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EDITORIAL COMMENT

Stressing the Circle

Circular RNA-LONP2 Role in Atherosclerosis*

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Atherosclerosis is a chronic inflammatory disease affecting the vascular wall of large- and medium-sized arteries. This disease leads to myocardial infarction, ischemic stroke, and peripheral arterial disease, representing the leading cause of morbidity and mortality in developed countries.[1] Hypercholesterolemia, hypertension, and diabetes mellitus are among the systemic risk factors predisposing to atherosclerosis. Atheromas preferentially form in arterial regions in which the blood flow is disturbed, whereas arterial regions exposed to stable flow remain healthy.[2] In particular, early atherosclerotic lesions appear nonrandomly in the artery, with a strikingly localized pattern that follows the geometry of branched or curved points. In light of this finding, it is possible to classify the near-wall shear stress profiles in atheroprone and atheroprotected arterial geometries.[2]

The blood flow imposes a stress on the endothelium with frictional forces or wall shear stresses. There are several blood flow patterns, ranging from a time-averaged unidirectional laminar flow, usually occurring in unbranched arterial regions, to a disturbed flow, occurring in branched or curved arteries. These flow patterns result in laminar shear stress (LSS) and low-magnitude and oscillatory shear stress (OSS), respectively.

Shear stress stimulates various mechanosensors, such as ion channels, nicotinamide adenine dinucleotide phosphate oxidase and xanthine oxidase, receptor tyrosine kinases, G proteins, and cell/cell and cell/matrix adhesion complexes. These mechanotransduction mechanisms link the externally applied mechanical stress to intracellular and intranuclear events.[3]

Different spatial and temporal patterns of shear stresses, despite the common mechanosensors and mechanotransducers, trigger distinguishable gene expression patterns. Indeed, LSS upregulates atheroprotective genes, such as endothelial nitric oxide synthase, the Krüppel-like factor family (*KLF2* and *KLF4*), nuclear factor erythroid 2-related factor 2-like bZIP transcription factor 2 (*NRF2* or *NFE2L2*), and superoxide dismutases (*MN-SOD* and *EC-SOD*), and downregulates proatherogenic genes, such as those related to coagulation, leukocyte diapedesis, and smooth muscle cell proliferation. Conversely, OSS enhances proatherogenic genes, such as vascular cell adhesion protein 1 and intercellular adhesion molecule 1 and suppresses atheroprotective genes. Thus, LSS plays a crucial role in vascular homeostasis, whereas OSS leads to vascular dysfunction and disease.

Although the interconnection between hemodynamics, endothelial pathobiology, and atherogenesis has been extensively investigated, more in-depth research is needed to understand the molecular mechanisms underpinning the responses to different flow patterns. Specifically, among the emerging players, noncoding RNAs (ncRNAs) are particularly promising[4]; ncRNAs are mainly categorized according to their sequence length into small or long ncRNAs.[5] Small ncRNAs include microRNAs (miRNAs), and long ncRNAs include linear long ncRNAs and circular RNAs (circRNAs). circRNAs identify single-strand covalently closed molecules generated through the back-splicing of linear RNA precursors. Accordingly, circRNAs are resistant to exonucleases, which makes them significantly more stable than their linear counterparts and promising biomarkers. circRNAs can act as miRNA sinks or sponges, thus actively

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competing with miRNA targets.[6] Several flow-sensitive miRNAs and few long ncRNAs have been found to regulate endothelial function and atherosclerosis,[4] whereas the role of circRNAs remains unclear.

In this issue of *JACC: Basic to Translational Science*, Wang et al[7] report that flow-sensitive circRNA-LONP2 modulates the inflammatory response induced by shear stress and the development of atherosclerosis both in vitro and in vivo. By microarray profiling in human umbilical vein endothelial cells (ECs), they identified several circRNAs modulated by LSS compared to the static condition, among which circRNA-LONP2 was significantly downregulated. Reverse transcription quantitative polymerase chain reaction experiments showed instead that the linear form of LONP2 was not modulated, indicating independent circRNA-LONP2 modulation, a telltale of significant biological function. Importantly, the circRNA exon structure was confirmed by divergent primers and Sanger's sequencing, whereas the exonucleases degradation resistance proved the circular structure, and the in situ hybridization results indicated the cytoplasmic localization.

The atheroprotective LSS and the atheropromoting OSS had a different impact on circRNA-LONP2 expression; the former reduced and the latter increased the circRNA expression. Gain- and loss-of-function experiments showed that in human ECs the inhibition of circRNA-LONP2 expression reduced the messenger RNA levels of the proinflammatory *VCAM-1* and *ICAM-1* and increased the messenger RNA levels of the antioxidative stress and anti-inflammatory genes *NRF2* and heme oxygenase 1, whereas circRNA-LONP2 overexpression had opposite effects. The increase of *ICAM-1* and *VCAM-1* messenger RNA levels mediated by OSS was counteracted by circRNA silencing. The effects of the circRNA-LONP2-mediated generation of reactive oxygen species on endothelial inflammation was demonstrated by silencing cirRNA-LONP2 expression, which attenuated the tumor necrosis factor alpha-induced expression of *VCAM-1* and *ICAM-1* in ECs, reduced reactive oxygen species levels, and reduced the adhesion of monocytes to the cocultured ECs. These data suggest that circRNA-LONP2 regulates flow-dependent inflammatory responses.

The ability of circRNAs to act as a miRNA sink or sponge, thus actively competing with miRNA targets, has been demonstrated in several cardiovascular systems.[5,6] An MS2-RNA tagging strategy was used to pull down miRNAs interacting with circRNA-LONP2; among them, miR-200a-3p was particularly promising and was further characterized. First, the phenotype induced by miR-200a-3p that mimics transfection in ECs was similar to that induced by circRNA-LONP2 silencing. Moreover, miR-200a-3p inhibition prevented the anti-inflammatory effects of circRNA-LONP2 silencing. miR-200a-3p levels did not change in circRNA-LONP2 gain- and-loss-of function or under LSS, indicating that circRNA-LONP2 inflammatory action is mediated by miR-200a-3p interaction without affecting the expression of the miRNA. This is an interesting mechanistic detail because miRNA/circRNA interaction has often been reported to lead to the decrease of miRNA levels.[5,6]

Under normal conditions, NRF2 is retained in the cytoplasm by kelch-like Ech-associated protein 1 (KEAP1), which triggers NRF2 proteasomal degradation. Upon ECs exposure to LSS, NRF2 dissociates from KEAP1 and translocates into the nucleus, inducing the expression of antioxidant genes and inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway through multiple mechanisms.

KEAP1 messenger RNA was experimentally demonstrated as a circRNA-LONP2 target; accordingly, miR-200a-3p mimics significantly downregulated KEAP1 and upregulated NRF2, indicating that circRNA-LONP2 induced endothelial inflammation through a miR-200a-3p/KEAP1/NRF2 pathway in humans.

The apolipoprotein E-deficient ($ApoE^{-/-}$) mouse model displays impaired lipoprotein clearance causing the increase of cholesterol ester-enriched particles in the blood, which, in turn, facilitates the development of atherosclerotic plaques. In the experimental model adopted by Wang et al,[7] $ApoE^{-/-}$ mice, 6 weeks after the partial ligation of the left common carotid artery, developed a plaque-laden area. The overexpression of circRNA-Lonp2 by an endothelial-retargeted adeno-associated virus vector 9 under the control of the endothelial-specific Tie2 promoter expanded the plaque area, the neointima size, and the lipid deposition. Moreover, the circRNA-forced expression increased the proinflammatory markers Vcam-1 and Icam-1 and the mononuclear phagocytes marker Mac-3, whereas it reduced Nrf2 and Ho-1 without affecting the expression of Keap1. Interestingly, circRNA-Lonp2 regulated Nrf2 expression in mice via an alternative pathway (present in mice and humans). The mechanotransducer and transcription factor yes-associated protein 1 (Yap1) regulates the transcription of enhancer of zeste homolog 2 (Ezh2), a histone-H3 Lys27 (H3K27) trimethyl transferase that represses the transcription of Nrf2.[8] Gain- and loss-of-function and rescue

experiments in mouse aortic ECs showed that circRNA-Lonp2 regulated oxidative stress and endothelial inflammation via a miR-200a-3p/Yap1/Ezh2 axis. The mechanistic redundancy of NRF2 regulation by circRNA-LONP2 is indicative of the importance of the identified pathway upon shear stress.

The study limitations are well considered by the authors, including the need for further investigations to evaluate the potential role of RNA-binding proteins in flow-mediated circRNA-LONP2 expression. Moreover, the clinical significance of circRNA-LONP2 dysregulation needs to be investigated in humans. However, this study constitutes a significant advancement in the field, being the first to identify an arterial flow-sensitive circRNA with a role in vascular remodeling, suggesting circRNA-LONP2 as a potential antiatherosclerotic therapeutic target. This is particularly relevant in the prospect of the rapidly evolving field of RNA therapy, exploring approaches to target the circRNA back-splicing junction with techniques such as RNA interference and antisense oligonucleotides.[9]

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