human plaque rupture and intraluminal thrombosis. Spontaneous plaque disruption has been reported in the brachiocephalic arteries of apolipoprotein E-deficient and $LDLR^{-/-}$ mice; however, the frequency of plaque disruption in these strains is not high enough to allow interventional studies. Additionally, we merely measured the end-point serum concentrations of Lp-PLA_2, which may not reflect changes in Lp-PLA_2 over time. Further study is needed to clarify these details.

In conclusion, LV-mediated RNAi effectively knocked down *Lp-PLA_2* gene expression in apolipoprotein E-deficient mice, resulting in decreased plaque area and reduced plaque vulnerability, independent of the plasma lipoprotein profile. Our work indicates that LV-mediated gene silencing of Lp-PLA_2 may provide a novel therapeutic approach for the treatment of vulnerable plaques.

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