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The suppression of ox-LDL-induced inflammatory response and apoptosis of HUVEC by lncRNA XIAT knockdown *via* regulating miR-30c-5p/PTEN axis

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Abstract. – OBJECTIVE: Exposure of oxidized low-density lipoprotein (ox-LDL) could cause dysfunction of HUVEC, thus leading to atherosclerosis development, which is a common inflammatory vascular disease. Long noncoding RNA X-inactive specific transcript (XIST) has been reported to be implicated in atherosclerosis. However, the mechanism by which this lncRNA participates in the progression of atherosclerosis is poorly defined.

MATERIALS AND METHODS: HUVEC challenged by ox-LDL were used as a cellular model of atherosclerosis. Cell viability, apoptosis, LDH release, and inflammatory cytokines secretion were detected by MTT, flow cytometry, and ELISA assays. The expression levels of XIST, microRNA (miR)-30c-5p, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) were measured by quantitative Real Time-Polymerase Chain Reaction and Western blot. The target interaction between XIST and miR-30c-5p or miR-30c-5p and PTEN was validated by the Luciferase reporter assay and RNA immunoprecipitation.

RESULTS: Treatment of ox-LDL induced cell apoptosis and inflammatory cytokines release in HUVEC. XIST expression was enhanced in HUVEC treated by ox-LDL, and its knockdown decreased cell apoptosis and inflammatory response in ox-LDL-treated cells. MiR-30c-5p was a target of XIST and its overexpression suppressed cell apoptosis and inflammatory response induced by ox-LDL, which was weakened by the introduction of XIST. PTEN was a target of miR-30c-5p, and its interference led to great inhibition of cell apoptosis and inflammatory response induced by ox-LDL in HUVEC, while this effect was attenuated by miR-30c-5p deficiency or XIST overexpression.

CONCLUSIONS: XIST knockdown suppresses inflammatory response and apoptosis of HUVEC stimulated by ox-LDL by increasing miR-30c-5p and decreasing PTEN.

Key Words:

Atherosclerosis, Inflammatory response, Apoptosis, XIST, MiR-30c-5p, PTEN.

Abbreviations

ox-LDL=oxidized low-density lipoprotein, XIST= X-inactive specific transcript, PTEN=phosphatase and tensin, ox-LDL=Oxidized low-density lipoprotein, NOD2=nucleotide-binding oligomerization domain 2, HUVEC=Human umbilical vein endothelial cells, LDH=lactate dehydrogenase.

Introduction

Atherosclerosis is an inflammation-related cardiovascular event, which could arise to coronary artery disease with high morbidity and mortality[1,2]. Vascular endothelium is the key for the cardiovascular system, and the dysfunction of endothelial cells contributes to atherosclerosis[3]. Oxidized low-density lipoprotein (ox-LDL) is associated with atherosclerosis development and is widely used to establish the cellular model of atherosclerosis by exposing to HUVEC[4-7]. Moreover, the inflammatory response mediated by ox-LDL is an important event during atherosclerosis[8]. However, the mechanism underlying how ox-LDL-mediated endothelial cells dysfunction remains poorly understood.

Noncoding RNAs, which are not translated into proteins, are implicated in the regulation of endothelial function in atherosclerosis[9]. Long noncoding RNAs (lncRNAs) are the RNA molecules with more than 200 nucleotides in length, which play important roles in the development of cardiovascular diseases[10]. Especially, lncRNAs are involved in the progression of atherosclerosis by regulating vascular function and inflammation[11]. Moreover, lncRNAs could participate in atherosclerosis by regulating endothelial cell function by lncRNA/microRNA (miRNA)/mRNA axes as competing endogenous RNAs (ceRNAs)[12]. For example, Cao et al[7] reported that lncRNA H19 promotes atherosclerosis progression by exacerbating HUVEC in-

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jury induced by ox-LDL *via* interacting with miR-let-7. Zhu et al[13] suggested that lncRNA activated by tumor growth factor-β (lncRNA ATB) contributes to viability, migration, and angiogenesis of endothelial cells by regulating miR-195. Apart from these, lncRNA lymphoid enhancer-binding factor 1 antisense RNA 1 (LEF1-AS1) promotes the development of atherosclerosis *via* regulating vascular smooth muscle cell function by miR-544a/phosphatase and tensin homolog deleted on chromosome 10 (PTEN) axis[14]. X-inactive specific transcript (XIST) as a lncRNA is associated with poor prognosis and cancer development in multiple malignancies[15,16]. However, it is insufficient for understanding how and whether XIST regulates atherosclerosis development except for the report of Xu et al[17], which revealed that XIST knockdown decreases endothelial cell apoptosis induced by ox-LDL *via* regulating miR-320 and nucleotide-binding oligomerization domain 2 (NOD2).

The database of DIANA tools predicted that XIST and PTEN have the complementary sequences of miR-30c-5p, which stimulated us to assume a potential ceRNA hypothesis of XIST/miR-30c-5p/PTEN. Hence, this study aimed to explore the role of XIST in apoptosis and inflammatory response in HUVEC stimulated by ox-LDL and investigate the target association between miR-30c-5p and XIST or PTEN, thus disclosing a novel mechanism for understanding the pathogenesis of atherosclerosis.

Materials and Methods

Cell Culture and Treatment

Human umbilical vein endothelial cells (HUVEC) were purchased from American Tissue Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. For the establishment of the atherosclerosis model, HUVEC were treated with ox-LDL (Solarbio, Beijing, China) for the indicated time and the cells without treatment of ox-LDL were regarded as the control group.

MTT

HUVEC (1 x 10^4/well) in a 96-well plate in triplicate were exposed to different concentrations (20, 50, or 100 μg/ml) of ox-LDL for 24 h or 50 μg/ml ox-LDL for different time points (12, 24, or 48 h). At the ending point, the mediums containing ox-LDL were replaced with fresh mediums with 0.5% MTT solution (Beyotime, Shanghai, China). Following another culture for 4 h, the mediums were removed, and dimethyl sulfoxide (DMSO) solutions (100 μl/well) purchased from Sigma-Aldrich (St. Louis, MO, USA) were added into each well. After the dissolution of formazan, the absorbance at 570 nm was determined using a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was expressed as percentage by normalizing the control (non-treated with ox-LDL) group.

Flow Cytometry

Flow cytometry was performed to assess cell apoptosis using Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, USA). Transfected or non-transfected (blank) HUVEC (2 x 10^5/well) were seeded into 12-well plates and exposed to 50 μg/ml ox-LDL for 24 h. Each sample was prepared in triplicate. Subsequently, the cells were collected and incubated with Annexin V-FITC and PI solution for 10 min in the dark, followed by detection of the stained cells using flow cytometry. The apoptotic rate of the cells included early and late apoptosis were presented as percentage of cells with Annexin V-FITC positive and PI negative or positive.

LDH Release and ELISA Assays

LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China) was used to measure the activity of lactate dehydrogenase (LDH) released from the damaged cells. Transfected or non-transfected (blank) HUVEC (1 x 10^4/well) were placed into 96-well plates and every sample was prepared in triplicate. After exposure of 50 μg/ml ox-LDL for 24 h, LDH activity of cells was analyzed by the kit according to the manufacturer's instructions and calculated by the standard curves.

To detect the inflammatory cytokines secretion, the mediums of the treated cells were collected and analyzed using Human High Sensitivity ELISA Kit for IL-6 and IL-1β (Thermo Fisher Scientific, Waltham, MA, USA). After the reaction was performed following the manufacturer's instructions, the intensity of the color was determined at 450 nm and calculated *via* the standard curves.

Cell Transfection

The XIST overexpression vectors were generated by inserting the full-length sequences into pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA, USA), and pcDNA3.1 empty vectors

(pcDNA) were used as a corresponding control. siRNA against XIST (si-XIST) (5'-GCACAAUAUCUUUGAACUA-3'), siRNA against PTEN (si-PTEN) (5'-GGUGAAACUAUACUUUACATT-3'), siRNA negative control (si-NC) (5'-UUCUCCGAACGUGUCACGUTT-3'), miR-30c-5p mimic (miR-30c-5p) (5'-UGUAAACAUCCUACACUCUCAGC-3'), miRNA negative control (miR-NC) (5'-CGAUCGCAUCAGCAUCGAUUGC-3'), miR-30c-5p inhibitor (in-miR-30c-5p) (5'-GCUGAGAGUGUAGGAUGUUUACU-3') and inhibitor negative control (in-miR-NC) (5'-CUAACGCAUGCACAGUCGUACG-3') were generated by GenePharma (Shanghai, China). The transfection of the constructed oligonucleotides with 30 nM concentration in HUVEC was received *via* Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h. Blank is the non-transfected group.

qRT-PCR

Transfected or treated HUVEC were washed and lysed in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The RNA (1 μg) was reversely transcribed to cDNA using the PrimeScript RT Reagent Kit (TaKaRa, Otsu, Shiga, Japan) and cDNA product was used for qRT-PCR using SYBR mix (TaKaRa, Otsu, Shiga, Japan). The primers were listed as: XIST (Forward, 5'-ACGCTGCATGTGTCCTTAG-3'; Reverse, 5'-GAGCCTCTTATAGCTGTTTG-3'); PTEN (Forward, 5'-AGAACTTATCAAACCCTT-3'; Reverse, 5'-GTCCTTACTTCCCCAT-3'); miR-30c-5p (Forward, 5'-GCCGCTGTAAACATCCTACACT-3'; Reverse, 5'-GTGCAGGGTCCGAGGT-3'). GAPDH (Forward, 5'-AGAAGGCTGGGGCTCATTTG-3'; Reverse, 5'-AGGGGCCATCCACAGTCTTC-3') and U6 (Forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; Reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3') were regarded as internal control for XIST, PTEN, or miR-30c-5p, respectively. The relative expression levels of XIST, PTEN, and miR-30c-5p were analyzed by the $2^{-\Delta\Delta Ct}$ method[18].

Luciferase Reporter Assay and RIP

DIANA tools were used to search the targets of XIST or miR-30c-5p. The sequences of XIST or 3'UTR of PTEN containing the complementary sequences with miR-30c-5p were inserting into the pmirGLO Luciferase reporter vectors (Promega, Madison, WI, USA) to generate the wild-type Luciferase reporter vectors (XIST WT and PTEN 3'UTR WT). Meanwhile, the corresponding mutants (XIST MUT and PTEN 3'UTR MUT) were obtained *via* mutating the seed sites with miR-30c-5p. HUVEC were co-transfected with constructed Luciferase reporter vectors together with miR-30c-5p or miR-NC. After 24 h post-transfection, HUVEC were collected and analyzed by the Luciferase reporter system (Promega, Madison, WI, USA) for the Luciferase activity analysis. For RIP assay, HUVEC transfected with miR-30c-5p or miR-NC were analyzed with a Magna RNA immunoprecipitation kit (Millipore, Billerica, MA, USA). The enrichment level of XIST in Ago2 or IgG RIP complex was assessed by qRT-PCR.

Western Blot

After the indicated transfection, HUVEC were washed and collected for protein extraction. Equal amounts (30 μg) of protein lysates were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and membrane transfer using polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blockage *via* 5% non-fat milk at room temperature for 1 h, the membranes were incubated with primary antibodies against PTEN (ab31392, 1:1000 dilution, Abcam, Cambridge, MA, USA) overnight and the secondary antibody (ab97051, 1:20000 dilution, Abcam, Cambridge, MA, USA) for 2 h. The experiments were repeated three times and β-actin (ab227387, 1:10000 dilution) was used as a loading control. After the development of the enhanced chemiluminescence solution (Beyotime, Shanghai, China), the relative expression of PTEN was normalized to indicate the control group.

Statistical Analysis

GraphPad Prism 7.0 (La Jolla, CA, USA) was used to process the data of three independent experiments and the data were presented as mean ± standard deviation. The comparison between the two groups was conducted by the Student's *t*-test and the differences between three or more groups were analyzed by ANOVA with the Tukey's post-hoc test. *p*-value less than 0.05 was considered to be statistically significant.

Results

XIST Expression is Enhanced in ox-LDL-Treated HUVEC

First, the model of atherosclerosis was established using HUVEC stimulated by ox-LDL *in*

vitro. As shown in Figure 1A, the activity of HUVEC was decreased after the treatment of ox-LDL for 24 h in a concentration-dependent manner. Meanwhile, the exposure of ox-LDL also induced a great reduction of cell viability in a time-dependent manner (Figure 1B). Moreover, after treatment of 50 μg/ml ox-LDL for 24 h, the cell apoptosis (Figure 1C), LDH release (Figure 1D), and secretion of IL-6 and IL-1β (Figures 1E and 1F) were significantly increased in HUVEC compared with those in the control group. Furthermore, the abundance of XIST was significantly increased 2.9-fold in HUVEC after stimulation of ox-LDL (Figure 1G). These data suggested that the high expression of XIST might be associated with ox-LDL-induced HUVEC injury in atherosclerosis.

Knockdown of XIST Inhibits Apoptosis and Inflammatory Response in ox-LDL-Treated HUVEC

To explore the role of XIST in dysfunction of HUVEC induced by ox-LDL, its abundance in HUVEC was knocked down using siRNA, which was confirmed with a 65% reduction of XIST level in si-XIST group in comparison to si-NC group (Figure 2A). Moreover, the data of flow cytometry showed that XIST knockdown remarkably reduced the apoptotic rate of HUVEC induced by ox-LDL (Figure 2B). Meanwhile, the LDH release in HUVEC stimulated by ox-LDL was notably decreased by silencing XIST (Figure 2C). In addition, the secretion of IL-6 and IL-1β triggered by ox-LDL was evidently suppressed in HUVEC (Figures 2D and 2E). These results indicated that XIST knockdown attenuated ox-LDL-induced HUVEC dysfunction.

XIST is a Sponge of MiR-30c-5p

To explore the mechanism mediated by XIST, DIANA tools were used and provided the potential complementary sequences between XIST and miR-30c-5p (Figure 3A). To confirm the interaction between XIST and miR-30c-5p, the Luciferase reporter assay and RIP were performed in HUVEC. As displayed in Figure 3B, the Luciferase activity was notably decreased by 70% in XIST WT group by transfection of miR-30c-5p compared with that of miR-NC, while the activity was not changed in the XIST MUT group. Moreover, RIP assay described that miR-30c-5p overexpression led to an 8.2-fold increase of XIST level enriched by Ago2 RIP, whereas IgG showed little enrichment (Figure 3C). In addition, the expression of miR-30c-5p in HUVEC was remarkably decreased by 67% by insult of ox-LDL (Figure 3D). Besides, the qRT-PCR assay revealed that the level of miR-30c-5p in HUVEC was significantly elevated by XIST silence and decreased by XIST overexpression (Figure 3E). These findings suggested that miR-30c-5p was targeted and negatively regulated by XIST in HUVEC.

XIST Regulates Apoptosis and Inflammatory Response by Sponging miR-30c-5p in ox-LDL-Treated HUVEC

In order to investigate the effect of miR-30c-5p in ox-LDL-treated HUVEC, the cells were transfected with miR-30c-5p or miR-NC before treatment of ox-LDL. As shown in Figure 4A, the overexpression of miR-30c-5p greatly inhibited cell apoptosis induced by ox-LDL in HUVEC. Furthermore, the LDH release caused by ox-LDL in the cells was evidently suppressed by miR-30c-5p addition (Figure 4B). Furthermore, the addition of miR-30c-5p significantly decreased the secretion of IL-6 and IL-1β in HUVEC challenged by ox-LDL (Figures 4C and 4D). Besides, to explore whether these events were mediated by XIST, the cells were transfected with pcDNA or XIST in the presence of miR-30c-5p. As displayed in Figures 4A-4D, the suppressive effect mediated by miR-30c-5p on cell apoptosis, the LDH release, and the inflammatory cytokines secretion were abated by the introduction of XIST. Together, XIST regulated ox-LDL-induced HUVEC dysfunction by mediating miR-30c-5p.

PTEN is a Target of MiR-30c-5p

DIANA tools also predicted the targets of miR-30c-5p and it displayed the binding sites of miR-30c-5p and PTEN (Figure 5A). To validate this prediction, the wild-type and mutant Luciferase reporter vectors targeted PTEN were constructed and transfected into HUVEC. The results showed that miR-30c-5p overexpression resulted in a 62% reduction of the Luciferase activity in PTEN 3'UTR WT group, while it did not affect the activity when the seed sites were mutated in PTEN 3'UTR MUT group (Figure 5B). Moreover, the mRNA level of PTEN in HUVEC was significantly increased 2.7-fold after treatment of ox-LDL (Figure 5C). In addition, the expression of PTEN protein in HUVEC was markedly decreased *via* miR-30c-5p overexpression but increased by miR-30c-5p exhaustion (Figure 5D). Meanwhile, Western blot also described that

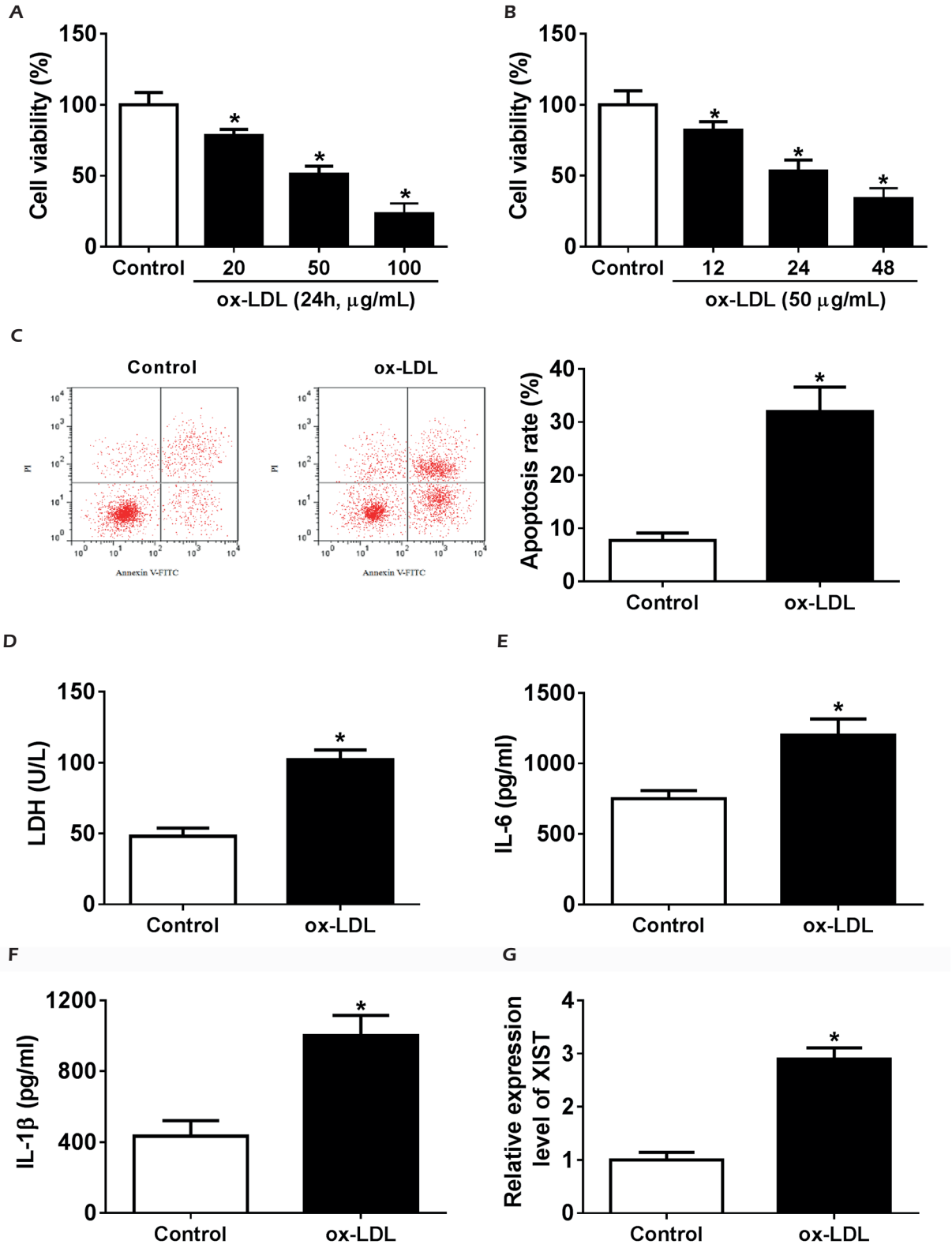

Figure 1. The expression of XIST is increased in ox-LDL-induced atherosclerosis model *in vitro*. ***A,*** and ***B,*** HUVEC were stimulated with different concentrations of ox-LDL for 24 h or 50 μg/ml ox-LDL for different time points and then cell viability was determined by MTT. Cell apoptosis ***(C)*** and ***(D)*** and inflammatory response ***(E)*** and ***(F)*** in HUVEC treated by ox-LDL were measured by flow cytometry, LDH release, and ELISA detection kit. ***G,*** qRT-PCR was performed to detect the level of XIST in HUVEC treated by ox-LDL. *$p<0.05$ compared with the control (non-treated with ox-LDL) group.

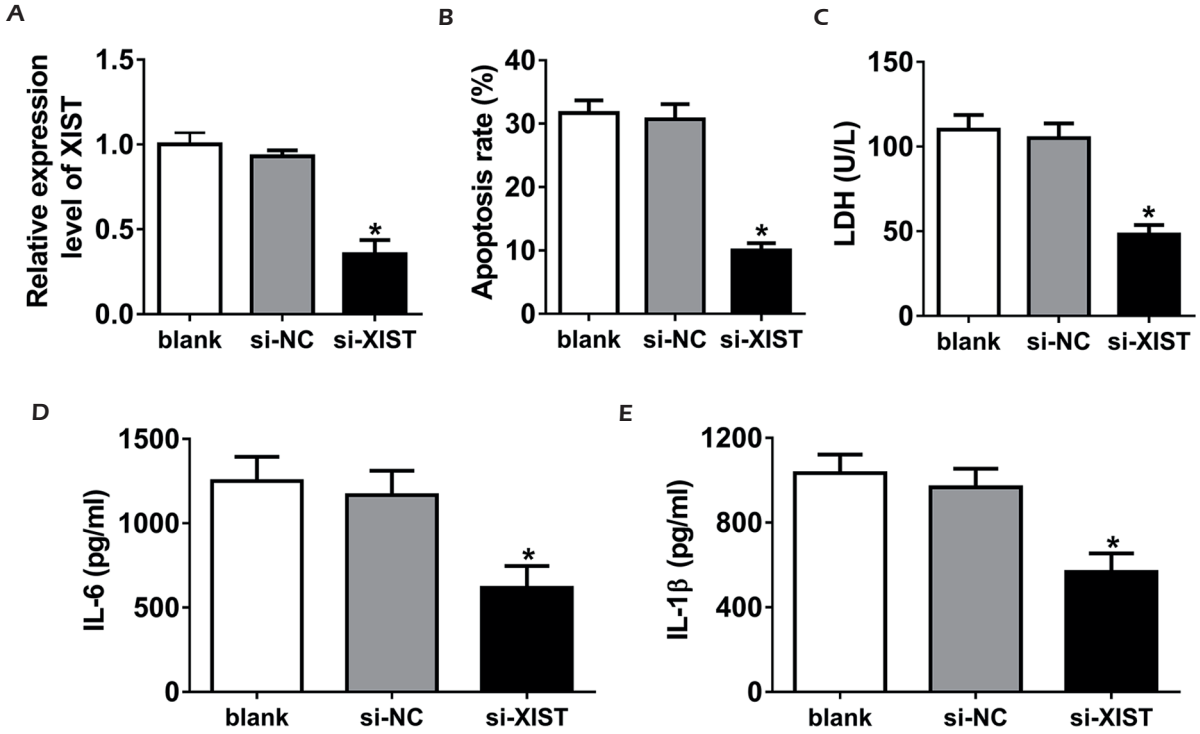

Figure 2. XIST knockdown represses cell apoptosis and inflammatory response in HUVEC stimulated by ox-LDL. ***A,*** The expression of XIST in HUVEC transfected with si-XIST or si-NC was measured by qRT-PCR after treatment of ox-LDL. Cell apoptosis ***(B)***, LDH release ***(C)***, IL-6 ***(D)***, and IL-1β ***(E)*** levels in HUVEC transfected with si-XIST or si-NC were determined after stimulation of ox-LDL. Blank is the non-transfected group. * $p<0.05$ compared with si-NC group.

PTEN protein level in HUVEC was positively regulated by XIST (Figure 5E). These data revealed that PTEN was a target of miR-30c-5p in HUVEC.

MiR-30c-5p and XIST Regulate Apoptosis and Inflammatory Response by Targeting PTEN in ox-LDL-Treated HUVEC

To evaluate the effect of PTEN in ox-LDL-treated HUVEC, the cells were transfected with si-NC or si-PTEN and then treated by ox-LDL. As shown in Figures 6A-6D, the knockdown of PTEN significantly inhibited cell apoptosis, LDH release, and levels of IL-6 and IL-1β in HUVEC stimulated by ox-LDL. Moreover, to explore whether PTEN was required for miR-30c-5p-mediated regulation of HUVEC function, the cells were co-transfected with si-PTEN and in-miR-NC or in-miR-30c-5p. As displayed in Figures 6A-6D, the silence of PTEN-mediated inhibition of HUVEC injury was abolished by miR-30c-5p deletion. In addition, to explore whether PTEN-mediated HUVEC dysfunction was regulated by XIST, HUVEC were co-transfected with si-PTEN and pcDNA or XIST and then treated by ox-LDL. The results showed that the introduction of XIST mitigated the effect of PTEN knockdown on HUVEC injury induced by ox-LDL (Figures 6E-6H). These results uncovered that miR-30c-5p and XIST regulated ox-LDL-induced HUVEC dysfunction by targeting PTEN.

Discussion

LncRNAs exhibit vital roles in the regulation of endothelial dysfunction and cardiovascular disorders[19]. Previous studies[6,7,13,14,20] on atherosclerosis have indicated multiple promising lncRNAs, including metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), H19, ATB, LEF1-AS1, and zinc finger e-box binding homeobox 1 antisense 1 (ZEB1-AS1). However, we known a little about the biological role and mechanism of XIST in atherosclerosis. This investigation used ox-LDL-induced model to investigate the role of

this lncRNA in endothelial cells dysfunction and first confirmed the potential ceRNA network of XIST/miR-30c-5p/PTEN.

In the present work[5,6], we first established the cellular model of atherosclerosis using HUVEC treated by ox-LDL and found that XIST expression was enhanced in HUVEC after treatment of ox-LDL, which was consistent with the former work[17]. This suggested that XIST might be associated with ox-LDL-induced injury in HUVEC. Moreover, the pro-apoptotic role of XIST in HUVEC has been previously reported[17]. However, the regulatory effect of XIST on the inflammatory response in atherosclerosis remains elusive. Inflammatory cytokines, especially IL-1β, play key roles in atherosclerosis and could act as a target for atherosclerosis therapy[21,22]. We found that the knockdown of XIST attenuated the inflammatory response mediated by ox-LDL, revealed by reduction of IL-1β and IL-6 secretion and indicating the anti-inflammatory role of XIST silence in atherosclerosis, which is also in agreement with that in human fibroblast challenged by lipopolysaccharide[23]. Our research demonstrated the therapeutic effect of XIST inhibition on atherosclerosis by decreasing endothelial cells apoptosis and inflammatory injury.

Previous reports[16,24,25] suggested that XIST could serve as a ceRNA for miRNAs to regulate cell processes. To explore the potential mechanism addressed by XIST in atherosclerosis, we explored the target miRNAs and observed the interaction between XIST and miR-30c-5p. The former work demonstrated that the downregula-

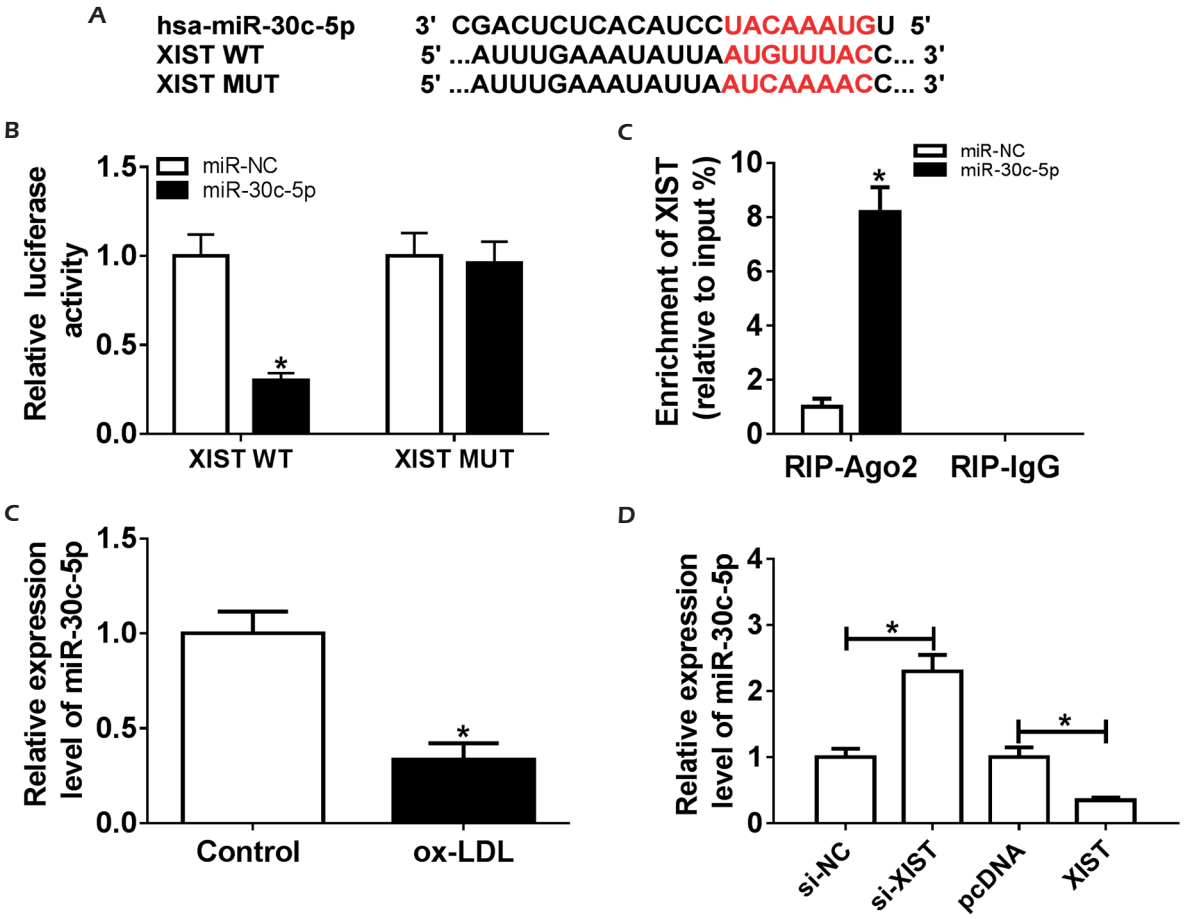

Figure 3. XIST is a decoy of miR-30c-5p. ***A,*** The binding sites of XIST and miR-30c-5p. ***B,*** and ***C,*** Luciferase reporter assay and RIP assay were performed in HUVEC transfected with miR-30c-5p or miR-NC. ***D,*** The expression of miR-30c-5p was detected in HUVEC after treatment of ox-LDL. ***E,*** The effect of XIST on miR-30c-5p expression in HUVEC was analyzed by qRT-PCR. * $p<0.05$ compared with miR-NC group for B, C, with control (non-treated with ox-LDL) group for D, and with si-NC or pcDNA group for E.

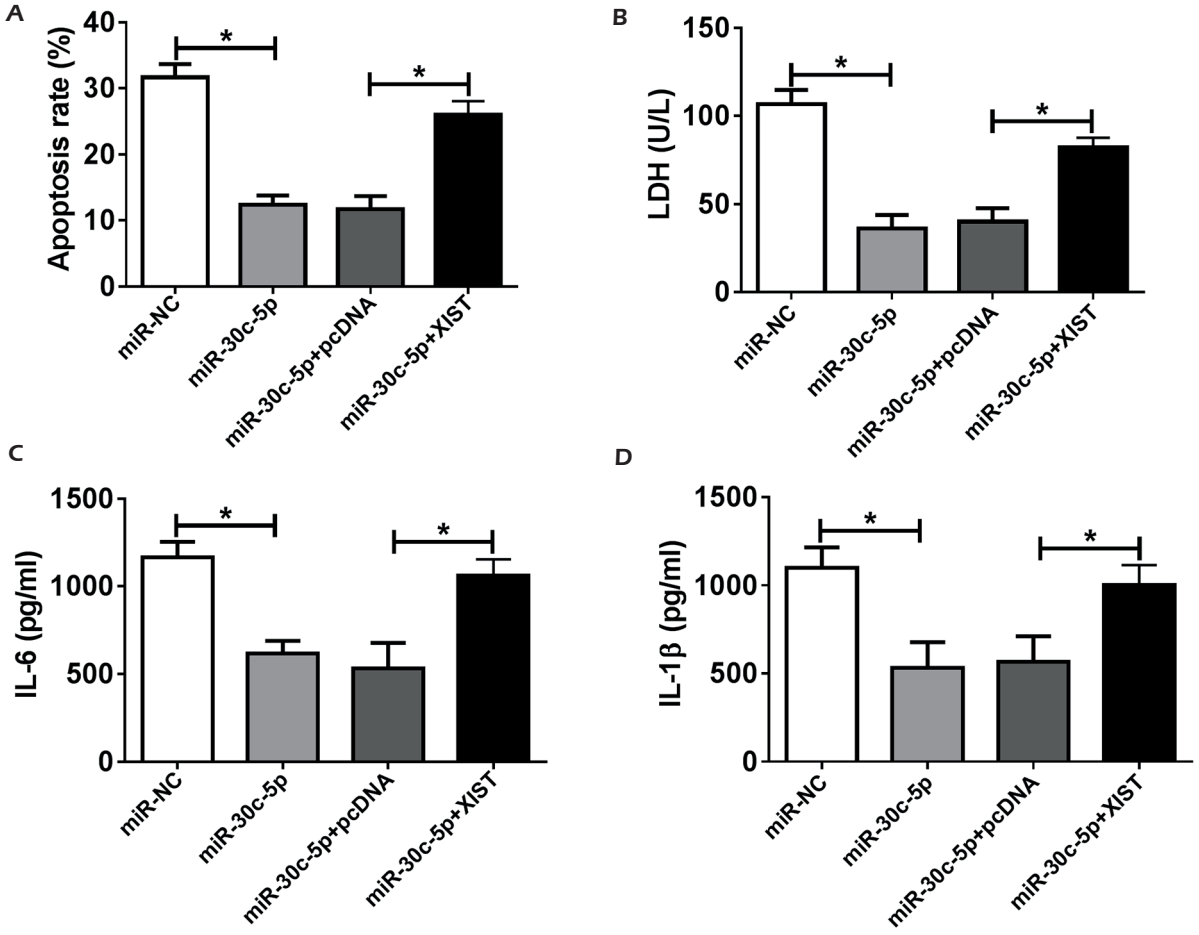

Figure 4. XIST sponges miR-30c-5p to regulate cell apoptosis and inflammatory response in HUVEC stimulated by ox-LDL. Cell apoptosis ***(A)***, LDH release ***(B)***, IL-6 ***(C)***, and IL-1β ***(D)*** levels in HUVEC transfected with miR-NC, miR-30c-5p, miR-30c-5p, and pcDNA or XIST were examined after the treatment of ox-LDL. *$p<0.05$ compared with miR-NC or miR-30c-5p + pcDNA group.

tion of miR-30c-5p was responsible for ox-LDL-induced injury in atherosclerosis[26]. Moreover, Li et al[27] revealed that miR-30c-5p suppressed NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, as well as its mediated pyroptosis in ox-LDL-treated endothelial cells, suggesting the anti-inflammatory and anti-apoptotic role of miR-30c-5p in atherosclerosis. In our work, we also found that miR-30c-5p overexpression decreased apoptosis and inflammatory response induced by ox-LDL in HUVEC, which is also in agreement with the findings of Li et al[27]. Besides, miR-30c-5p was negatively regulated by XIST and the rescue experiments uncovered that the effect of XIST on regulation of ox-LDL-induced injury was realized by sponging miR-30c-5p.

As a result of binding miRNAs, lncRNAs could regulate mRNA and the protein expression. We used DIANA tools to predict that PTEN has similar seed sites of miR-30c-5p with XIST and validated PTEN as a target of miR-30c-5p using the Luciferase reporter assay. Previous investigations[14,28] reported the promoting role of PTEN in atherosclerosis development by inducing the vascular smooth muscle cell proliferation and migration. Moreover, PTEN could promote the apoptosis of vascular endothelial cells in tumor necrosis factor-α-induced atherosclerosis model[29]. Similarly, our analysis also displayed the poor effect of PTEN on atherosclerosis development, which its knockdown inhibited apoptosis and inflammatory response in HUVEC treated by ox-LDL. Furthermore, we found that PTEN protein

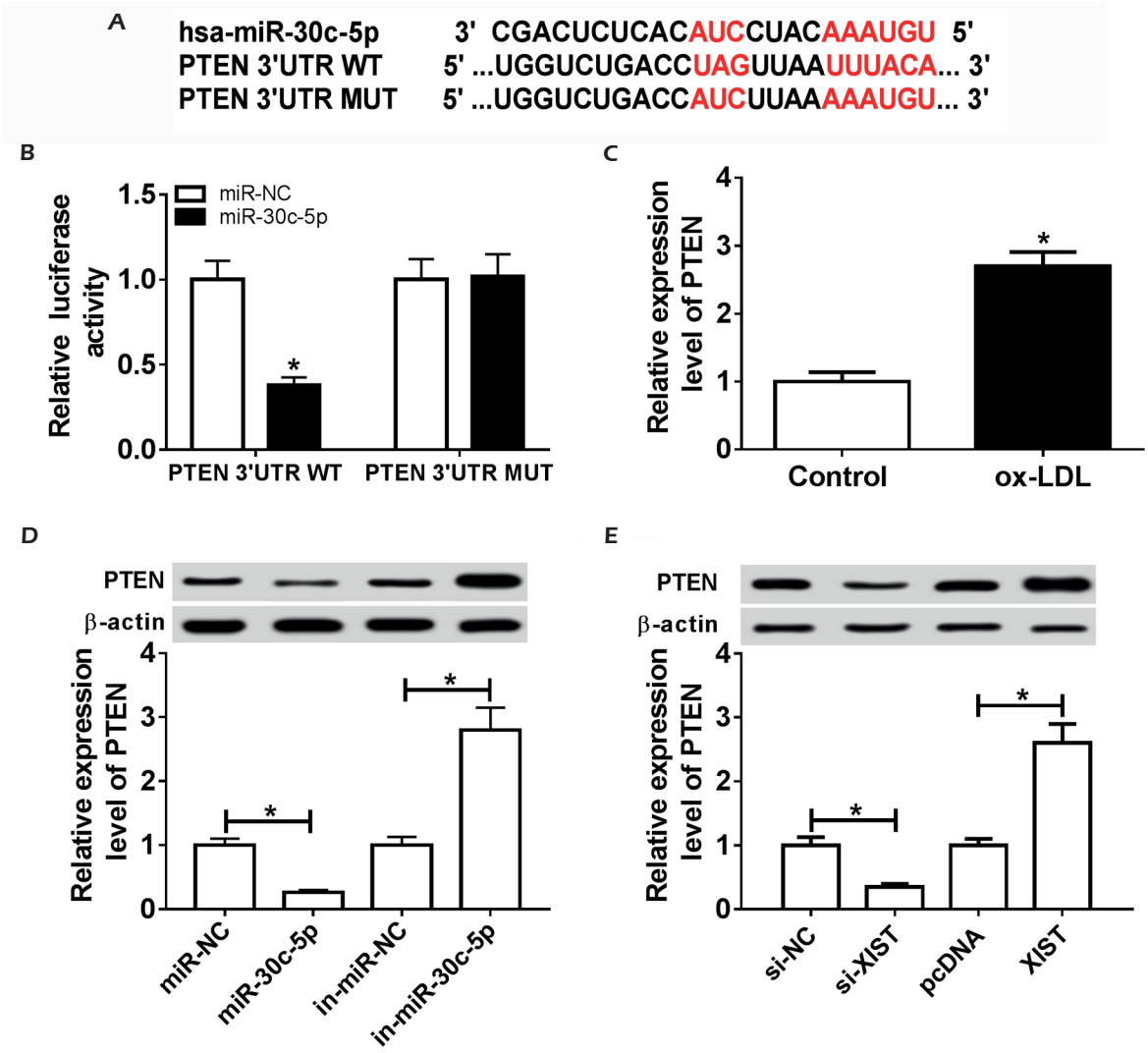

Figure 5. PTEN is a target of miR-30c-5p. ***A,*** The binding sites of miR-30c-5p and PTEN. ***B,*** Luciferase activity was analyzed in HUVEC co-transfected with miR-NC or miR-30c-5p and PTEN 3'UTR WT or PTEN 3'UTR MUT. ***C,*** qRT-PCR assay was conducted to detect the mRNA level of PTEN in HUVEC after treatment of ox-LDL. ***D,*** and ***E,*** Western blot was carried out to detect the effect of miR-30c-5p or XIST on PTEN protein expression in HUVEC. * $p<0.05$ compared with miR-NC group for B, with control (non-treated with ox-LDL) group for C, with miR-NC or in-miR-NC for D, and with si-NC or pcDNA group for E.

was negatively regulated by miR-30c-5p and positively regulated by XIST. Meanwhile, the function of PTEN inhibition on HUVEC dysfunction was abated by miR-30c-5p exhaustion or XIST introduction, which indicated that XIST could regulate HUVEC dysfunction by targeting PTEN *via* competitively sponging miR-30c-5p.

Conclusions

The expression level of XIST was increased in ox-LDL-induced atherosclerosis model *in vitro*. Moreover, the silence of XIST attenuated cell apoptosis and the inflammatory response of HUVEC induced by ox-LDL, which might be associated with miR-30c-5p/PTEN axis. This research elucidated a new mechanism for endothelial cells dysfunction during atherosclerosis and indicated a novel target for the treatment of atherosclerotic cardiovascular disease.

Conflict of Interests

The Authors declare that they have no conflict of interests.

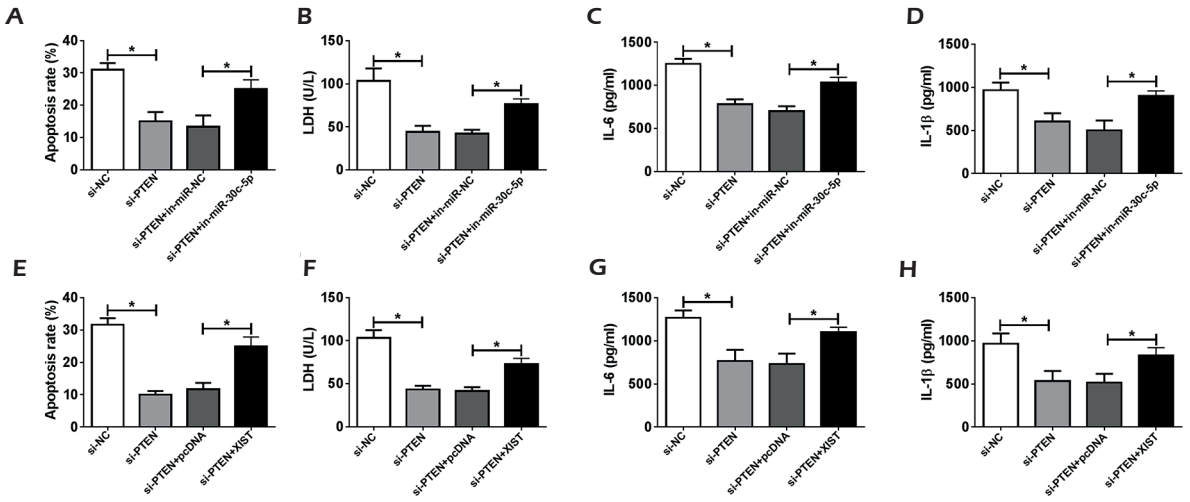

Figure 6. MiR-30c-5p and XIST target PTEN to regulate cell apoptosis and inflammatory response in HUVEC stimulated by ox-LDL. Cell apoptosis ***(A)***, LDH release ***(B)***, IL-6 ***(C)*** and IL-1β ***(D)*** levels in HUVEC transfected with si-NC, si-PTEN, si-PTEN, and in-miR-NC or in-miR-30c-5p were examined after treatment of ox-LDL. Cell apoptosis ***(E)***, LDH release ***(F)***, IL-6 ***(G)***, and IL-1β ***(H)*** levels in HUVEC transfected with si-NC, si-PTEN, si-PTEN, and pcDNA or XIST were detected after treatment of ox-LDL. * $p<0.05$ compared with si-NC, si-PTEN + in-miR-NC or si-PTEN + pcDNA group.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University. The methods used in this study were performed in accordance with the relevant guidelines and regulations.

Consent for Publication

All authors of this work consent this manuscript to be published.

Availability of Data and Materials

All original data and materials are available from the corresponding author upon request.

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