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LncRNA HOXA-AS2 represses endothelium inflammation by regulating the activity of NF-κB signaling

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HIGHLIGHTS

- We identify lncRNA HOXA-AS2 as a critical repressor of endothelium inflammation.
- HOXA-AS2 has a significant correlation with carotid artery atherosclerosis.
- HOXA-AS2 represses the activation of NF-κB signaling by controlling IκBα degradation and RelA acetylation at K310 site.
- NF-κB inversely activates the transcription elongation of HOXA-AS2 by establishing a NF-κB/HOXA-AS2 negative feedback loop.

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ABSTRACT

Background and aims: Endothelium inflammation, which can lead to endothelial activation and dysfunction, is widely accepted as the major event in multiple vascular disorders. The lncRNA HOXA-AS2 was previously reported to be involved in multiple inflammation-linked cancers. However, the role of HOXA-AS2 in endothelium inflammation is poorly understood. This study aims to determine the regulatory role of HOXA-AS2 in endothelium inflammation and related vascular diseases.

Methods: High throughput mRNA sequencing was performed to establish expression profiles after HOXA-AS2 depletion. We extracted total RNAs of human peripheral blood mononuclear cells from normal control group and experimental group with carotid artery atherosclerosis, and performed qRT-PCR to assay the correlation between HOXA-AS2 expression and inflammatory vascular diseases.

Results: Inhibition of HOXA-AS2 can induce the activation of NF-κB signaling and subsequent inflammatory response. More importantly, HOXA-AS2 is inversely found to be inversely regulated by NF-κB in a negative feedback manner by helping recruit BRD4/P-TEFb complex to HOXA-AS2 promoter region, therefore facilitating release of the promoter-proximal paused RNA polymerase II and activating transcription elongation.

Conclusions: We identify HOXA-AS2 as a critical repressor of endothelium inflammation. Moreover, this study offers us a new way to balance the NF-κB signaling-driven excessive endothelium inflammation by establishing a NF-κB/HOXA-AS2 negative feedback loop. Based on these findings, we conclude that HOXA-AS2 may serve as a crucial therapeutic target for various vascular disorders which are significantly associated with endothelium inflammation.

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1. Introduction

Endothelium is widely considered as a critical integrator and transducer for signaling transduction in response to diverse stimulis. When continuously exposed to proinflammatory stimulation, endothelium will become dysfunctional because of the endothelial inflammation, ultimately resulting in a number of vascular disorders [1–5]. Previous investigations reported that multiple signaling pathways are involved in endothelium inflammation [6–9], among which NF-κB is the major inflammatory signaling. NF-κB, a key proinflammatory transcription factor, is composed of homo- or hetero-dimers of RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) or p52/p100(NF-κB2) [10–13], among which p65/p50 heterodimer is the major form functionally involved in endothelial inflammation. Aberrant activation of NF-κB can lead to seriously inflammatory lesions in the endothelium [14,15]. Hence, increasing studies focused on the regulation of NF-κB activity. Numerous evidence revealed NF-κB activity can be regulated at multiple levels [12,16]. First, NF-κB is restrained in the cytoplasm by the inhibitor of κB (IκB) proteins. As the major inhibitor protein of IκB family, IκBα was shown to be phosphorylated by its upstream IκB kinase (IKK), which will then lead to its rapid degradation via proteasome and subsequent nuclear translocation of NF-κB [12]. Another way for the regulation of NF-κB activity is through the potential modification of the RelA subunit of NF-κB. RelA contains several phosphorylation or acetylation sites which are responsible for NF-κB activity, especially the K310 acetylation site. It is reported that acetylation at the K310 site is significantly associated with the transcriptional activation of NF-κB by regulating the recruitment of the bromodomain-containing protein BRD4, which is required for P-TEFb-mediated transcriptional pause release and productive elongation [17].

Transcription of protein-coding genes in eukaryotic cells generally consists of several steps including initiation, elongation and termination. Of note, transcription elongation is regarded as a rate-limiting step, which could be tightly regulated by transcription elongation factors and a number of cofactors [18–22]. Among these transcriptional regulators, the positive elongation factor b (P-TEFb) accounts for the leading position, which could be recruited to the genes' promoter region by its partner bromodomain containing 4 (BRD4) and phosphorylate the C-terminal domain (CTD) of RNA polymerase II at serine 2 site, therefore simulating productive elongation [23–27]. Increasing studies revealed multiple coregulators are involved in the regulation of transcription elongation, such as transcription factor NF-κB which can interact with BRD4 via the acetylated K310 site of RelA, thus facilitating the BRD4/P-TEFb complex recruitment to genes' promoters and activating transcription elongation [28].

Long non-coding RNAs (LncRNAs) are a set of non-coding transcripts, which generally are more than 200 bases in length [29–31]. To date, most of the lncRNAs remain poorly understood, to a large extent, due to the low expression levels. Even so, some identified lncRNAs are found to be significantly involved in the regulation of endothelial inflammation and vascular diseases; for example, the proinflammatory lncRNA E330013P06 can regulate foam cell formation by controlling inflammatory gene expression [32], another lncRNA, named ANRIL, plays a regulatory function in the growth of vascular smooth muscle cells [33,34].

In this study, we first characterize HOXA-AS2, a recently reported lncRNA, which is tightly associated with inflammation-linked cancers [35–38], as a critical regulator of endothelium inflammation and related vascular disorders by repressing the activation of NF-κB signaling. We found HOXA-AS2 has an inhibitory effect on proteasome-mediated IκBα degradation and RelA acetylation at the K310 site. More importantly, the expression of HOXA-AS2 was found to be inversely regulated by NF-κB in a negative feedback manner by activating transcription elongation. Hence, the lncRNA HOXA-AS2 may coordinate with NF-κB to form a negative feedback loop in the regulation of endothelium inflammation.

2. Materials and methods

2.1. Cell culture and transfection

Both HAECs and HUVECs were cultured in completed endothelial cell medium (ECM) (ScienCell, Carlsbad, CA, USA), human monocyte THP-1 and human embryonic kidney HEK293T cell lines were cultured in RPMI (HyClone) and DMEM (Gibco) medium, respectively, supplemented with 10% (v/v) fetal calf serum (Gibco) and 100 units ml^{-1} streptomycin and penicillin (Millipore, Billerica, MA, USA), at 37 °C in a humidified 5% CO_2 incubator. HAECs and HUVECs were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). HEK293T and THP-1 cells were purchased from American Type Culture Collection (Manassas, VA, USA). For transfection, HEK293T cells were transiently transfected with indicated plasmids or *in vitro* synthesized siRNAs using lipofectamine 3000 transfection reagent (#L3000015) purchased from Invitrogen, according to manufacturers' instructions.

2.2. Antibodies and reagents

Anti-p65 antibody (#sc-109) was purchased from Santa Cruz (Santa Cruz, CA, USA), anti-VCAM1 (#66294-1-Ig), anti-ICAM1 (#10831-1-AP), anti-CCL2 (#66272-1-Ig), anti-IκBα (#10268-1-AP) and anti-Lamin B1(#12987-1-AP) antibodies were purchased from Proteintech Group Inc. Anti-GAPDH (#2118) and anti-ac-310 RelA (#3045s) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti-BRD4 and CDK9 antibodies were reported previously [20]. The chemical reagent TNF-α (#H8916) and Bay 11–7082 (#B5556) were obtained from Sigma, CellTracker™ Green CMFDA (#C2925) was from Invitrogen.

2.3. Plasmid constructions and lentiviral infection

The short hairpin RNAs (shRNAs) targeting human *HOXA-AS2, BRD4* and *CDK9* RNAs were cloned into a modified pLV-H1-Puro lentiviral vector. The corresponding sequences for these shRNAs were: shAS2-1, 5′-AAACCTTGTAGATAGCTTGAGCTGG-3′ shAS2-2, 5′-GAGTTCAGCTCAAGTTGAACATACA-3′, shBRD4, 5′-GAACCTCCCTGATTACTATAA-3′ and shCDK9, 5′-CTACTACATCCACAGAAACAA-3’. The human *HOXA-AS2*, amplified using reverse transcription PCR, was inserted into a modified pLV-EF1α lentiviral vector as previously described [18]. For lentiviral infection, experimental procedures were conducted as previously described [18].

2.4. Quantitative RT-PCR and unbiased mRNA sequencing

Total RNAs were isolated from cells using Trizol reagent according to manufacturers' instructions. Reverse transcription was performed with 1 μg total RNA using a qPCR RT Kit (# FSQ-201) purchased from TOYOBO. Real-time quantitative PCR was performed using an EvaGreen qPCR Master Mix from Applied Biological Materials Inc. The relative changes of gene expression were determined by the 2-ΔΔCT method. The primer sequences used in qRT-PCR analysis are shown in Supplementary Table S1.

The mRNA sequencing experiments were performed by OE Biotech (Shanghai, China). Briefly, total RNAs were extracted using Trizol reagent and then subject to library construction according to standard Illumina protocols. The libraries were sequenced with Illumina HigSeq × Ten sequence platform using the paired-end RNA-seq approach. For data analysis, raw reads were aligned to reference genome, using HTSeq-count, and processed by Cufflinks, which used the normalized RNA-seq fragement counts to measure the relative abundances of the transcripts. The RNA-seq data have been deposited in the Sequence Read Archive (SRA) database with accession number SRP155583.

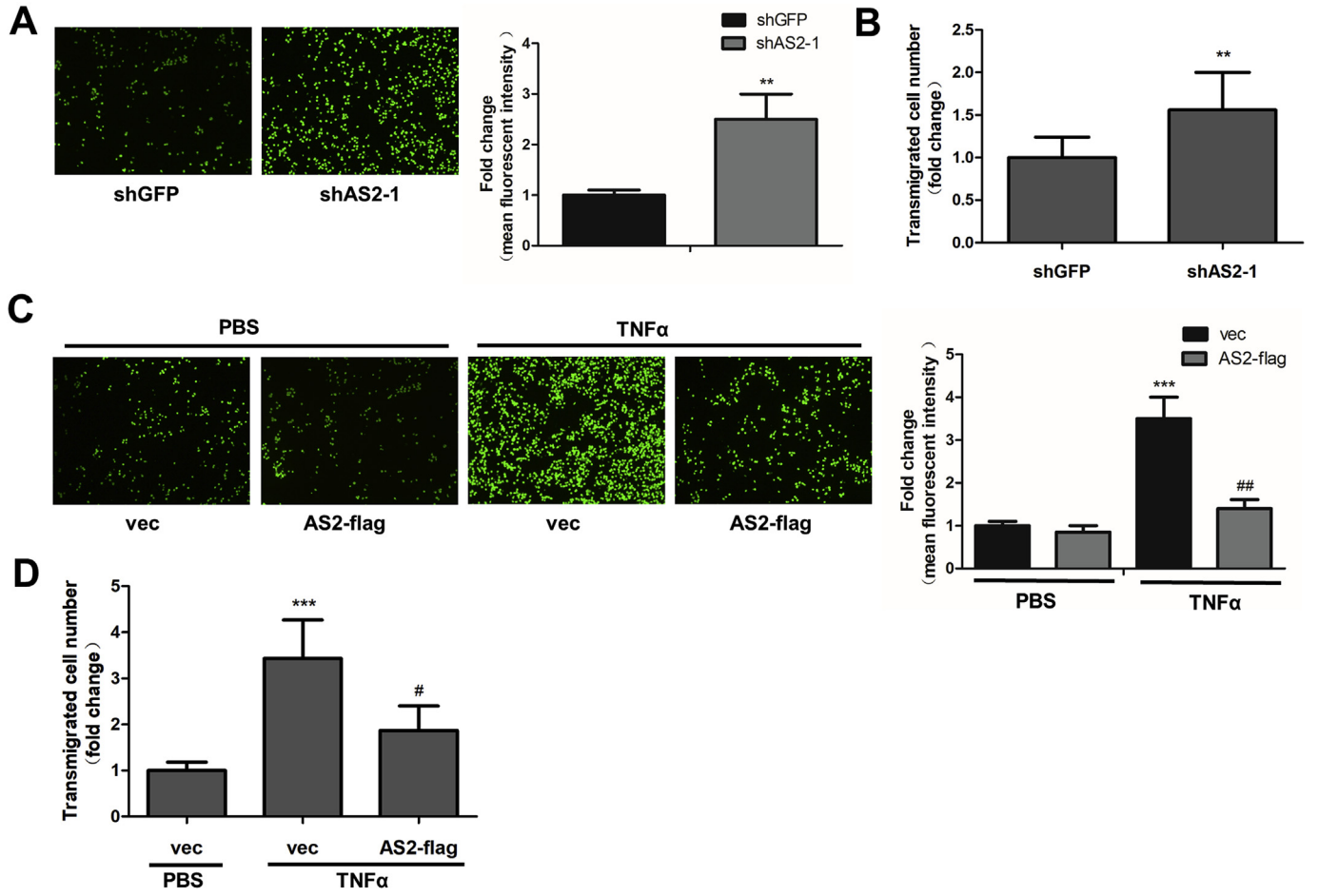

Fig. 1. HOXA-AS2 has an inhibitory effect on endothelial activation.
(A) Fluorescence microscopy images showing adhesion of CMFDA-labeled THP-1 cells to HUVECs, with or without HOXA-AS2 knockdown, quantified using ImageJ software. (B) The effect of HOXA-AS2 on THP-1 cell migration through HUVEC monolayers, measured by transendothelial migration assay. *HOXA-AS2* was silenced in HUVECs before incubation with THP-1 cells. (C) Fluorescence microscopy images showing adhesion of CMFDA-labeled THP-1 cells to control or HOXA-AS2-overexpressed HUVECs under basal and TNFα-treated (20 ng/ml, 3 h) conditions, quantified using ImageJ software. (D) The effect of aberrant overexpression of HOXA-AS2 on transmigrating capacity of THP-1 cells through HUVEC monolayers, determined by monocyte transendothelial migration assay. The lentivirus-mediated overexpression of HOXA-AS2 was conducted in HUVECs, then treated with TNFα for 3 h, followed by incubation with THP-1 cells. All results are from biological triplicates and data shown are the mean ± SD. $n = 3$. $^{**}p < 0.01$ *versus* shGFP; $^{***}p < 0.001$ *versus* vec; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* TNFα-treated vec.

2.5. Monocyte adhesion assay

HUVEC monolayers transfected or infected with indicated plasmids were cultured in a 6-well plate and treated with 10 ng/ml TNFα for 3 h. In parallel, THP-1 monocytes were labeled with 8 μM of CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) at 37 °C for 30 min according to the manufacturer's instructions. Each well of HUVECs was exposed to the 0.2 ml CMFDA-labeled THP-1 cells (10^7 cells/ml) and then incubated at 37 °C for 2 h. After incubation, each well was washed three times with 2.0 ml ECM medium, the adherent monocytes were imaged by fluorescent microscopy and the quantification was done by ImageJ software.

2.6. Transendothelial migration assay

HUVEC monolayers transfected or infected with indicated plasmids were seeded on the upper chamber of a 24-multiwell double-chamber system (porosity 3 μm) and then treated with TNFα for 6 h, medium was dsicarded and cells were washed with fresh RPMI medium for three times. 6×10^5 THP-1 cells per well were added to the upper chamber and allowed to migrate through the endothelial monolayer overnight. Finally, the migrated cell number was counted in the lower chamber.

2.7. Chromatin immunoprecipitation (ChIP)

Briefly, 10^7 HUVEC cells were cross-linked with 1% formaldehyde for 10 min at room temperature and then quenched with glycine at the concentration of 125 mM. After washing two times with PBS, the cells were lysed by a lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1%SDS, 0.5 mM PMSF), subsequently sonicated with a sonicator so that the chromatin DNA was sheared into fragments from 100 to 500bp. The sonicated lysates were then cleared and incubated with indicated antibodies for 6 h at 4 °C. 30 μl protein A beads were added and incubated for 1 h at 4 °C with gentle rotation. Immunoprecipitates were washed three times with the lysis buffer and one time with a high salt buffer (50 mM Tris-HCl pH7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1%SDS, 0.5 mM PMSF). DNA was eluted in an elution buffer (100 mM NaHCO3, 1%SDS) and cross-links were reversed overnight. RNA and protein were digested using RNase A and Proteinase K, respectively. The immunoprecipitated DNAs were determined by qRT-PCR. The primer sequences for ChIP assays are presented in Supplementary Table S2.

2.8. Clinicopathological sample analysis

The clinical PBMC samples used in this study were all obtained with informed consent from the First Affiliated People's Hospital of Xinxiang Medical University. Carotid artery atherosclerotic group, which contains patients with more than 80% carotid artery stenosis, and the normal control group without clinically significant carotid artery occlusion were recruited. All experiments were conducted with the approval of the Xinxiang Medical University Review Board.

2.9. Statistical analysis

Student' s *t*-test was used for comparisons between two groups. For a comparison of ≥3 groups, 1-way ANOVA was used. The correlation of HOXA-AS2 expression and clinicopathological characteristics was analyzed by chi-squared test. p-value < 0.05 was considered statistically significant. All data are representative of at least three independent experiments and presented as mean ± SD.

3. Results

3.1. HOXA-AS2 functions as a repressor for endothelial activation

It was previously reported that lncRNA HOXA-AS2 is critically associated with inflammation-linked multiple cancers [35–38]. Therefore, we hypothesized that HOXA-AS2 plays an important role in endothelium inflammation. To examine the regulatory function of HOXA-AS2 in endothelial activation, we first employed monocyte adhesion assay using control or HOXA-AS2-depleted human umbilical vein endothelial cells (HUVECs). We found the adhesive THP-1 cells to HUVEC monolayer with HOXA-AS2 knockdown were markedly increased, compared with a control vector-treated HUVECs (Fig. 1A). Next, we performed transendothelial migration assay to further characterize the role of HOXA-AS2 in endothelial activation. As a consequence, inhibition of HOXA-AS2 was found to have a promoted effect on monocyte transendothelial migration through HUVECs (Fig. 1B). Moreover, we generated a lentiviral cDNA vector of HOXA-AS2 and conducted HOXA-AS2 ectopic overexpression to observe the effect of HOXA-AS2 on endothelial activation. Consistently, HOXA-AS2 overexpression was shown to dramatically inhibit TNFα-induced THP-1 cell adhesion to HUVEC monolayers and transendothelial migration through endothelial cells (ECs) (Fig. 1C and D). Collectively, these observations suggest that lncRNA HOXA-AS2 could act as a suppressor for endothelium inflammation.

3.2. HOXA-AS2 mediates the expressions of inflammatory factors

To understand the role of HOXA-AS2 at molecular level, we established expression profiles of transcriptome in HUVECs, with or without HOXA-AS2 knockdown, to observe the HOXA-AS2 downstream-regulated genes. The mRNA high-throughput sequencing result showed 308 and 573 genes were down- and up-regulated more than two folds, respectively, after HOXA-AS2 knockdown (Fig. 2A). Notably, HOXA-AS2 was found to be functionally involved in inflammatory response, including regulation of chemokine and cytokine activity, using gene ontology (GO) analysis (Fig. 2A and Supplementary Fig. 2A). Further analysis revealed that expressions of a number of inflammatory factors were remarkably increased after inhibition of HOXA-AS2 (Fig. 2B). To validate the functional involvement of HOXA-AS2 in regulation of the inflammatory factors' expressions, we performed qRT-PCR and Western blot analysis to detect expressions of the canonical inflammatory genes in HUVECs and human arterial endothelial cells (HAECs), respectively, with or without HOXA-AS2 knockdown. As excepted, expressions of these inflammatory genes were significantly augmented when *HOXA-AS2* was silenced both in HUVECs and HAECs (Fig. 2C and D and Supplementary Fig. 2B). Furthermore, lentivirus-mediated aberrant overexpression of HOXA-AS2 was carried out in HUVECs, then subject to qRT-PCR analysis. Consequently, highly expressed HOXA-AS2 was found to have a markedly inhibitory effect on expressions of these inflammatory factors (Fig. 2E). Taken together, these findings identify HOXA-AS2 as a critical regulator for controlling expressions of multiple inflammatory factors.

3.3. HOXA-AS2 regulates the activation of NF-κB signaling

Given the critical role of HOXA-AS2 in inflammatory response, we further analyzed HOXA-AS2 downstream-regulated genes and discovered that numerous genes are NF-κB transcriptional targets. Moreover, we performed KEGG pathway analysis for the RNA-seq data and found HOXA-AS2 is critically associated with NF-κB signaling pathway and other signaling pathways, which have significant crosstalk with NF-κB pathway, like TNF signaling pathway and toll-like receptor signaling pathway (Supplementary Fig. 3A). Thus, we hypothesized that HOXA-AS2 is implicated in the regulation of NF-κB activity. To prove this notion, we first determined expression of the NF-κB inhibitor protein IκBα in control and HOXA-AS2-depleted cells. Interestingly, the expression of IκBα was reduced in HOXA-AS2-depleted HUVECs and HAECs, compared with the control sample (Fig. 3A and Supplementary Fig. 3B), indicating HOXA-AS2 plays an important role in the regulation of IκBα expression and subsequent nuclear translocation of NF-κB. Not only that, the acetylation of RelA at K310, widely accepted as the key modification site for controlling NF-κB activity [28,39], was shown to be obviously elevated in both HUVECs and HAECs when HOXA-AS2 was knocked down (Fig. 3A and Supplementary Fig. 3B). Next, we employed nuclear fractionation experiments to observe the effect of HOXA-AS2 on the cellular localization of NF-κB. Western bolt analysis showed the inhibition of HOXA-AS2 facilitated the release of NF-κB from cytoplasm, meanwhile inducing NF-κB accumulation in nuclear fraction (Fig. 3B). However, HOXA-AS2 almost has no effect on the distribution of the cytoplasmic protein β-actin and nuclear protein Lamin B1 (Fig. 3B). Moreover, we performed ChIP assay in HUVECs after HOXA-AS2 depletion, with an antibody against RelA, to observe whether HOXA-AS2 could influence NF-κB occupancies at the promoters of its target genes. The ChIP results showed that attenuation of HOXA-AS2 induced NF-κB enrichment at its target gene promoters (Fig. 3C).

To further confirm the regulatory function of HOXA-AS2 for NF-κB activity, aberrant overexpression of HOXA-AS2 was employed in HUVECs. We found highly expressed HOXA-AS2 significantly increased IκBα expression level, while it had an inhibitory effect on RelA acetylation at K310 site (Fig. 3D). Furthermore, anti-RelA ChIP assay revealed overexpressed HOXA-AS2 markedly abolished TNFα-induced RelA binding to promoters of these NF-κB transcriptional targets. (Fig. 3E). These data suggest that HOXA-AS2 functions as a critical repressor for the activation of NF-κB signaling.

3.4. HOXA-AS2 is regulated by NF-κB in a negative feedback manner

It is widely reported that negative feedback regulation mechanisms universally exist in signaling transduction, as a self-protective manner. Thus, we asked whether NF-κB could inversely mediate the expression of HOXA-AS2. To test this, we first performed data mining analysis of the ChIP-seq data which was reported by Brown et al. [40]. Intriguingly, the anti-RelA ChIP-seq result revealed TNFα stimulation induced the enriched RelA occupancy near the *HOX-AS2* promoter (Supplementary Fig. 4A). To further validate this result, we performed ChIP-qPCR in HUVECs singly activated with TNFα for 3 h or pre-treated with NF-κB inhibitor Bay 11–7082 for 1 h and additional co-treatment with TNFα for 3 h, with an antibody against RelA. As a consequence, RelA distribution at *HOXA-AS2* promoter was obviously induced by TNFα (Fig. 4A). However, the Bay 11–7082 pre-treatment disrupted the TNFα-activated RelA enrichment at *HOXA-AS2* promoter (Fig. 4A).

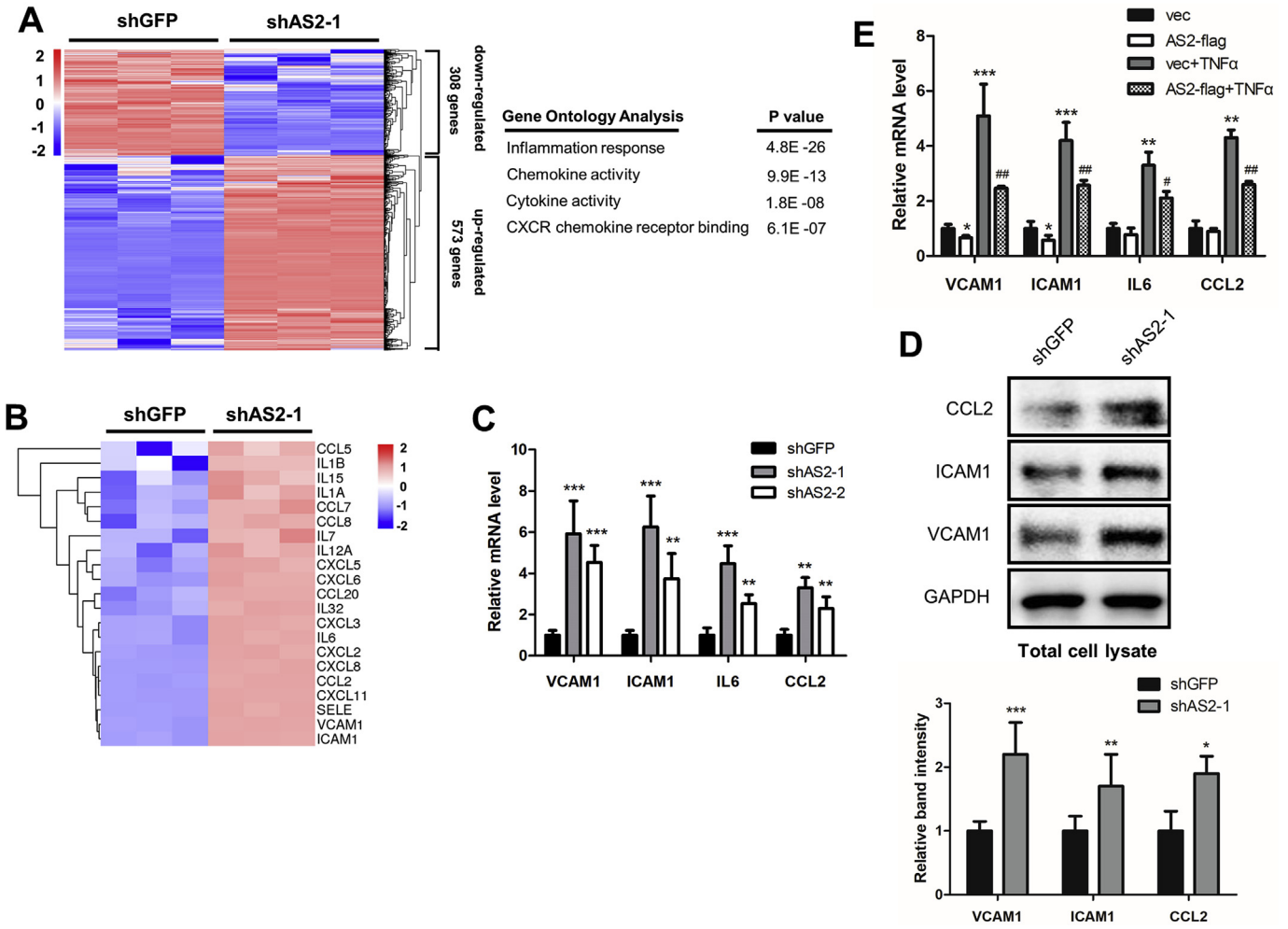

Fig. 2. HOXA-AS2 regulates the expressions of inflammatory factors.
(A) Hierarchical clustering analysis showing the differentially expressed genes in HUVECs after inhibition of HOXA-AS2, which were subject to gene ontology (GO) analysis. (B) Heat map showing significantly upregulated inflammatory factors in HOXA-AS2-depleted HUVECs, compared with the control sample. (C) qRT-PCR analysis to confirm the effect of HOXA-AS2 on mRNA expressions of four representative inflammatory genes. (D) The effect of HOXA-AS2 on the protein expression levels of four representative inflammatory genes, determined by Western blotting in HUVECs with or without HOXA-AS2 knockdown, quantified using ImageJ software. (E) qRT-PCR assay was performed to examine the expressions of four representative inflammatory genes in control and HOXA-AS2-overexpressed HUVECs in the presence or absence of TNFα treatment (20 ng/ml, 1 h). All results are from biological triplicates and data shown are the mean ± SD. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* shGFP or vec; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* TNFα-treated vec.

These observations indicate that NF-κB may serve as a critical transcriptional regulator for mediating HOXA-AS2 expression. Next, qRT-PCR analysis was employed to examine the effect of NF-κB on expression of HOXA-AS2. Inhibition of NF-κB was found to have a significantly inhibitory effect on HOXA-AS2 expression (Fig. 4B and Supplementary Fig. 4B).

Prior studies showed NF-κB is involved in the regulation of transcriptional pause release by helping BRD4-mediated P-TEFb recruitment to gene promoter, releasing the promoter-proximal paused RNA polymerase II (Pol II) and facilitating transcription elongation [17]. Therefore, we suspected that NF-κB is likely to modulate HOXA-AS2 expression in such manner. To test this hypothesis, we conducted ChIP assays to investigate the influence of NF-κB on BRD4 or P-TEFb binding capacities with the *HOXA-AS2* promoter. Interestingly, neither shRNA-mediated RelA knockdown nor Bay 11–7082 treatment markedly repressed TNFα-induced BRD4 or CDK9 enrichment at *HOXA-AS2* promoter (Fig. 4C and D and Supplementary Fig. 4C and D). To further confirm the role of BRD4 and P-TEFb in regulation of HOXA-AS2 expression, qRT-PCR analysis was carried out to detect HOXA-AS2 expression after BRD4 or CDK9 knockdown. As expected, both BRD4 and CDK9 attenuation dramatically reduced HOXA-AS2 expression under the condition of TNFα treatment. (Fig. 4E). Collectively, these findings demonstrate that lncRNA HOXA-AS2 can be mediated by a NF-κB-dependent regulatory mechanism for promoter-proximal pausing.

3.5. *HOXA-AS2 is significantly correlated with inflammation-linked vascular disease*

To emphasized the importance of HOXA-AS2 in inflammatory vascular disease, we extracted total RNAs of peripheral blood mononuclear cells (PBMCs) from patients diagnosed with carotid artery atherosclerosis and age-matched control group, subsequently measured the expression of HOXA-AS2. qRT-PCR analysis revealed expressions of HOXA-AS2, inflammatory factors VCAM1 and CCL2 were critically elevated in patients with atherosclerotic lesions, compared with the normal group (Fig. 5 and Supplementary Fig. 5A and B), suggesting a significant correlation between HOXA-AS2 and inflammatory vascular disease.

4. Discussion

As one of the most important inflammatory signaling, NF-κB

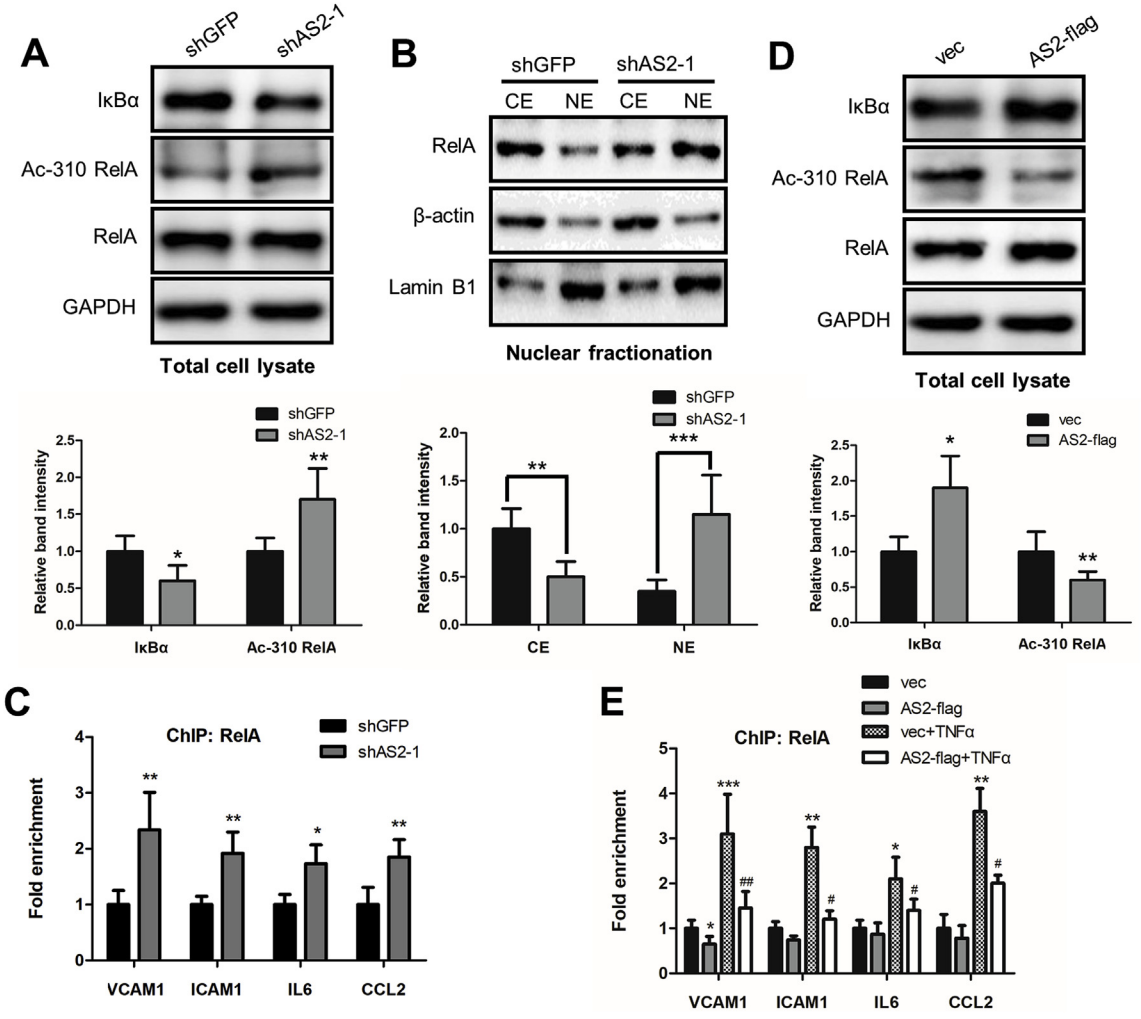

Fig. 3. HOXA-AS2 represses NF-κB activity.
(A) The effect of HOXA-AS2 inhibition on degradation of IκBα and RelA acetylation at K310 site, examined by Western blot analysis in HUVECs, quantified using ImageJ software. (B) Nuclear fractionation analysis in HUVECs showing the effect of HOXA-AS2 on NF-κB nuclear translocation, quantified using ImageJ software. (C) Anti-RelA ChIP assay was performed in HUVECs to investigate NF-κB binding affinity at the promoters of four NF-κB transcriptional targets. (D) The effect of HOXA-AS2 overexpression on NF-κB activity, measured by Western blot analysis in HUVECs treated with TNFα for 1 h, quantified using ImageJ software. (E) ChIP assay was performed in HUVECs, with an antibody against RelA, to detect the effect of overexpressed HOXA-AS2 on RelA binding capacity at four NF-κB target gene promoter regions, with or without TNFα stimulation (20 ng/ml, 1 h). All results are from biological triplicates and data shown are the mean ± SD. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* shGFP or vec; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* TNFα-treated vec.

pathway is widely reported to be involved in regulation of endothelial function. Aberrant activation of NF-κB signaling can lead to endothelial inflammation and dysfunction [41–43]. Endothelium serves as a critical integrator and transducer for signaling transduction in response to a number of stimuli, whereas endothelial dysfunction is significantly associated with diverse inflammatory vascular diseases [44–48]. In this study, we characterize the lncRNA HOXA-AS2 as a crucial regulator to protect the endothelium from inflammatory lesions. We demonstrated the inhibitory role of HOXA-AS2 in endothelium inflammation, using monocyte adhesion and transendothelial migration assays *in vitro*; attenuation of HOXA-AS2 was found to markedly promote the monocyte adhesion to ECs and transendothelial migration through HUVEC monolayers. More importantly, *In vivo* studies showed HOXA-AS2 has a significant correlation with carotid artery atherosclerosis and its expression in atherosclerotic patients is highly increased, compared with the normal control group, emphasizing the functional importance of HOXA-AS2 when suffering from inflammatory vascular lesions.

Prior studies revealed that NF-κB activity can be regulated at multiple levels [10,12]. In resting condition, NF-κB is primarily sequestered in the cytoplasm by an NF-κB inhibitor protein IκBα. The degradation of IκBα can facilitate NF-κB release from the inhibition of IκBα and entry into the nucleus to participate in transcription regulation. Moreover, RelA subunit of NF-κB can be modified by specific kinases or acetyltransferases, which greatly contribute to the activity of NF-κB; the acetyltransferase p300-mediated RelA acetylation at K310 site was widely reported to enhance the transcriptional activity of NF-κB by interacting with the coactivator BRD4, activating transcription elongation of the NF-κB target genes. Our investigations uncovered that the lncRNA HOXA-AS2 could function as a repressor to protect IκBα from degradation and therefore inhibit NF-κB nuclear translocation (Fig. 6). On the other hand, HOXA-AS2 was found to mediate RelA acetylation at K310 site, HOXA-AS2 depletion could enhance the acetylation status at RelA K310 site (Fig. 6). Collectively, these observations demonstrate that HOXA-AS2 can act as a critical repressor to negatively regulate the activity of NF-κB signaling.

Negative feedback regulation is a universal event in a variety of cellular processes including signal transduction, e.g. the activation of NF-κB signaling can elevate the expression of ubiquitin-editing enzyme TNFAIP3, however, the upregulated TNFAIP3 could inversely inhibit NF-κB activity. By such a self-protective manner, NF-κB signaling is

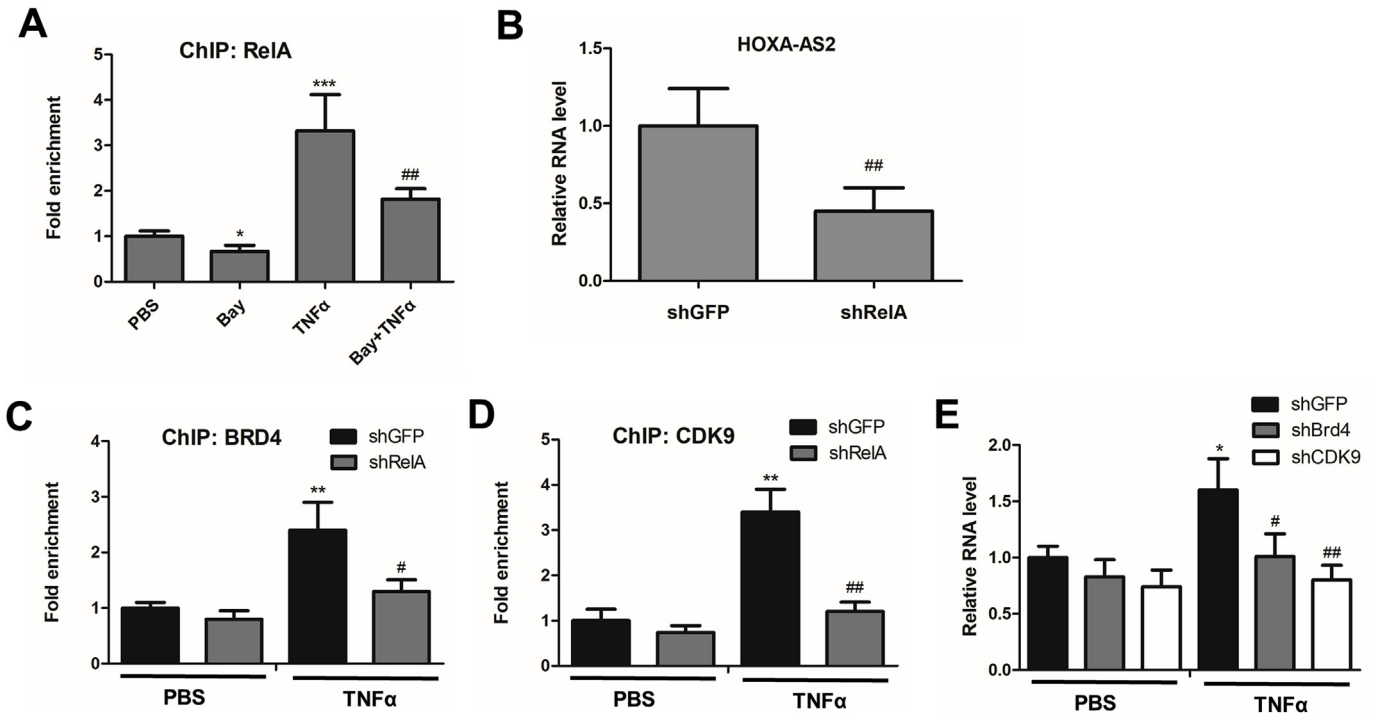

Fig. 4. NF-κB enhances HOXA-AS2 expression by activating its transcription elongation.
(A) Anti-RelA ChIP assay in HUVECs, treated with TNFα for 3 h or pre-treated with Bay 11–7082 for 1 h and additional co-treatment with TNFα for 3 h, to demonstrate the distribution of NF-κB near the promoter region of *HOXA-AS2*. (B) qRT-PCR analysis was performed to measure the expression of HOXA-AS2 after knocking down RelA in HUVECs activated with TNFα for 1 h. (C–D) ChIP assay was performed in HUVECs in the presence or absence of TNFα activation (20 ng/ml, 1 h), with an antibody against BRD4 or CDK9, to examine the BRD4 and CDK9 occupancy at *HOXA-AS2* promoter, respectively, with or without RelA knockdown. (E) qRT-PCR analysis showing the effect of BRD4 and CDK9 on the expression of HOXA-AS2, respectively, which was performed in HUVECs with or without TNFα treatment (20 ng/ml, 1 h) after BRD4 or CDK9 knockdown. All results are from biological triplicates and data shown are the mean ± SD. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* PBS or shGFP; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* TNFα or TNFα-treated shGFP.

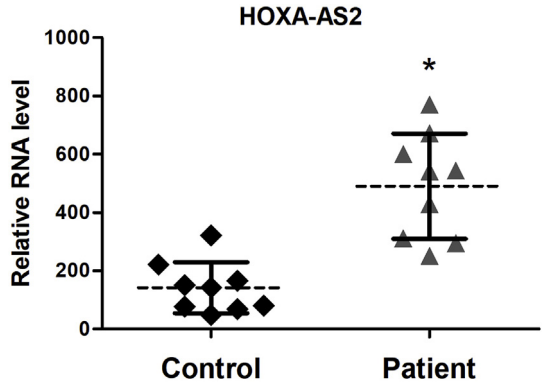

Fig. 5. The expression of HOXA-AS2 is highly increased in patients with atherosclerotic lesions.
qRT-PCR analysis was performed to detect the expression of HOXA-AS2, as well as VCAM1 and CCL2, in the normal control and atherosclerotic patients. Total RNAs were extracted from peripheral blood mononuclear cells derived from the control group and patients with atherosclerosis. The solid and dashed horizontal lines represent standard deviation (SD) and mean, respectively. $n = 9$. for each experimental group. $^{*}p < 0.05$ *versus* the normal control group.

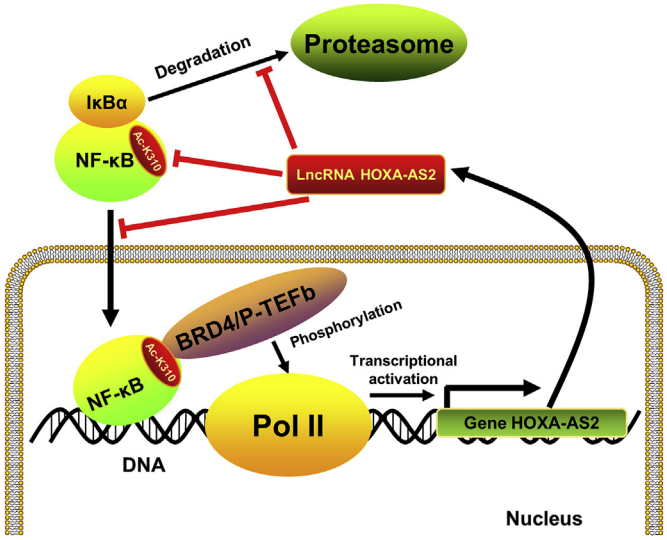

Fig. 6. Schematic representation of a critical molecular mechanism by which HOXA-AS2 regulates endothelium inflammation.
When endothelial cells are exposed to pro-inflammatory stimuli, NF-κB is activated and enters the nucleus, inducing the transcriptional activation of HOXA-AS2 by helping recruiting the transcription elongation complex BRD4/P-TEFb to *HOXA-AS2* promoter and stimulating the BRD4/P-TEFb-mediated phosphorylation of Pol II C-terminal domain. However, the upregulated HOXA-AS2 will in turn repress NF-κB activity by blocking the proteasome-mediated IκBα degradation and RelA K310 site acetylation. Taken together, NF-κB can coordinate with HOXA-AS2 to establish a negative feedback loop, by which the endothelium can avoid overactivation of NF-κB signaling and excessive inflammation.

prevented from excessive activation in response to pro-inflammatory stimuli. Here, we identify HOXA-AS2 as another feedback regulator of NF-κB. Mechanistically, NF-κB is found to help recruit BRD4/P-TEFb complex to *HOXA-AS2* promoter region, therefore facilitating release of the paused RNA polymerase II and the subsequent activation of transcription elongation (Fig. 6). The upregulated HOXA-AS2 will then in turn have an inhibitory effect on NF-κB activation by suppressing the proteasome-mediated IκBα degradation and RelA K310 acetylation

(Fig. 6), consequently relieving endothelium inflammation and related vascular disorders. NF-κB can upregulated HOXA-AS2, while the increased HOXA-AS2 will inversely repress NF-κB activity. By this negative feedback manner, HOXA-AS2 coordinates with NF-kB to establish a balanced state, therefore preventing inflammatory vascular disorders from further activation of NF-κB signaling and subsequent inflammatory lesions. This may be the main reason why inflammatory atherosclerosis is usually accompanied by highly expressed HOXA-AS2. In summary, these observations offer us a new way to balance the NF-κB signaling-driven excessive inflammation and we conclude that HOXA-AS2 may serve as a promising therapeutic target for treating endothelial inflammation-linked vascular diseases in the near future.

Conflicts of interest

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

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Author contributions

Juntang Lin, Yizhou Jiang and Xinxing Zhu conceived and designed the project. Xinxing Zhu, Yanli Liu and Jinjin Yu performed most of the experiments. Genshen Zhong helped analyzing the clinicopathological samples. Yanyan Feng performed cDNA cloning and shRNA preparation. Xinxing Zhu wrote the manuscript. All authors contributed to discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.12.012.

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