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ORIGINAL RESEARCH - PRECLINICAL

Shear-Sensitive circRNA-LONP2 Promotes Endothelial Inflammation and Atherosclerosis by Targeting NRF2/HO1 Signaling

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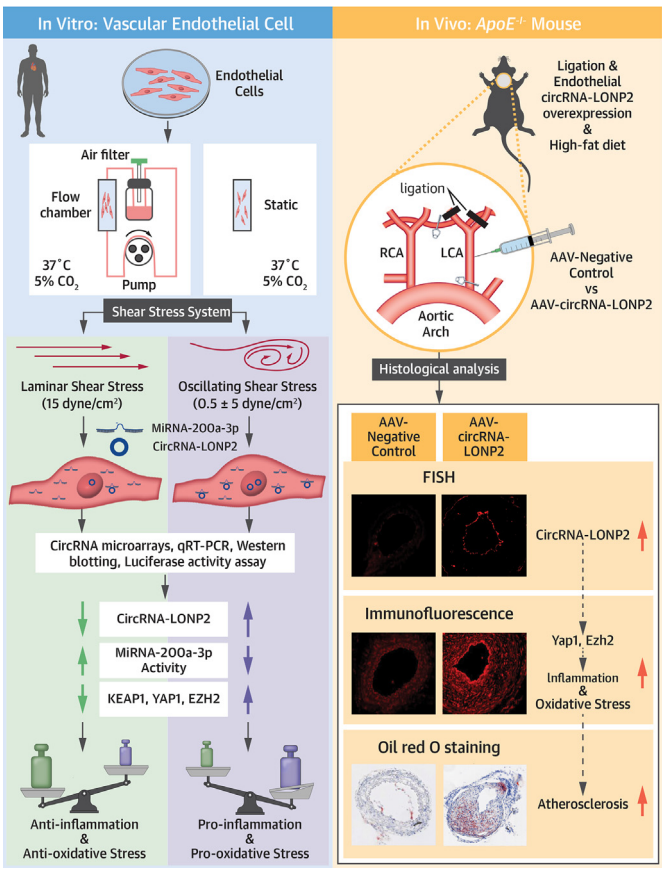
VISUAL ABSTRACT



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HIGHLIGHTS

- **CircRNA-LONP2 modulates the flow-dependent inflammatory response.**
- **CircRNA-LONP2 accelerates atherosclerosis progression.**
- **CircRNA-LONP2 induces oxidative stress and endothelial inflammation through miR-200a-3p.**
- **Species-specific differences exist in circRNA-LONP2-regulated nuclear factor erythroid 2-related factor 2 signaling.**

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SUMMARY

Hemodynamic shear stress is a frictional force that acts on vascular endothelial cells and is essential for endothelial homeostasis. Physiological laminar shear stress (LSS) suppresses endothelial inflammation and protects arteries from atherosclerosis. Herein, we screened differentially expressed circular RNAs (circRNAs) that were significantly altered in LSS-stimulated endothelial cells and found that circRNA-LONP2 was involved in modulating the flow-dependent inflammatory response. Furthermore, endothelial circRNA-LONP2 overexpression promoted endothelial inflammation and atherosclerosis in vitro and in vivo. Mechanistically, circRNA-LONP2 competitively sponged miR-200a-3p and subsequently promoted Kelch-like ECH-associated protein 1, Yes-associated protein 1, and enhancer of zeste homolog 2 expression, thereby inactivating nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling, promoting oxidative stress and endothelial inflammation, and accelerating atherosclerosis. LSS-induced down-regulation of circRNA-LONP2 suppresses endothelial inflammation, at least in part, by activating the miR-200a-3p-mediated nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling pathway. CircRNA-LONP2 may serve as a new therapeutic target for atherosclerosis. (J Am Coll Cardiol Basic Trans Science 2024;9:652-670)

ABBREVIATIONS AND ACRONYMS

AAV9 = adeno-associated virus vector 9

ceRNA = competing endogenous RNA

EC = endothelial cell

EZH2 = enhancer of zeste homolog 2

HO = heme oxygenase

HUVEC = human umbilical vein endothelial cell

ICAM = intercellular cell adhesion molecule

KEAP1 = Kelch-like ECH-associated protein 1

LCA = left carotid artery

MAEC = mouse aortic endothelial cell

mRNA = messenger RNA

miRNA = microRNA

MUT = mutant

LSS = laminar shear stress

OSS = low oscillating shear stress

NF-κB = nuclear factor-κB

NRF2 = nuclear factor erythroid 2-related factor

qRT-PCR = quantitative reverse transcription-polymerase chain reaction

siRNA = small interfering RNA

TNF = tumor necrosis factor

VCAM = vascular cell adhesion molecule

YAP1 = Yes-associated protein 1

WT = wild-type

Hemodynamic shear stress is a frictional force that acts on vascular endothelial cells (ECs) and is essential for endothelial homeostasis under normal physiological conditions. The physiological level of laminar shear stress (LSS) inhibits atherosclerosis by reducing the expression of inflammatory molecules, including adhesion molecules, that guide leukocytes to migrate through arterial walls.[1] Conversely, low oscillating shear stress (OSS) promotes endothelial inflammation and the development of atherosclerosis.[2-4] However, the molecular mechanisms underlying the flow-dependent regulation of vascular function are not fully understood.

Circular RNAs (circRNAs) are a novel class of endogenous noncoding RNAs that present a covalently closed loop structure via back-splicing the 3′ end of RNAs with the 5′ end.[5-8] Previous studies have shown that circRNAs regulate gene expression through various mechanisms, including acting as microRNA (miRNA) sponges, interacting with proteins, and encoding proteins.[9-11] circRNAs play crucial regulatory roles in cardiovascular diseases, including atherosclerosis.[12-14] However, the role of circRNAs in mediating the effects of shear stress on endothelial inflammation remains unclear.

In the current study, we first screened differentially expressed circRNAs that were significantly altered in LSS-stimulated ECs and identified that circRNA-LONP2 may be involved in modulating the flow-dependent inflammatory response. Furthermore, endothelial circRNA-LONP2 overexpression promoted endothelial inflammation and the development of atherosclerosis in vitro and in vivo. Mechanistically, we found that circRNA-LONP2 acts as a competing endogenous RNA (ceRNA) to sponge miR-200a-3p, thereby increasing Kelch-like ECH-associated protein 1 (KEAP1), Yes-associated protein 1 (YAP1), and enhancer of zeste homolog 2 (EZH2) expression, inactivating nuclear factor erythroid 2-related factor (NRF2) signaling and promoting oxidative stress and endothelial inflammation.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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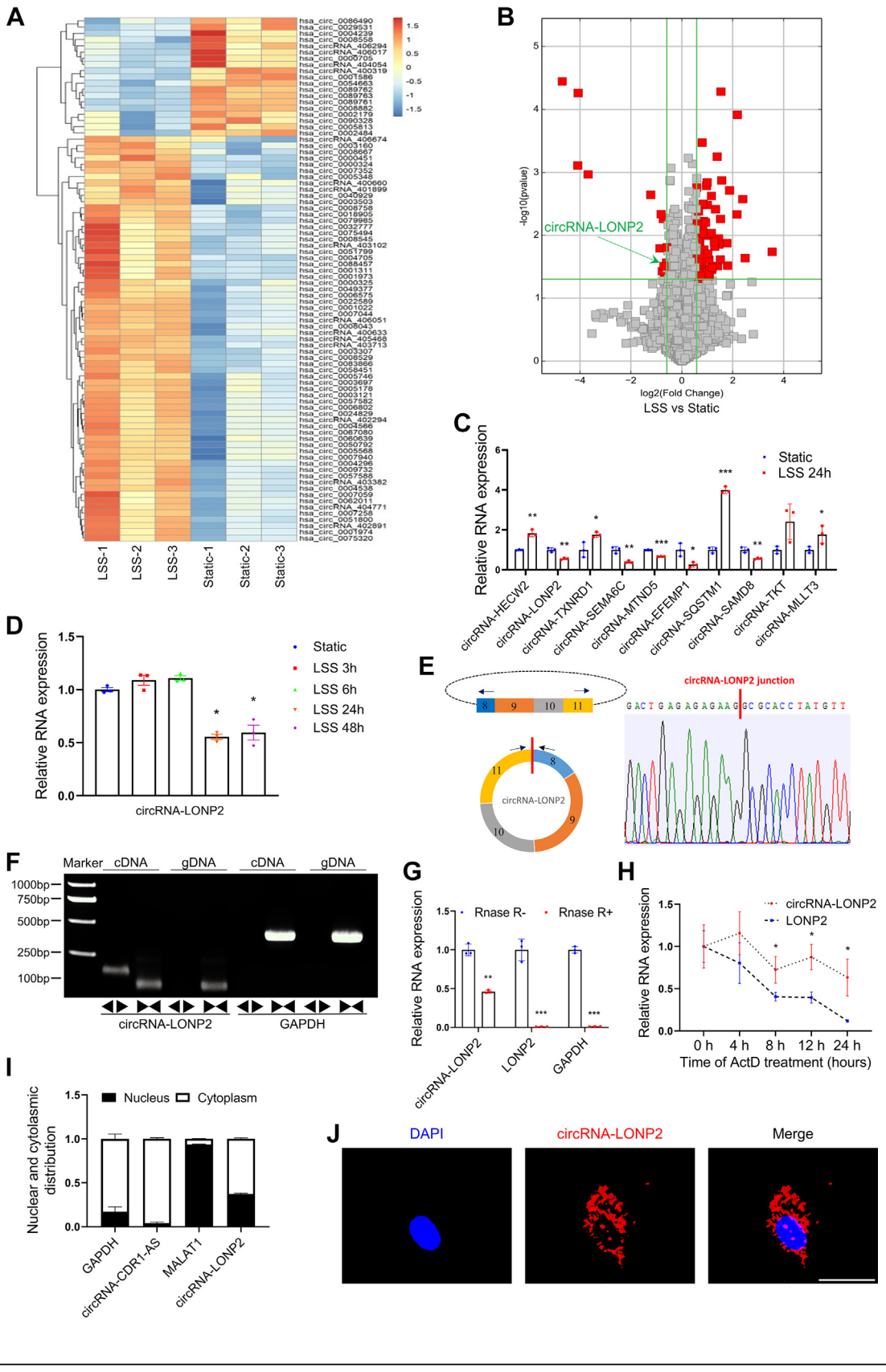
FIGURE 1 CircRNA-LONP2 Is Down-Regulated by Atheroprotective LSS



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METHODS

CELL CULTURE. Human umbilical vein ECs (HUVECs) (catalog no. 8000) and human aortic ECs (catalog no. 6100) were purchased from ScienCell Research Laboratories and cultured in an EC culture medium containing 5% fetal bovine serum, 1% EC growth supplement, and 1% penicillin/streptomycin (catalog no. 1001; ScienCell Research Laboratories). HUVECs from passages 5 to 7 were used for all experiments. The cells were cultured in a carbon dioxide incubator (5% carbon dioxide) at 37°C.

Mouse aortic ECs (MAECs) (catalog no. BNCC359881; Bena Culture Collection) and Lenti-X 293T cells (catalog no. 632180; Takara Bio) were cultured in high-glucose Dulbecco's modified Eagle medium (catalog no. C11995500BT; Gibco) containing 10% fetal bovine serum (catalog no. A31608-02; Gibco) and 1% penicillin/streptomycin (catalog no. 15140122; Gibco).

THP-1 cells (catalog no. SCSP-567) were purchased from National Collection of Authenticated Cell Cultures and cultured in RPMI 1640 (catalog no. 21875034; Gibco) containing 10% fetal bovine serum, 1% penicillin/streptomycin (catalog no. 15140122; Gibco), 250 ng/mL amphotericin B, 1 mM glutamine, and 0.05 mM 2- mercaptoethanol (catalog no. 31350010; Gibco).

ANIMAL STUDIES. Animal experiments complied with animal welfare regulations. The animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (Sun Yat-Sen University, approval no. SYSU-IACUC-2021-000608) and by the Animal Research Committee of the Eighth Affiliated Hospital of Sun Yat-sen University (approval no. 2021-010-01). Male $ApoE^{-/-}$ genetically engineered mice (7-8 weeks old, weighing 32 ± 3 g) were bred following the guidelines of the China Animal Protection Association. $ApoE^{-/-}$ mice were carefully kept in a specific pathogen-free grade animal facility at the Animal Center of Sun Yat-Sen University and fed a high-fat diet from the time of purchase.

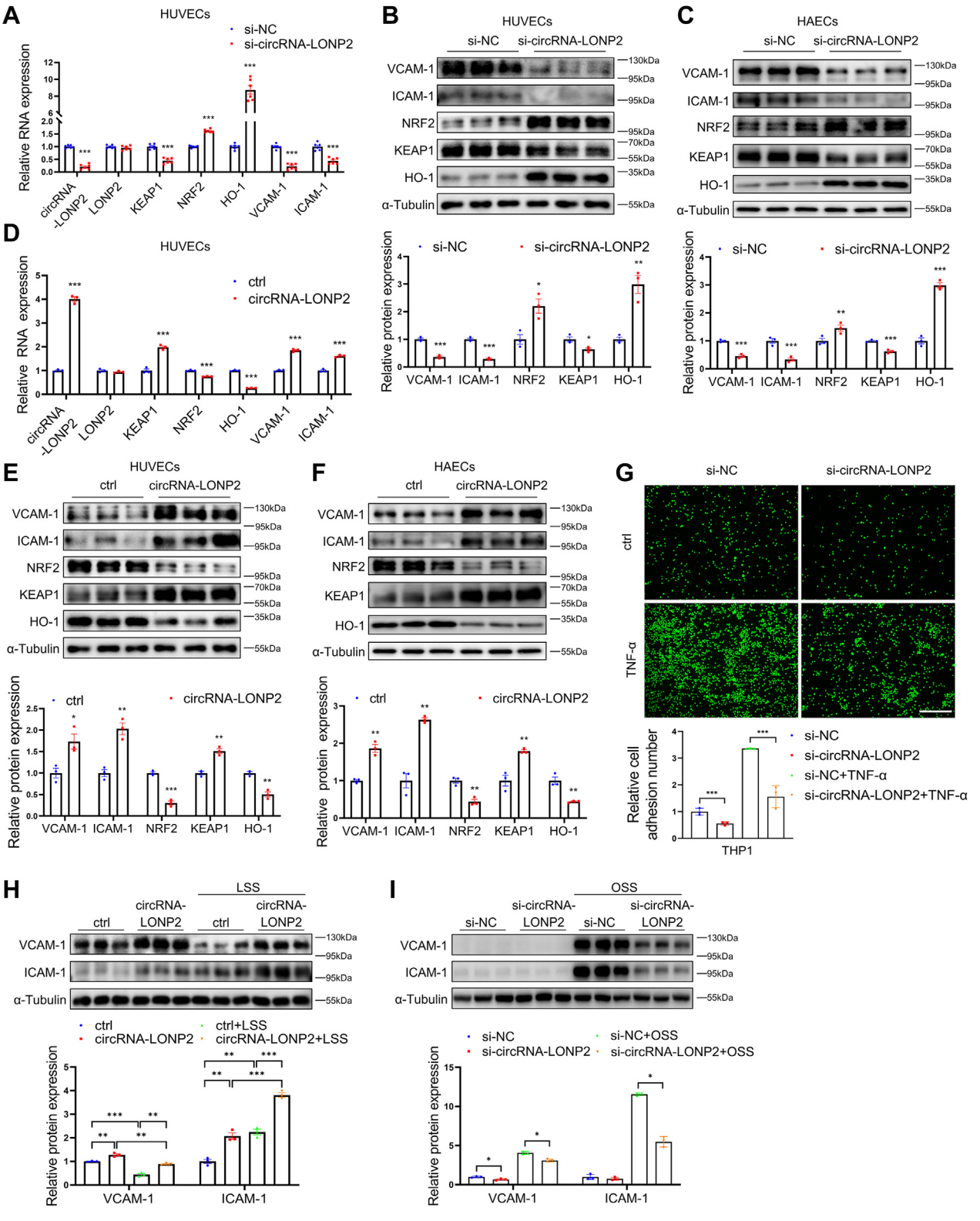
$ApoE^{-/-}$ mice were subjected to partial carotid artery ligation surgery[15] on the left carotid artery (LCA) 5 weeks later; they were simultaneously injected in situ with recombinant adeno-associated virus vector 9 (AAV9) with SLRSPPS peptide modification ($AAV9^{SLRSPPS}$)-Tie2-circRNA-LONP2 (1×10^{10} vector genomes) or $AAV9^{SLRSPPS}$-Tie2-control (1×10^{10} vector genomes). High-fat feeding continued for 6 weeks postoperatively. The $ApoE^{-/-}$ mice were then sacrificed, and the bilateral carotid arteries were harvested for further histologic and immunofluorescent staining analyses. Atherosclerotic plaques of the LCA and right coronary artery were identified by using Oil Red O staining; the intima and media areas of the LCA and right coronary artery were examined by using hematoxylin and eosin staining. Further details are provided in the Supplemental Appendix.

STATISTICAL ANALYSIS. All experiments were independently repeated at least 3 times, and the data were statistically processed with GraphPad Prism 8.0 (GraphPad Software). Data were tested for normality by using the Shapiro-Wilk test. Data are presented as mean ± SEM, and differences between groups were tested for significance by using the two-tailed Student's *t*-test (two groups). One-way analysis of variance (>2 groups) or 2-way analysis of variance

FIGURE 1 Continued

(A) Heatmap of the top 84 differentially expressed circular RNAs (circRNAs) in human umbilical vein endothelial cells (HUVECs) exposed to atheroprotective laminar shear stress (LSS) (15 dyne/cm^2 for 24 hours). (B) Volcano plots showing the variation in circRNA expression between the 2 groups. The vertical lines indicated 1.5 fold change (log2 scaled) up and down, respectively, and the horizontal line represents the *P* value of 0.05, as determined by the Student's *t*-test (−log10 scaled). The red dots in the volcano plot indicate the differentially expressed circRNAs that were statistically significant. (C) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) verification of 10 significantly expressed circRNAs. (D) Expression of circRNA-LONP2 in HUVECs was detected after exposure to atheroprotective LSS (15 dyne/cm^2) for 3, 6, 24, and 48 hours. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. (E) Exon information of circRNA-LONP2 (circBase identifier: hsa_circ_0008558). The back-splice junction sequences of circRNA-LONP2 were amplified by divergent primers and verified using Sanger sequencing. The red vertical line indicates the backsplice site. Divergent and convergent primers are indicated by the arrows. (F) Divergent primers amplify circRNA-LONP2 in complementary DNA (cDNA) but not in genomic DNA (gDNA). GAPDH was used as a linear control. (G) Relative RNA levels of circRNA-LONP2, LONP2, and GAPDH were detected by qRT-PCR after treatment with RNase R. (H) Relative RNA levels of circRNA-LONP2 and LONP2 were detected by qRT-PCR after treatment with actinomycin D (ActD) (5 ng/mL) at the indicated time points in HUVECs. (I) Cellular RNA fractionation assays were used to analyze cellular distribution of circRNA-LONP2. MALAT1 served as a positive control for the nucleus, and circRNA-CDR1-AS1 and GAPDH served as positive controls for the cytoplasm. (J) RNA-fluorescence in situ hybridization assay was performed to detect circRNA-LONP2 expression in HUVECs using 5′ and 3′ Cy-3 labeled probes (circRNA-LONP2). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar = 25 μm. Statistical analyses were performed applying unpaired 2-tailed Student's *t*-test (C, G, H, and I) or 1-way analysis of variance with Tukey's post hoc test (D). Values are mean ± SEM. n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control.

FIGURE 2 CircRNA-LONP2 Modulation of the Flow-Dependent Inflammatory Response



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(>2 groups) with Tukey's post hoc test were used for multiple pairwise comparisons. A P value <0.05 was considered statistically significant.

An expanded Methods section and Supplemental Tables 1 to 6 are presented in the Supplemental Appendix.

RESULTS

CircRNA-LONP2 IS DOWN-REGULATED BY ATHEROPROTECTIVE LSS. To identify the changes in circRNA expression patterns after shear stress stimulation, we conducted circRNA microarray analysis using RNA extracted from HUVECs cultured for 24 hours under static or LSS conditions. A total of 84 differentially expressed circRNAs were detected with >1.5 fold change and $P < 0.05$, including 65 up-regulated and 19 down-regulated circRNAs (**Figures 1A and 1B**). We confirmed that several circRNAs do have differential expression, including the highly abundant circRNA-LONP2 (circBase identifier: hsa_circ_0008558), which was significantly down-regulated in LSS-stimulated ECs compared with the static control (**Figure 1C**). circRNA-LONP2 was selected for further investigation because of its relatively high expression level in ECs (Supplemental Figure 1) and important impact on EC function (**Figure 2**, Supplemental Figure 2).

Upon examining ECs at different time points after LSS stimulation, we found that long-term (24 hours and 48 hours) LSS stimulation reduced circRNA-LONP2 expression, whereas short-term LSS (3 hours and 6 hours) had no effect (**Figure 1D**). The levels of linear LONP2 messenger RNA (mRNA) did not change in ECs that received LSS stimulation for 24 hours compared with those in static controls. These results suggest that circRNA-LONP2, but not the linear LONP2 transcripts, may be related to mediate the effects of LSS.

Several RNA-binding proteins have been reported to be involved in circRNA production, including quaking (QKI),[16] fused in sarcoma (FUS),[17] double-stranded RNA-specific adenosine deaminase (ADAR), and ATP-dependent RNA helicase A (DHX9).[18] Therefore, we investigated the mRNA expression of these RNA-binding proteins in response to different shear stress stimuli. Our results reveal the opposite effects of LSS and OSS on the mRNA levels of ADAR, DHX9, FUS, and QKI in ECs (Supplemental Figure 3), suggesting that shear stress regulates circRNA-LONP2 expression through the aforementioned proteins.

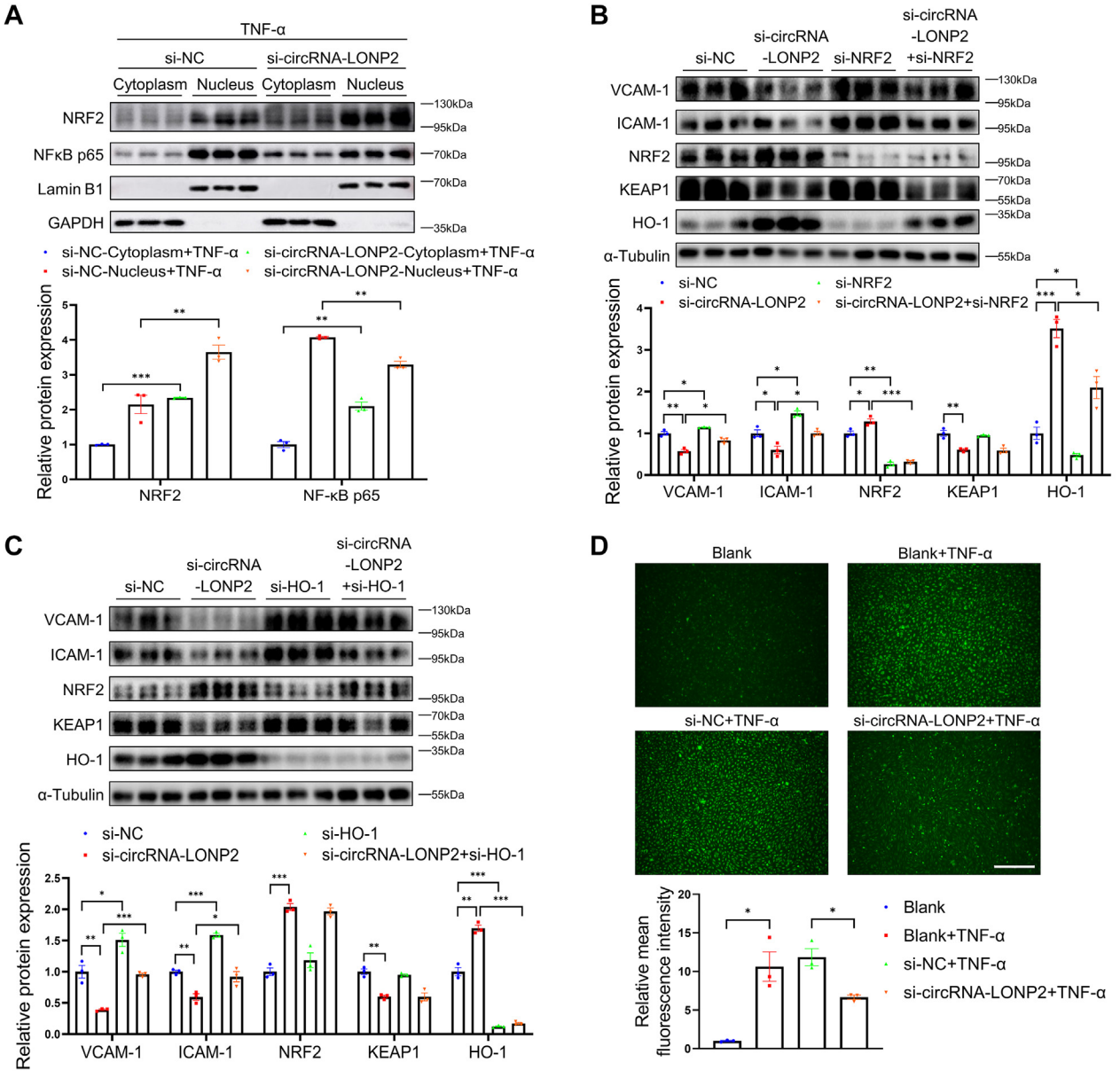
We then assessed the exon structure of circRNA-LONP2, which is derived from exons 8, 9, 10, and 11 of LONP2. Using divergent primers and Sanger sequencing, we confirmed head-to-tail splicing of circRNA-LONP2 and validated the whole structure of circRNA-LONP2 (**Figure 1E**, Supplemental Figure 4). Polymerase chain reaction analysis revealed that divergent primers could amplify circRNA-LONP2 in reverse-transcribed RNA (complementary DNA), but not in genomic DNA, indicating that this RNA is circular in form (**Figure 1F**). **Figures 1G and 1H** illustrate the resistance of circRNA-LONP2 to exonucleolytic degradation, confirming its circular nature. In addition, nuclear-cytoplasmic fractionation (**Figure 1I**) and RNA-fluorescence in situ hybridization (**Figure 1J**) showed that circRNA-LONP2 was preferentially located in the cytoplasm of ECs, indicating that the cytoplasm is the main site for the biological effects of circRNA-LONP2.

CircRNA-LONP2 MODULATION OF THE FLOW-DEPENDENT INFLAMMATORY RESPONSE. To investigate the biological functions of circRNA-LONP2, small interfering RNAs (siRNAs) targeting the back-spliced junction of circRNA-LONP2 were used to specifically knock down circRNA-LONP2 expression in ECs. Quantitative reverse transcription-polymerase chain reaction

FIGURE 2 Continued

(A) RNA levels of circRNA-LONP2, LONP2, Kelch-like ECH-associated protein 1 (KEAP1), nuclear factor erythroid 2–related factor (NRF2), heme oxygenase (HO)-1, vascular cell adhesion molecule (VCAM)-1, and intercellular cell adhesion molecule (ICAM)-1 in HUVECs transfected with cirRNA-LONP2 small interfering RNA (siRNA) (si-cirRNA-LONP2) or control siRNA (si-NC) were measured by using qRT-PCR. n = 6. (B and C) Protein levels of KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 in endothelial cells transfected with si-cirRNA-LONP2 or si-NC were detected by using Western blot analysis. n = 3. (D) RNA levels of circRNA-LON2, LONP2, KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 in HUVECs transfected with circRNA-LONP2 or control (ctrl) lentivirus were measured by RT-qPCR. GAPDH served as an internal reference. n = 3. (E and F) Protein levels of KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 in endothelial cells transfected with circRNA-LONP2 or ctrl lentivirus were evaluated by using Western blot analysis. n = 3. (G) HUVECs were transfected with si-cirRNA-LONP2 or si-NC for 48 hours and then exposed to tumor necrosis factor (TNF)-α (5 ng/mL) for 4 hours. A monocyte adhesion assay was performed by incubating HUVECs with fluorescently labeled THP-1 cells. Scale bar: 1 mm. n = 3. (H) HUVECs were transfected with circRNA-LONP2 or ctrl lentivirus for 72 hours and subsequently exposed to atheroprotective LSS (15 dyne/cm^2) for another 24 hours. Protein levels of VCAM-1 were detected by using Western blot analysis. n = 3. (I) HUVECs were transfected with si-cirRNA-LONP2 or si-NC for 24 hours and were subsequently exposed to oscillatory shear stress (OSS) (±5 dyne/cm^2 at 1 Hz) for another 24 hours. Protein levels of VCAM-1 and ICAM-1 were detected by using Western blot analysis. n = 3. Statistical analyses were performed applying the unpaired 2-tailed Student's t-test (A to G) or 1-way analysis of variance with Tukey's post hoc test (H and I). Values are mean ± SEM. n ≥ 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus ctrl. Abbreviations as in **Figure 1**.

FIGURE 3 CircRNA-LONP2 Regulated Endothelial Inflammation via NRF2/HO-1 Pathway



(A) HUVECs were transfected with si-cirRNA-LONP2 or si-NC for 48 hours and subsequently treated with TNF-α (5 ng/mL) for an additional 4 hours. Western blot analysis on cellular proteins separated into nuclear and cytoplasmic fractions from HUVECs. Lamin B1 and GAPDH were considered as markers for nucleus and cytoplasm, respectively. (B) HUVECs were transfected with si-cirRNA-LONP2 or si-NC with or without NRF2 siRNA (si-NRF2) for 48 hours. Protein levels of KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 were determined by Western blot analysis. (C) HUVECs were transfected with si-cirRNA-LONP2 or si-NC with or without HO-1 siRNA (si-HO-1) for 48 hours. Protein levels of KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 were determined by Western blot analysis. (D) HUVECs were transfected with si-cirRNA-LONP2 or si-NC for 48 hours and then exposed to TNF-α (5 ng/mL) for 4 hours. Intracellular reactive oxygen species levels were detected by 2,7-dichlorodihydrofluorescein diacetate and were observed with a fluorescence microscope. Scale bar: 1 mm. Statistical analyses were performed by applying the unpaired 2-tailed Student's *t*-test (A and D) or the 1-way analysis of variance with Tukey's post hoc test (B and C). Values are mean ± SEM. n = 3. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control. Other abbreviations as in Figures 1 and 2.

(qRT-PCR) showed that circRNA-LONP2 silencing reduced the mRNA levels of KEAP1, and inflammatory genes vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) increased the mRNA levels of the antioxidative stress and anti-inflammatory genes NRF2 and heme oxygenase-1 (HO-1), and had no significant effects on the mRNA levels of LONP2 (**Figure 2A**). Interestingly, Western blot analysis revealed that circRNA-LONP2 silencing reduced the protein levels of VCAM-1, ICAM-1, and KEAP1 while promoting the protein expression of NRF2 and HO-1 in HUVECs and human aortic ECs (**Figures 2B and 2C**).

After confirming the high circularization efficiency (approximately 75%) (Supplemental Figure 5) and high splicing accuracy (approximately 92.5%) (Supplemental Figure 6) of transcripts generated from the circRNA-LONP2 overexpression vector, we investigated whether circRNA-LONP2 overexpression had opposite effects. As expected, circRNA-LONP2 overexpression significantly up-regulated the mRNA and protein levels of VCAM-1, ICAM-1, and KEAP1 but down-regulated those of NRF2 and HO-1 (**Figures 2D to 2F**).

To distinguish the effects of circRNA-LONP2 from its linear counterpart, a plasmid with the reverse circularization sequence deletion (R-deletion) was constructed, allowing the plasmid to produce only linear transcripts for use as a control in circRNA-LONP2 overexpression experiments (Supplemental Figure 7A). qRT-PCR showed that the wild-type (WT) vector, but not the R-deletion mutant (MUT), significantly overexpressed circRNA-LONP2, whereas the R-deletion MUT significantly overexpressed the linear transcript (Supplemental Figure 7B). As expected, no significant differences were detected between the R-deletion group and the control group (Supplemental Figures 7B and 7C), suggesting that the linear counterpart of circRNA-LONP2 does not modulate endothelial inflammation and oxidative stress. Furthermore, circRNA-LONP2 silencing significantly reversed the inflammation and oxidative stress of ECs induced by circRNA-LONP2 overexpression but had no effect on the expression of the linear transcripts (Supplemental Figures 7D and 7E). These results suggest that circRNA-LONP2, but not its counterpart, is the major cause of inflammation and oxidative stress in ECs.

In addition, knockdown of endogenous circRNA-LONP2 attenuated tumor necrosis factor-α (TNF-α)-induced protein expression of VCAM-1 and ICAM-1 in ECs, which brought about reduced adhesion of monocytes to ECs (**Figure 2G**). Consistently, circRNA-LONP2 overexpression partially counteracted LSS-induced repression of VCAM-1, monocyte chemoattractant protein 1, and E-Selectin (SELE) expression (**Figure 2H**, Supplemental Figure 8). Considering that oscillatory shear stress (OSS, 0.5 ± 5 dyne/cm^2) up-regulated the expression levels of circRNA-LONP2 (Supplemental Figure 9), we investigated whether knockdown of circRNA-LONP2 could partially reverse the pro-inflammatory effects of OSS. As expected, circRNA-LONP2 silencing partially attenuated the OSS-induced VCAM-1 and ICAM-1 protein expression in ECs (**Figure 2I**). Taken together, our results suggest that circRNA-LONP2 regulates flow-dependent inflammatory responses.

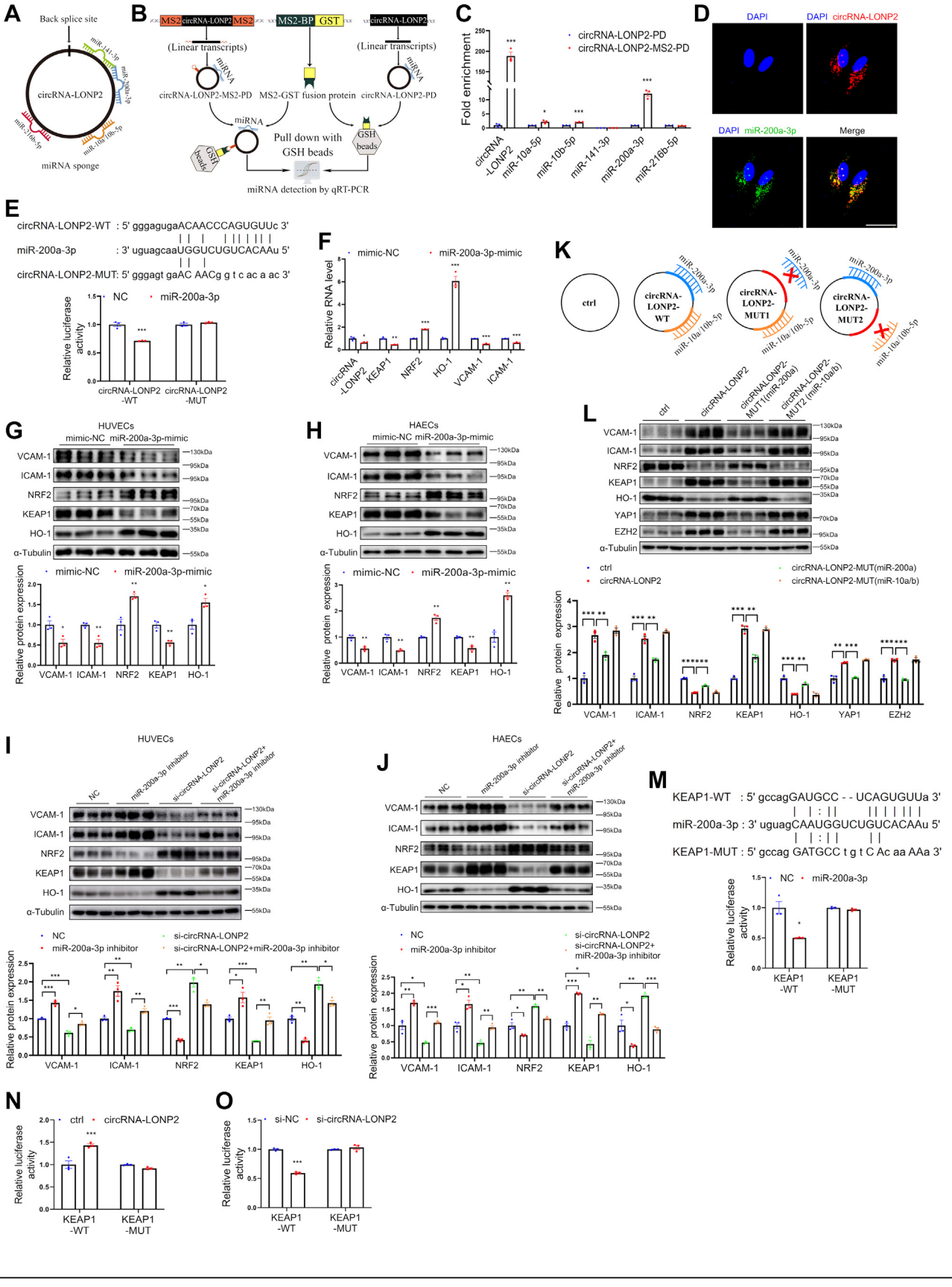
CircRNA-LONP2 REGULATED ENDOTHELIAL INFLAMMATION VIA THE NRF2/HO-1 AXIS. Because circRNA-LONP2 is derived from the back-splicing of its linear transcript, we tested whether the linear LONP2 transcript mediates the function of circRNA-LONP2. Our data show that silencing or overexpression of circRNA-LONP2 has no effect on mRNA levels of linear LONP2 (**Figures 2A and 2D**). Consistently, LSS down-regulated the expression of circRNA-LONP2 without affecting the mRNA level of linear LONP2 (Supplemental Figure 10). These results suggest that shear-sensitive circRNA-LONP2 modulates endothelial inflammation independently from linear LONP2.

To investigate the possible mechanisms underlying circRNA-LONP2-induced endothelial inflammation, we further performed Western blot analysis of the nuclear and cytoplasmic fractions of ECs transfected with circRNA-LONP2 siRNA or control siRNA. The purity of the nuclear and cytosolic fractions was confirmed by Western blot analysis using antibodies against the nuclear (Lamin B1) and cytoplasmic (GAPDH [glyceraldehyde 3-phosphate dehydrogenase]) marker proteins. As expected, the knockdown of circRNA-LONP2 attenuated TNF-α-stimulated nuclear factor-κB (NF-κB) p65 translocation into the nucleus, while promoting NRF2 nuclear translocation, suggesting that circRNA-LONP2 may induce endothelial inflammation by modulating the nuclear translocation of NRF2 and NF-κB (**Figure 3A**).

A previous study reported that the NRF2/HO-1 pathway has anti-inflammatory properties.[19] We therefore explored whether circRNA-LONP2 regulates endothelial inflammation through the NRF2/HO-1 axis. As expected, inhibition of NRF2 or its downstream target, HO-1, by siRNA significantly abrogated the anti-inflammatory effects of circRNA-LONP2 siRNA in ECs (**Figures 3B and 3C**). Collectively, these data show that circRNA-LONP2 regulates endothelial inflammation via the NRF2/HO-1 axis.

Given that circRNA-LONP2 works through the NRF2/HO-1 axis, we performed a reactive oxygen

FIGURE 4 CircRNA-LONP2 Induced Endothelial Inflammation Through the miR-200a-3p/KEAP1/NRF2 Pathway



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species assay to determine if oxidative stress was reduced after circRNA-LONP2 silencing in HUVECs. As shown in **Figure 3D**, silencing of circRNA-LONP2 reduced the TNF-α-induced reactive oxygen species levels in ECs. These results suggest that circRNA-LONP2 modulates endothelial inflammation by regulating the oxidative stress state of ECs.

CircRNA-LONP2 INDUCED ENDOTHELIAL INFLAMMATION THROUGH THE miR-200A-3p/KEAP1/NRF2 PATHWAY. Because circRNA-LONP2 was observed to be preferentially located in the cytoplasm of ECs, we hypothesized that circRNA-LONP2 may regulate gene expression by acting as miRNA sponges. The starBase database[20] was used to predict the targets of circRNA-LONP2, and 5 broadly conserved miRNAs (miR-10a-5p, miR-10b-5p, miR-141-3p, miR-200a-3p, and miR-216b-5p) that directly bind to circRNA-LONP2 were selected for further analysis (**Figure 4A**). To identify miRNAs that might bind to circRNA-LONP2, we designed an MS2 RNA pull-down assay. A vector was constructed expressing circRNA-LONP2 labeled with MS2 RNA hairpins (circRNA-LONP2-MS2-PD) and a control vector expressing circRNA-LONP2 (circRNA-LONP2-PD). By labeling circRNA-LONP2 with the MS2 structure and co-expressing MS2-binding protein (ie, MS2-BP) fused to a GST tag (ie, MS2-BP-GST), we were able to use glutathione magnetic beads to pull down miRNAs that interact with circRNA-LONP2.

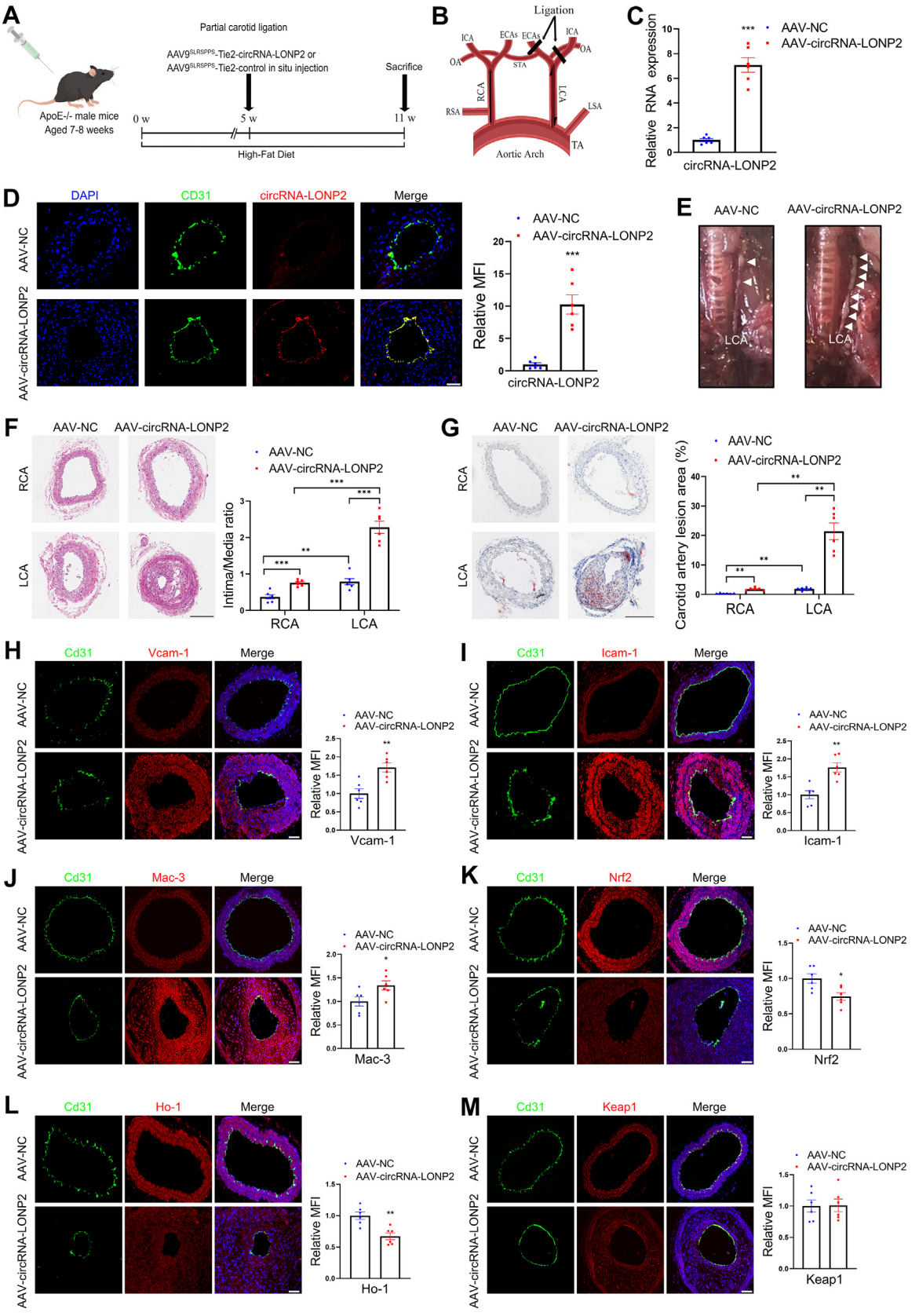
In addition, to avoid nonspecific pull-down of linear transcripts, one-half of the MS2 sequence was cloned at the 5′ end of circRNA-LONP2 and the other half at the 3′ end; thus, only circRNA-LONP2 (whose 5′ end and 3′ end are connected to form a closed loop by back-splicing) transcripts, rather than its linear transcripts, can form intact MS2 (**Figure 4B**, Supplemental Figure 11A). The results showed specific enrichment of circRNA-LONP2 and miR-200a-3p, miR-10a-5p, and miR-10b-5p in the MS2-labeled circRNA-LONP2-MS2-PD group compared with the control (**Figure 4C**, Supplemental Figure 11B). Because miR-200a-3p was the most enriched miRNA in the circRNA-LONP2 pull-down assay, fluorescence in situ hybridization assays were performed to confirm the interaction, revealing the co-localization of circRNA-LONP2 and miR-200a-3p (**Figure 4D**). We further performed dual-luciferase assays using reporters containing WT and MUT miR-200a-3p binding sites of circRNA-LONP2 to verify the interaction between circRNA-LONP2 and miR-200a-3p. The results proved that miR-200a-3p mimics could significantly down-regulate the luciferase activity of the WT group, but not the MUT group, indicating that circRNA-LONP2 may indeed bind miR-200a-3p (**Figure 4E**).

Given the apparent interaction between circRNA-LONP2 and miR-200a-3p, we investigated the biological function of miR-200a-3p. We found that transfection of ECs with miR-200a-3p mimics significantly down-regulated the RNA levels of circRNA-LONP2, KEAP1, VCAM-1, and ICAM-1 while up-regulating the RNA levels of NRF2 and HO-1 (**Figure 4F**). In addition, overexpression of miR-200a-3p promoted NRF2 and HO-1 protein expression while suppressing the protein expression of VCAM-1, ICAM-1, and KEAP1 (**Figures 4G and 4H**). The

FIGURE 4 Continued

(A) Starbase online database provided predicted putative binding sites of several broadly conserved miRNAs with circRNA-LONP2. (B) Schematic illustrating that circRNA-LONP2 with MS2 stem-loop was pulled down by glutathione (GSH) magnetic beads. circRNA-LONP2 without MS2 stem-loop structure served as a control. (C) Fold enrichment of miRNAs pulled down by magnetic beads was detected by using qRT-PCR. (D) RNA fluorescence in situ hybridization assay illustrating the co-localization of circRNA-LONP2 and miR-200a-3p in HUVECs using 5′ and 3′ Cy-3 labeled probes (circRNA-LONP2) and 5′ and 3′ FAM-labeled probes (miR-200a-3p). Nuclei were stained with DAPI. Scale bar = 25 μm. (E) The predicted wild-type (WT) or mutated (MUT) miR-200a-3p binding site in circRNA-LONP2. Luciferase reporter activity of circRNA-LONP2-WT or circRNA-LONP2-MUT in Lenti-X 293T cells co-transfected with miR-200a-3p mimic or mimic control (mimic-NC) was detected by the Dual-Luciferase Reporter Assay System. (F) RNA levels of circRNA-LONP2, KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 in HUVECs transfected with miR-200a-3p-mimic or mimic-NC for 48 hours were measured by RT-qPCR. (G and H) ECs were transfected with miR-200a-3p-mimic or mimic-NC for 48 hours. KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 protein levels were detected by using Western blot analysis. (I and J) ECs were transfected with si-cirRNA-LONP2 or si-NC with or without miR-200a-3p inhibitor for 48 hours. Protein levels of KEAP1, NRF2, HO-1, VCAM-1, and ICAM-1 were determined by Western blot analysis. (K) The schematic depicts WT circRNA-LONP2 (circRNA-LONP2-WT), circRNA-LONP2 with a MUT miR-200a-3p binding site (circRNA-LONP2-MUT1), and circRNA-LONP2 with a MUT miR-10a/10b-5p binding site (circRNA-LONP2-MUT2). Circ-pLO5 served as a ctrl. (Diagrams obtained using Figdraw) (L) Protein levels of VCAM-1, ICAM-1, NRF2, KEAP1, HO-1, Yes-associated protein 1 (YAP1), and enhancer of zeste homolog 2 (EZH2) in HUVECs transfected with circRNA-LONP2, circRNA-LONP2-MUT (miR-200a), circRNA-LONP2-MUT (miR-10a/b), or ctrl lentivirus were analyzed by Western blot analysis. (M) Schematic representation of predicted WT or MUT miR-200a-3p binding sites in KEAP1 mRNA. Luciferase reporter activity of KEAP1-WT or KEAP1-MUT in Lenti-X 293T cells co-transfected with miR-200a-3p mimic or mimic ctrl was detected by using the Dual-Luciferase Reporter Assay System. (N) Luciferase reporter activity of KEAP1-WT or KEAP1-MUT in Lenti-X 293T cells co-transfected with circRNA-LONP2 or control plasmids was detected by using the Dual-Luciferase Reporter Assay System. (O) Luciferase reporter activity of KEAP1-WT or KEAP1-MUT in Lenti-X 293T cells co-transfected with si-cirRNA-LONP2 or si-NC was detected by using the Dual-Luciferase Reporter Assay System. Statistical analyses were performed by applying the unpaired 2-tailed Student's *t*-test (C, E, F, G, H, M, N, and O) or the 2-way analysis of variance with Tukey's post hoc test (I, J and L). Values are mean ± SEM. n = 3. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus control. A, B, and K were created by using Figdraw. Abbreviations as in **Figures 1 and 2**.

FIGURE 5 CircRNA-LONP2 Accelerates the Progression of Atherosclerosis



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cell phenotype induced by miR-200a-3p was similar to that induced by circRNA-LONP2 silencing, suggesting that miR-200a-3p mediates the regulatory effect of circRNA-LONP2 on EC function. As expected, transfection with miR-200a-3p inhibitors significantly rescued the inhibitory effects of circRNA-LONP2 silencing on KEAP1, VCAM-1, and ICAM-1 protein expression, while abolishing circRNA-LONP2 silencing-induced NRF2 and HO-1 protein expression (**Figures 4I and 4J**). Thus, the antioxidative and anti-inflammatory effects of circRNA-LONP2 silencing in ECs were mediated by miR-200a-3p.

Interestingly, we found that miR-10a-5p and miR-10b-5p could also be pulled down by circRNA-LONP2, although not as efficiently as miR-200a-3p. Previous studies have shown that miR-10a exerts anti-inflammatory effects in cultured human aortic ECs.[21-23] To test whether circRNA-LONP2 also regulates endothelial inflammation by sponging miR-10a-5p and miR-10b-5p and to further show that the anti-inflammatory effects of circRNA-LONP2 silencing in ECs are mediated by miR-200a-3p, circRNA-LONP2-MUT1 (circRNA-LONP2 with mutated miR-200a-3p binding sites) vectors and circRNA-LONP2-MUT2 (circRNA-LONP2 with mutated miR-10a/b-5p binding sites) vectors (**Figure 4K**) were constructed. Western blot analysis revealed that a mutation in the miR-200a-3p binding site largely blocked the pro-oxidative and pro-inflammatory effects of circRNA-LONP2, whereas a mutation in the miR-10a/b binding site had no effect (**Figure 4L**), suggesting that circRNA-LONP2 promotes oxidative stress and endothelial inflammation by sponging miR-200a-3p.

Notably, previously studies reported that circRNA-LONP2 directly interacts with pri-miR-17 and miR-27b-3p.[24,25] Consistent with these findings, our data indicated that primary miR-17 and miR-27b-3p, but not miR-17-3p or miR-17-5p, were pulled down by circRNA-LONP2 in HUVECs, albeit with lower enrichment compared with miR-200a-3p (Supplemental Figure 11B). To examine the roles of pri-miR-17 and miR-27b-3p in circRNA-LONP2-induced endothelial oxidative stress and inflammation, circRNA-LONP2-MUT3 (circRNA-LONP2 with mutated miR-27b-3p binding sites) vectors and circRNA-LONP2-MUT4 (circRNA-LONP2 with mutated pri-miR-17 binding sites) vectors (Supplemental Figure 11C) were constructed. Western blot analysis revealed that these mutations had no effect on the pro-oxidative and pro-inflammatory effects of circRNA-LONP2 (Supplemental Figure 11D), suggesting that pri-miR-17 and miR-27b-3p are not involved in circRNA-LONP2-induced endothelial oxidative stress and inflammation.

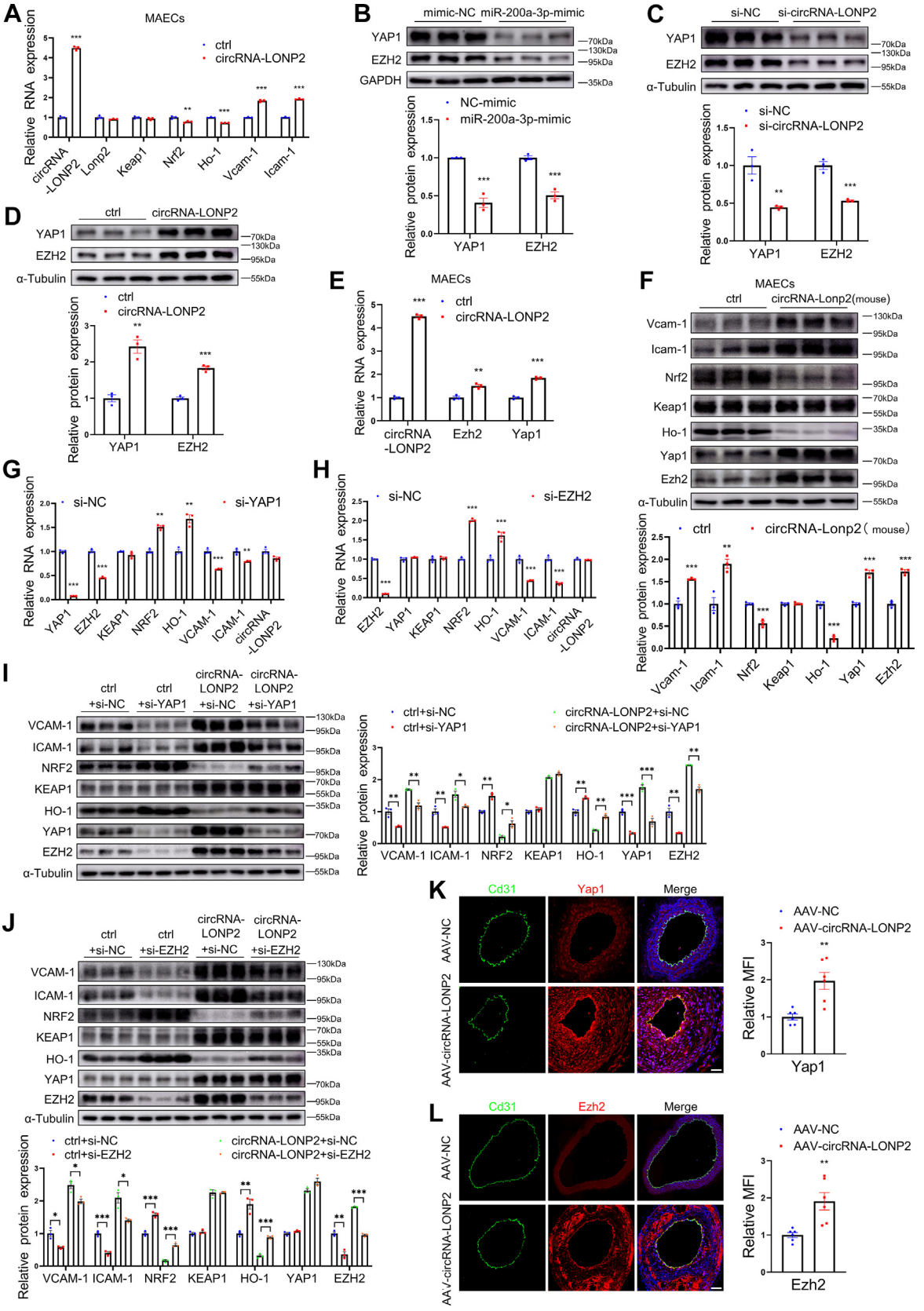
We further investigated whether the expression levels of miR-200a-3p can be regulated by circRNA-LONP2 or LSS. As shown in Supplemental Figures 12 and 13A to 13C, although circRNA-LONP2 was more abundant than miR-200a-3p, knockdown or overexpression of circRNA-LONP2 had no effect on the expression of miR-200a-3p. Furthermore, LSS had no effect on the expression of miR-200a-3p (Supplemental Figure 13D). These data indicate that shear-sensitive circRNA-LONP2 modulates endothelial inflammation without affecting the expression of miR-200a-3p.

Because miR-200a-3p was found to target KEAP1,[26] we hypothesized that circRNA-LONP2 induced endothelial inflammation through the miR-200a-3p/KEAP1/NRF2 pathway. To confirm that KEAP1 is regulated by miR-200a-3p, luciferase reporters containing WT or MUT 3′-untranslated region of KEAP1 were constructed. Dual-luciferase reporter assays showed that the luciferase activities of the KEAP1 WT

FIGURE 5 Continued

(A) Schematic figure showing the experimental strategy (by Figdraw). Seven- to eight-week-old male *ApoE*$^{-/-}$ mice were fed a high-fat diet and subjected to partial ligation of the left common carotid artery (LCA) at week 5. During the ligation, the LCA was infused with Endo-AAV9-Tie2-circRNA-LONP2 or Endo-AAV9-Tie2-control lentiviruses. (B) Schematic representation of partial left carotid artery ligation (Diagrams obtained using Figdraw). (C) qRT-PCR analysis of circRNA-LONP2 in arterial tissues from mice treated with Endo-AAV9-Tie2-circRNA-LONP2 or Endo-AAV9-Tie2-control lentiviruses; n = 6. (D) RNA fluorescence in situ hybridization for circRNA-LONP2 and immunofluorescence staining for Cd31 and DAPI in the ligated LCA of *ApoE*$^{-/-}$ mice. Scale bar: 40 μm. Quantification of relative mean fluorescent intensity of circRNA-LONP2 (n = 6). (E) After 6 weeks, the LCAs were separated to examine atherosclerotic lesions. Representative images of carotid arteries are shown. White arrows indicate plaque lesions. (F) Carotid arteries were harvested 6 weeks after ligation for hematoxylin and eosin staining (left). Representative images are shown. The areas of the intima and media were quantified by using ImageJ software (right). Scale bar: 200 μm, n = 6. (G) Oil Red O staining of carotid arteries (left). Representative images are shown. Plaque areas were quantified by using ImageJ software (right). Scale bar: 200 μm; n = 6. (H to M) Immunofluorescence staining for Vcam-1 (H), Icam-1 (I), Mac-3 (J), Nrf2 (K), Ho-1 (L), Keap1 (M), Cd31, and DAPI in the ligated LCA of *ApoE*$^{-/-}$ mice. Scale bar: 40 μm. Quantification of relative mean fluorescent intensity of Vcam-1, Icam-1, Mac-3, Nrf2, Ho-1, and Keap1 (n = 6). The unpaired 2-tailed Student's *t*-test was used for statistical analyses (C, D, and F to M). Values are mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control. A was created by using Figdraw. AAV9 = adeno-associated virus vector 9; RCA = right carotid artery; other abbreviations as in **Figures 1 and 2**.

FIGURE 6 CircRNA-LONP2 Modulates Oxidative Stress and Endothelial Inflammation via the miR-200a-3p/YAP1/EZH2 Axis



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reporter (but not MUT reporter) were significantly decreased when transfected with miR-200a-3p mimics compared with the control group (Figure 4M). In addition, we found that circRNA-LONP2 overexpression or knockdown further increased or reduced the luciferase activity of the KEAP1 WT reporter (Figures 4N and 4O). These results revealed that circRNA-LONP2 serves as a sponge for miR-200a-3p to regulate KEAP1 and promote endothelial inflammation via the ceRNA mechanism in ECs.

CircRNA-LONP2 accelerates the progression of atherosclerosis. A previous report showed that AAV9^{SLRSPPS} could efficiently target ECs.[27] To study the function of endothelial circRNA-LONP2 in atherogenesis, *ApoE*$^{-/-}$ mice with partial ligation of the LCA were infected with AAV9^{SLRSPPS}-Tie2-circRNA-LONP2 or AAV9^{SLRSPPS}-Tie2-control (Figures 5A and 5B). The murine Tie2 promoter was used to drive endothelial circRNA-LONP2 expression in *ApoE*$^{-/-}$ mice. Endothelial overexpression of circRNA-LONP2 was confirmed by qRT-PCR and fluorescence in situ hybridization (Figures 5C and 5D). Six weeks after ligation, the plaque area, neointima size, and intimal lipid deposition in *ApoE*$^{-/-}$ mice infected with AAV9^{SLRSPPS}-Tie2-circRNA-LONP2 were significantly aggravated compared with those in the control group (Figures 5E to 5G, Supplemental Figure 14). In addition, immunofluorescence staining showed that circRNA-LONP2 increased the protein levels of the pro-inflammatory markers Vcam-1 (Figure 5H) and Icam-1 (Figure 5I) and mononuclear phagocytes marker Mac-3 substantially (Figure 5J) in the ligated LCA of *ApoE*$^{-/-}$ mice, while decreasing the protein levels of antioxidative and anti-inflammatory Nrf2 (Figure 5K) and Ho-1 (Figure 5L), without significantly affecting the protein expression of Keap1 (Figure 5M). These data suggest that circRNA-LONP2 accelerates the progression of atherosclerosis by inducing oxidative stress and endothelial inflammation. Furthermore, these results reveal that there is an additional pathway by which circRNA-LONP2 regulates NRF2 signaling without affecting KEAP1 expression.

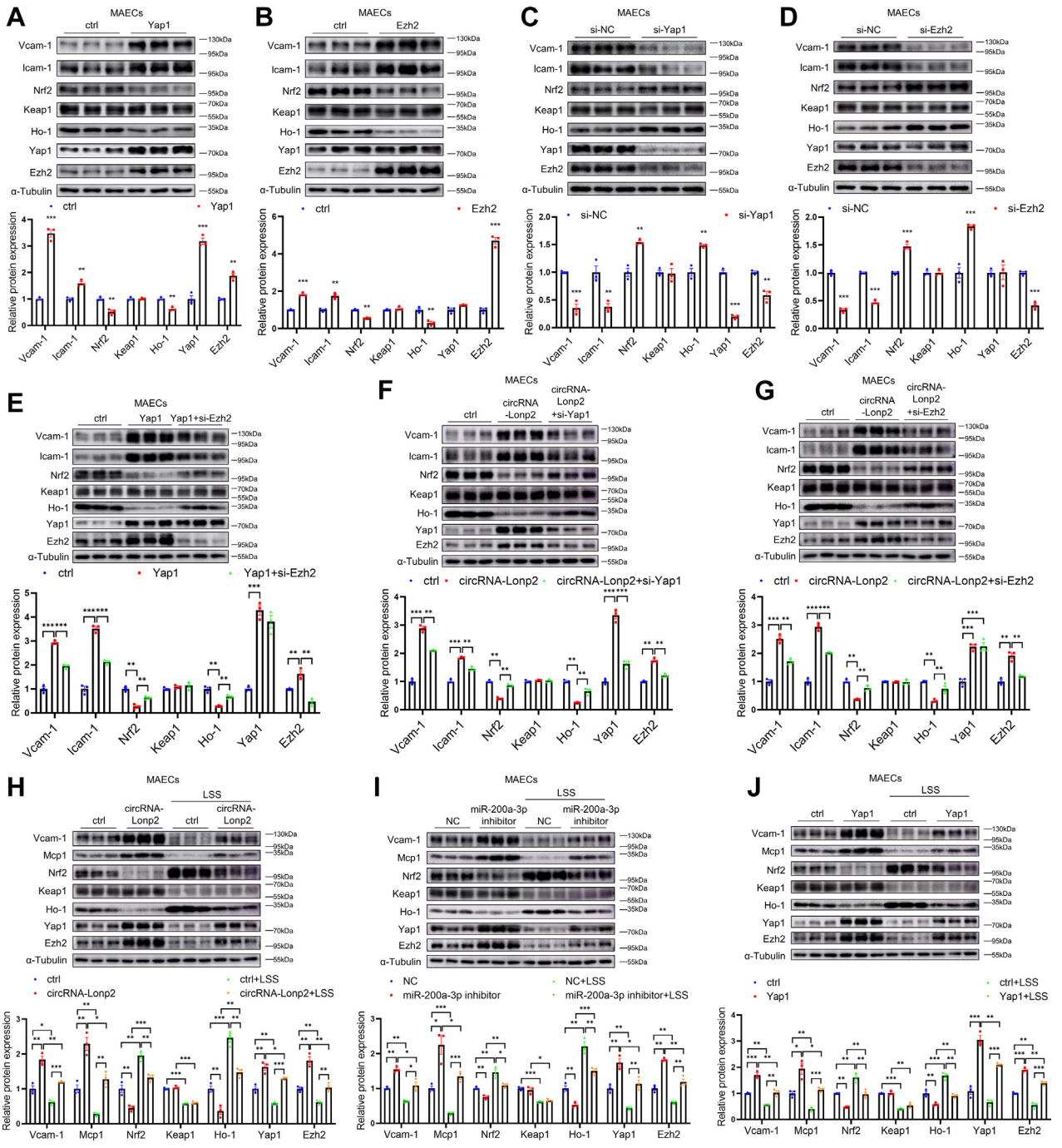
CircRNA-LONP2 modulates oxidative stress and endothelial inflammation via the miR-200a-3p/YAP1/EZH2 axis. Although miR-200a-3p was conserved across most vertebrates, the miR-200a-3p binding site was not conserved in mouse *Keap1*, suggesting that mouse *Keap1* mRNA is not a target of miR-200a-3p. Indeed, although the overexpression of circRNA-LONP2 increased the mRNA levels of Vcam-1 and Icam-1 while reducing the mRNA levels of Nrf2 and Ho-1, it did not affect the mRNA levels of Keap1 in MAECs (Figure 6A). Consistently, immunofluorescence staining showed that circRNA-LONP2 has no effect on protein levels of Keap1 in mice (Figure 5M). These data suggested that the regulatory effects of circRNA-LONP2 on the progression of atherosclerosis in mice are not mediated by Keap1 but by other miR-200a-3p targets.

A previous report has shown that miR-200a-3p inhibits inflammation and the formation of atherosclerotic lesions by targeting EZH2.[28] EZH2 functions as a histone H3 Lys27 (H3K27) trimethyltransferase that transcriptionally represses NRF2 expression in lung cancer.[29] In addition, YAP1, which promotes the development of atherosclerotic lesions,[30] has been identified as a target of miR-200a-3p.[31] YAP1/TAZ promotes the transcription of EZH2 in lung cancer.[32] Therefore, we further explored whether EZH2 and YAP1 are involved in the pro-oxidative and pro-inflammatory effects of circRNA-LONP2. As expected, overexpression of miR-200a-3p decreased EZH2 and YAP1 protein expression (Figure 6B). Consistently, silencing circRNA-LONP2 down-regulated YAP1 and EZH2 (Figure 6C), whereas the overexpression of circRNA-LONP2 had the opposite effect

FIGURE 6 Continued

(A) RNA levels of circRNA-LON2, Lonp2, Keap1, Nrf2, Ho-1, Vcam-1, and Icam-1 in mouse aortic endothelial cells (MAECs) transfected with human circRNA-LONP2 or control (ctrl) lentivirus were measured by RT-qPCR. Gapdh served as an internal reference; n = 3. (B) HUVECs were transfected with miR-200a-3p-mimic or mimic-NC (control) for 48 hours. YAP1 and EZH2 protein levels were detected by Western blot analysis; n = 3. (C) Protein levels of YAP1 and EZH2 in HUVECs transfected with si-cirRNA-LONP2 or si-NC (control) were detected by Western blot analysis; n = 3. (D) Protein levels of YAP1 and EZH2 in HUVECs transfected with circRNA-LONP2 or ctrl lentivirus were analyzed by Western blot analysis; n = 3. (E) RNA levels of circRNA-LON2, Yap1, and Ezh2 in MAECs transfected with human circRNA-LONP2 or ctrl lentivirus were measured by RT-qPCR. Gapdh served as an internal reference; n = 3. (F) Protein levels of Keap1, Nrf2, Ho-1, Vcam-1, Icam-1, Yap1, and Ezh2 in MAECs transfected with mouse circRNA-Lonp2 (mouse) or ctrl lentivirus were detected by using Western blot analysis; n = 3. (G and H) RNA levels of YAP1, EZH2, KEAP1, NRF2, HO-1, VCAM-1, ICAM-1, and circRNA-LONP2 in HUVECs transfected with YAP1 or EZH2 siRNA (si-YAP1 or si-EZH2) or si-NC were measured by RT-qPCR; n = 3. (I and J) HUVECs overexpressing circRNA-LONP2 were co-transfected with si-YAP1 or si-EZH2 for 72 hours. Protein levels of KEAP1, NRF2, HO-1, VCAM-1, ICAM-1, YAP1, and EZH2 were determined by Western blot analysis; n = 3. (K and L) Immunofluorescence staining for Yap1 (K), Ezh2 (L), Cd31, and DAPI in the ligated LCA of *ApoE*$^{-/-}$ mice. Scale bar: 40 μm. Quantification of relative mean fluorescent intensity of Yap1 and Ezh2 (n = 6). Statistical analyses were performed by using the unpaired 2-tailed Student's *t*-test (A-H, K, and L) or the 1-way analysis of variance with Tukey's post hoc test (I and J). Values are mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control. Abbreviations as in Figures 1 to 3.

FIGURE 7 Shear Stress Modulates MAEC Oxidative Stress and Inflammation Via the circRNA-LONP2/miR-200a-3p/YAP1/EZH2 Axis



(A and B) Protein levels of Keap1, Nrf2, Ho-1, Vcam-1, Icam-1, Yap1, and Ezh2 in MAECs transduced with mouse Yap1 (A), mouse Ezh2 (B), or ctrl lentivirus were analyzed by using Western blot analysis. (C and D) Protein levels of Keap1, Nrf2, Ho-1, Vcam-1, Icam-1, Yap1, and Ezh2 in MAECs transfected with mouse Yap1 siRNA (si-Yap1), mouse Ezh2 siRNA (si-Ezh2), or si-NC were analyzed by using Western blot analysis. (E) MAECs transduced with mouse Yap1 or mCherry (ctrl) lentivirus for 24 hours were transfected with mouse Ezh2 siRNA or control siRNA for an additional 48 hours. Protein levels of Keap1, Nrf2, Ho-1, Vcam-1, Icam-1, Yap1, and Ezh2 were determined by using Western blot analysis. (F and G) MAECs transduced with mouse circRNA-Lonp2 or mCherry (ctrl) lentivirus for 24 hours were transfected with mouse si-Yap1 (F), mouse si-Ezh2 (G), or control siRNA for an additional 48 hours. Protein levels of Keap1, Nrf2, Ho-1, Vcam-1, Icam-1, Yap1, and Ezh2 were determined by using Western blot analysis. (H to J) MAECs transfected with circRNA-Lonp2 or ctrl lentivirus (H), miR-200a-3p inhibitor or negative control (NC) inhibitor (I), Yap1 or ctrl lentivirus (J) were exposed to atheroprotective LSS (15 dyne/cm^2) for 24 hours. The protein levels of Vcam-1, Mcp1, Nrf2, Keap1, Ho-1, Yap1, and Ezh2 were detected by using Western blot analysis. Unpaired 2-tailed Student's *t*-test was used for statistical analyses. Values are mean ± SEM. n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control. Abbreviations as in Figures 1, 2, and 6.

(Figure 6D). Furthermore, the overexpression of human circRNA-LONP2 increased mRNA levels of Yap1 and Ezh2 in MAECs (Figure 6E). Collectively, these data suggest that EZH2 and YAP1 are conserved downstream targets of circRNA-LONP2.

We further explored whether mouse circRNA-Lonp2 has a similar function. Although there were some mismatches between the human and mouse Lonp2 genome sequences, the binding sites of miR-200a-3p in human circRNA-LONP2 and mouse circRNA-Lonp2 were conserved (Supplemental Figure 15). We found that circRNA-Lonp2 was expressed in MAECs and in the mouse aorta (Supplemental Figure 16). The overexpression of mouse circRNA-Lonp2 up-regulated Ezh2, Yap1, Vcam-1, and Icam-1, while down-regulating Nrf2 and Ho-1 at the protein level, without affecting Keap1 expression in MAECs (Figure 6F). These data suggest that, in mice, circRNA-Lonp2 promotes oxidative stress and endothelial inflammation by targeting Yap1 and Ezh2 rather than Keap1.

Next, we investigated whether circRNA-LONP2 functions by targeting YAP1/EZH2 in both humans and mice. Silencing YAP1 or its downstream target EZH2 decreased the mRNA and protein levels of VCAM-1 and ICAM-1, while increasing the mRNA and protein levels of NRF2 and HO-1, without affecting the mRNA and protein levels of KEAP1 in HUVECs (Figures 6G to 6J). Moreover, silencing of YAP1 or EZH2 by siRNA significantly abrogated the pro-oxidative and pro-inflammatory effects of circRNA-LONP2 in HUVECs (Figures 6I and 6J). Consistent with these data, immunofluorescence staining showed that circRNA-LONP2 significantly up-regulated Yap1 and Ezh2 in mice (Figures 6K and 6L). Collectively, these data suggest that circRNA-LONP2 functions by targeting YAP1/EZH2 in both humans and mice.

To show the causal relationship between Yap/Ezh2 expression and Nrf2 activation in mouse ECs, we conducted a series of gain-of-function and loss-of-function experiments as well as rescue experiments. Overexpression of Yap1 or its downstream target Ezh2 increased the protein levels of Vcam-1 and Icam-1, while decreasing the protein levels of Nrf2 and Ho-1, without affecting Keap1 protein expression in MAECs (Figures 7A and 7B). Silencing of Yap1 or Ezh2 had the opposite effect (Figures 7C and 7D). Moreover, silencing of Ezh2 significantly abrogated the pro-oxidative and pro-inflammatory effects of Yap1 in MAECs (Figure 7E). Consistent with these data, silencing of Yap1 or Ezh2 significantly abrogated the pro-oxidative and pro-inflammatory effects of mouse circRNA-Lonp2 in MAECs (Figures 7F and 7G). In addition, circRNA-Lonp2 overexpression partially counteracted the LSS-induced repression of Vcam-1, Mcp-1, Yap1, and Ezh2 protein expression, as well as partially attenuated the LSS-induced up-regulation of Nrf2 and Ho1 protein expression, with no effect on Keap1 protein expression in MAECs (Figure 7H). Consistently, miR-200a-3p inhibitor transfection or Yap1 overexpression partially counteracted the anti-oxidative and anti-inflammatory effects of LSS in MAECs (Figures 7I and 7J). Taken together, these results indicate that shear-sensitive circRNA-LONP2 regulates oxidative stress and endothelial inflammation via a conserved signaling mechanism, the miR-200a-3p/YAP1/EZH2 axis.

DISCUSSION

LSS inhibits endothelial inflammation and protects arteries from atherosclerosis;[33-35] however, the mechanism of this effect is unclear. CircRNAs are key regulators of vascular homeostasis and atherosclerosis,[36,37] but their role in mediating the action of LSS has not been explored. In this study, we first revealed that LSS down-regulated circRNA-LONP2 expression, inhibiting oxidative stress and endothelial inflammation by activating the miR-200a-3p-mediated NRF2/HO-1 signaling pathway. These findings provide strong evidence for the atherogenic effects of endothelial circRNA-LONP2 and suggest it as potential target for atherosclerotic therapy.

Although atherosclerosis is associated with many systemic risk factors, such as hypercholesterolemia, hypertension, diabetes, smoking, and aging, this process primarily occurs near arterial branches, bifurcations, and curvatures, where the blood flow is disturbed, generating OSS that drives atherosclerosis by inducing vascular inflammation and oxidative stress.[38] OSS triggers endothelial inflammation by up-regulating the expression of endothelial adhesion molecules (VCAM-1 and ICAM-1), facilitating monocyte adhesion to ECs.[39] Activation of NF-κB signaling is crucial in OSS-induced inflammation.[40] NRF2 is activated by LSS, which induces activation of anti-inflammatory and antioxidant gene expression and protects cells from oxidative stress and inflammatory damage.[41-43] NRF2 activates the transcription of a series of antioxidant genes and is the main regulator of the cellular antioxidant defense system.[44] Under normal conditions, NRF2 is sequestered in the cytoplasm by KEAP1, which prevents NRF2 from nuclear translocation and facilitates its proteasomal degradation.[45] In response to LSS stimulation, NRF2 is activated by dissociation from KEAP1 and

translocation into the nucleus, which induces the expression of antioxidant genes.[41,43]

In addition to its antioxidant effects, NRF2 inhibits the NF-κB signaling pathway through a variety of mechanisms. First, NRF2 inhibits oxidative stress-mediated NF-κB activation by reducing intracellular reactive oxygen species levels.[46] Second, NRF2 induces up-regulation of cellular HO-1 expression, preventing degradation of IκB-α.[47] Thus, targeted activation of NRF2 may protect the arterial vascular system from atherosclerosis through its antioxidant and anti-inflammatory effects. We found that LSS-induced down-regulation of circRNA-LONP2 suppresses endothelial inflammation, at least in part by activating the NRF2/HO-1 signaling pathway. These findings suggest that circRNA-LONP2 can serve as a therapeutic target in atherosclerosis.

miRNAs are a class of small, noncoding RNAs, each about 22 nucleotides long, that are active in post-transcriptional gene silencing.[48,49] miRNAs respond to shear stress regulation and participate in vascular EC inflammation.[50-52] miR-200a-3p is highly conserved among vertebrate species and reportedly targets KEAP1,[26,53-55] YAPI,[31,56,57] and EZH2.[28] However, the role of miR-200a-3p in mediating the effects of shear stress on endothelial inflammation remains unclear. LSS suppresses YAP1, which interrupts inflammatory signaling pathways and prevents atherosclerosis progression.[58] EZH2 is down-regulated by LSS to prevent apoptosis in ECs[59]; EZH2 down-regulation also enhances activation of atherosclerosis-protective MAPK7 signaling, conferring an antiatherosclerotic effect.[60] Consistent with these previous findings, we found that miR-200a-3p targets and inhibits KEAP1, YAP1, and EZH2 expression in ECs and that transfection with miR-200a-3p mimics activated NRF2 signaling. In addition, knockdown of NRF2 or its downstream HO-1 significantly attended the anti-inflammation effect of circRNA-LONP2 knockdown. Together, these results establish the previously unknown functions of circRNA-LONP2 in regulating NRF2 signaling via sponging miR-200a-3p and endothelial inflammation via NRF2 signaling. Interestingly, we uncovered 2 mechanisms (conserved and species-specific mechanisms) by which circRNA-LONP2 modulates NRF2 signaling. First, circRNA-LONP2 regulates the nuclear transport of NRF2 via the miR-200a-3p/KEAP1 axis (human-specific regulatory mechanism). Second, circRNA-LONP2 regulates NRF2 expression via the miR-200a-3p/YAP1/EZH2 axis (a conserved regulatory mechanism in both humans and mice). Our findings highlight species-specific differences in circRNA-LONP2–regulated NRF2 signaling.

Previous studies reported that circRNA-LONP2 directly interacts with and promotes the processing of primary microRNA-17 in colorectal carcinoma cells[24] and directly interacts with miR-27b-3p to accelerate the progression of esophageal squamous cell carcinoma through the miR-27b-3p-ZEB1 axis.[25] Although our data confirm that circRNA-LONP2 can interact with pri-miR-17 or miR-27-3p, the mutation experiments rule out a role for pri-miR-17 or miR-27-3p in mediating circRNA-LONP2–induced vascular endothelial inflammation and oxidative stress. Further research is needed to explore the role of circRNA-LONP2 in vascular ECs through its interaction with pri-miR-17 or miR-27-3p.

STUDY LIMITATIONS. First, although most of the circRNA-LONP2 was located in the cytoplasm, a small number of circRNA-LONP2 were still located in the nucleus. Further experiments are required to explore the function of circRNA-LONP2 in the nucleus. Second, the mechanism of how LSS mediates circRNA-LONP2 reduction remains unclear. Further studies are needed to explore the potential role of ADAR, DHX9, FUS, and QKI in shear stress–mediated circRNA-LONP2 regulation. Third, the ceRNA hypothesis suggests that gene expression is optimally regulated when miRNA and ceRNA are expressed almost equally.[61,62] However, our study showed that circRNA-LONP2 is approximately twice as abundant as miR-200a-3p in HUVECs. Further studies are needed to explore the effects of other factors on ceRNA activity, such as the subcellular location of circRNA-LONP2 and miR-200a-3p, miR-200a-3p/circRNA-LONP2 affinity, RNA editing, and RNA-binding proteins. Finally, although our in vitro and in vivo experiments showed that circRNA-LONP2 promotes oxidative stress and endothelial inflammation and accelerates the progression of atherosclerosis, further research is needed to explore the clinical value of circRNA-LONP2.

CONCLUSIONS

The current study found that antiatherosclerotic LSS significantly down-regulated the expression of circRNA-LONP2, and the reduction of circRNA-LONP2 alleviated oxidative stress and endothelial inflammation by enhancing the activity of miR-200a-3p, which targets KEAP1, YAP1, and EZH2 mRNA for degradation and subsequently activates the NRF2/HO-1 pathway. Our findings suggest that

circRNA-LONP2 could serve as a new therapeutic target for atherosclerosis.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Hemodynamic shear stress is a frictional force that acts on vascular ECs and is essential for endothelial homeostasis under normal physiological conditions. circRNAs are powerful regulators of vascular homeostasis and atherosclerosis; however, their roles in mediating the effects of LSS remain unexplored. This study provides the first evidence that circRNA-LONP2 modulates the flow-dependent endothelial inflammatory response and accelerates the progression of atherosclerosis, suggesting a clinical significance of circRNA-LONP2 in atherosclerosis.

TRANSLATIONAL OUTLOOK: OSS induces circRNA-LONP2 expression and promotes atherosclerosis; however, LSS inhibits circRNA-LONP2 expression and has anti-atherosclerosis properties. We propose that endothelial-specific knockdown of circRNA-LONP2, or its downstream targets KEAP1, YAP1 or EZH2, might be a promising therapeutic strategy to prevent or treat atherosclerosis.

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KEY WORDS atherosclerosis, circRNA-LONP2, endothelial inflammation, microRNA-200a-3p, shear stress

APPENDIX For an expanded Methods section and supplemental figures, please see the online version of this paper.