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MicroRNA-3188 targets ETS-domain protein 4 and participates in RhoA/ ROCK pathway to regulate the development of atherosclerosis

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We aimed to elucidate the roles and regulatory mechanism of miR-3188 in oxidized low-density lipoprotein (ox-LDL)-induced cell injury in THP-1 derived macrophages, thus providing a new insight for the treatment of atherosclerosis (AS). A total of 85 AS patients and 45 healthy controls were enrolled. The levels of miR-3188 and lipoprotein-associated phospholipase A2 (Lp-PLA$_2$) in AS patients and healthy controls were detected. Then ox-LDL was used to treat human THP-1 derived macrophages. The effects of overexpression and suppression of miR-3188 on regulating ox-LDL-induced cell injury in THP-1 derived macrophages were investigated. Additionally, the potential target of miR-3188 was identified, which was verified by luciferase reporter assay. Besides, the relationship between miR-3188 and RhoA/ROCK pathway was explored. miR-3188 was downregulated in AS patients, while the levels of Lp-PLA$_2$ in AS patients were increased. Ox-LDL significantly induced cell injury by decreasing cell viability, inducing cell apoptosis and increasing the production of inflammatory cytokines, including IL-1β, IL-6, MCP-1 and TNF-α. In addition, miR-3188 was significantly downregulated after ox-LDL treatment. Overexpression of miR-3188 alleviated ox-LDL-induced cell injury, while inhibition of miR-3188 had opposite effects. ETS-domain protein 4 (ELK4) was a target of miR-3188. The effects of miR-3188 inhibition on ox-LDL-induced cell injury were markedly reversed by knockdown of ELK4. Besides, inhibition of miR-3188 enhanced ox-LDL-activated RhoA/ROCK pathway, while knockdown of ELK4 suppressed this pathway. Downregulation of miR-3188 may contribute to AS development via negatively regulating Lp-PLA$_2$, targeting ELK4 and activating RhoA/ROCK pathway. miR-3188 may serve as a target for AS treatment.

1. Introduction

Atherosclerosis (AS) is a chronic inflammatory disease characterized by the abnormal accumulation of lipid-laden macrophages in atherosclerotic lesions (Hansson et al. 2006; Harangi et al. 2016). The endothelial dysfunction is an early step in the progression of AS, which subsequently results in the amplification of inflammatory response because a large number of inflammatory cytokines are released by dysfunctional endothelial cells (Mudau et al. 2012; Vanhoutte 2009). Moreover, the accumulation of macrophages is considered as a major contributor to the inflammatory response (Moore et al. 2013). Inflammation has been found to play an important role of in all phases of AS (Siegel et al. 2013). Seriously, AS and its clinical complications, including peripheral artery disease, stroke and myocardial infarction, lead to high morbidity and mortality in Western societies (Feinberg and Moore 2016). Therefore, a better understanding of key mechanisms underlying AS pathogenesis has great significance for the development of potential therapeutic strategies.

MicroRNAs (miRNAs), a family of small non-coding RNAs, can be implicated in several cellular processes via regulating the expression of their target mRNAs (Arunachalam et al. 2015). miRNAs have been identified to participate in the atherosclerotic process of AS, including atherosclerotic plaque initiation, progression, and rupture (Andreou et al. 2015). miRNAs are also found to have a key role in controlling the functions of endothelial cell and macrophages, thus contributing to the progression of AS (Madrigalmatute et al. 2013). In addition,miRNA-27b can promote plaque stability in AS through regulating the activity of the CCL20/CCR6 axis via targeting Naa15 (Qun et al. 2016). miR-93 is upregulated and contributes to the pathogenesis of coronary AS via targeting ABCA1 (He et al. 2015). Recently, miR-3188 is reported to regulate FOXO1-mediated repression of tumorigenesis and chemotherapy resistance in nasopharyngeal carcinoma through p-PI3K/p-AKT/c-JUN signaling pathway (Zhao et al. 2016a). miR-3188 may affect inflammation to play a key role in regulating the severity of the acute lung injury (Liu et al. 2015). Nevertheless, the roles of miR-3188 in the development of AS has not been characterized.

In the present study, we detected the levels of miR-3188 and lipoprotein-associated phospholipase A2 (Lp-PLA$_2$) in AS patients and healthy controls. In addition, it has been reported that the uptake of oxidized low-density lipoprotein (ox-LDL) by macrophages contributes to the development of AS (Dai et al. 2014). We thus used ox-LDL to treat human THP-1 derived macrophages, thus to stimulate the pathologic process of AS. Then miR-3188 was overexpressed and suppressed to further investigate its role in ox-LDL-induced cell injury in THP-1 derived macrophages. Besides, the potential target of miR-3188 was identified and the relationship between miR-3188 and RhoA/ROCK pathway was explored. The objective of our study was to investigate the effects and regulatory mechanism of miR-3188 in ox-LDL-induced cell injury in THP-1 derived macrophages, thus to provide a broader perspective for the treatment of AS.

2. Investigations and results

2.1. Demographic, clinic and laboratory characteristics between groups

In this study, 85 AS patients and 45 healthy controls were enrolled. The demographic, clinic and laboratory characteristics of the above two groups were collected and compared. As shown in the Table, there were no significant differences in the sex, ages, body mass index, total cholesterol, HDL-C, LDL-C, triglyceride, fasting blood-glucose, heart rate, SBP and DBP between the above two groups (all $P > 0.05$).

Table: Demographic, clinic and laboratory characteristics between groups

Variables	Control (n=85)	Atherosclerosis (n=45)	P value
Males/Females	45/40	25/20	0.8972
Ages (years)	53.65±5.36	55.63±5.72	0.0525
Body mass index (kg/m^2)	24.32±1.27	24.68±1.45	0.1459
Total cholesterol (mg/dl)	187.46±32.29	189.78±36.39	0.7099
HDL-C (mg/dl)	48.58±12.28	49.83±11.95	0.5783
LDL-C (mg/dl)	125.49±32.78	127.65±30.63	0.7154
Triglyceride (mg/dl)	155.47±55.86	160.49±57.83	0.6309
Fasting blood-glucose (mmol/l)	4.92±0.43	4.89±0.52	0.7258
Heart rate (per minute)	74.32±8.68	75.57±9.38	0.4489
SBP (mm Hg)	124.35±5.43	125.32±6.89	0.3800
DBP (mm Hg)	77.84±7.73	76.68±6.48	0.3919

2.2. Plasma miR-3188 and Lp-PLA$_2$ levels

We then detected the levels of miR-3188 and Lp-PLA$_2$ in AS patients and healthy controls. The results showed that miR-3188 expression in AS patients were significantly lower than healthy control ($P < 0.05$, Fig. 1A). However, ELISA assay showed that the levels of Lp-PLA$_2$ in AS patients were significantly higher than healthy controls ($P < 0.05$, Fig. 1B). The correlation between miR-3188 expression and Lp-PLA$_2$ concentration was investigated. Expected results were obtained that negative correlation existed between miR-3188 expression and Lp-PLA$_2$ concentration ($R^2 = 0.7719$, $P < 0.001$, Fig. 1C). These data suggest that miR-3188 may be involved in the development of AS via negatively regulating Lp-PLA$_2$.

2.3. Ox-LDL induced cell injury and downregulated miR-3188 expression

To investigate the roles of miR-3188 in AS, ox-LDL was used to activate human THP-1 derived macrophages to stimulate the pathologic process of AS. The results showed that, in comparison with control group, ox-LDL treatment significantly decreased cell viability ($P < 0.05$, Fig. 2A), induced cell apoptosis by down-regulating the expressions of Bcl-2, pro-caspase-3 and pro-caspase-9 and up-regulating the expressions of Bax, cleaved-caspase-3 and cleaved-caspase-9 (Fig. 2B and 2C) in an experiment period of 48 h. Moreover, the production of inflammatory cytokines, including IL-1β, IL-6, MCP-1 and TNF-α, was significantly increased after 24 h of ox-LDL treatment ($P < 0.05$, Fig. 2D). Besides, we found that compared with the control group, the expression of miR-3188 was significantly decreased after ox-LDL treatment in a time-dependent manner during an experiment period of 48 h ($P < 0.05$, Fig. 2E).

2.4. Overexpression of miR-3188 alleviated ox-LDL-induced cell injury in THP-1 derived macrophages

To further investigate the roles of miR-3188 in AS, we overexpressed and suppressed the expression of miR-3188 in THP-1 derived macrophages. As shown in Fig. 3A, miR-3188 expression was significantly upregulated in miR-3188 mimic transfected cells and markedly downregulated in miR-3188 inhibitor transfected cells in comparison with their corresponding controls ($P < 0.05$). Moreover, in comparison with mimic NC transfected control cells or ox-LDL-treated cells, overexpression of miR-3188 significantly promoted the viability of miR-3188 mimic transfected control cells or ox-LDL-treated cells (Fig. 3B), while suppression of miR-3188 had opposite effects (Fig. 3C). Furthermore, overexpression of miR-3188 inhibited cell apoptosis by regulating apoptosis-related proteins ($P < 0.05$, Fig. 3D), and suppression of miR-3188 induced cell apoptosis ($P < 0.05$, Fig. 3E). Besides, overexpression of miR-3188 decreased the production of inflammatory cytokines, including IL-1β, IL-6, MCP-1 and TNF-α ($P < 0.05$, Fig. 3F) and suppression of miR-3188 had opposite effects on the production of inflammatory cytokines ($P < 0.05$, Fig. 3G). These data indicated that overexpression of miR-3188 alleviated ox-LDL-induced cell injury in THP-1 derived macrophages.

2.5. ELK4 was a target of miR-3188

The targets of miR-3188 were also analyzed and detected. As shown in Fig. 4A, ELK4 was identified as a possible target of miR-3188 based on the information of TargetScanHuman. Luciferase reporter assay was then carried out to verify this result. Expected results were obtained that miR-3188 mimic significantly inhibited the luciferase activity of WT-ELK4 ($P < 0.05$, Fig. 4B), but could not target MUT-ELK4. Moreover, the expression of ELK4 in miR-3188 mimic group was significantly lower than mimic NC group ($P < 0.05$, Fig. 4C), while markedly increased in miR-3188 inhibitor group than inhibitor NC group ($P < 0.05$, Fig. 4C). Similar results were obtained by western blot (Fig. 4D). These findings confirmed that ELK4 was a target of miR-3188 and was negatively regulated by miR-3188.

2.6. miR-3188 overexpression alleviated ox-LDL-induced cell injury by targeting ELK4

We the detected whether miR-3188 overexpression alleviated ox-LDL-induced cell injury via targeting ELK4. As shown in Fig. 5A and 5B, ELK4 was successfully knocked down in ox-LDL treated cells ($P < 0.05$). In addition, knockdown of ELK4 significantly alleviated ox-LDL-induced cell injury by increasing cell viability (Fig. 5C), inhibiting cell apoptosis by regulating several apoptosis-regulated proteins ($P < 0.05$, Fig. 5D), and decreasing the production of IL-1β, IL-6, MCP-1 and TNF-α ($P < 0.05$, Fig. 5E). Besides, the effects of miR-3188 inhibition on decreased

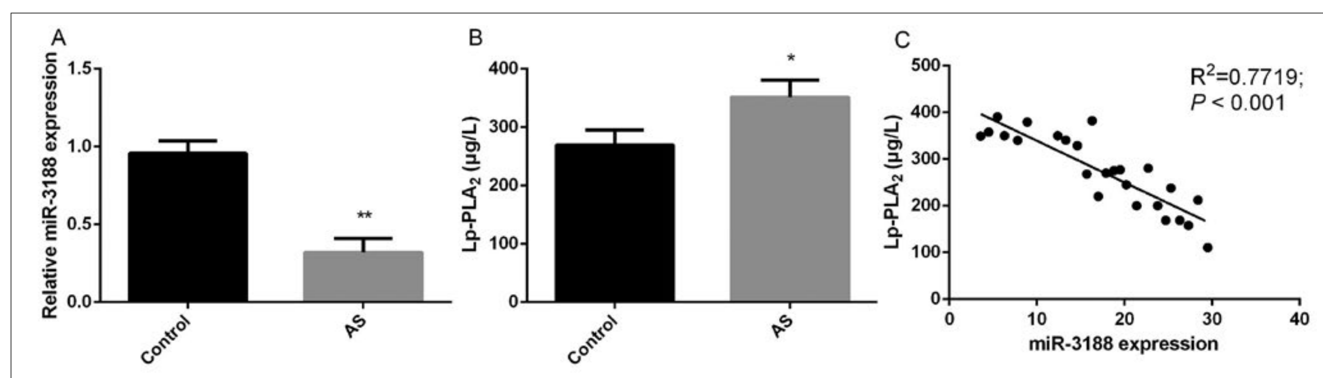

Fig. 1: The levels of miR-3188 and Lp-PLA$_2$ in AS patients and healthy controls. A: The expression of miR-3188; B: The concentration of Lp-PLA$_2$; C: The correlation existed between miR-3188 expression and Lp-PLA$_2$ concentration ($R^2 = 0.7719$, $P < 0.001$). Data were expressed as mean ± SD. *, $P < 0.05$; **, $P < 0.01$ compared with control.

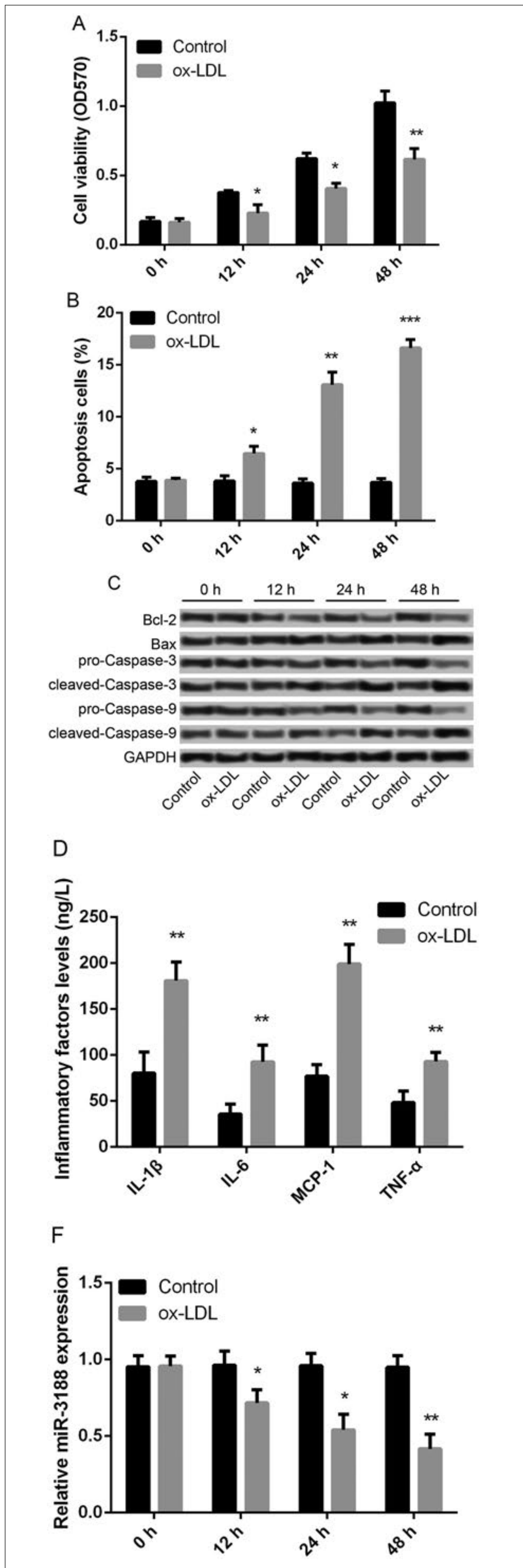

Fig. 2: Ox-LDL induced cell injury and downregulated miR-3188 expression. A: Cell viability of different treated groups in an experiment period of 48 h; B: The percentage of apoptosis cells of different treated groups in an experiment period of 48 h; C: The expressions of Bcl-2, Bax, pro-caspase-3, cleaved-caspase-3, cleaved-caspase-9 and pro-caspase-9 in different treated groups during an experiment period of 48 h; D: The production of inflammatory cytokines after 24 h of ox-LDL treatment, including IL-1β, IL-6, MCP-1 and TNF-α; E: The expression of miR-3188 after different times of ox-LDL treatment. Data were expressed as mean±SD. *, P <0.05; **, P< 0.01 compared with control.

viability, induced apoptosis and promoted production of inflammatory cytokines were markedly reversed by knockdown of ELK4 (Fig. 5F-H).

2.7. *miR-3188 protected against AS by regulating RhoA/ROCK pathway*

To further explore the regulatory mechanism of miR-3188 in the development of AS, we detected the relationship between miR-3188 and RhoA/ROCK pathway. The results showed that the expression levels of RhoA, ROCK1 and ROCK2 were significantly increased after ox-LDL treatment ($P < 0.05$, Fig. 6), indicating that ox-LDL induced the activation of RhoA/ROCK pathway. In addition, the expression levels of RhoA, ROCK1 and ROCK2 were further increased in ox-LDL+ miR-3188 inhibitor group compared with ox-LDL+inhibitor NC group ($P < 0.05$, Fig. 6). However, the expression levels of RhoA, ROCK1 and ROCK2 were decreased in ox-LDL+si-ELK4 group compared with ox-LDL+si-NC group ($P < 0.05$, Fig. 6).

3. Discussion

In this study, we investigated the potential roles of miR-3188 in AS development. The results showed that miR-3188 was downregulated in AS patients, and a negative correlation between miR-3188 expression and Lp-PLA_2 concentration was observed. In addition, miR-3188 was significantly downregulated after ox-LDL treatment. Overexpression of miR-3188 significantly alleviated ox-LDL-induced cell injury in THP-1 derived macrophages, while inhibition of miR-3188 had opposite effects. In addition, ELK4 was a target of miR-3188. The effects of miR-3188 inhibition on ox-LDL-induced cell injury were markedly reversed by knockdown of ELK4. Besides, inhibition of miR-3188 could enhance ox-LDL-activated RhoA/ROCK pathway, while knockdown of ELK4 had opposite effects on this pathway. These findings primarily elucidate the possible roles and regulatory mechanism in AS and merit further discussion.

Lp-PLA_2 is a calcium-independent phospholipase A2 which can be co-localized with macrophages in atherosclerotic plaques (Tselepis et al. 2011). Lp-PLA_2 is found to have a powerful inflammatory and atherogenic action to regulate carotid AS and arterial in the development of coronary artery disease (Ikonomidis et al. 2014). The lysophosphatidylcholine (lysoPC) generated by Lp-PLA_2 is shown to play a key role in the inflammation and vulnerability of human atherosclerotic plaques (Gonçalves et al. 2012). It is reported that the level of Lp-PLA2 is higher in patients with atherothrombosis ischemic stroke and may reflect the severity and early prognosis in patients with atherothrombosis acute ischemic stroke of this disease (Zhao et al. 2016b). Notably, a reversible oral Lp-PLA2 inhibitor, sarapladib, is observed to prevent necrotic core expansion, thus reducing coronary atherosclerotic plaque in humans (Serruys et al. 2008). In this study, miR-3188 was downregulated in AS patients, and miR-3188 was negatively correlated with Lp-PLA_2. Given the key role of Lp-PLA_2 in atherosclerotic plaques, we hypothesize that miR-3188 may be implicated in AS development via negatively regulating Lp-PLA_2.

Furthermore, one interesting finding of this study was that ELK4 was a target of miR-3188. It has been reported that ELK4 is involved in the disease development. ELK4 can regulate distinct serum response factor to facilitate acute responses to external infection

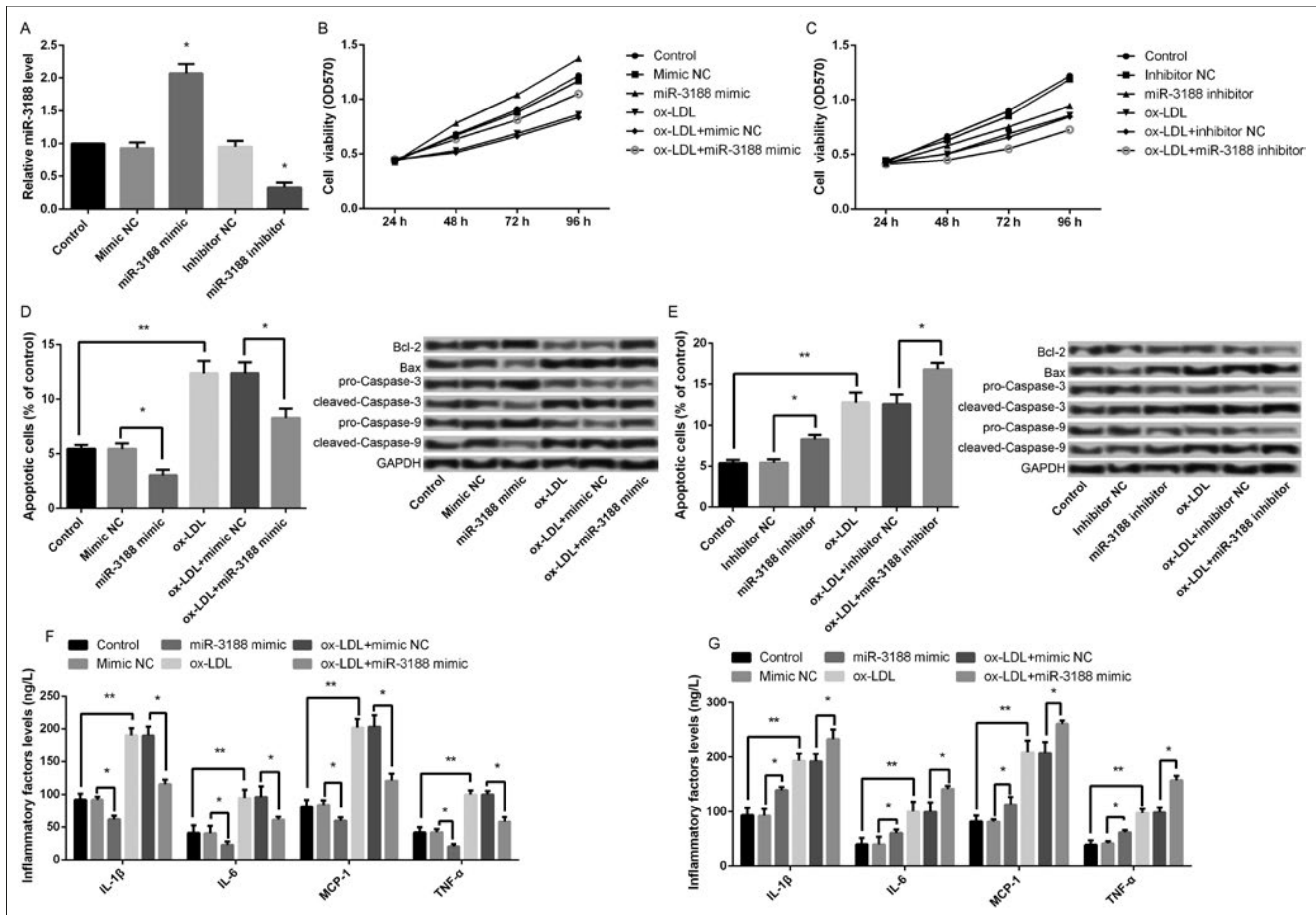

Fig. 3: Overexpression of miR-3188 alleviated ox-LDL-induced cell injury in THP-1 derived macrophages. A: The expression of miR-3188 in different transfected groups; B and C: Cell viability of different groups; D and E: Cell apoptosis of different groups; F and G: The production of IL-1β, IL-6, MCP-1 and TNF-α in different groups. Data were expressed as mean ± SD. *, P <0.05; **, P< 0.01 compared with corresponding control.

in macrophages (Xie 2014). Downregulation of ELK4 is shown to increase sensitivity of glioblastoma to apoptosis and reduce tumor formation in gliomas (Day et al. 2011). In our study, we found that knockdown of ELK4 reversed the effects of miR-3188 inhibition on ox-LDL-induced cell injury. Although the roles of ELK4 in AS development have not been fully investigated, we hypothesize that ELK4 may be involved in ox-LDL-induced AS and miR-3188 may contribute to AS development by targeting ELK4.

Importantly, we also detected the relationship between miR-3188 and RhoA/ROCK pathway. In a previous study, RhoA/ROCK pathway is found to be correlated with a variety of cardiovascular diseases, including AS (Sun and Aiguo 2013). Deficiency of ROCK1 in macrophages prevents the development of AS through affecting key macrophage functions such as macrophage chemotaxis (Wang et al. 2007). The RhoA/ROCK pathway is also shown to play a crucial role of in the regulation of vascular function in AS and vascular disease (Zhou 2011). Matsumoto also confirmed that RhoA/ROCK pathway has inhibitory effects on atherogenesis in ApoE−/− mice (Matsumoto et al. 2013). Significantly, inhibition of Rho/ROCK pathway contributes to the anti-atherosclerotic

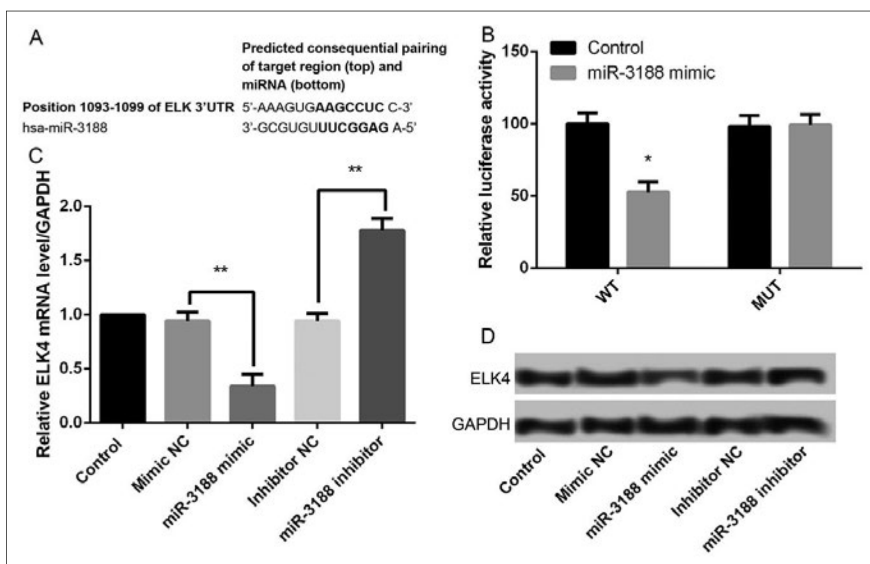

Fig. 4: ELK4 was a target of miR-3188 and was negatively regulated by miR-3188. A: The predicted binding sequence of ELK4 and miR-3188based on the information of TargetScanHuman; B: Luciferase reporter assay showed miR-3188 mimic significantly inhibited the luciferase activity of WT-ELK4, but could not target MUT-ELK4; C and D: The expression of ELK4 in different transfected groups. Data were expressed as mean±SD. *, P <0.05; **, P< 0.01 compared with corresponding control.

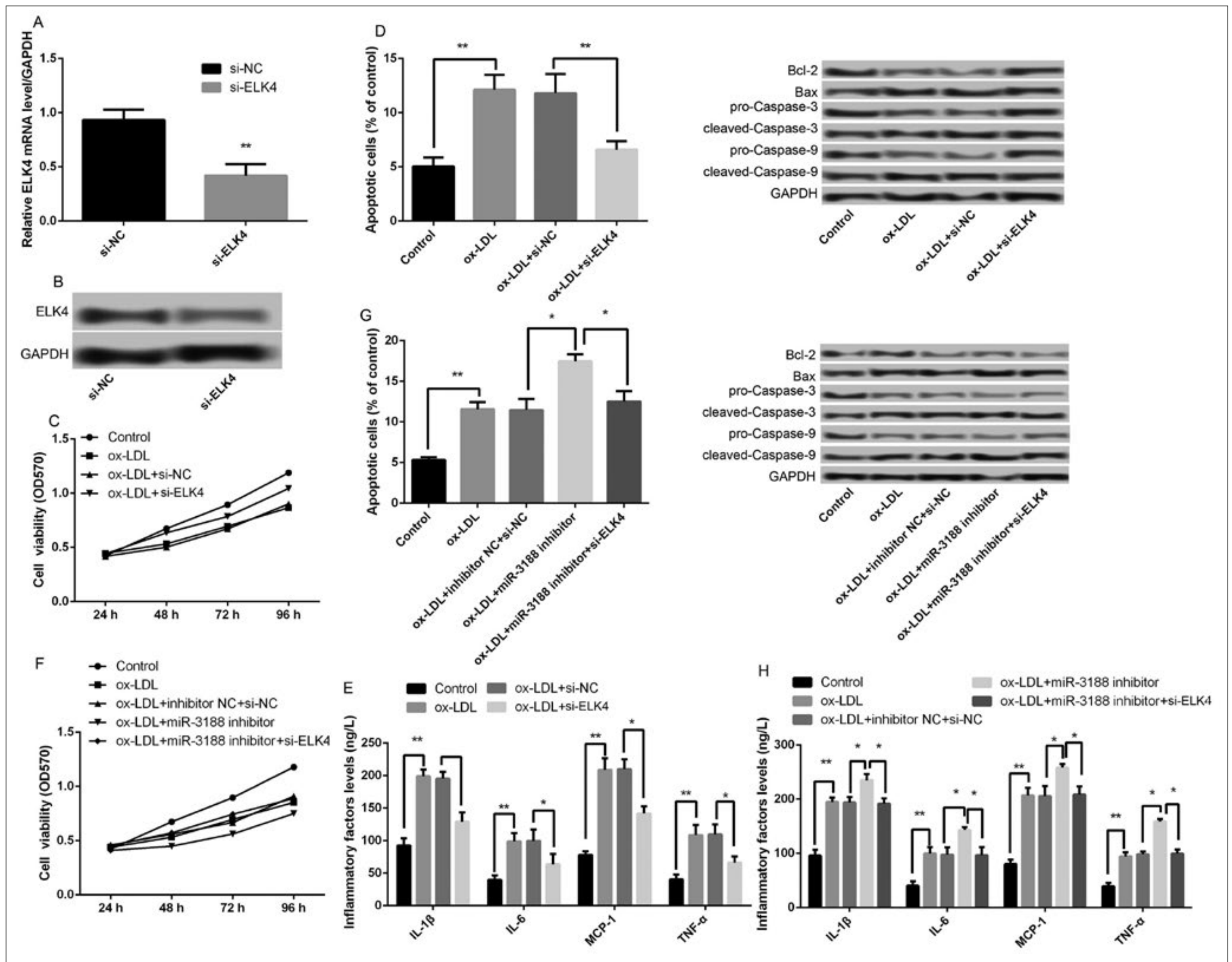

Fig. 5: miR-3188 overexpression alleviated ox-LDL-induced cell injury by targeting ELK4. A and B: The expression of ELK4 after siRNAs treatments; C: Cell viability of different siRNAs groups; D: Cell apoptosis of different siRNAs groups; E: The production of IL-1β, IL-6, MCP-1 and TNF-α in different siRNAs groups; F: Cell viability of different treated groups; G: Cell apoptosis of different treated groups; H: The production of IL-1β, IL-6, MCP-1 and TNF-α in different treated groups. Data were expressed as mean±SD. *, P <0.05; **, P< 0.01 compared with corresponding control.

effects of statins in AS and could be a potential therapeutic target for AS (SawadaNaoki and Liaojames 2014). The results of this study showed that inhibition of miR-3188 could enhance the ox-LDL-activated RhoA/ROCK pathway, while knockdown of ELK4 had opposite effects on this pathway, indicating that the RhoA/ROCK pathway may be a key mechanism to mediate the roles of miR-3188 and ELK4 in regulating AS development.

In conclusion, our study indicates that downregulation of miR-3188 may contribute to AS development via negatively regulating Lp-PLA2, targeting ELK4 and activating the RhoA/ROCK pathway. Targeting miR-3188 may provide a more attractive target for the treatment of AS.

4. Experimental

4.1. Study population

From January 2015 to December 2016, a total of 130 participants, including 45 patients (25 males and 20 females, aged 55.63±5.72 years) with AS and 85 healthy volunteers (45 males and 40 females, aged 53.65±5.36 years) without AS, were selected continuously from Hebei general hospital. All participants underwent office blood pressure monitoring, physical examination, and carotid artery Doppler ultrasonography. The cIMT of the common carotid artery was used to evaluate whether participants had AS, which was measured by ATL HDI 3000 ultrasound system (Advanced Technology Laboratories, Bothell, WA) equipped with a 5-MHz linear array transducer. According to the 2007 Guidelines for the management of arterial hypertension (Mancia et al. 2007), a cIMT ≥ 1.2 mm was defined as AS. The cIMT in carotid arteries was measured by a trained sonographer with registered diagnostic medical sonography certification and its value was used for analyses. Participants were excluded from the study if they had a history of diabetes mellitus, hypertension, coronary heart disease, thyroid diseases, tumor, neck surgery, heavy smokers, and relevant medications (lipid-lowering drugs, antiplatelet or antihypertensive drugs). This study was approved by the Ethics Committee of Hebei General Hospital (Shijiazhuang, China) and conducted following the Declaration of Helsinki. All participants provided their informed consent.

4.2. Sample collection

Fasting blood samples were collected from each participant in the morning. Plasma was then collected after centrifuging at 3,000 rpm for 10 min. Plasma was divided into aliquots, and then frozen at −80 °C for storage until use. Fasting blood glucose, blood lipid, routine laboratory tests and renal function were tested.

4.3. Cell culture

Human monocytic cell line THP-1 was purchased from American Type Culture Collection, Bethesda, MD, USA) and then cultured in RPMI 1640 medium (Gibco, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin (Sigma, St Louis, MO, USA) and 10 mmol/L HEPES (Sigma, St Louis, MO, USA). Human THP-1 cells were then cultured in 100 nmol/L phorbol12-myristate 13-acetate (PMA, Calbiochem, San Diego, CA, USA) for 48 h to induce the differentiation of monocytes into macrophages. After starvation treatment with RPMI1640 medium containing 0.5% FBS for 6 h, human THP-1 derived macrophages were exposed with 50 μg/mL oxLDL for 24 h for inflammasome stimulation.

4.4. Cell transfection and treatment

MiR-3188 mimics, inhibitor and scramble control were obtained from GenePharma Co., Ltd. and then transfected into cells using lipofectamine 2000 (Invitrogen). In addition, the RNA oligonucleotides for small interference RNA (siRNA) against

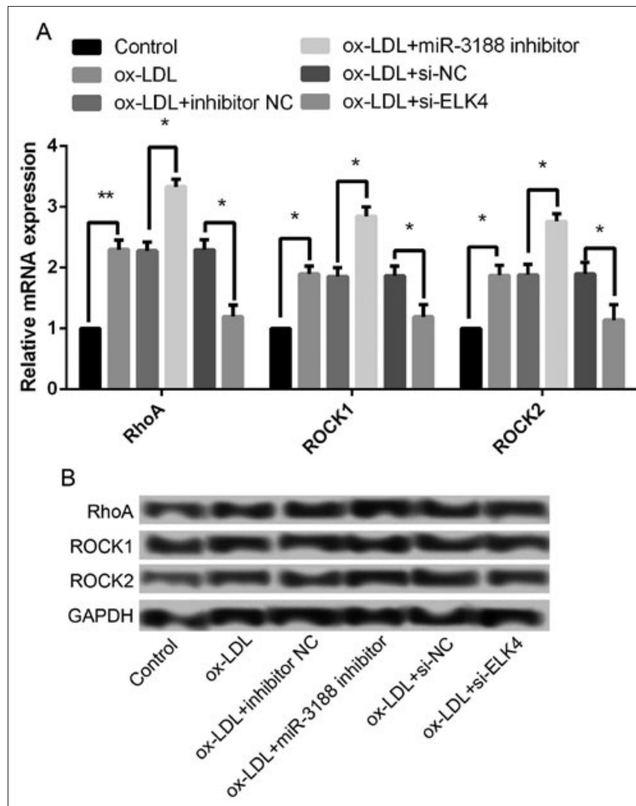

Fig. 6: miR-3188 protected against AS by regulating RhoA/ROCK pathway. A: The mRNA expression levels of RhoA, ROCK1 and ROCK2 in different treated groups; B: The protein expression levels of RhoA, ROCK1 and ROCK2 in different treated groups. Data were expressed as mean±SD. *, $P < 0.05$; **, $P < 0.01$ compared with corresponding control.

ETS-domain protein 4 (ELK4) (si-ELK4) were synthesized by GenePharma and transfected into cells using the same method. Six hours after transfection, the cells were exposed to 50 μg/mL of oxLDL for 24 h and then harvested for subsequently experiments.

4.5. *Flow cytometry analysis of apoptosis*

Human THP-1 derived macrophages were seeded into 6-well plates and the treated with 50 μg/ml of ox-LDL for 0, 12, 24, and 48 h. Cells were harvested and then double stained with annexin V and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Jiang-su, China) following the manufacturer's protocol. The number of apoptotic cells was determined by FCM with a FACS Calibur flow cytometer (Becton–Dickinson).

4.6. *Quantitative RT-PCR*

Total mRNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription into cDNA was then performed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA). Quantitative RT-PCR analyses for miRNAs and mRNAs were conducted using TaqMan miRNA assays (Ambion) and PrimeScript RT-PCR kits (Takara), respectively. U6 small nuclear RNA and β-actin were used as an endogenous control for data normalization and the relative expression of miRNAs and mRNAs was then calculated using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

4.7. *Luciferase reporter assay*

Luciferase reporter vectors of the wild type (WT)-ELK4-3'UTR containing miR-3188 target site and mutated (MUT)-ELK4-3'UTR containing identical flanking nucleotides of miR-3188 target site were synthetized by GenePharma Co., Ltd.. Then luciferase reporter vectors of WT-ELK4-3'UTR and MUT-ELK4-3'UTR were co-transfected with miR-3188 mimic in oxLDL-stimulated macrophages. To monitor transfection efficiency, cells were also transfected with the pmirGLO-control vector. miR-3188 scramble control was transfected as a control. After 24 h of transfection, firefly luciferase activity was determined using the dual luciferase reporter assay system (Promega) by means of a GloMAX 20/20 Luminometer (Promega). The reporter activity of Renilla was used as an internal control for normalization.

4.8. *Enzyme-linked immunosorbent assay (ELISA) assay*

Plasma levels of Lp-PLA2 were determined with a commercially available ELISA kit (PLACTM Test; diaDexus Inc., South San Francisco, CA, USA) and its lower detection limit of Lp-PLA2 was 2 ng/ml. Moreover, the levels of secreted pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein 1 (MCP-1) and tumor necrosis factor α (TNF-α) in the supernatants were also detected using ELISA assay according to the manufacturer's instructions.

4.9. *Western blot analysis*

The total protein was extracted with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors (Roche, Guangzhou, China). Protein extracts were then denatured, subjected to electrophoresis on 10% polyacryl amide SDS gels and transferred into polyvinylidene difluoride (PVDF) membranes. Primary antibodies against Bcl-2, Bax, pro-caspase-3, cleaved-caspase-3, pro-caspase-9, cleaved-caspase-9, ELK4, RhoA, ROCK1, ROCK2 and GAPDH (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were prepared in 5% blocking buffer and then incubated with the membranes at 4 °C overnight. Followed by the incubation with goat anti-rabbit secondary antibody labeled with far-red-fluorescent Alexa Fluor 680 dye, the immunofluorescence signal of each band were detected using a SuperSignal West Femto Maximum Sensitivity Substrate kit (ThermoPierce). The intensity of each band was finally quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.10. *Statistical analysis*

All data were expressed as mean ± SD and tested for the normal distribution using Shapiro-Wilk test. The difference analysis for variables between patients and controls was performed using Student t-test. Pearson's correlation was used for analyzing the correlation between miR-3188 and Lp-PLA_2. All statistical analyses were made using version 17.0 software (SPSS Inc., Chicago, IL, USA) and two-sided $P < 0.05$ indicated statistical significance.

Conflicts of interest: None declared.

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