miR-291b-3p mediated ROS-induced endothelial cell dysfunction by targeting HUR

XIAOFANG SUI1*, SHUQIAN YU1*, LIN DOU2*, XIEHUI CHEN3*, XUEJIE LI4, JUN YANG4, YANAN SU4, SHUYUE WANG5, FENGLING WANG1 and JIAN LI2

1Department of Geriatrics, The First Affiliated Hospital of Jiamusi University, Jiamusi, Heilongjiang 154002; 2The Ministry of Health Key Laboratory of Geriatrics, Beijing Hospital, National Center of Gerontology, Beijing 100730; 3Department of Geriatric Cardiovascular Medicine, Shenzhen Sun Yat-Sen Cardiovascular Hospital, Shenzhen, Guangdong 518112; 4Department of Geriatrics, Clinical Medical School, Jiamusi University, Jiamusi, Heilongjiang 154002; 5National Engineering Laboratory for Druggable Gene and Protein Screening, Northeast Normal University, Changchun, Jilin, P.R. China

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Correspondence to: dr Fengling Wang, Department of Geriatrics, The First Affiliated Hospital of Jiamusi University, 348 Dexiang Road, Jiamusi, Heilongjiang 154002, P.R. China
E-mail: fucongtianjiang@163.com

Dr Jian Li, The Ministry of Health Key Laboratory of Geriatrics, Beijing Hospital, National Center of Gerontology, 1 Dahua Road, Beijing 100730, P.R. China
E-mail: lijian@bjhmoh.cn

*Contributed equally

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Abstract. Endothelial dysfunction is an early marker of atherosclerosis. Previous studies have indicated that microRNA (miR)-291b-3p regulates the metabolism of lipids and glucose in the liver via targeting adenosine monophosphate-activated kinase α1 and transcription factor p65. The present study investigated whether miR-291b-3p mediated H2O2-mediated endothelial dysfunction. The level of apoptosis of EOMA mouse endothelial cells was analyzed by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling staining. The mRNA levels of miR-291b-3p, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) were determined by quantitative polymerase chain reaction. The level of phosphorylated extracellular signal-regulated kinase, and levels of B-cell lymphoma 2 (Bcl-2)-associated X protein and Bcl-2 protein were detected by western blot analysis. The treatment of H2O2 induced the apoptosis and increased the mRNA levels of miR-291b-3p, ICAM-1 and VCAM-1 in EOMA cells. It was also demonstrated that the overexpression of miR-291b-3p promoted EOMA cell apoptosis and dysfunction. In contrast, the downregulation of miR-291b-3p rescued the effect of H2O2 on EOMA cell dysfunction. In addition, Hu antigen R (HuR) was identified as a target gene of miR-291b-3p in EOMA cells. The overexpression of HuR reversed the endothelial dysfunction induced by miR-291b-3p mimics. The present study provides novel insight into the critical role of miR-291b-3p on the endothelial dysfunction induced by H2O2. miR-291b-3p may mediate H2O2-induced endothelial dysfunction via targeting HuR.

Introduction

Atherosclerosis is a chronic inflammatory disease of the medium- and large-sized arteries, which is associated with the interactions between endothelial cells, vascular smooth muscle cells, macrophages, platelets and cytokines (1). Endothelial dysfunction and inflammation are early markers of atherosclerosis (2). Normally, endothelial cells prevent interaction between vascular muscle cells and circulating monocytes and lymphocytes (3). Oxidative stress and inflammation may injure endothelial cells and promote the development of atherosclerosis (4). The damaged endothelial cells synthesize and release pro-inflammatory factors, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which stimulate the inflammatory cells to attach at the dysfunctional endothelial cells (5,6). Therefore, protecting endothelial function is an important therapeutic strategy for the prevention of atherosclerosis.

MicroRNAs (miRNA/miR) are small, noncoding RNAs, that negatively regulate target gene expression at the post-transcriptional level by directly binding to 3’-untranslated regions (UTRs) (7). It was demonstrated previously that miRNAs may participate in the development of atherosclerosis (8,9). For example, the miR-17-92 cluster was significantly downregulated among patients with atherosclerosis (8,9). The miR-17-92 cluster was significantly downregulated among patients with atherosclerosis (8,9).

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acute and chronic vascular disease states (8,11,12). miR-21 suppressed the apoptosis and death of vascular smooth muscle cells induced by hydrogen peroxide (H2O2) via regulating programmed cell death 4 (13). miR-429 promoted endothelial cell apoptosis in high-fat diet mice through suppressing B-cell lymphoma 2 (Bcl-2) expression (14).

miR-291b-3p is a member of the miR-290 cluster. It was demonstrated that miR-291b-3p promoted hepatocyte apoptosis via the downregulation of RNA-binding protein Hu antigen R (HuR) (15). Additionally, miR-291b-3p may also regulate the metabolism of lipids and glucose in the liver via targeting adenosine monophosphate-activated kinase α1 and transcription factor p65 (16,17). However, it remains unclear whether the miR-291b-3p is associated with endothelial dysfunction. Therefore, the present study explored the role of miR-291b-3p in endothelial dysfunction. The present study demonstrated that the treatment of H2O2 induced the apoptosis and increased the mRNA levels of miR-291b-3p, ICAM-1 and VCAM-1 in EOMA cells. And overexpression of miR-291b-3p promoted EOMA cell apoptosis and dysfunction. In addition, HuR was identified as a target gene of miR-291b-3p in EOMA cells. The overexpression of HuR reversed the endothelial dysfunction induced by an miR-291b-3p mimic. It was hypothesized that miR-291b-3p may be involved in the endothelial cell dysfunction induced by H2O2 via targeting HuR.

Materials and methods

Cell culture. The EOMA mouse endothelial cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in high-glucose Dulbecco’s modified Eagle’s medium (H-DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 units/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) and 0.1 mg/ml streptomycin (HyClone; GE Healthcare Life Sciences) at 37˚C with humidified air and 5% CO2. EOMA cells were treated with 100 µM H2O2 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 24 h.

Transfection of miR-291b-3p mimics and inhibitors in EOMA cells. The sequences of negative control (NC), microRNA inhibitor negative control (NCl), miR-291b-3p mimic (291m) and inhibitor (291i) were as follows (5’-3’): NC sense, UUC UCCGAAGUGUACCGUTT; NC antisense, ACGUGACAC GUUCCGGAATT; NCI, CAGUACUUUUGUGUAAG CAA; 291m sense, AAGUGUCACUCAUUUGUUGU; 291m antisense, AAACAAAUGGAGCAUUUUU; and 291i, ACAAAACAAUGGAGCAUUU. All the siRNA oligos were purchased from Shanghai GenePharma co., Ltd. (Shanghai, China). According to the manufacturer’s protocol of HiperFect transfection reagent (Qiagen, GmbH, Hilden, Germany), EOMA cells were seeded in 6-well plates with 1x104 cell/well with 2 ml H-DMEM medium containing serum and antibiotics prior to transfection. 5 µl 20 µM NC, NCI, 291m or 291i sequences were pre-incubated with HiperFect transfection reagent at room temperature for 10 min. Then, the solutions were added into the EOMA cells at a final concentration of 50 nM. The cells were then cultured at 37˚C for an additional 48 h.

Adenovirus construction. Recombinant adenovirus vectors expressing mouse HuR (AD-HuR) and control adenovirus vectors containing green fluorescent protein (GFP) (AD-CON) were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). A total of 15 µl AD-HuR (1x109 pfu/ml) was transfected into EOMA cells at a multiplicity of infection of 100 for 48 h at 37˚C. A total of 15 µl AD-CON (1x109 pfu/ml) was added to the control groups at a multiplicity of infection of 100 to maintain a consistent viral load.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was harvested from EOMA cells using TRIZol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols. A stem-loop reverse transcription primer and avian myeloblastosis virus transcriptase (Takara Biotechnology Co., Ltd., Dalian, China) were used to quantify mature miRNAs. A total of 1 µg RNA was reversed transcribed into first-strand cDNA using random primers (Takara Biotechnology Co., Ltd.). qPCR was performed to determine the mRNA levels of miR-291b-3p, VCAM-1, ICAM-1 and HuR. The relative gene expression was normalized to U6 or 18s. Each reaction was performed in triplicate, and analysis was performed by the 2ΔΔCt method (18). The relative level of miR-291b-3p was normalized by U6 small nucleolar RNA, which was used as the housekeeping gene. The sequences of RT primers were as follows (5’-3’): miR-291b-3p forward, GCTGATCC AGTGCAGGTCCGGATTTGCAGCTTGCAGAACA CAAAC; U6 forward, GCGGCT CCGAAGGGTTT; U6 reverse, CGTCGTATCTCGACTGAGGTTCGACGAGATCCACCAAA; ICAM-1 forward, CTTTCTACCTCTTGTGCAGCGAGATCCACCAAA; ICAM-1 reverse, CGTCGTATCTCGACTGAGGTTCGACGAGATCCACCAAA; 18s forward, GGA AGGGCACCACGAGAT; and 18s reverse, TGGACGCCCC GCACATCTAAC.

Western blot analysis. Western blot analysis was performed as previously described (17). The cells were lysed with radioimmunoprecipitation assay lysis buffer (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Following centrifugation at 10,000 x g for 15 min at 4˚C, the supernatants were collected. The proteins were quantified using a bicinchoninic acid kit (Thermo Fisher Scientific, Inc.). Cell lysates containing 15 µg protein were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in TBS with Tween-20 (pH 8.0) (Beijing Solarbio Science and Technology, Co., Ltd.) and probed with the primary antibodies (1:1,000) at 4˚C overnight. Then, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (cat. no. ABCA2103366; Ab-mart, Inc., Berkeley Heights, NJ, USA) for 2 h at room temperature, followed by detection with a Immobilon
Western chemiluminescence kit (EMD Millipore). The antibodies against HuR (cat. no. 12582), Bcl-2 (cat. no. 3498), Bcl-2-associated X protein (Bax; cat. no. 5023), GAPDH (cat. no. 5174), phosphorylated extracellular signal-regulated kinase (p-ERK1/2; cat. no. 4370), ERK1/2 (cat. no. 4695) and GAPDH were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Image J version 1.42 (National Institutes of Health, Bethesda, MD, USA) was used to calculate the band intensity.

**Luciferase assay.** To determine the target gene of miR-291b-3p, the 3'-UTR and coding region of HuR containing miR-291b-3p binding sites were amplified from NCTC 1469 cells (American Type culture collection, Manassas, VA, USA) by the following primers (restriction sites are underlined): HuR-coding region-F-Xhol: 5'-CCCTCGAGCTGGCTCTGGAATCAT TGCT-3'; HuR-coding region-R-Xhol: 5'-CCCTCGAGAGGC AGTCTTGGTTTCCTGA-3'; HuR-UTR-F-Xhol: 5'-CCCTCG AGCTATATGGGGTGCTTCCTCA-3'; HuR-UTR-R-Xhol: 5'-CCCTCGAGCAAACAGCCTTCTTTTCTG-3'.

PCR was performed with genomic DNA isolated from EOMA cells. The thermocycler conditions for PCR were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 45 sec and 72°C for 30 sec. A total of 2 µg PCR products were digested with 1 µl Xhol (New England Biolabs. Inc., Ipswich, MA, USA) and inserted into Xhol-linearized luciferase reporter vector pmiRGL (Promega Corporation, Madison, WI, USA). To perform the luciferase reporter assay, EOMA cells were cultured in 96-well plates at 5,000 cells/well in 100 µl H-dMEM culture medium. The luciferase reporter plasmid was pmiRGL from Promega Corporation. Subsequently, the recombinant luciferase vector (0.1 µg) and miR-291b-3p mimic (5 ng) were transfected into EOMA cells with Effectene reagent (Qiagen...
H₂O₂ treatment induced the phosphorylation of ERK and EOMA endothelial cells. It was demonstrated previously that H₂O₂ treatment led to induced apoptosis in EOMA cells (19). To investigate the effects of miR-291b-3p on endothelial cell apoptosis, the level of miR-291b-3p was determined in the EOMA cells treated with 100 µM H₂O₂ for 24 h. TUNEL staining confirmed that the level of miR‑291b‑3p was increased significantly compared with those transfected with miRNA inhibitor control (Fig. 2E). Transfection with miR-291b-3p inhibitors rescued the apoptosis rate and downregulated mRNA expression levels of ICAM-1 and VCAM-1 in EOMA cells (Fig. 2F and 2G). In addition, transfection with miR-291b-3p inhibitors in EOMA cells led to decreased p-ERK and BAX protein levels, accompanied by increased Bcl-2 protein expression (Fig. 2H). The results demonstrated that miR-291b-3p may modulate apoptosis and the expression of ICAM-1 and VCAM-1 in EOMA cells.

**Results**

### H₂O₂ promotes miR-291b-3p expression and apoptosis in EOMA endothelial cells.

It has been confirmed that H₂O₂ induces endothelial cell apoptosis (19). To investigate the effects of miR-291b-3p on endothelial cell apoptosis, the level of miR-291b-3p was determined in the EOMA cells treated with 100 µM H₂O₂ for 24 h. TUNEL staining confirmed that H₂O₂ treatment led to induced apoptosis in EOMA cells (Fig. 1A). Compared with the control group, the mRNA levels of miR-291b-3p, ICAM-1 and VCAM-1 were increased in EOMA cells treated with H₂O₂ (Fig. 1B and C). Additionally, H₂O₂ treatment induced the phosphorylation of ERK and upregulated Bax expression, accompanied by decreased Bcl-2 protein expression (Fig. 1D). These results suggested that miR-291b-3p may be involved in the process of endothelial cell injury.

### miR-291b-3p modulates endothelial cell dysfunction.

Next, the effects of miR-291b-3p on EOMA cell dysfunction were observed. 291m and 291i were transfected into EOMA cells for 48 h. The results of the qPCR assay indicated that the level of miR-291b-3p was increased significantly in EOMA cells transfected with 291m compared with those transfected with miRNA mimic controls (Fig. 2A). Overexpression of miR-291b-3p induced apoptosis and upregulated ICAM-1 and VCAM-1 mRNA expression levels in EOMA cells (Fig. 2B and C). In EOMA cells transfected with miR-291b-3p mimics, the levels of p-ERK and Bax proteins were increased, whilst Bcl-2 protein expression was decreased (Fig. 2D). In contrast, the level of miR-291b-3p was decreased to 40-50% in EOMA cells transfected with the miR-291b-3p inhibitor compared with those transfected with the miRNA inhibitor control (Fig. 2E). Transfection with miR-291b-3p inhibitors decreased the apoptosis rate and downregulated mRNA expression levels of ICAM-1 and VCAM-1 in EOMA cells (Fig. 2F and 2G). In addition, transfection with miR-291b-3p inhibitors in EOMA cells led to decreased p-ERK and BAX protein levels, accompanied by increased Bcl-2 protein expression (Fig. 2H). The results demonstrated that miR-291b-3p may modulate apoptosis and the expression of ICAM-1 and VCAM-1 in EOMA cells.

**HuR, as a target gene of miR-291b-3p, modulates endothelial apoptosis and dysfunction.**

It was demonstrated previously that HuR was a target gene of miR-291b-3p in mouse hepatocytes (15). To confirm the effect of HuR on H₂O₂-induced endothelial dysfunction, the HuR protein and mRNA levels in EOMA cells treated with H₂O₂ were analyzed. The results indicated that H₂O₂ treatment only decreased HuR protein levels but did not affect HuR mRNA levels (Fig. 4A). A previous study had suggested that miR-291b-3p contained 2 binding sites in the coding region from 4,289-4,312 bp (15). The results of the luciferase assay indicated that the overexpression of miR-291b-3p significantly decreased the luciferase activity in EOMA cells transfected with the luciferase reporter vector containing the HuR coding region. However, the luciferase activity was only slightly decreased when the EOMA cells were co-transfected with the luciferase reporter vector containing HuR-3’-UTR and miR-291b-3p mimic (Fig. 4B). Therefore, miR-291b-3p may directly bind at the coding region of HuR from 902-923 bp. The overexpression of miR-291b-3p decreased HuR levels (Fig. 4C), whilst the downregulation of miR-291b-3p led to increased HuR levels (Fig. 4D). However, miR-291b-3p did not alter the mRNA levels of HuR (Fig. 4E). In addition, the overexpression of HuR decreased the rate of apoptosis and the mRNA expression of ICAM-1 and VCAM-1 in EOMA cells (Fig. 4F and G). In EOMA cells transfected with AD-HUR, the p-ERK and BAX levels were...
Figure 2. miR-291b-3p modulates EOMA cell dysfunction. (A) In EOMA cells, transfection with miR-291b-3p mimic increased miR-291b-3p levels and (B) cell apoptosis rate, accompanied by elevated (C) mRNA expression of ICAM-1 and VCAM-1. (D) The levels of p-ERK/ERK and Bax were raised and the protein level of Bcl2 was reduced in EOMA cells transfected with the miR-291b-3p mimic. (E) In EOMA cells, transfection with miR-291b-3p inhibitors decreased miR-291b-3p levels and (F) cell apoptosis rate, accompanied by reduced mRNA expression of (G) ICAM-1 and VCAM-1. (H) The levels of p-ERK/ERK and Bax were decreased and the protein level of Bcl2 was increased in EOMA cells transfected with miR-291b-3p mimic. Data are presented as the mean ± standard error of the mean (n=5). *P<0.05, **P<0.01 and ***P<0.001 vs. the control.

miR, microRNA; NC, negative control; ICAM‑1, intercellular adhesion molecule‑1; VCAM‑1, vascular cell adhesion molecule‑1; ERK, extracellular signal‑regulated kinase; p‑ERK, phosphorylated ERK; Bcl‑2, B‑cell lymphoma 2; Bax, Bcl‑2‑associated X protein; NCI, miRNA inhibitor negative control; 291m, miR‑291b‑3p mimic; 291i, miR‑291b‑3p inhibitor.
miR-291b-3p regulates apoptosis and dysfunction of EOMA cells via targeting HuR. In order to additionally assess whether miR-291b-3p regulated EOMA cell apoptosis via targeting HuR, miR-291b-3p mimics and AD-HUR were co-transfected into EOMA cells for 48 h. The results indicated that transfection with AD-HUR rescued the miR-291b-3p mimic-induced apoptosis and the increased mRNA expression of ICAM-1 and VCAM-1 (Fig. 5A and B). However, overexpression of miR-291b-3p did not affect the activation of ERK and the expression levels of Bax and Bcl-2 in EOMA cells transfected with AD-HUR (Fig. 5C). Taken together, these results suggested that miR-291b-3p participated in endothelial dysfunction via regulating HuR protein expression.
Figure 4. HuR modulated endothelial apoptosis and dysfunction. (A) The levels of HuR protein and mRNA were measured in EOMA cells treated with H₂O₂. (B) The luciferase activity was analyzed in EOMA cells transfected with luciferase reporter vector containing the HuR coding region or 3'-UTR. The protein level of HUR was analyzed in EOMA cells transfected with (C) 291m or (D) 291i. (E) The mRNA level of HuR was analyzed by qPCR in EOMA cells transfected with 291m or 291i. (F) The levels of apoptosis in EOMA cells transfected AD-HUR or control AD-CON vectors was measured by TUNEL staining. (G) The mRNA levels of ICAM-1 and VCAM-1 in EOMA cells transfected with AD-HUR or AD-CON were measured by qPCR. (H) The levels of p-ERK, Bax and Bcl-2 were analyzed by western blot analysis. Data are presented as the mean ± standard error of the mean (n=5). *P<0.05, **P<0.01 and ***P<0.001 vs. control. Con/NC, negative control; miRNA, microRNA; NCI, miRNA inhibitor negative control; qPCR, quantitative polymerase chain reaction; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; HuR, Hu antigen R; AD-CON, recombinant adenovirus vector expressing mouse HuR; AD-HUR, adenovirus vector containing green fluorescent protein; H₂O₂, hydrogen peroxide; 291m, miR-291b-3p mimics; 291i, miR-291b-3p inhibitor; UTR, untranslated region.
Discussion

In the present study, it was demonstrated that miR-291b-3p participated in endothelial dysfunction via targeting HuR. In particular, the results indicated that: i) H₂O₂ treatment increased miR-291b-3p expression; ii) miR-291b-3p may serve an important role in endothelial dysfunction, which is involved in the H₂O₂-induced endothelial dysfunction; and iv) miR-291b-3p regulated endothelial function via targeting HuR.

Endothelial dysfunction is a major cause of atherosclerosis. It was demonstrated that H₂O₂ damaged endothelial function by promoting cell apoptosis and inflammation (20). In the
present study, EOMA cells were treated with H₂O₂ to establish cell models of endothelial dysfunction. In this cell model, the levels of miR-291b-3p and apoptosis were increased, accompanied by increased mRNA levels of ICAM-1 and VCAM-1.

The association between H₂O₂ and endothelial dysfunction remains incompletely characterized. Accumulating evidence has suggested that miRNAs are involved in endothelial dysfunction (21). miR-291b-3p belongs to the miR-290 cluster, which contains miR-290-3p, miR-291a-3p, miR-291b-3p, miR-292-3p, miR-294 and miR-295 (22). It was reported that miR-291b-3p may serve important roles in differentiation of embryonic stem cells, and the metabolism of lipids and glucose in the liver (15-17,23). In the present study, miR-291b-3p mimics and inhibitors were transfected into EOMA cells to additionally investigate the role of miR-291b-3p in H₂O₂-induced endothelial dysfunction. The results suggested that miR-291b-3p served as an effector molecule of H₂O₂-associated endothelial dysfunction. miR-291b-3p may modulate the protein levels of p-ERK, Bax, Bcl-2 and mRNA expression of ICAM-1 and VCAM-1 in EOMA cells. VCAM-1 and ICAM-1 are secreted by dysfunctional endothelial cells, leading to attachment of inflammatory cells to the damaged endothelial cells. The activated ERK pathway induces endothelial cells to generate excessive levels of ICAM-1 and VCAM-1, which are major factors responsible for the infiltration of inflammatory cells to the atheroma-prone sites (5,24).

Next, the present study additionally identified that miR-291b-3p regulated endothelial function via targeting HuR. It was demonstrated previously that miR-29ib contributed to hepatocyte apoptosis by regulating the expression of HuR, which in turn increased Bcl-2 mRNA stability (25). In the present study, it was identified that miR-29ib-3p may negatively modulate HuR protein levels, and that the overexpression of HuR inhibited the effects of miR-29ib-3p mimics on the endothelial functions. HuR is an RNA binding protein widely expressed in mammalian cells. AU-rich elements (AREs)-mediated transcript degradation is considered to be an important gene regulation mechanism at the post-transcriptional level (26). HuR may specifically recognize and bind to AREs to adjust mRNA stability and translation. HuR may also be transported between the nucleus and cytoplasm. This translocation allows HuR to efficiently modulate the mRNA stability (27). HuR may modulate gene expression in two distinctive mechanisms: Through one mechanism, HuR may positively regulate gene expression by stabilizing target mRNA, including cyclooxygenase-2, cyclin D1 and cyclin-dependent kinase inhibitor 1 (28-30). Through the other mechanism, HuR may also negatively modulate gene expression by decreasing the translation efficiency of mRNA, including tumor necrosis factor-α, myc proto-oncogene protein and cyclin-dependent kinase inhibitor 1B (31-33). In a previous study, it was suggested that HuR upregulated Bcl-2 expression by stabilizing its mRNA (15). In the present study, it was identified that the levels of p-ERK and Bax were also decreased in EOMA cells transfected with AD-HUR. However, the mechanism through which HuR regulates ERK phosphorylation, and Bax and Bcl-2 expression, requires additional study.

In conclusion, the present study provides novel data that miR-291b-3p contributes to H₂O₂-induced endothelial dysfunction via targeting HUR. The present study may provide a novel therapeutic strategy for the prevention of atherosclerosis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
XS, SY and LD planed the experiments, XC and XL performed the cellular experiments, JY, YS and SW analyzed the data. FW and JL were involved in the study conception and design, analysis and interpretation of data, drafting and critical revision of the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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