Circular RNA HMGCS1 sponges miR-4521 to aggravate type 2 diabetes-induced vascular endothelial dysfunction

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Abstract

Noncoding RNA plays a pivotal role as novel regulators of endothelial cell function. Type 2 diabetes, acknowledged as a primary contributor to cardiovascular diseases, plays a vital role in vascular endothelial cell dysfunction due to induced abnormalities of glucolipid metabolism and oxidative stress. In this study, aberrant expression levels of circHMGCS1 and miR-4521 were observed in diabetes-induced vascular endothelial dysfunction. The overexpression of circHMGCS1 or silencing of miR-4521 expedited the onset of diabetes and aggravated vascular endothelial dysfunction. Mechanistically, circHMGCS1 upregulated arginase 1 by sponging miR-4521, leading to decrease in vascular nitric oxide secretion and inhibition of endothelial nitric oxide synthase activity, and an increase in the expression of adhesion molecules and generation of cellular reactive oxygen species, reduced vasodilation and accelerated the impairment of vascular endothelial function. Collectively, these findings illuminate the physiological role and interacting mechanisms of circHMGCS1 and miR-4521 in diabetes-induced cardiovascular diseases, suggesting that modulating the expression of circHMGCS1 and miR-4521 could serve as a potential strategy to prevent diabetes-associated cardiovascular diseases. Furthermore, our findings provide a novel technical avenue for unraveling ncRNAs regulatory roles of ncRNAs in diabetes and its associated complications.

Impact Statement:

Cardiovascular disease, the top cause of diabetic deaths, progresses via vascular endothelial dysfunction. This study highlights the role of circHMGCS1 and miR-4521 in diabetic vascular function and may be markers for the development of diabetic vascular endothelial dysfunction.

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in patients with type 2 diabetes mellitus (T2DM). The incidence of CVD in T2DM individuals was estimated to be at least two to four times higher than that in non-diabetic individuals (Gregg et al, 2016; Yun & Ko, 2021). T2DM gives rise to abnormal metabolic conditions, including chronic hyperglycemia, dyslipidemia, insulin resistance, inflammation, and oxidative stress (Roden & Shulman, 2019). These processes ultimately lead to vascular endothelial damage, which disrupts the maintenance of vascular homeostasis, thereby promoting the development of CVD (Lundberg & Weitzberg, 2022; Niemann et al, 2017). Vascular endothelial dysfunction (VED) is considered the underlying basis for vascular complications at all stages of T2DM (Eelen et al, 2015; Meigs et al, 2004). However, the mechanism by which the key molecules trigger endothelial dysfunction in diabetes-induced vascular impairment remains unknown.

Endothelial cells form a continuous monolayer lining the arterial, venous, and lymphatic vessels, playing a crucial physiological role in maintaining vascular homeostasis (Bazzoni & Dejana, 2004). Moreover, endothelial cells actively regulate vascular tone, preserve endothelial integrity, and modulate platelet activity, contributing to hemostasis and endothelial repair (Li et al, 2019). The leading cause of T2DM is a prolonged high-sugar, high-fat diet (HFHG), commonly known as a Western diet, which inevitably leads to VED, characterized by reduced content of nitric oxide (NO), uncoupling of endothelial nitric oxide synthase (eNOS), increased formation of reactive oxygen species (ROS) and upregulation of endothelial-related adhesion molecules such as intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), and endothelin 1 (ET-1). These alterations ultimately lead to elevated blood pressure and impaired vascular arteriolar relaxation, contributing to CVD development (Xu et al, 2021; Yeh et al, 2022; Zheng et al, 2018). Despite this understanding, the identification of pivotal regulatory factors

governing gene expression in diabetes-induced VED remains a formidable challenge.

circRNAs are generated through back-splicing of precursor messenger RNAs (pre-mRNAs), exhibiting features such as a covalently closed loop structure, high stability, conservation, and tissue-specific expression. These molecules primarily function as miRNA sponges, regulators of transcription, and interactors with proteins (Kristensen et al, 2019; Liu & Chen, 2022). Conversely, miRNAs are single-stranded small RNA molecules of 21–23 nucleotides, characterized by high conservation, temporal and tissue-specific expression, and relatively poor stability. miRNAs—critical regulators of gene expression—control a wide array of cellular processes (Gebert & MacRae, 2019). The interplay between miRNAs and circRNAs forms a complex regulatory network, profoundly influencing diverse biological pathways and disease states. miRNAs serve as guides, targeting specific mRNAs to induce their degradation or translational repression, thereby suppressing gene expression post-transcriptionally (Treiber et al, 2019). By contrast, circRNAs contain miRNA binding sites, competitively inhibiting miRNA activity and thereby reducing miRNA binding to other mRNAs. This competitive adsorption effect modulates intracellular miRNA levels, affecting miRNA-mediated regulation of other target genes (Cheng et al, 2019; Huang et al, 2019; Shen et al, 2019; Zeng et al, 2021).

The expression levels of circRNA in diabetic patients exhibit abnormalities, and its potential as a biomarker for early diagnosis or therapeutic target has been gradually being substantiated (Jiang et al, 2022; Stoll et al, 2020; Tian et al, 2018). However, research on circRNA in the context of diabetes-induced VED is scarce. Current research has predominantly focused on the regulatory roles of circRNA in diabetic cardiomyopathy (Yuan et al, 2023), diabetic nephropathy (Yang & Liu, 2022), gestational diabetes (Du et al, 2022), and diabetic retinopathy (Zhu et al, 2019). Investigations into circRNA regulation of VED have only described the phenomenon and analyzed the correlation of the results, lacking the precision to infer

causation accurately (Jiang et al, 2020; Liu et al, 2017; Ma et al, 2023). Moreover, comprehension of VED induced by the complex multifactorial processes of diabetes pathogenesis remains relatively inadequate. Additionally, the interaction between circRNA and miRNA in diabetes-induced VED remains a subject of research controversy (Cheng et al, 2019; Liu et al, 2019; Pan et al, 2018). Therefore, a more comprehensive research approach is required to elucidate the specific mechanisms by which circRNA operates in diabetes-related CVDs.

The present study elucidated the mechanisms underlying the interaction between circRNA and miRNA in regulating diabetes-associated VED. Through the screening strategy, we successfully identified circHMGCS1—a circRNA originating from the pre-mRNA of HMGCS1. We demonstrated that the upregulation of circHMGCS1 and downregulation of miR-4521 significantly promoted diabetes-induced VED. Moreover, in-depth mechanistic investigations revealed that circHMGCS1 functioned as a miR-4521 sponge, increasing arginase 1 (ARG1) expression in vascular endothelial cells and accelerating VED progression. Furthermore, the expression patterns and interaction mechanisms between circHMGCS1 and miR-4521 in endothelial cells were identified for the first time in these findings, which also highlight the circHMGCS1/miR-4521/ARG1 axis as a novel therapeutic target for interventions designed to protect patients from diabetes-induced VED.

Results

Global expression analysis of endotheliocyte circRNAs

To explore the potential role of circRNAs in regulating endotheliocyte function, we characterized the transcriptome of circRNA derived from total RNA in human umbilical vein endothelial cells (HUVECs) stimulated by high palmitate and high glucose (PAHG), which allowed us to investigate the potential role of circRNAs in diabetes-induced VED (Figures S1A-S1C). A total of 17,179 known circRNAs were identified, with 66 of them exhibiting differential expression (GEO submission: GSE237597). Our main focus was on circRNAs that shared identical genomic and splicing lengths that exhibited fold changes greater than 2 or less than -2 ($p < 0.01$) in PAHG-treated HUVECs (Figure 1A). Specifically, 48 circRNAs were upregulated, whereas 18 were downregulated (Figure S1D). Furthermore, approximately 66.65% of circRNAs were found to be produced through reverse splicing of exons (Figure S1E).

The observed changes in circRNA levels were confirmed through the real-time quantitative reverse transcription PCR (qRT-PCR) analysis of the five most upregulated circRNAs, suggesting that the results of the RNA-seq data are credible. Among them, circHMGCS1 (hsa_circ_0008621, 899 nt in length, identified as circHMGCS1 in subsequent studies because of its host gene being HMGCS1 (Figure S1F-S1H) (Liang et al, 2021)) exhibited significant upregulation compared with the non-PAHG-treated condition (Figure 1B). The circHMGCS1 sequence is situated on chromosome 5 of the human genome, specifically at 43292575-43297268 with no homology to mouse sequences. Subsequently, divergent primers were designed to amplify circHMGCS1, whereas convergent primers were designed to amplify the corresponding linear mRNA from cDNA and genomic DNA (gDNA) in HUEVCs. amplification of circHMGCS1 was observed in cDNA but not in gDNA (Figure 1C), which confirmed the circularity of circHMGCS1. Sanger sequencing of the amplified product of circHMGCS1 confirmed its sequence alignment with the

annotated circHMGCS1 in circBase (Figure 1D). This observation confirms the origin of circHMGCS1 from exons 2 to 7 of the HMGCS1 gene (Figure 1E).

RNA-Fluorescence *in situ* hybridization (RNA-FISH) confirmed the robust expression of cytoplasmic circHMGCS1 in HUVECs (Figure 1F). The qRT-PCR analysis of nuclear and cytoplasmic RNA revealed the predominant cytoplasmic expression of circHMGCS1 in HUVECs (Figure 1G). qRT-PCR also demonstrated that circHMGCS1 displayed a stable half-life exceeding 24 h, whereas the linear transcript HMGCS1 mRNA had a half-life of less than 8 h (Figure 1H). Furthermore, circHMGCS1 exhibited resistance to digestion by ribonuclease R (an exonuclease that selectively degrades linear RNA (Xiao & Wilusz, 2019), also known as RNase R), whereas linear HMGCS1 mRNA was easily degraded upon RNase R treatment (Figure 1I). These findings indicate that the higher expression of circHMGCS1 in VED is more than just a byproduct of splicing and suggestive of functionality.

Upregulation of circHMGCS1 promotes diabetes-induced VED

To explore the potential role of circHMGCS1 in regulating endothelial cell function, we cloned exons 2–7 of HMGCS1 into lentiviral vectors for ectopic overexpression of circHMGCS1 (Figure S2). We found that circHMGCS1 was successfully overexpressed in HUVECs without significant changes in HMGCS1 mRNA expression, and the level of the circular transcripts increased nearly 60-fold than the level of the linear transcripts (Figure 2A). These results confirm that the circularization is efficient and that circHMGCS1 has no effect on HMGCS1 expression. Further experiments demonstrated that the overexpression of circHMGCS1 stimulated the expression of adhesion molecules (VCAM1, ICAM1, and ET-1) (Figures 2B and 2C), suggesting that circHMGCS1 is involved in VED promotion. We then used PAHG to stimulate HUVEC-overexpressing circHMGCS1. NO levels were significantly decreased after PAHG stimulation for 24 h, and circHMGCS1 overexpression further decreased NO levels (Figure 2D), indicating that increased circHMGCS1 expression inhibits endothelial diastolic function. eNOS

activity was inhibited by PAHG, and upregulated circHMGCS1 expression further decreased eNOS activity (Figure 2E). Superoxide is one of the major ROS known to promote atherosclerosis (Griendling et al, 2016). Therefore, we used DHE to assess superoxide levels in endothelial cells. The overexpression of circHMGCS1 enhanced PAHG-induced ROS generation (Figure 2F), increasing oxidative stress in endothelial cells. The expression levels of adhesion molecules (VCAM1, ICAM1, and ET-1) were further elevated with circHMGCS1 overexpression (Figure 2G and 2H). These findings suggest that elevated expression of circHMGCS1 may contribute to the development of endothelial cell dysfunction.

miR-4521 protects endothelial cells from PAHG-induced dysfunction

circRNAs may function as ceRNAs to sponge miRNAs, thereby modulating miRNA target depression and imposing an additional level of post-transcriptional regulation in human diseases (Zhong et al, 2018). Accordingly, we conducted miRNA sequencing in PAHG-stimulated HUVECs to identify the potential miRNA targets of circHMGCS1. The deep sequencing analysis revealed 98 significantly differentially expressed miRNAs (Figures 3A and 3B, GEO submission: GSE237295). We then used the miRanda database and ceRNA theory to obtain four candidate miRNAs (miR-4521, miR-3143, miR-98-5p, and miR-181a-2-3p) (Figures 3C and 3D). qRT-PCR confirmed that all four miRNAs were downregulated (Figure 3E). Next, we observed that each of the four miRNA mimics induced a significantly increase in the expression of their corresponding miRNAs in HUVECs through the application of synthetic miRNA mimics (Figure S3A). Relative to the other three miRNAs, miR-4521 mimics significantly inhibited adhesion molecules (ICAM1, VCAM1, and ET-1) expression in HUVECs (Figures 3F and 3G), making it a suitable candidate for further endothelial function studies. We then investigate the effect of miR-4521 on PAHG-stimulated endothelial cell function using synthetic miR-4521 mimics and miR-4521 inhibitor in HUVECs (Figures S3B and S3C). Enhanced miR-4521 levels effectively restored PAHG-induced reductions in NO content and eNOS activity in

HUEVCs, while miR-4521 inhibition led to opposite results (Figures 3H and 3I). whereas miR-4521 mimics significantly inhibited PAHG-induced expression of ROS and adhesion molecules (VCAM1, ICAM1, and ET-1) (Figure 3J), whereas the miR-4521 inhibitor increased the PAHG-induced expression of ROS and adhesion molecules (VCAM1, ICAM1, and ET-1) (Figures 3K and 3L, and Figure S3D and S3E). These findings suggest that miR-4521 is involved in PAHG-induced endothelial cell dysfunction.

circHMGCS1 acts as a miR-4521 sponge to regulate endothelial dysfunction

We then explored the interaction mechanism between circHMGCS1 and miR-4521. As displayed in Figure S4A and S4B, the overexpression of circHMGCS1 reduced the expression level of miR-4521, whereas knockdown of circHMGCS1 resulted in an upregulation of miR-4521. Meanwhile, bioinformatics analysis revealed the predicted binding sites between miR-4521 and circHMGCS1 (Figure S4B). We constructed a dual-luciferase reporter by inserting the wild-type (WT) or mutant (MUT) linear sequence of circHMGCS1 into the pmirGLO luciferase vector. The co-transfection of miR-4521 mimics with the circHMGCS1 luciferase reporter gene in HEK293T cells, reducing luciferase activity; mutations in the binding sites between circHMGCS1 and miR-4521 abrogated the effect of miR-4521 mimics on the activity of the circHMGCS1 luciferase reporter gene mutant (Figure 4A). Furthermore, AGO2 immunoprecipitation in HUVECs transfected with miR-4521 or its mutants demonstrated that miR-4521 facilitates the association of AGO2 with circHMGCS1 in HUVECs (Figure 4B). Meanwhile, we used biotinylated miR-4521 mimics in HUVECs stably overexpressing circHMGCS1 to further validate the direct binding of miR-4521 and circHMGCS1. The qRT-PCR results revealed that miR-4521 captured endogenous circHMGCS1, and the negative control with a disrupting putative binding sequence failed to coprecipitate circHMGCS1 (Figure 4C). Consistently, the use of biotin-labeled circHMGCS1 effectively captured both miR-4521 and AGO2 (Figure S4D and S4E). Moreover, the RNA-FISH assay

showed colocalization of miR-4521 and circHMGCS1 in the cytoplasm (Figure 4D). Altogether, these findings indicate that circHMGCS1 functions as a molecular sponge for miR-4521.

Rescue experiments were conducted to investigate whether circHMGCS1 regulates endothelial cell function through miR-4521 sponging. We achieved miR-4521 inhibition by transfecting HUVECs with a recombinant adeno-associated virus 9 (AAV9) vector carrying miR-4521 sponges. Notably, miR-4521 sponges significantly promoted the PAHG-induced reduction in NO content and eNOS activity. Under this condition, increased circHMGCS1 expression did not interfere with the changes in NO content and eNOS activity (Figures 4E and 4F). Moreover, miR-4521 sponges elevated ROS expression in PAHG treated HUVECs, whereas no further additional effect on ROS expression was observed upon the overexpression of circHMGCS1 when miR-4521 was sponged (Figure 4G). Further evaluation of endothelial functional molecules revealed that circHMGCS1 failed to stimulate the expression of these factors when miR-4521 was sponged (Figures 4H-4J). Furthermore, the reduction in NO content and eNOS activity induced by circHMGCS1 overexpression was reversed by miR-4521 mimics (Figures 4K and 4L), and the circHMGCS1-mediated increase in ROS content was inhibited by miR-4521 mimics (Figure 4M). Furthermore, the expression of adhesion molecules (ICAM1, VCAM1, and ET-1) was increased in circHMGCS1-transfected HUVECs, and this effect was significantly ameliorated by miR-4521 mimics (Figures 4N-4P). These findings demonstrate that circHMGCS1 acts as a sponge for miR-4521, thereby regulating endothelial function.

circHMGCS1 serves as a miR-4521 sponge to regulate diabetes-induced VED

To further explore the interaction between miR-4521 and circHMGCS1 in diabetes-induced VED, we administered exogenous miR-4521 agomir (mimics) via tail vein injection to mice, whereas agomir NC, expressing random sequence, served as the negative control in HFHG-induced diabetes to examine its effect on diabetes

and associated VED (Figure S5A). Compared with to non-diabetic WT (Control) mice, DM mice exhibited a significant increase in body weight, upon miR-4521 agomir application, the body weight of diabetic mice significantly decreased compared with the untreated diabetic mice (Figure 5A), and elevated fasting blood glucose levels in DM mice were likewise substantially inhibited (Figure 5B). By contrast, the combined treatment of circHMGCS1 and miR-4521 agomir did not significantly affect the body weight and blood glucose levels. OGTT and ITT experiments demonstrated that miR-4521 agomir considerably enhanced glucose tolerance and insulin resistance in diabetic mice (Figures 5C, 5D, and Figure S5B and S5C). Blood lipid biochemistry analysis revealed that miR-4521 agomir restored abnormal blood lipid levels in diabetic mice (Figures S5D-S5G). Notably, the abundant presence of circHMGCS1 in the body can negate the inhibitory effect of miR-4521 on diabetes development. These results suggest that miR-4521 can inhibit the occurrence of diabetes, whereas circHMGCS1 specifically dampens the function of miR-4521, weakening its protective effect against diabetes.

To investigate the roles of miR-4521 and circHMGCS1 in mouse vascular relaxation, we conducted an *ex vivo* culture of thoracic aortas from mice subjected to different treatments. A significant impairment in acetylcholine (Ach)-induced vascular relaxation response in the thoracic aorta of diabetic mice was markedly improved by 4521 agomir, emphasizing the critical involvement of miR-4521 in vascular relaxation. Conversely, circHMGCS1 inhibited the protective function of miR-4521 on vascular relaxation (Figure 5E). Across all experimental groups, the smooth muscle exhibited a normal endothelium-dependent relaxation response to the nitric oxide donor sodium nitroprusside (SNP) (Figure S5H), revealing intact smooth muscle function. Meanwhile, diabetic mice displayed a significant increase in SBP compared with normal mice, which was significantly restrained by miR-4521 agomir treatment. Nevertheless, this beneficial effect was nullified in the presence of circHMGCS1 (Figure 5F). Furthermore, miR-4521 agomir inhibited intimal thickening and smooth muscle cell proliferation in diabetic mice, whereas

upregulation of circHMGCS1 abrogated this effect (Figure 5G). These findings demonstrate that circHMGCS1 and miR-4521 play specific roles in regulating vascular endothelial function in diabetic mice.

We measured the NO level to further investigate the interaction between miR-4521 and circHMGCS1 in regulating diabetes-induced VED. Compared with the control group, the NO level decreased in the thoracic aorta of diabetic mice; however, miR-4521 agomir treatment increased its content (Figure 5H). Furthermore, miR-4521 treatment effectively enhanced eNOS activity (Figure 5I), whereas circHMGCS1 hindered miR-4521 regulatory function on NO content and eNOS activity. Furthermore, miR-4521 reduced ROS production in the blood vessels (Figures 5J and 5K) and inhibited the expression of endothelial adhesion molecules (ICAM1, ET-1, and VCAM1) (Figure 5L). Nonetheless, in the presence of circHMGCS1, the effect of miR-4521 was abrogated. These results indicated that circHMGCS1 acts as a miR-4521 sponge, participating in diabetes-induced VED.

ARG1 is a direct target of miR-4521 to accelerate endothelial dysfunction

To investigate whether circHMGCS1-associated miR-4521 regulated VED-related gene expression by targeting the 3'-untranslated region (UTR), we conducted predictions of mRNAs containing miR-4521 binding sites using the GenCard, mirDIP, miRWalk, and Targetscan databases. Through bioinformatics analyses, we identified binding sites for miR-4521 on KLF6, ARG1, and MAPK1 genes (Figure 6A). Previous studies have demonstrated the involvement of elevated arginase activity in diabetes-induced VED. Arginase can modulate the production of NO synthesized by eNOS through competitive utilization of the shared substrate L-arginine (Jung et al, 2010; Romero et al, 2008). Moreover, miR-4521 possesses the capability to bind to the 3' untranslated region of ARG1 in both human and mouse genomes (Figure S6A). The protein expression of ARG1 was increased in the aorta of diabetic mice and PAHG-treated HUVECs (Figures 6B and Figure S6B). The luciferase reporter assay was applied to verify the targeting ability through the

pmirGLO vector, which included either the WT or MUT 3'-UTR of ARG1 (Figure S6C). The overexpression of miR-4521 reduced the luciferase activities of the WT reporter vector but not the MUT reporter vector (Figure 6C). We next investigated the function of miR-4521 and ARG1 to regulate the function of HUVECs. miR-4521 mimics recovered the reduced levels of NO and the compromised activity of eNOS triggered by ARG1 overexpression (Figures 6D and 6E). Meanwhile, it inhibited the upregulation in ROS levels induced by ARG1 overexpression (Figures 6F). The mRNA and protein levels of adhesion molecules (ET-1, ICAM1, and VCAM1) through ARG1 in HUVECs. The rescue experiment results demonstrated that miR-4521 mimics reversed the promotional effect of ARG1 on the mRNA and protein expression of adhesion molecules *in vitro* (Figures 6G and 6H). Subsequently, we evaluated the gene and protein expression levels of ARG1 using qRT-PCR and western blotting. miR-4521 mimics repressed ARG1 transcription and protein expression, whereas miR-4521 inhibition upregulated ARG1 expression (Figures 6I-6K). Taken together, these results suggest that miR-4521 inhibits VED through sponging ARG1.

To explore the regulatory effect of the interaction between circHMGCS1 and miR-4521 on ARG1 expression. we firstly used HUVECs employing overexpression of circHMGCS1. circHMGCS1 overexpression significantly further promoted ARG1 transcription and protein expression during the PAHG treatment (Figures 6L and 6M), confirming the regulatory function of circHMGCS1 in VED-related ARG1 expression. We next studied the function of miR-4521, which acts as a sponge for circHMGCS1 to regulate ARG1 in HUVECs. The mRNA and protein levels of ARG1 were significantly increased when HUVECs were transfected with circHMGCS1 (Figures 6N and 6O). However, miR-4521 overexpression counteracted the effects of circHMGCS1 on ARG1 mRNA and protein expression. Conversely, knockdown of circHMGCS1 reduced ARG1 expression and increased miR-4521 expression in HUVECs (Figures 6P, 6Q and and Figure S4B). Besides, the inhibition of miR-4521 using a miR-4521 sponge in HUVECs further increased

ARG1 expression under the PAHG treatment. Notably, elevated circHMGCS1 expression did not affect ARG1 regulation (Figure 6R). Moreover, compared with diabetic mice, circHMGCS1 overexpression dampened the function of miR-4521 agomir on ARG1 (Figure 6S). These findings indicate that circHMGCS1 serves as a sponge for miR-4521, facilitating ARG1 regulation and VED promotion.

ARG1 is essential for circHMGCS1 and miR-4521 to regulate diabetes-induced VED

To delve further into how circHMGCS1 and miR-4521 regulated endothelial cell function via ARG1, we used AAV9 expressing ARG1 shRNA (ARG1 shRNA) or GFP (NC shRNA, used as the control) to reduce ARG1 levels. ARG1 shRNA mitigated the effects of PAHG on NO content and eNOS activity (Figures 7A and 7B), and inhibited PAHG-induced ROS in endothelial cells (Figure 7C). Additionally, ARG1 shRNA significantly reduced the elevated expression of adhesion molecules (ICAM1, VCAM1, and ET-1) induced by PAHG (Figures 7D and 7E). F Further rescue experiments revealed that miR-4521 and circHMGCS1 exhibited no significant regulatory effect on endothelial function in the presence of ARG1 shRNA. These findings demonstrate that ARG1 plays a crucial regulatory effect on miR-4521 and circHMGCS1 in modulating endothelial cell function.

Considering the role of ARG1 in regulating endothelial cell function through circHMGCS1 and miR-4521, we downregulated ARG1 expression in mice by administering AAV9 ARG1 shRNA through tail vein injection (Figure S7A). As anticipated, AAV9 ARG1 shRNA-infused mice exhibited decreased body weight and lower fasting blood glucose levels compared with diabetic mice (Figures 7F and 7G). Moreover, OGTT and ITT demonstrated that reduced ARG1 levels in AAV9 ARG1 shRNA-treated mice preserved glucose tolerance and insulin sensitivity (Figures 7H, 7I, and Figures S7B and S7C). The lowered ARG1 levels also reversed serum lipid levels (Figure S7D-S7G). However, the overexpression of circHMGCS1 or miR-4521 had no significant effect on glucose and lipid metabolism in AAV9 ARG1

shRNA-expressing mice. These findings confirm that ARG1 is an indispensable regulator for circHMGCS1 and miR-4521 in regulating diabetes.

We next investigated whether ARG1 regulates vascular endothelial cell function in the context of diabetes. Knocking down ARG1 in the thoracic aorta of mice expressing AAV9 ARG1 shRNA restored diastolic function of the thoracic aorta in diabetic mice (Figure 7J), emphasizing the vital role of ARG1 in VED. Furthermore, all experimental groups exhibited a normal endothelium-dependent relaxation response to the NO donor SNP, suggesting intact smooth muscle function (Figure S7H). Moreover, AAV9 ARG1 shRNA effectively inhibited diabetes-induced intima thickening and inhibited smooth muscle cell proliferation (Figure 7K), and which also counteracted the elevation of SBP induced by diabetes (Figure 7L). However, the regulatory effects of circHMGCS1 and miR-4521 on vascular dilation were ineffective in the presence of ARG1 shRNA. We next measured VED-related functional factors. Compared with diabetic mice, the thoracic aorta of mice expressing AAV9 ARG1 shRNA exhibited restored NO content and eNOS activity (Figures 7M and 7N). Furthermore, knocking down ARG1 reduced the high expression levels of vascular endothelial adhesion molecules (ICAM1, ET-1, and VCAM1) induced by diabetes (Figure 7O). Immunofluorescence analysis demonstrated a significant decrease in ROS content in the thoracic aorta of mice expressing AAV9 ARG1 shRNA compared with diabetic mice (Figures 7P and 7Q). Notably, circHMGCS1 and miR-4521 lost their regulatory capacity on vascular endothelial cell function in the presence of ARG1 shRNA. These results indicate that ARG1 plays a critical role in circHMGCS1 and miR-4521 to modulate diabetes-induced VED.

Discussion

The field of ncRNA biology has garnered considerable attention and has witnessed intensive research over the last few years. ncRNAs possess the capacity to function as novel regulators in various physiological systems and disease contexts (Esteller, 2011; Matsui & Corey, 2017; Statello et al, 2021). However, most studies have explored the expression patterns and functions of ncRNAs within single disease settings (Arcinas et al, 2019; Shan et al, 2017). The present study contributes to the understanding of ncRNAs and their involvement in diabetes-associated CVD, characterized by disruption of lipid metabolic homeostasis and redox balance in vascular endothelial cells under diabetic conditions. A comprehensive genome-wide analysis revealed 17,179 known circRNA loci transcribed in diabetic endothelial cells. Based on conservation, endothelial specificity, and abundance criteria, five circRNAs were selected for validation, indicating their predominant upregulation in the diabetic endothelial environment. Among them, we discovered a novel circRNA, circHMGCS1, exhibiting high abundance, stability, and cell specificity in diabetic endothelial cells and contributing to endothelial function regulation. Our findings establish that circHMGCS1 promotes VED progression, and its overexpression exacerbates endothelial cell dysfunction. Our study highlights the pivotal role of circHMGCS1 in regulating endothelial cell function during diabetes.

circRNAs play regulatory roles by acting as microRNA sponges or interacting with proteins to regulate alternative splicing, transcription, and epigenetic modifications (Arcinas et al, 2019; Hansen et al, 2013; Memczak et al, 2013). To investigate the mechanism of circHMGCS1 in VED regulation, we analyzed miRNA sequencing results and prediction software to identify miR-4521 as a novel endothelial-expressed miRNA. miR-4521 was found to possess a stable binding site with circHMGCS1, and its expression was significantly decreased in endothelial cells within a diabetic environment. Our functional studies revealed that miR-4521 mimics attenuated VED development in diabetes, whereas its inhibition exacerbated

VED progression. These findings preliminarily demonstrate the regulatory ability of miR-4521 in VED. The interaction between circHMGCS1 and miR-4521 was further characterized through luciferase reporter gene assays, RNA pull-down, and RNA immunoprecipitation. AAV9-mediated circHMGCS1 overexpression in endothelial cells dampened miR-4521 expression and accelerated diabetes-induced VED. Conversely, restoring miR-4521 expression effectively alleviated VED progression, highlighting the critical role of miR-4521 in counteracting circHMGCS1-mediated promotion of diabetes-induced VED. Moreover, the absence of miR-4521 aggravated diabetes-induced VED, whereas exogenous circHMGCS1 addition did not affect VED development. Intriguingly, exogenous miR-4521 significantly restrained diabetes-induced VED exacerbation, which was attenuated upon the subsequent addition of exogenous circHMGCS1. These findings underscore the essential regulatory role of the circHMGCS1 and miR-4521 interaction in maintaining diabetic vascular homeostasis.

Finally, we investigated the downstream targets crucial for circHMGCS1-mediated endothelial cell function. Arginase is a dual-nucleus manganese metalloenzyme that catalyzes the hydrolysis of L-arginine, primarily producing L-ornithine and urea (Kanyo et al, 1996). ARG1 and ARG2—two isoforms of arginase—exist in vertebrates, including mammals. Although ARG1 and ARG2 share the same catalytic reaction, they are encoded by different genes and exhibit distinct immunological characteristics (Hara et al, 2020; Pudlo et al, 2017; Su et al, 2021). Elevated ARG1 activity in endothelial cells has been identified as a significant contributor to VED in T2DM individuals. This dysfunction is attributed to the excessive formation of ROS caused by the competition between ARG1 and eNOS for L-arginine. Consequently, oxidases or mitochondrial complexes become overactivated, resulting in reduced NO levels and subsequent VED (Caldwell et al, 2018). By contrast, inhibiting arginase has been shown to considerably enhance endothelial function in T2DM patients (Mahdi et al, 2018; Shemyakin et al, 2012; Zhou et al, 2018). Our findings suggest that T2DM enhances ARG1 activity and

expression in vascular endothelial cells. Conversely, ARG1 inhibition in endothelial cells delayed the onset of diabetes and preserved NO metabolite levels in the aorta. Additionally, Ach-induced vasodilation remains intact in the absence of ARG1 in the aortic ring, as ARG1 inhibition promotes eNOS-dependent relaxation, thereby preventing VED. Meanwhile, ROS content in the aorta of diabetic mice could be reduced under ARG1 inhibition, thereby suppressing the occurrence of oxidative stress. AAV9-mediated overexpression of circHMGCS1 increases ARG1 expression, which is inhibited by exogenous miR-4521 intervention. In the absence of miR-4521, circHMGCS1 cannot regulate ARG1 expression, whereas exogenous miR-4521 intervention inhibits the diabetes-induced increase in ARG1 expression *in vivo*, and this effect is counteracted by circHMGCS1 overexpression. Both *in vitro* and *in vivo* data suggest that the miR-4521 or circHMGCS1 fails to regulate the effect of diabetes-induced VED in the absence of ARG1. Therefore, ARG1 may serve as a promising VED biomarker, and circHMGCS1 and miR-4521 play a key role in regulating diabetes-induced VED by ARG1.

It would be intriguing to elucidate the mechanisms through which circHMGCS1 and miR-4521 exert their regulatory roles in endothelial cell function. The present study elucidated the potential VED-associated mechanisms in diabetes, with a specific focus on the complicated mutual effects between circHMGCS1 and miR-4521. The ceRNA network involving circHMGCS1-miR-4521-ARG1 can become a novel and significant regulatory pathway for preventing and potentially treating diabetes-induced VED. Our findings suggest that targeted inhibition of circHMGCS1 or the overexpression of miR-4521 could serve as effective strategies in mitigating diabetes-induced VED. These insights underscore the promising function of ncRNAs in developing therapeutic interventions for diabetes-associated VED.

Experimental Section

Cell Culture

HUVECs were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 μg/mL of streptomycin, and 100 U/mL of penicillin. The cells were cultured at 37 °C in a humidified incubator with 95% air and 5% CO_2. To simulate the elevated glucose levels observed in diabetes, we cultured HUVECs in a PAHG medium. The medium contained 25 mM glucose and 250 μM saturated free fatty acid (FFA) palmitate (16:C; Sigma, USA) and was incubated for 24 h. For *in vitro* experiments, HUVECs were subcultured in six-well plates. The cells were then transfected with miR-4521 mimics, miR-inhibitor, or miR-NC and incubated for 48 h. Subsequently, the cells were treated with PAHG for another 24 h. Samples were collected to determine the corresponding indices.

Animal design

Male C57BL/6J mice (6–8 weeks old) were obtained from SLAC Laboratory Animal Co., Ltd. (SLAC ANIMAL, China). The mice were housed at a temperature of 22 ± 1 °C with a 12-h light/dark cycle. To induce diet-induced diabetes, we fed wild type littermates either a standard chow (Control) or a high fat-high sucrose (HFHG) diet, where the diet composition consisted of 60% fat, 20% protein, and 20% carbohydrate (H10060, Hfkbio, China). The dietary regimen was maintained for 14 weeks. Throughout this period, body weight and fasting blood glucose (FBG) levels were measured on a weekly basis. An FBG level of ≥11.1 mmol/L was used as the criterion for a successful diabetic model. To study the role of ARG1 in diabetes-induced endothelial dysfunction, we established an HFHG-induced diabetic model (DM), and then pAAV9-ARG1 shRNA (1×10^{12} vg/mL, 100 μL) was injected into the tail vein. Subsequently, we observed changes in relevant indicators. To investigate the regulatory relationship among ARG1, miR-4521, and circHMGCS1 in diabetes-induced endothelial dysfunction, we injected miR-4521 agomir or

pAAV9-circHMGCS1 via the tail vein, following the procedure described in Figure S5A. The mice were sacrificed after a 12-h fast, and blood and other tissues were collected and stored at –80 °C until further analysis. All animal experiments were conducted in strict accordance with the guidelines and laws governing the use and care of laboratory animals in China (GB/T 35892-2018 and GB/T 35823-2018) and NIH Guide for the Care and Use of Laboratory Animals. The experimental procedures involving animals were performed at the Animal Experiment Center of Zhejiang Chinese Medical University in Hangzhou, China. The animal protocol was conducted in compliance with the guidelines set forth by the Ethics Committee for Laboratory Animal Care at Zhejiang Chinese Medical University (Approval No. 2022101345).

Vascular function

After sacrificing the mice, the thoracic aortae and mesenteric arteries were carefully removed and dissected in an oxygenated ice-cold Krebs solution. The changes in the isometric tone of the aortic rings and second-order mesenteric arteries were recorded using a wire myograph (Danish Myo, DK). The arterial segments were stretched to achieve an optimal baseline tension (3 mN for the aorta) and allowed to equilibrate for 1 h. Subsequently, the segments were contracted using 60 mmol/L of KCl and rinsed with Krebs solution. Endothelium-dependent relaxation (EDR) was evaluated by assessing the concentration-responses to cumulative additions of acetylcholine (ACh) in noradrenaline (NE, 10^{-3} mol/L) precontracted rings.

Blood pressure measurements

An intelligent non-invasive blood pressure monitor (BP-2010AUL) was used to measure blood pressure in different mouse groups simultaneously. Prior to measurement, the mice were placed in a blood pressure room until reaching a temperature of 25 ± 1 °C. After a 15-min equilibration period, the mice were gently positioned in a mice jacket set at a constant temperature of 40 °C, with their tail inserted into a pulse sensor. Throughout the measurements, a quiet environment was maintained to minimize external disturbances. The instrument automatically

recorded the stabilized sensor signal to obtain the systolic blood pressure (SBP).

Serum lipid assays

After 5 weeks of blood collection from mouse orbits and 12 weeks of drug administration, blood samples were obtained from mouse arteries. The collected blood was centrifuged at 1,500 g for 15 min to separate the serum. Commercial kits and an automatic biochemical analyzer (Biobase BK-600) were used to measure triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c).

Construction of circHMGCS1 and ARG1 plasmids and stable transfection using lentivirus

circHMGCS1-overexpressing lentiviruses (pLV-circHMGCS1) were obtained from Hangzhou Ruipu Biotechnology Co., Ltd (Guangzhou, China). The circHMGCS1 sequence [NM_001098272: 43292575-43297268], the splice site AG/GT and ALU elements were inserted into the pCDH-circRNA-GFP vector (upstream ALU: AAAGTGCTGAGATTACAGGCGTGAGCCACCACCCCCGGCCCACTTTTTGT AAAGGTACGTACTAATGACTTTTTTTTTATACTTCAG, downstream ALU: GTAAGAAGCAAGGAAAAGAATTAGGCTCGGCACGGTAGCTCACACCTGT AATCCCAGCA). The restriction enzyme sites selected were EcoRI and NotI. Lentiviruses containing an empty vector and expressing GFP were used as the negative control (pLV-NC) (Liang & Wilusz, 2014). ARG1-overexpressing lentiviruses (pLV-ARG1 OE) were acquired from Genomeditech (China). HUVECs were cultured in 6-well plates until reaching 60% confluence and then infected with lentivirus particles in the presence of 5 μg/ml of polybrene at a multiplicity of infection of 30. The cells were cultured for at least 3 days before further experiments were conducted. The overexpression efficiency of circHMGCS1 or ARG1 was evaluated using quantitative real-time PCR or western blottings.

Cloning and production of circHMGCS1, miR-4521 sponge, circHMGCS1 shRNA and ARG1 shRNA

The pAAV-circHMGCS1-ZsGreen1 vector was constructed by inserting the full-length circHMGCS1 into the pAAV-circRNA-ZsGreen1 vector. circHMGCS1 shRNA vector was constructed by inserting circHMGCS1 shRNA (5'-ACAUAGCAACUGAGGGCUUCG-3') into pLKO.1 GFP shRNA plasmid. To achieve AAV-mediated ARG1 gene silencing, we obtained a shRNA sequence targeting ARG1 from Sigma-Aldrich Mission RNAi (TRCN0000101796). The oligo sequences 5’GATCCGCCTTTGTTGATGTCCCTAATCTCGAGATTAGGGACATCAACAAAGGCTTTTTA-3’ and 5’-AGCTTAAAAAGCCTTTGTTGATGTCCCTAATCTCGAGATTAGGGACATCAACAAAGGCG-3’ were synthesized, annealed, and ligated to the pAAV-ZsGreen-shRNA shuttle vector, resulting in the construction of pAAV-shARG1-ZsGreen1. The miR-4521 sponge cassette was cloned into the pAAV-ZsGreen1 expression vector to generate pAAV-miR-4521 sponge-ZsGreen1. Recombinant AAVs (rAAV9s) were produced by transfecting HEK293T cells plated at a density of 1×10^7 cells/15 cm plate one day prior to transfection using polyethyleneimine (Linear PEI, MW 25 kDa, Sigma-Aldrich) as the transfection reagent. For each 15-cm plate, the following reagents were added: 20 μg of pHelper, 10 μg of pAAV-RC9, and 10 μg of pAAV-circHMGCS1-ZsGreen1, pAAV-shARG1-ZsGreen1, or pAAV-miR-4521 sponge-ZsGreen1. These plasmids were combined with 500 μL of serum-free and antibiotic-free DMEM and 100 μL of PEI reagent (1 mg/mL, pH 5.0). The DNA-PEI reagent was added drop-wise to the cells without changing the media on the 15-cm plates. After three days, the cells were collected and lysed through repeated cycles of freezing and thawing at −80 °C and 37 °C, respectively. The rAAV9s were purified using the ViraTrap™ Adenovirus Purification Maxiprep Kit (BW-V1260-02, Biomiga). The virus titer was determined

using qPCR and adjusted to 1×10^{12} viral genomes (vg)/mL in PBS containing 4% sucrose.

Transfection of miRNA mimic or inhibitor

miR-4521 mimics, miRNA negative control (miR-NC), miR-4521 inhibitor, and miRNA inhibitor negative control (miR-NC inhibitor) were obtained from Tingske (China). To investigate the functional role of miR-4521 in HUVECs, we performed transfections using Hieff Trans® *in vitro* siRNA/miRNA Transfection Reagent with miR-4521 mimics, miR-NC, miR-NC inhibitor, or miR-NC inhibitor at a concentration of 50 nM, following the manufacturer's protocols. After 48 h of transfection, proteins and RNA were collected for further analysis. The sequences used in the experiments are listed as follows: miRNA negative control (miR-NC): 5'-UUCUCCGAACGUGUCACGUTT-3', 5'-ACGUGACACGUUCGGAGAATT-3';

hsa-miR-98-5p-mimics: 5'-UGAGGUAGUAAGUUGUAUUGUU-3', 5'-CAAUACAACUUACUACCUCAUU-3'; Hsa-miR-3143-mimics: 5'-UAACAUUGUAAAGCGCUUCUUU-3', 5'-AGAAGCGCUUUACAAUGUUAUU-3'; Hsa-miR-181a-2-3p-mimics: 5'-ACCACUGACCGUUGACUGUACC-3', 5'-UACAGUCAACGGUCAGUGGUUU-3'; hsa-miR-4521-mimics: 5'-GAGCACAGGACUUCCUUAGCUU-3', 5'-GCUAAGGAAGUCCUGUGCUCAG-3'; hsa-miR-4521-inhibitor: 5'-CUGAGCACAGGACUUCCUUAGC-3'; miRNA inhibitor negative control (miR-NC inhibitor): 5'-CAGUACUUUUGUGUAGUACAA-3'; miR-4521 agomir: 5'-GAGCACAGGACUUCCUUAGCUU: 5'-GCUAAGGAAGUCCUGUGCUCAG-3'.

NO assays

The supernatant fluid from HUVECs treated with the indicated reagents and a tissue

homogenate of aortic rings were collected. NO concentrations were determined using the Griess reagent and a total nitric oxide assay kit (Beyotime, China) following the manufacturer's instructions.

eNOS activity assay

The cellular eNOS activity was assessed as per previously described protocol. (Leopold et al, 2007). Briefly, Cells were exposed to a PBS buffer (400 μL/well) containing 1.5 Ci/ml [3H] L-arginine for 15 minutes to initiate eNOS activity measurement. The reaction was stopped using 1 N ice-cold TCA (500 μL/well). After freeze-thawing in liquid nitrogen, cells were scraped, treated with water-saturated ether three times, and neutralized with 1.5 ml of 25 mM pH 8.0 HEPES. Dowex AG50WX8 columns (Tris form) were used for elution with a 1 ml solution of 40 mM pH 5.5 HEPES supplemented with 2 mM EDTA and 2 mM EGTA. The eluate was collected for [3H] L-citrulline quantification using liquid scintillation spectroscopy. Additionally, the assessment of eNOS activity in cell lysates and tissues was performed through the previously outlined immunoprecipitation approach (Du et al, 2001). Briefly, the samples were divided into two tubes: one for protein blotting and the other for eNOS activity measurement. The eNOS immunocomplexes, linked to protein A-Sepharose beads, were reconstituted in assay buffer, and eNOS activity was gauged by tracking the transformation of [3H] L-arginine into [3H] L-citrulline. Equal enzyme quantities in each incubation were validated via Western blotting.

Detection of ROS for tissue and cell

To assess the level of ROS in tissues, we used a red fluorescent reactive oxygen probe called DHE (dihydroethidium) (Karim et al, 2022; Su et al, 2018). In brief, 10 μL of the DHE probe was combined with 190 μL of tissue homogenate supernatant, thoroughly mixed, and subsequently incubated in darkness at 37 °C for 30 min. Following incubation, the samples were promptly imaged under a fluorescent microscope.

For ROS detection in cells, the treated cells were washed once by PBS, then 20 μM DHE was added, and incubated at 37°C for 30 min away from light, then washed three times by PBS and then colorless DMEM medium was added, followed by fluorescence microscopy for observation.

CircRNA sequencing

Total RNA was extracted from HUVECs, both with and without PAHG stimulation, utilizing Trizol (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The extracted total RNA was then assessed for quality and quantity using a denaturing agarose gel, Nano Drop, and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, USA). For the construction of RNA-seq libraries to obtain sequence information of linear transcripts, 3 μg of total RNA samples underwent treatment with the epicenter Ribo-ZeroTM Kit (Illumina, San Diego, USA) to remove rRNA. Linear RNA was subsequently eliminated using RNase R (Epicentre Technologies, USA). The resulting cleaved RNA fragments were reverse-transcribed to generate cDNA, which served as the template for the synthesis of U-labeled second-stranded DNAs. This synthesis step involved the use of E. coli DNA polymerase I, RNase H, and dUTP, facilitated by the PrimeScript RT regent Kit (TaKaRa, Japan). Purification of the synthesized second-stranded DNAs was accomplished using AMPureXP beads. All subsequent steps were conducted in accordance with the manufacturer's protocols. To assess the quality of the libraries, an Agilent 2100 Bioanalyzer was employed, and the paired-end sequencing was performed using an Illumina Hiseq 4000 (LC Bio, China), adhering to the recommended protocol provided by the vendor.

Treatment of circRNA raw sequencing data

The sequencing data was filtered with Cutadapt by removing reads containing adaptor contamination, poly-N or low quality and undetermined bases, and then sequence quality was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Kechin et al, 2017).

Bowtie2 and Hisat2 to map reads to the genome of species (Kim et al, 2015; Langmead & Salzberg, 2012). CIRCExplorer2 and CIRI were used to *denovo* assemble the mapped reads to circular RNAs (Kim & Salzberg, 2011; Zhang et al, 2016). Then, back splicing reads were identified in unmapped reads by tophat-fusion. All samples were generated unique circular RNAs. The expression level was calculated according to the junction reads per billion mapped reads (RPB). Differentially expressed circRNAs analysis was performed with log2 (fold change) >1 or log2 (fold change) <-1 and with statistical significance (p value < 0.05) by R package–edgeR (Robinson et al, 2010).

microRNA microarray assay

Total RNA was extracted from HUVECs treated with either PAHG or PBS using TRIzol reagent (Invitrogen, USA). The μParaflo MicroRNA microarray Assay by LC Sciences (LC Sciences, USA) using miRBase version 22.0 was performed. After 3'-extension with a poly(A) tail and ligating an oligonucleotide tag, hybridization was done overnight on a μParaflo microfluidic chip. Cy3 dye facilitated dye staining after RNA hybridization. Fluorescence images were acquired using the GenePix 400B laser scanner (Molecular Devices, USA). Data were analyzed by background subtraction and signal normalization using a LOWESS filter. miRNA expression with a two-tailed p-value of <0.05 was considered significant. Intensities below 500 were considered false positives.

Network analysis of competing endogenous RNA

The miRNA targets of circHMGCS1 were computationally predicted using three bioinformatic tools: miRanda (http://www. microrna.org/microrna/home.do), whereas the potential target genes of miR-4521 were envisaged using four distinct databases: TargetScan (http://www.targetscan.org/), GenCard (https://www.genecards.org/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/), and mirDIP (http://ophid.utoronto.ca/mirDIP/).

Isolation of RNA and miRNA for quantitative Real Time-PCR (qRT-PCR) analysis

RNA was extracted utilizing a total RNA extraction kit (Yeason, 19221ES50, China) and a miRNA extraction kit (Yeason, 19331ES50, China) according to the corresponding manufacturer's protocol. The RNA concentration was determined at 260 nm using a spectrophotometer. Reverse transcription was conducted using a reverse transcription kit (TAKARA, Japan) with random primers according to the manufacturer's protocol to synthesize cDNA and circRNA. Quantitative real-time PCRs (qRT-PCRs) were conducted on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using the standard protocol provided by the SYBR Green PCR Kit (Yeason, China). For miRNA expression detection, reverse transcription was executed, and microRNAs were detected using stem-loop primers procured from Tsingke (China). The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All qRT-PCR assays were repeated thrice. The primers used for the

qRT-PCR assays are listed as follows: Hsa-miR-98-5p: 5'-CGCGCGTGAGGTAGTAAGTTGT-3', 5'-AGTGCAGGGTCCGAGGTATT-3'; Hsa-miR-3143: 5'-GCGATAACATTGTAAAGCGCTT-3', 5'-AGTGCAGGGTCCGAGGTATT-3'; Hsa-miR-181a-2-3p: 5'-GCGACCACTGACCGTTGAC-3', 5'-AGTGCAGGGTCCGAGGTATT-3'; Has-miR-4521: 5'-CGGCTAAGGAAGTCCTGTGC-3', 5'-CGCAGGGTCCGAGGTATTC-3'; U6: 5'-ATTGGAACGATACAGAGAAGATT-3' , 5'-GGAACGCTTCACGAATTTG-3'; ET-1: 5'-GCCTGCCTTTTCTCCCCGTTAAA-3', 5'-CAAGCCACAAACAGCAGAGA-3'; ICAM1: 5'-TGACCGTGAATGTGCTCTCC-3', 5'-TCCCTTTTTGGGCCTGTTGT-3'; ARG1: 5'-TTCTCAAAGGGACAGCCACG-3', 5'-CGCTTGCTTTTCCCACAGAC-3'; ICAM1:

5’-ATGCCCAGACATCTGTGTCC-3’, 5’-GGGGTCTCTATGCCCAACAA-3’; VCAM1: 5’-AAATCGAGACCACCCCAGAA-3’, 5’-AGGAAAAGAGCCTGTGGTGC-3’; β-actin: 5’-CTCACCATGGATGATGATATCGC-3’, 5’-CACATAGGAATCCTTCTGACCCA-3’; HMGCS1: 5’-TCGTGGGACACATATGCAAC-3’, 5’-TGGGCATGGATCTTTTTGCAG-3’; hsa_circ_0000992: 5’-TCACACGGGAGAGTGTTACC-3’, 5’-GTCCATCGAGAAAAGCTGATGC-3’; hsa_circ_0004036: 5’-GGCTCACAAGCAGCCTTTAC-3’, 5’-GGTGATACAGGAGCGGGTAG-3’; hsa_circ_0004889: 5’-GTCATATATGGGAAAGAGGGCTT-3’, 5’-GGACAAGCCTTCATGGGCTC-3’; hsa_circ_0006719: 5’-CCTCCCTCGGTGTTGCCTTC-3’, 5’-TCCAGGCCAGGTAGACAGAAC-3’; hsa_circ_0008621: 5’-CAAACATAGCAACTGAGGGCTTC-3’, 5’-TAATGCACTGAGGTAGCACTG-3’; GAPDH: 5’-GAAAGCCTGCCGGTGACTAA-3’, 5’-GCATCACCCGGAGGAGAAAT-3’.

circRNA Validation by PCR

DNA and gDNA templates were PCR amplified using BioRad Mastercyclers following the manufacturer’s protocol. Subsequently, the PCR products were visualized using a 1% agarose gel stained with GelRed. To validate the PCR results, we purified the products using the EZ-10 Column DNA Purification Kit (Sangon Biotech, China), and direct PCR product Sanger sequencing was performed. circHMGCS1-full length: 5′-AGCAACTGAGGGCTTCGT-3′, 5′-ATGTTTGAATGCACAAGTCCTAC-3′; circHMGCS1-divergent primers: 5′-TTATGGTTCTGGTTTGGCTGC-3′, 5′-TTCAGCGAAGACATCTGGTGC-3′; circHMGCS1-convergent primers: 5′-ATAGCAACTGAGGGCTTCGTG-3′, 5′-GCGGTCTAATGCACTGAGGT-3′.

Cell nuclear/cytoplasmic fractionation

RNA extracts from HUVECs were subjected to nuclear/cytoplasmic isolation using the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific, USA) following the manufacturer's protocol.

RNase R treatment

For RNase R treatment, approximately 2 μg of total RNAs from HUVECs were incubated with or without 20 U of RNase R (Geneseed, China) at 37°C for 30 min. The resulting RNAs were then purified using an RNA Purification Kit (Qiagen, USA).

Actinomycin D assay

HUVECs were seeded equally in 24-well plates at a density of 5×10^4 cells per well. Subsequently, the cells were exposed to actinomycin D (2 μg/mL; Abcam, USA) for different time intervals: 0, 4, 8, 12, and 24 h. After the respective exposure times, the cells were collected for RNA extraction. The relative RNA levels of circHMGCS1 and HMGCS1 mRNA were analyzed using qRT-PCR and normalized to the values obtained from the control group (0 h).

RNA immunoprecipitation

RIP assay was conducted using the PureBinding®RNA Immunoprecipitation Kit (GENESEED, China) as per the manufacturer's guidelines. HUVECs were lysed in complete RNA immunoprecipitation lysis buffer containing 1% proteinase inhibitor and 1% RNase inhibitor after being infected with circNC or circHMGCS1. The cell extract was then incubated with magnetic beads conjugated with anti-Argonaute 2 (AGO2) or anti-IgG antibody (Abcam, USA) overnight at 4 °C. The beads were subsequently washed, and the proteins were removed using columns. Eventually, the isolated RNA was extracted using TRIzol Reagent, and the purified RNA was utilized for subsequent qRT-PCR analysis.

Pull-down Assay

A pull-down assay was conducted using the PureBinding®RNA-Protein pull-down Kit (GENESEED, P0201, China) following the manufacturer's protocol. The pull-down assay with biotinylated miR-4521 or circHMGCS1 was performed as previously described (Wang et al, 2020). Briefly, HUVECs were transfected with biotinylated RNA or mutants (Tingke, 50 nmol/L) using the Hieff Trans® *in vitro* siRNA/miRNA Transfection Reagent (Yeason, China). Following a 48-h transfection period, the cells were collected, PBS-washed, and incubated for 10 min in a capture buffer on ice. A 10% fraction of the cell lysates was retained as input. The remaining lysates were subsequently exposed to streptavidin magnetic beads for 30 min at 4 °C. After treatment with wash buffer and the RNeasy Mini Kit (Qiagen), the captured RNAs were extracted for subsequent qRT-PCR analysis , while the associated proteins were processed for Western blot analysis.

The sequences are listed as follows: Biotin-miR-4521-MUT: 5'-GCAUUCCUUCAGGAGUGCUCAG-3', Biotin-miR-4521-WT: 5'-GCUAAGGAAGUCCUGUGCUCAG-3'. Biotin-circHMGCS1-WT: 5'-ATGTGTCCCACGAAGCCCTCAGTTGCTATGTTTGAA-3', Biotin-circHMGCS1-MUT: 5'-GACGTCGTGTGCGTCGGTGCTAAGCTTCACAGATAC-3'.

Western blot analysis

Western blot was performed following previously described procedures (Xu et al, 2022). Protein extraction from cells or tissues was carried out using protein lysis buffer containing protease and phosphatase inhibitor cocktail (Beyotime, China). The concentrations of the protein lysates were determined using the Beyotime BCA Protein Assay Kit (Beyotime, China) according to the manufacturer's instructions. Equal quantities of proteins (20 μg/lane) were loaded onto 8% or 10% SDS-PAGE gels and subsequently transferred to PVDF membranes (Millipore, USA). The membranes were then incubated with primary antibodies overnight at 4°C. The primary antibodies used were as follows: anti-ARG1 (diluted 1:1,000; Abcam,

ab133543), anti-ARG2 (diluted 1:1,000; Abcam, ab264066), anti-ET-1 (diluted 1:1,000; Abcam, ab2786), anti-VCAM1 (diluted 1:1,000; Abcam, ab134047), anti-ICAM1 (diluted 1:1,000; Abcam, ab222736), anti-AGO2(diluted 1:2,000; proteintech, 67934-1-Ig), anti-β-actin (diluted 1:2,000; Abcam, ab5694), and anti-GAPDH (diluted 1:2,000; Abcam, ab8245). After incubation with the corresponding secondary antibodies for 2 hours at room temperature, the membranes were washed. Antibody binding was detected using an ECL detection reagent (Millipore, USA). Digital images were captured using a Gel DocTM XR+ System with ImageLab software (BioRad, USA). The quantification of the labeled bands was performed using ImageJ 1.55.

RNA Fluorescent *In Situ* Hybridization (RNA-FISH)

The RNA-FISH assay was conducted in HUVECs. Cy3-labeled circHMGCS1 probe and FAM-labeled miR-4521 probe were custom-designed and synthesized by RiboBio (China). The signals emanating from the probes were detected using the Ribo^{TM} FISH Kit (RiboBio) in accordance with the manufacturer's protocol. Briefly, frozen sections were fixed with 4% paraformaldehyde for 10 minutes, followed by PBS washing. The fixed sections were treated with PBS containing 0.5% triton X-100 at 4°C for 5 min, followed by incubation in pre-dehydration buffer at 37°C for 30 min. Subsequently, probes targeting circHMGCS1 and miR-4521 were applied to the sections. Hybridization was performed in a humid chamber at 37°C overnight. Post-hybridization washing was initially conducted with 4× saline sodium citrate containing 0.1% tween-20 at 42°C, followed by further washing with 2× saline sodium citrate at 42°C to eliminate non-specific and repetitive RNA hybridization. The slides were counterstained with DAPI (beyotime, China) and examined using an Olympus FV1200 (Olympus, Japan) confocal microscopy system. The mean fluorescent intensity (MFI) was determined using Image J software and subjected to statistical analysis. The probe

sequences are detailed as follows: Cy3-circHMGCS1:

5’-UCCCACGAAGCCCUCAGUUGCUAUG-3’, FAM-miR-4521: 5’-UUUGACUCGUGUCCUGAAGGAAUCG-3’.

Luciferase reporter assay

The complete sequence of circHMGCS1 and a mutant circHMGCS1 sequence lacking the miRNA binding site were chemically synthesized. These sequences were inserted into the Nhel and SaII sites of the pmirGLO vector to generate expression vectors denoted as circHMGCS1-WT and circHMGCS1-MUT. Transfection was carried out using lipofectamine 2000 (Invitrogen, USA) with 50 nM miR-4521 mimics, miR-NC, miR-4521 inhibitor, and miR-NC inhibitor. HEK293T cells were seeded in 96-well plates and cultured until reaching 50%-70% confluence prior to transfection. After 48-h of transfection, cells were harvested, and luciferase activity was quantified utilizing the Dual Luciferase Reporter Gene Assay Kit (Yeason, China). The pmirGLO vector expressing Renilla luciferase was utilized as an internal control for transfection, while the empty pmirGLO vector served as the negative control. Firefly luciferase activity of the pmirGLO vector was normalized with Renilla luciferase activity for comparison. Luciferase reporter assay was also used to investigate the regulation of miR-4521 on the expression of its target genes. For ARG1 and miR-4521, either wild-type or mutant ARG1 3’UTR fragments (433 bp) were inserted into NheI/SalI restriction sites of pmirGLO. 1 μg plasmids of ARG1 3’UTR-WT and ARG1 3’UTR-MUT, 50 nM miR-4521 mimics, miR-NC, miR-4521 inhibitor and miR-NC inhibitor were transfected. Luciferase activity was evaluated 48 hours post-transfection using the Dual Luciferase Reporter Gene Assay Kit (Yeason).

Histological and morphometric analysis

Aortic tissues were prepared by fixing them in 4% paraformaldehyde in PBS and subsequently embedding them in paraffin. The resulting paraffin-embedded tissues were then sectioned into 4-μm-thick slices. Hematoxylin-eosin (H&E) staining was performed on these sections to visualize the vascular tissue structures and assess the

degree of thickness in the thoracic aorta. The average thickness of five randomly selected regions of the thoracic aorta was measured using OlyVIA software (Olympus, Japan).

Statistical analysis

Statistical analyses were conducted using Prism software (GraphPad 8.0). Data are presented as mean ± standard deviation (SD) unless otherwise stated. Prior to statistical analysis, the normality of data distribution was evaluated using the Shapiro-Wilk test. For comparisons between the two groups, an unpaired two-tailed Student's t-test was used. When comparing more than the two groups, either a one-way or two-way analysis of variance (ANOVA) was conducted, followed by the Bonferroni multiple comparison post hoc test. Group differences were considered statistically significant at a p-value of <0.05. The level of significance was indicated by asterisks, with *, **, and *** denoting p-values lower than 0.05, 0.01, and 0.001, respectively.

Author contributions

Conceptualization: M.Z. and W.C. Methodology: M.Z., G.D., Y.X., W.C., and L.X. Investigation: M.Z., L.X., Y.X., and G.D. Formal analysis: M.Z. Validation: M.Z. Data curation: M.Z. and G.D. Writing (original draft): W.C. and M.Z. Writing (review and editing): W.C. and M.Z. Funding acquisition: W.C. Resources: W.C. Supervision: W.C. All authors contributed, read and approved the final version.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Unprocessed original image data have been deposited to Mendely Data and are available at: https://doi.org/10.17632/dxzvhnj7pm.1. Profiling by circRNA and miRNA Array in our work has been deposited in the GEO database as GSE237295 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE237295) and GSE237597 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE237597). Additional data related to this paper may be requested from the authors.

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Figure legends

Figure 1. **circHMGCS1 Upregulation and its Association with PAHG-induced Endothelial Dysfunction.** (A) Heat map illustrating the differential expression of circRNAs in HUVECs treated with PAHG for 24 hours (n=3). (B) qRT-PCR validation of five circRNAs in HUVECs treated with PAHG compared to normal cells, normalized to GAPDH (n=6). (C) PCR validation of circHMGCS1 presence in HUVECs, GAPDH was utilized as a negative control (n=6). (D) Alignment of circHMGCS1 sequence in CircBase (circular RNA database, upper) in agreement with Sanger sequencing results (lower). (E) Schematic representation of circularization of exons 2 to 7 of HMGCS1 (red arrow). (F) RNA-FISH analysis detecting circHMGCS1 expression in HUVECs using Cy3-labeled probes, Nuclei were counterstained with DAPI (red represents circHMGCS1, blue represents nucleus, scale bar = 50 μm, n=4). (G) qRT-PCR quantification of circHMGCS1 and HMGCS1 mRNA levels in the cytoplasm or nucleus of HUVECs. circHMGCS1 and HMGCS1 mRNA levels were normalized to cytoplasmic values (n=4). (H) qRT-PCR measure circHMGCS1 and HMGCS1 mRNA levels after actinomycin D treatment (n=4 at different time points). (I) circHMGCS1 expression was detected in RNase R-treated total RNA by qRT-PCR (n=4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, All significant difference was determined by unpaired two-tailed Student's t-test or one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Source data 1. Original file of the agarose gel shown in **Figure 1C**.

Figure 2. circHMGCS1 Overexpression Aggravates PAHG-induced Endothelial Dysfunction. (A) HUVECs were transfected with either circHMGCS1 overexpression lentivirus (pLV-circHMGCS1) or lentiviral circular RNA negative control vector (pLV-circNC). After 48 hours of infection, circHMGCS1 and

HMGCS1 expression levels were assessed by qRT-PCR and normalized to GAPDH (n=4). (B and C) Western blot and qRT-PCR were conducted to detect the expressions of adhesion molecules (VCAM1, ICAM1, and ET-1) in HUVECs between pLV-circHMGCS1 and pLV-circNC groups (n=4). (D) NO content in pLV-circHMGCS1-infected HUVECs after PAHG treatment (n=4). (E) eNOS activity in pLV-circHMGCS1-infused HUVECs after PAHG treatment (n=4). (F) DHE fluorescence was used to characterize ROS expression in different groups. Scale bar = 50 μm (n=8). (G and H) Relative expression of adhesion molecules (ICAM1, VCAM1 and ET-1) in HUVECs infected with pLV-circNC or pLV-circHMGCS1 after PAHG treatment were determined by Western blot and qRT-PCR (n=4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Source data 1. Original file for the western blot analysis shown in **Figure 2B**.

Source data 2. Original file for the western blot analysis shown in **Figure 2G**.

Figure 3. miR-4521 Prevents PAHG-induced Endothelial Dysfunction. (A) Heat map depicting differentially expressed miRNAs in HUVECs with or without PAHG treatment for 24 hours (n=3). (B) Volcano plot illustrating significant changes in miRNAs between control and PAHG-treated groups (Fold-change > 2 or < 0.5, $p \leq 0.01$). (C) Schematic illustration showing overlapping target miRNAs of circHMGCS1 predicated by miRanda and sequencing results. (D) The binding sites of circHMGCS1 were predicted using miRanda and involve hsa-miR-98-5p, hsa-miR-3143, hsa-miR-4521, and hsa-miR-181a-2-3p. (E) Relative expression of four miRNA candidates in HUVECs treated with PAHG was assessed using qRT-PCR (n=4). (F and G) The regulatory effects of four miRNA mimics on the protein expression levels of adhesion molecules (ICAM1, VCAM1, and ET-1) (n=4). (H) Regulation of NO content by miR-4521 mimic or miR-4521 inhibitor

under PAHG treatment (n=4). (I) Impact of miR-4521 mimic or miR-4521 inhibitor on eNOS activity during PAHG treatment. (n=4). (J) The ROS expression in miR-4521 mimic or miR-4521 inhibitor combined with PAHG treatment (n=8). (K) Adhesion molecules (ICAM1, VCAM1, and ET-1) expression in PAHG-treated HUVECs transfected with miR-4521 mimics was determined by Western blot (n=4). (L) Determination of adhesion molecules (ICAM1, VCAM1, and ET-1) expression via Western blot in PAHG-treated HUVECs transfected with miR-4521 inhibitor (n=4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Source data 1. Original file for the western blot analysis shown in **Figure 3F**.

Source data 2. Original file for the western blot analysis shown in **Figure 3K**.

Source data 3. Original file for the western blot analysis shown in **Figure 3L**.

Figure 4. **circHMGCS1 Regulates PAHG-induced Endothelial Dysfunction by Targeting and Sponging miR-4521**. (A) Luciferase reporter constructs containing wild-type (WT) or mutant (MUT) circHMGCS1 were cotransfected with miR-4521 mimics, miR-NC, miR-4521 inhibitor, or miR-NC inhibitor in HEK293T cells (n=4). (B) Immunoprecipitation shows AGO2-mediated binding of circHMGCS1 and miR-4521 (n=4). (C) Biotin-coupled miR-4521 or its mutant probe was employed for circHMGCS1 pull-down, and captured circHMGCS1 level was quantified by qRT-PCR (n=4). (D) RNA-FISH showing the colocalization of circHMGCS1 and miR-4521 in HUVECs (red represents circHMGCS1, green represents miR-4521, blue represents nucleus, scale bar = 50 μm, n=4). (E and F) circHMGCS1 exhibited no impact on NO content and eNOS activity in the presence of miR-4521 sponge (n=4). (G) circHMGCS1 demonstrated no influence on ROS expression with miR-4521 sponge (n=8). (H-J) circHMGCS1 had no effect on the expression of

adhesion molecules (ICAM1, VCAM1, and ET-1) in the presence of miR-4521 sponge which were determined by Western blot and qRT-PCR (n=4). (K and L) miR-4521 attenuated the reduction of NO content and eNOS activity induced by circHMGCS1 (n=4). (M) miR-4521 inhibited the increase of ROS expression caused by circHMGCS1 (n=8). (N-P) miR-4521 attenuated the increased expression of adhesion molecules (ICAM1, VCAM1 and ET-1) induced by circHMGCS1 which were determined by Western blot and qRT-PCR, respectively (n=4). *p < 0.05, **p < 0.01, ns means no significant. All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Source data 1. Original file for the western blot analysis shown in **Figure 4H**.

Source data 2. Original file for the western blot analysis shown in **Figure 4N**.

Figure 5. AAV9-mediated circHMGCS1 Overexpression Attenuates the Protective Effect of miR-4521 against Diabetes-induced VED. (A) Changes in body weight of mice from 0 to 14 weeks (n=8, ***p<0.001 DM versus control, #p<0.05 DM + agomir miR-4521 versus DM). (B) Alterations in fasting blood glucose in mice from 0 to 14 weeks (n=8, ***p<0.001 DM versus control, #p<0.05 DM + agomir miR-4521 versus DM). (C and D) ITT and OGTT were performed in the fasted mice (n=8, *p<0.05 DM versus control, #p<0.05 DM + agomir miR-4521 versus DM). (E) Relaxation responses of aortic rings from control, miR-4521 agomir, or miR-4521 agomir+AAV9 circHMGCS1 mice on DM diet (n=6). (F) SBP measured via tail-cuff method in different groups (n=8). (G) Hematoxylin and eosin staining (H&E) was performed on serial cross-sections of thoracic aortas from differently treated mice to assess vessel wall thickness. Scale bar: 100 μm (n=6). (H and I) NO content and eNOS activity in thoracic aorta after 6 weeks of DM diet with miR-4521 agomir or miR-4521 agomir + circHMGCS1 treatment (n=6). (J and K) Detection of ROS expression in thoracic aorta using DHE after miR-4521 agomir or

miR-4521 agomir+AAV9 circHMGCS1 injection (red represents ROS, green represents GFP, blue represents nucleus, scale bar = 100 μm, n=6). (L) Expression levels of adhesion molecules (ICAM1, VCAM1, and ET-1) in thoracic aorta after 6 weeks of DM diet with miR-4521 agomir or miR-4521 agomir + circHMGCS1 treatment (n=6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Source data 1. Original file for the western blot analysis shown in **Figure 5L**.

Figure 6. circHMGCS1 Functions as a miR-4521 Sponge in HUVECs to Modulate ARG1 Expression. (A) Schematic depiction illustrating the intersection of predicted target genes of miR-4521 from TargetScan, miRWalk, mirDIP, and GeneCard. (B) ARG1 expression in thoracic aorta of DM by western blot (n=6). (C) HEK293T cells were cotransfected with miR-4521 mimics, miR-NC, miR-4521 inhibitor, or miR-NC inhibitor, along with luciferase reporter constructs containing WT or MUT 3′-untranslated region of ARG1 (n=4). (D and E) Modulatory effect of miR-4521 mimic on NO content and eNOS activity under ARG1 overexpression (n=4). (F) Regulatory impact of miR-4521 mimic on ROS content under ARG1 overexpression (n=4). (G and H) ARG1-overexpressing HUVECs were generated using lentivirus and transfected with miR-4521 mimics for 24h to evaluate adhesion molecule expression (ICAM1, VCAM1, and ET-1) by Western blot and qRT-PCR (n=4). (I-K) ARG1 expression was significantly decreased by miR-4521 mimics and increased by miR-4521 inhibitor, as determined by qRT-PCR and Western blot (n=4). (L and M) ARG1 expression was significantly increased by circHMGCS1 overexpression, as determined by Western blot and qRT-PCR ($n \geq 4$). (N and O) circHMGCS1-overexpressed HUVECs, created with lentivirus and transfected with miR-4521 mimics for 48h, were examined for ARG1 expression by Western blot and qRT-PCR (n=4). (P and Q) ARG1 expression was significantly reduced by

circHMGCS1 shRNA, as determined by Western blot and qRT-PCR (n=3). (R) AAV9 miR-4521 sponge was used to inhibit the miR-4521 expression in HUVECs, followed by circHMGCS1 transfection, and then treated with PAHG for 24 hours to evaluate the expression of ARG1 by Western blot (n=4). (S) Detection of ARG1 expression in the thoracic aorta of DM treated with AAV9 miR-4521 agomir combined with AAV9 circHMGCS1 by Western blot (n=4). *$p < 0.05$, **$p < 0.01$, ns means no significant. All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Source data 1. Original file for the western blot analysis shown in **Figure 6B**.

Source data 2. Original file for the western blot analysis shown in **Figure 6G**.

Source data 3. Original file for the western blot analysis shown in **Figure 6J and Figure 6K**.

Source data 4. Original file for the western blot analysis shown in **Figure 6L**.

Source data 5. Original file for the western blot analysis shown in **Figure 6O**.

Source data 6. Original file for the western blot analysis shown in **Figure 6P**.

Source data 7. Original file for the western blot analysis shown in **Figure 6R**.

Source data 8. Original file for the western blot analysis shown in **Figure 6S**.

Figure 7. ARG1 is Inseparable from circHMGCS1 and miR-4521 Regulating Diabetes-Induced VED. (A and B) circHMGCS1 and miR-4521 had no effect on NO content and eNOS activity expression in the absence of ARG1 (n=4). (C) ROS expression remained unaffected by circHMGCS1 and miR-4521 in the absence of ARG1 (n=4). (D and E) Relative expression of adhesion molecules (ICAM1, VCAM1, and ET-1) remained unchanged in the absence of ARG1, despite the presence of circHMGCS1 and miR-4521, as determined by qRT-PCR and Western

blot (n=4). (F) Changes in mice body weight over the experimental period (n=8, **p<0.01, DM versus control, #p<0.05, DM + AAV9 ARG1 shRNA versus DM). (G) Fasting blood glucose levels in mice over time (n=8, **p<0.01, DM versus control, #p<0.05, DM + AAV9 ARG1 shRNA versus DM). (H and I) Blood glucose levels measured at week 13 (ITT) and week 14 (OGTT) (n=8, **p<0.01 DM versus control, #p<0.05, DM + AAV9 ARG1 shRNA versus DM). (J) Endothelium-dependent relaxations in aortic rings from different groups (n=8). (K) H&E performed on serial cross-sections of thoracic aortas from differently treated mice to evaluate vessel wall thickness. Scale bar = 100 μm (n=6). (L) SBP measured by the tail-cuff method in different groups (n=8). (M and N) NO content and eNOS activity expression in thoracic aort (n=6). (O) Relative expression of adhesion molecules (ICAM1, VCAM1, and ET-1) in thoracic aorta assessed by Western blot (n=6). (P and Q) ROS expression in the thoracic aorta using the DHE probe (Red represents ROS, Green represents GFP, Blue represents nucleus, scale bar: 100 μm, n=6). *p < 0.05, **p < 0.01, ns means no significant. All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Source data 1. Original file for the western blot analysis shown in **Figure 7E**.

Source data 2. Original file for the western blot analysis shown in **Figure 7O**.

Supplemental Figure legend

Figure S1. Distinct expression profiles of circRNAs in endothelial dysfunction of HUVECs. HUVECs exposed to palmitic acid and high glucose (PAHG) treatment for 24 hours were compared with untreated Control. (**A**) Alterations in NO content between the control and PAHG-treated groups (n=4). (**B**) Changes in eNOS activity between the control and PAHG-treated groups (n=4). (**C**) Relative expression levels of adhesion molecules (ICAM1, VCAM1, and ET-1) in HUVECs were assessed by Western blotting between the control and PAHG-treated groups (n=4). (**D**) Volcano plot illustrating significantly altered circRNAs between the control and PAHG-treated groups. Fold-change > 2 or < 0.5, $p \leq 0.001$. (**E**) Number of identified circRNAs in Control groups and PAHG-treated groups (n=3). (**F**) PCR amplification confirming the full-length circHMGCS1 (n=3). (**G**) Sanger sequencing validation of the full-length circHMGCS1 sequence (n=3). (**H**) Comparison results of circHMGCS1 sequences in Sanger sequencing and circbase database. *$p < 0.05$, **$p < 0.01$, All significant difference was determined by unpaired two-tailed Student's t-test, error bar indicates SD.

Figure supplement 1—source data 1. Original file for the western blot analysis shown in **Figure supplement 1C**.

Figure supplement 1—source data 2. Original file of the agarose gel shown in **Figure supplement 1F**.

Figure S2. A sketch map of circHMGCS1 plasmid construction.

Figure S3. miR-4521 inhibition aggravates diabetes-induced endothelial dysfunction. (A) Relative expression of hsa-miR-98-5p, hsa-miR-3143, hsa-miR-181a-2-3p and hsa-miR-4521 were detected in HUVECs after corresponding miRNA mimics treatment by qRT-PCR (n=4). (**B** and **C**) Relative

expression level of miR-4521 was determined by qRT-PCR in HUVECs transfected with miR-4521 mimics or miR-4521 inhibitor (n=4). (**D**) miR-4521 mimic inhibited the expression of adhesion molecules (ICAM1, VCAM1, and ET-1) in PAHG-treated HUVEC as detected by qRT-PCR (n=4). (**E**) miR-4521 inhibitor exacerbated the expression of adhesion molecules (ICAM1, VCAM1, and ET-1) in PAHG-treated HUVEC as detected by qRT-PCR (n=4). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Figure S4. Verification of the binding site of miR-4521 and circHMGCS1. HEK293T cells were used to package lentivirus overexpressing circHMGCS1, and then infected HUVECs cells, (**A**) Expression level of miR-4521 was assessed by qRT-PCR in pLV-circHMGCS1-transfected HEK293T cells (n=4). (**B**) The expression level of miR-4521 was assessed by qRT-PCR in HUVEC cells transfected with circHMGCS1 shRNA (n=3). (**C**) Schematic illustration of the predicted binding sites of miR-4521 on the circHMGCS1 transcript in human cells. (**D**) Biotin-coupled circHMGCS1 or its mutant probe was employed for miR-4521 pull-down assay, and captured miR-4521 level was quantified by qRT-PCR (n=4). (**E**) Western blot analysis of AGO2 protein expression captured by biotin-coupled circHMGCS1. **$p < 0.01$, ***$p < 0.001$, significant difference was determined by unpaired two-tailed Student's t-test, error bar indicates SD.

Figure supplement 4—source data 1. Original file for the western blot analysis shown in **Figure supplement 4E**.

Figure S5. circHMGCS1 counteracts the protective role of miR-4521 in diabetes-induced VED. (**A**) Timeline illustrating the HFHG feeding, AAV9 circHMGCS1, and miR-4521 agomir treatment schedule. (**B** and **C**) Quantification of

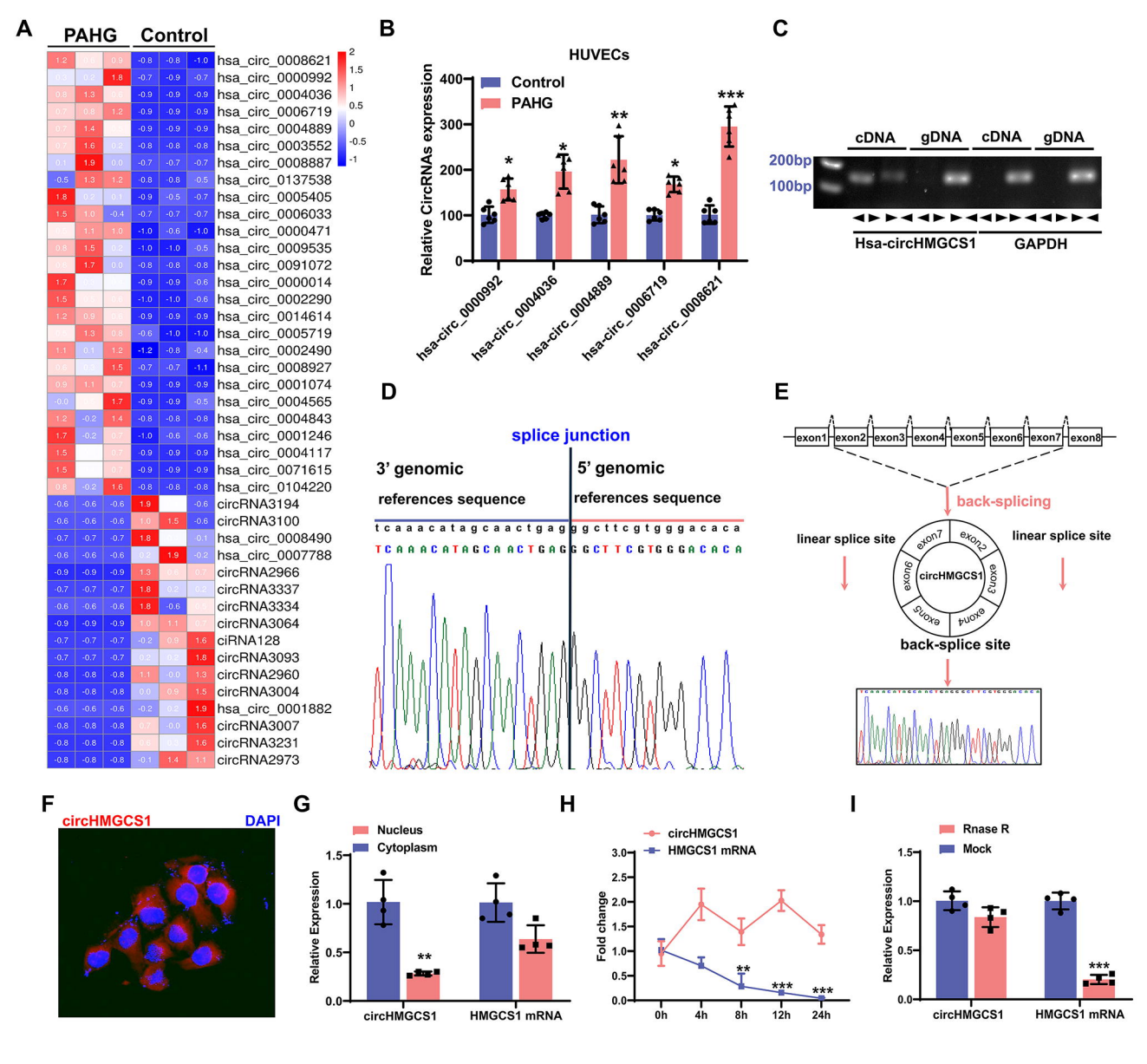
the area under the curve (AUC) for glucose and insulin tests (n=8). (**D**) Triglyceride (TG), (**E**) Total cholesterol (T-CHO), (**F**) High-density lipoprotein cholesterol (HDL-C), and (**G**) Low-density lipoprotein cholesterol (LDL-C) levels in the serum were measured using Automatic Biochemical Analyzer (n=6). (**H**) Effects on endothelium-independent vasorelaxation to SNP (n=6). *p < 0.05, **p < 0.01. All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Figure S6. miR-4521 modulates the expression of ARG1. (**A**) Schematic representation of the predicted binding sites and mutated sites for miR-4521 on ARG1 transcripts in both human cells and mice. (**B**) Western blot analysis assessing ARG1 and ARG2 expression in HUVECs after PAHG treatment (n=3). (**C**) Sequencing results displaying the mutation site in the dual luciferase assay. *p < 0.05, **p < 0.01, ns means no significant. All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.

Figure supplement 6—source data 1. Original file for the western blot analysis shown in **Figure supplement 6B**.

Figure S7. Loss of functional regulation of circHMGCS1 and miR-4521 on HUVECs in the absence of ARG1 *in vivo*. (**A**) Schematic outlining the timeline of HFHG feeding, AAV9 circHMGCS1 and miR-4521 agomir treatment, AAV9 ARG1 shRNA treatment, and experiment conclusion. (**B** and **C**) Blood glucose levels measured at week 13 (ITT) and week 14 (OGTT), the corresponding AUC for glucose or insulin tests was calculated (n=8, **p<0.01, DM versus control, #p<0.05, DM + AAV9 ARG1 shRNA versus HFHG). (**D**) TG, (**E**) T-CHO, (**F**) HDL-C, and (**G**) LDL-C levels in the serum of different treated groups (n=6). (**H**) Assessment of

endothelium-independent vasorelaxation to SNP (n=6). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ns means no significant. All significant difference was determined by one-way ANOVA followed by Bonferroni multiple comparison post hoc test, error bar indicates SD.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PAHG
Control
hsa_circ_0008621
hsa_circ_0000992
hsa_circ_0004036
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hsa_circ_0008887
hsa_circ_0137538
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hsa_circ_0006033
hsa_circ_0000471
hsa_circ_0009535
hsa_circ_0091072
hsa_circ_0000014
hsa_circ_0002290
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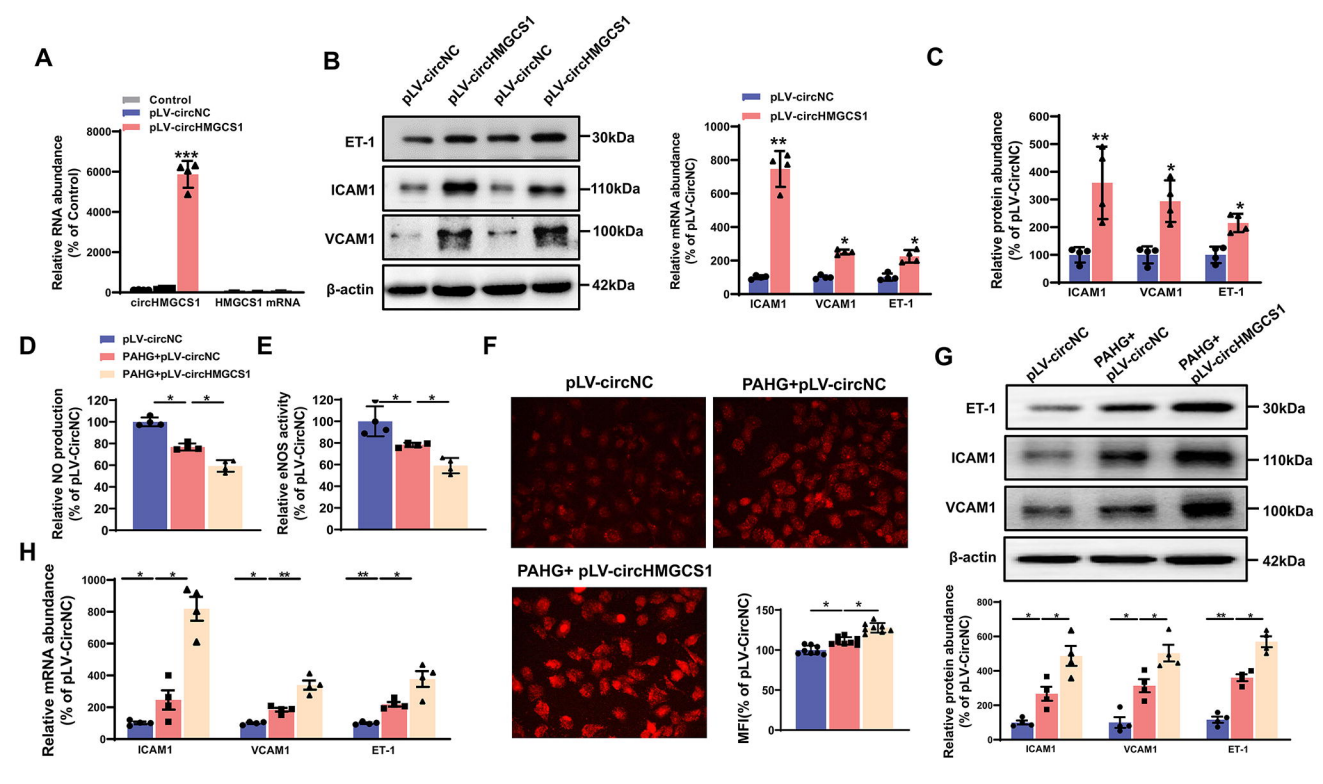
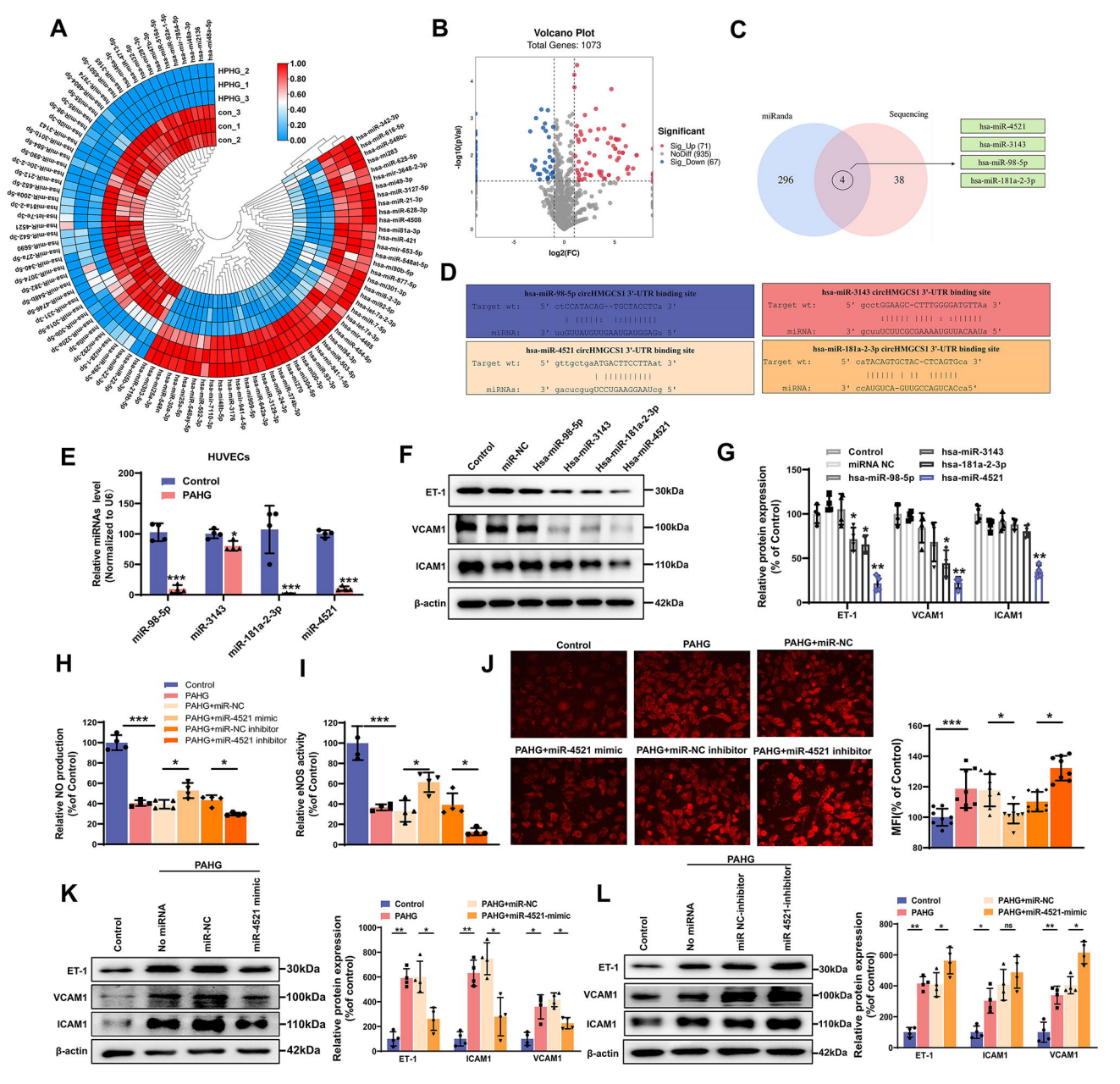
hsa_circ_0005719
hsa_circ_0002490
hsa_circ_0008927
hsa_circ_0001074
hsa_circ_0004565
hsa_circ_0004843
hsa_circ_0001246
hsa_circ_0004117
hsa_circ_0071615
hsa_circ_0104220
circRNA3194
circRNA3100
hsa_circ_0008490
hsa_circ_0007788
circRNA2966
circRNA3337
circRNA3334
circRNA3064
ciRNA128
circRNA3093
circRNA2960
circRNA3004
hsa_circ_0001882
circRNA3007
circRNA3231
circRNA2973
B
HUVECs
Control
PAHG
Relative CircRNAs expression
hsa-circ_0000992
hsa-circ_0004036
hsa-circ_0004889
hsa-circ_0006719
hsa-circ_0008621
C
cDNA
gDNA
cDNA
gDNA
200bp
100bp
Hsa-circHMGCS1
GAPDH
D
splice junction
3' genomic
references sequence
5' genomic
references sequence
E
exon1
exon2
exon3
exon4
exon5
exon6
exon7
exon8
back-splicing
linear splice site
linear splice site
circHMGCS1
back-splice site
F
circHMGCS1
DAPI
G
Nucleus
Cytoplasm
Relative Expression
circHMGCS1
HMGCS1 mRNA
H
circHMGCS1
HMGCS1 mRNA
Fold change
0h
4h
8h
12h
24h
I
Rnase R
Mock
Relative Expression
circHMGCS1
HMGCS1 mRNA


A
Control
pLV-circNC
pLV-circHMGCS1
Relative RNA abundance (% of Control)
circHMGCS1
HMGCS1 mRNA
B
pLV-circNC
pLV-circHMGCS1
pLV-circNC
pLV-circHMGCS1
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
Relative mRNA abundance (% of pLV-CircNC)
ICAM1
VCAM1
ET-1
C
Relative protein abundance (% of pLV-CircNC)
D
pLV-circNC
PAHG+pLV-circNC
PAHG+pLV-circHMGCS1
Relative NO production (% of pLV-CircNC)
E
Relative eNOS activity (% of pLV-CircNC)
F
pLV-circNC
PAHG+pLV-circNC
PAHG+ pLV-circHMGCS1
MFI(% of pLV-CircNC)
G
pLV-circNC
PAHG+ pLV-circNC
PAHG+ pLV-circHMGCS1
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
Relative protein abundance (% of pLV-CircNC)
H
Relative mRNA abundance (% of pLV-CircNC)
ICAM1
VCAM1
ET-1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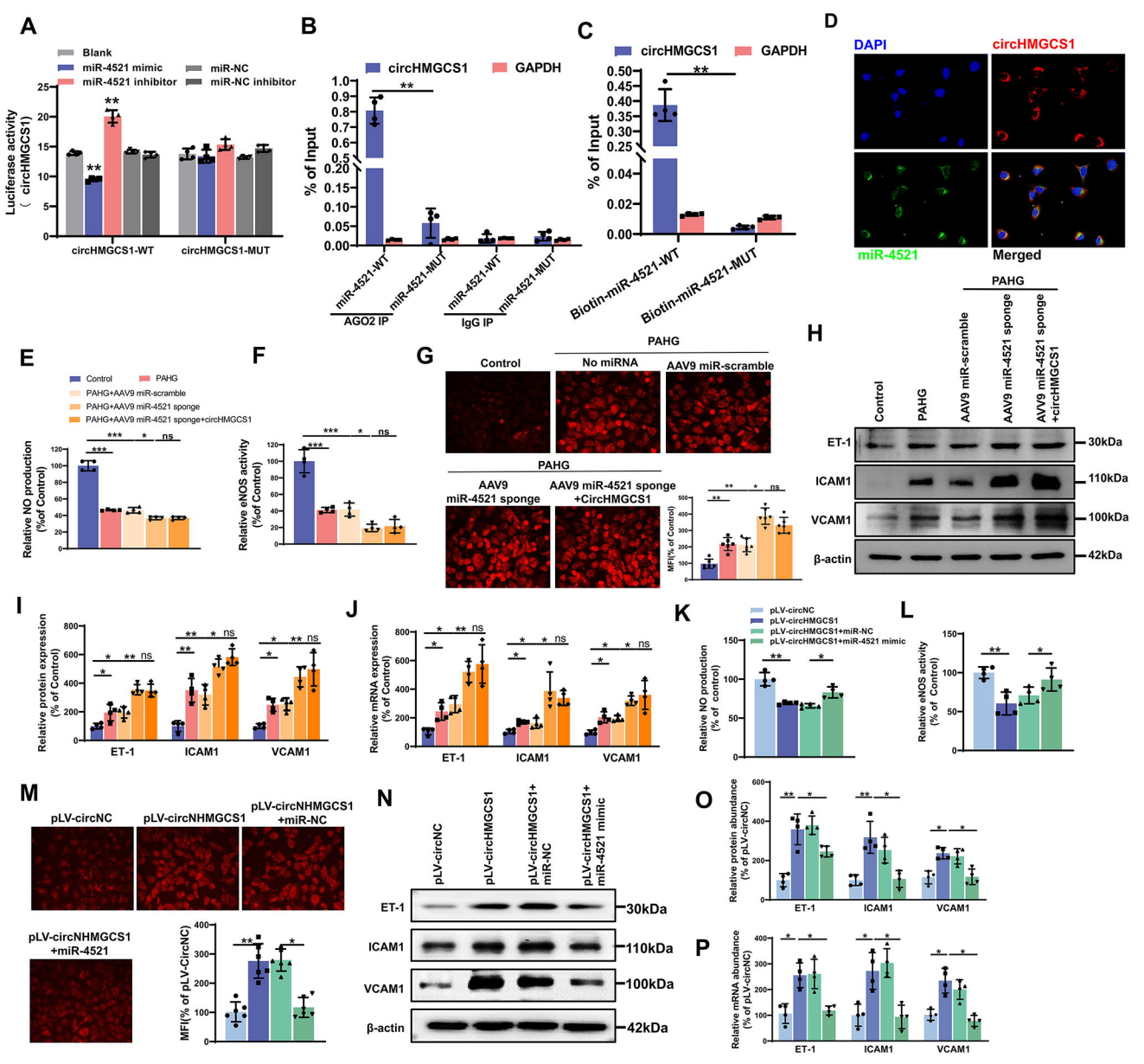
HPHG_2
HPHG_1
HPHG_3
con_3
con_1
con_2
1.00
0.80
0.60
0.40
0.20
0.00
B
Volcano Plot
Total Genes: 1073
-log10(pVal)
log2(FC)
Significant
Sig_Up (71)
NoDiff (935)
Sig_Down (67)
C
miRanda
Sequencing
296
4
38
hsa-miR-4521
hsa-miR-3143
hsa-miR-98-5p
hsa-miR-181a-2-3p
D
hsa-miR-98-5p circHMGCS1 3'-UTR binding site
Target wt: 5' ctCCATACAG--TGCTACCTCa 3'
miRNA: 3' uuGUUAUGUUGAAUGAUGGAGu 5'
hsa-miR-3143 circHMGCS1 3'-UTR binding site
Target wt: 5' gcctGGAAGC-CTTTGGGGATGTTAa 3'
miRNA: 3' gcuuUCUUCGCGAAAAUGUUACAAUa 5'
hsa-miR-4521 circHMGCS1 3'-UTR binding site
Target wt: 5' gttgctgaATGACTTCCTTAat 3'
miRNAs: 3' gacucgugUCCUGAAGGAAUcg 5'
hsa-miR-181a-2-3p circHMGCS1 3'-UTR binding site
Target wt: 5' caTACAGTGCTAC-CTCAGTGca 3'
miRNA: 3' ccAUGUCA-GUUGCCAGUCACca5'
E
HUVECs
Control
PAHG
Relative miRNAs level (Normalized to U6)
miR-98-5p
miR-3143
miR-181a-2-3p
miR-4521
F
Control
miR-NC
Hsa-miR-98-5p
Hsa-miR-3143
Hsa-miR-181a-2-3p
Hsa-miR-4521
ET-1
VCAM1
ICAM1
β-actin
30kDa
100kDa
110kDa
42kDa
G
Control
miRNA NC
hsa-miR-98-5p
hsa-miR-3143
hsa-181a-2-3p
hsa-miR-4521
Relative protein expression (% of Control)
ET-1
VCAM1
ICAM1
H
Control
PAHG
PAHG+miR-NC
PAHG+miR-4521 mimic
PAHG+miR-NC inhibitor
PAHG+miR-4521 inhibitor
Relative NO production (%of Control)
I
Relative eNOS activity (%of Control)
J
Control
PAHG
PAHG+miR-NC
PAHG+miR-4521 mimic
PAHG+miR-NC inhibitor
PAHG+miR-4521 inhibitor
MFI(% of Control)
K
PAHG
Control
No miRNA
miR-NC
miR-4521 mimic
ET-1
VCAM1
ICAM1
β-actin
30kDa
100kDa
110kDa
42kDa
Control
PAHG
PAHG+miR-NC
PAHG+miR-4521-mimic
Relative protein expression (%of control)
ET-1
ICAM1
VCAM1
L
PAHG
Control
No miRNA
miR NC-inhibitor
miR 4521-inhibitor
ET-1
VCAM1
ICAM1
β-actin
30kDa
100kDa
110kDa
42kDa
Control
PAHG
PAHG+miR-NC
PAHG+miR-4521-mimic
Relative protein expression (%of control)
ET-1
ICAM1
VCAM1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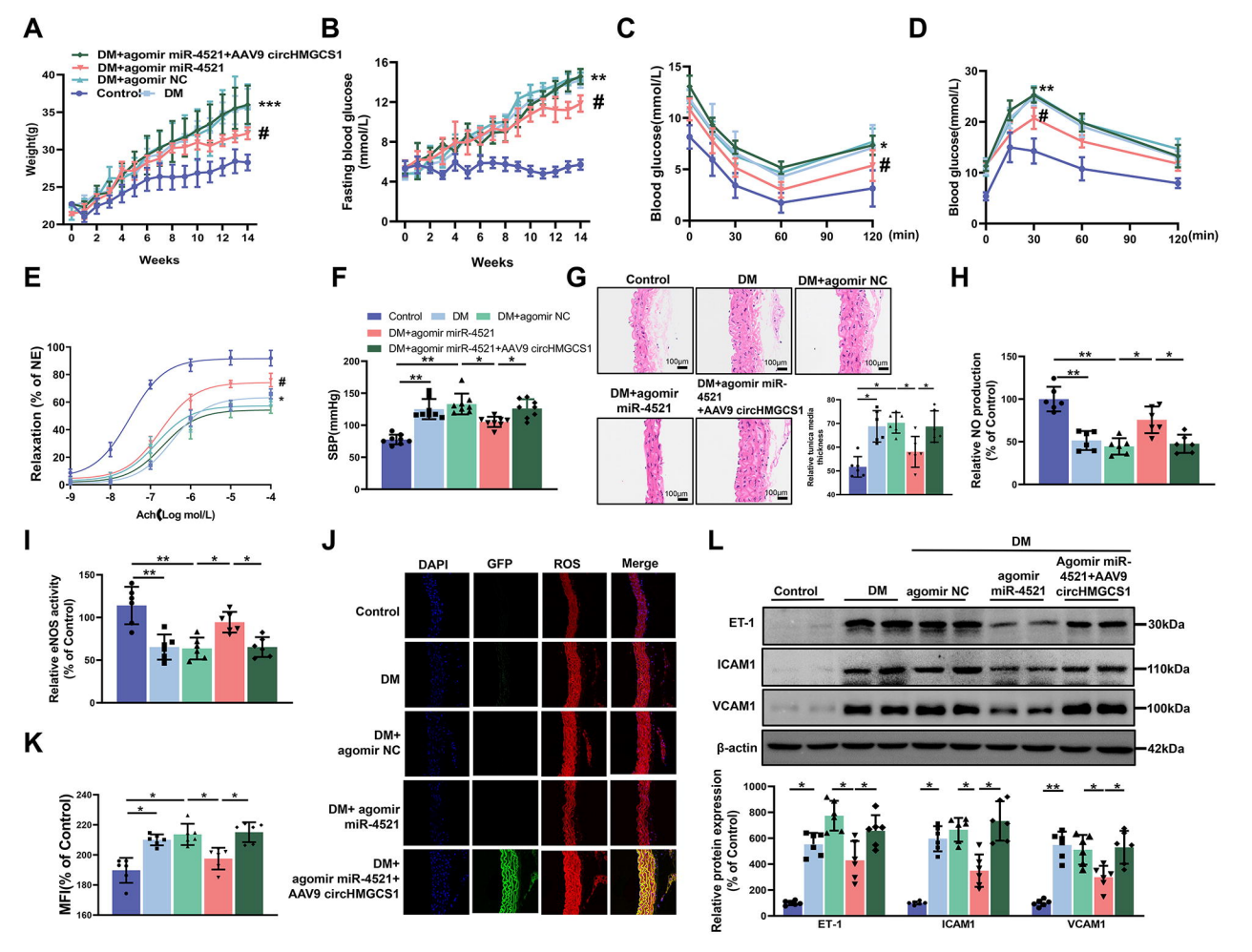
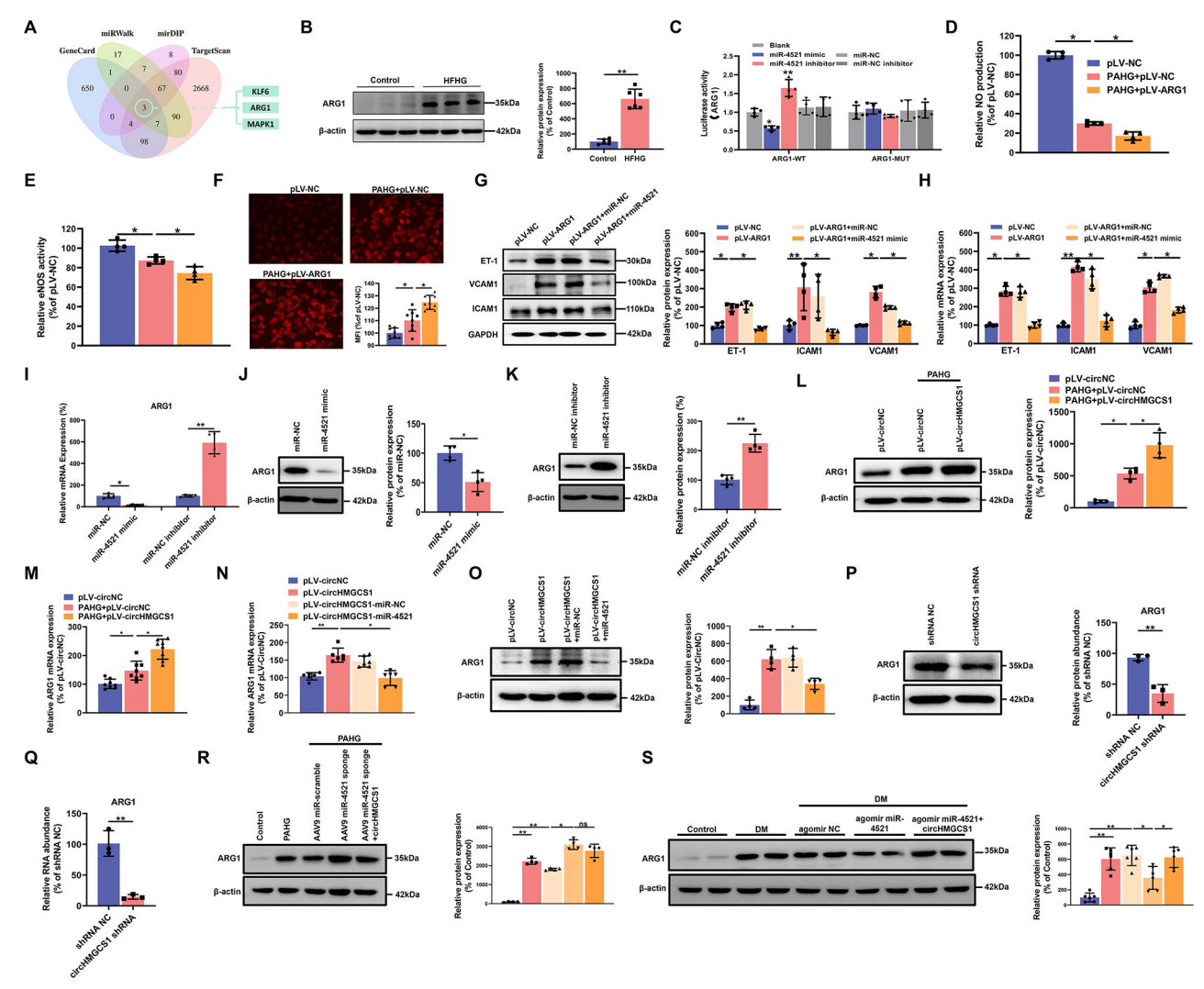
Blank
miR-4521 mimic
miR-4521 inhibitor
miR-NC
miR-NC inhibitor
Luciferase activity (circHMGCS1)
circHMGCS1-WT
circHMGCS1-MUT
B
circHMGCS1
GAPDH
% of Input
miR-4521-WT
miR-4521-MUT
AGO2 IP
IgG IP
C
circHMGCS1
GAPDH
% of Input
Biotin-miR-4521-WT
Biotin-miR-4521-MUT
D
DAPI
circHMGCS1
miR-4521
Merged
E
Control
PAHG
PAHG+AAV9 miR-scramble
PAHG+AAV9 miR-4521 sponge
PAHG+AAV9 miR-4521 sponge+circHMGCS1
Relative NO production (%of Control)
F
Relative eNOS activity (%of Control)
G
PAHG
Control
No miRNA
AAV9 miR-scramble
AAV9 miR-4521 sponge
AAV9 miR-4521 sponge +CircHMGCS1
MFI(% of Control)
H
PAHG
Control
AAV9 miR-scramble
AAV9 miR-4521 sponge
AAV9 miR-4521 sponge +circHMGCS1
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
I
Relative protein expression (% of Control)
ET-1
ICAM1
VCAM1
J
Relative mRNA expression (% of Control)
ET-1
ICAM1
VCAM1
K
pLV-circNC
pLV-circHMGCS1
pLV-circHMGCS1+miR-NC
pLV-circHMGCS1+miR-4521 mimic
Relative NO production (% of control)
L
Relative eNOS activity (% of Control)
M
pLV-circNC
pLV-circNHMGCS1
pLV-circNHMGCS1 +miR-NC
pLV-circNHMGCS1 +miR-4521
MFI(% of pLV-CircNC)
N
pLV-circNC
pLV-circHMGCS1
pLV-circHMGCS1+ miR-NC
pLV-circHMGCS1+ miR-4521 mimic
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
O
Relative protein abundance (% of pLV-circNC)
ET-1
ICAM1
VCAM1
P
Relative mRNA abundance (% of pLV-circNC)
ET-1
ICAM1
VCAM1


A
DM+agomir miR-4521+AAV9 circHMGCS1
DM+agomir miR-4521
DM+agomir NC
Control
DM
Weight(g)
Weeks
B
Fasting blood glucose (mmol/L)
Weeks
C
Blood glucose(mmol/L)
(min)
D
Blood glucose(mmol/L)
(min)
E
Relaxation (% of NE)
Ach(Log mol/L)
F
Control
DM
DM+agomir NC
DM+agomir mirR-4521
DM+agomir mirR-4521+AAV9 circHMGCS1
SBP(mmHg)
G
Control
DM
DM+agomir NC
DM+agomir miR-4521
DM+agomir miR-4521 +AAV9 circHMGCS1
Relative tunica media thickness
H
Relative NO production (% of Control)
I
Relative eNOS activity (% of Control)
J
DAPI
GFP
ROS
Merge
Control
DM
DM+ agomir NC
DM+ agomir miR-4521
DM+ agomir miR-4521+ AAV9 circHMGCS1
K
MFI(% of Control)
L
DM
Control
DM
agomir NC
agomir miR-4521
Agomir miR-4521+AAV9 circHMGCS1
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
Relative protein expression (% of Control)
ET-1
ICAM1
VCAM1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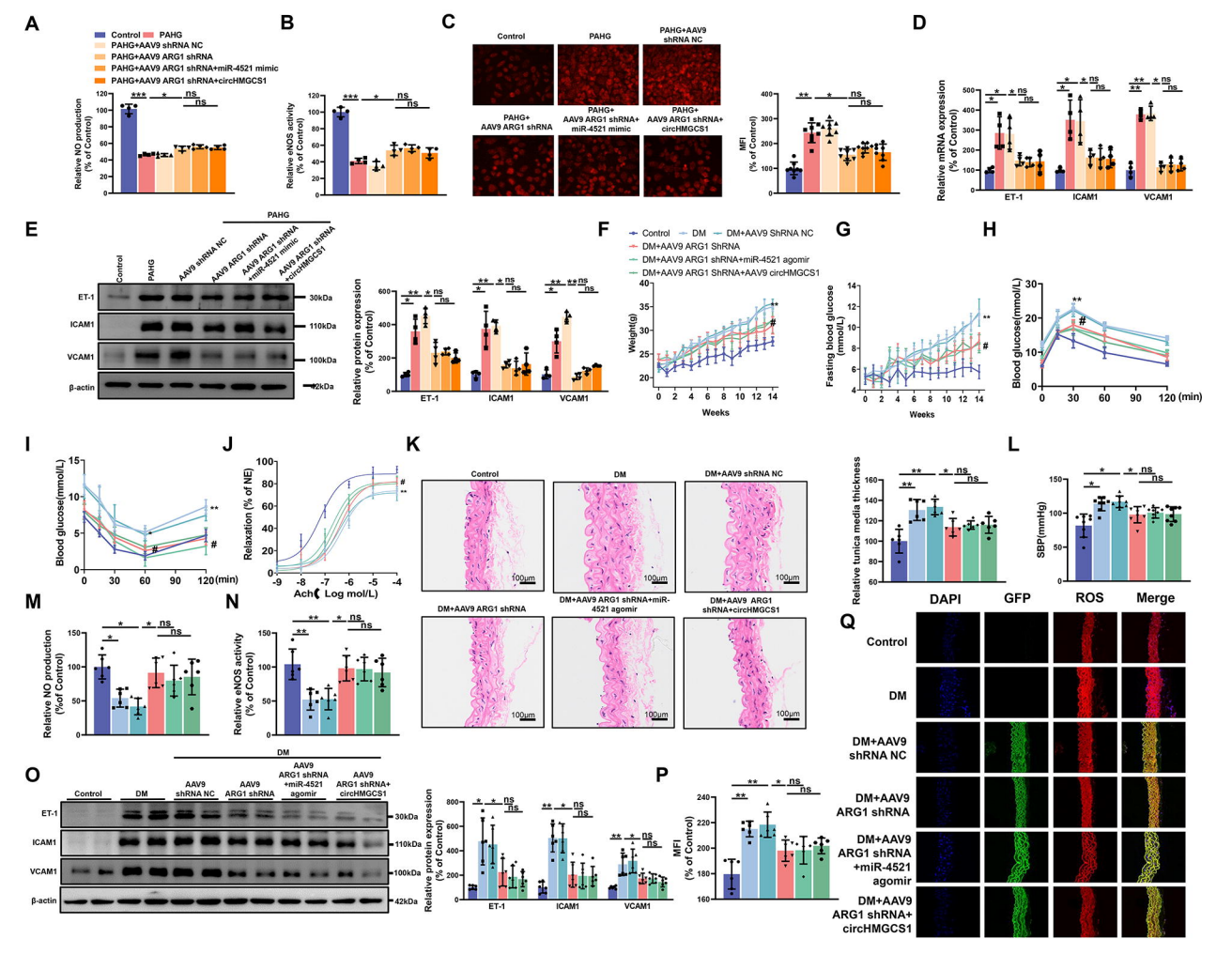
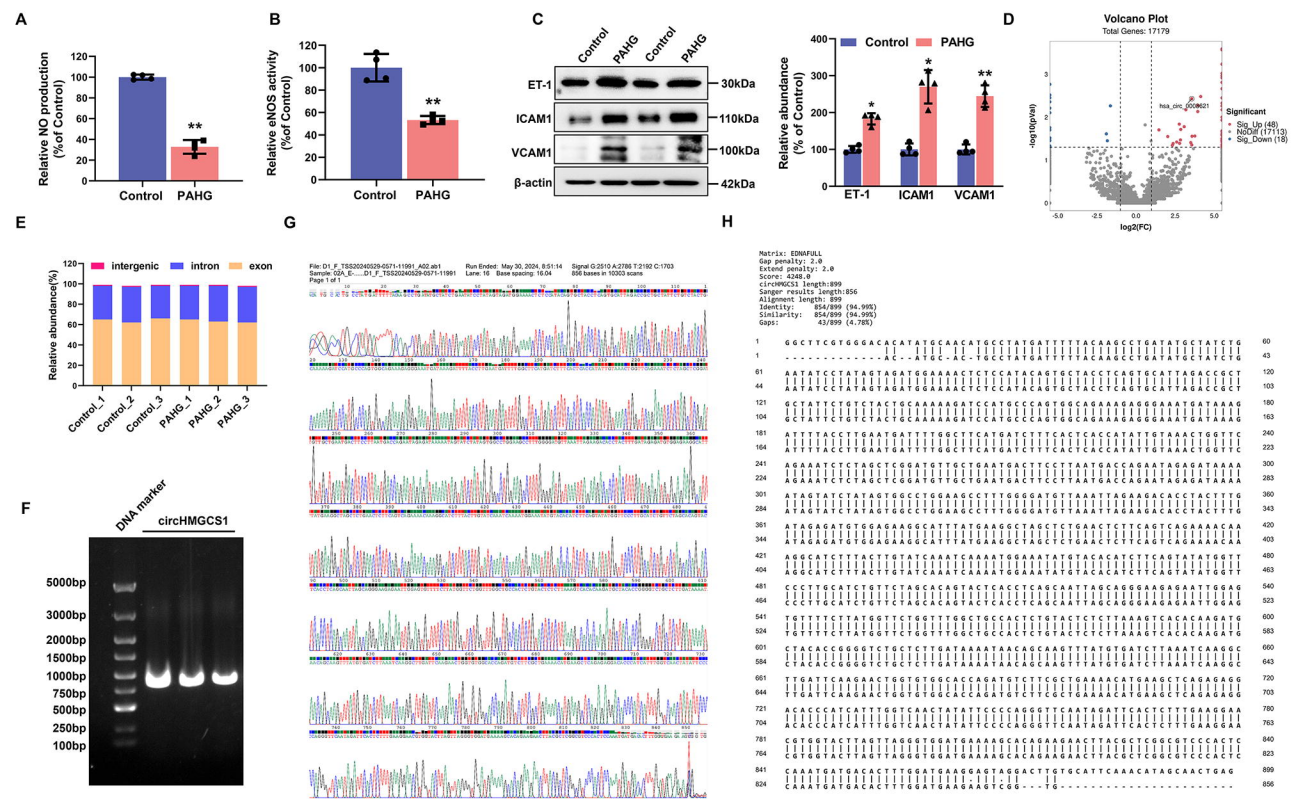
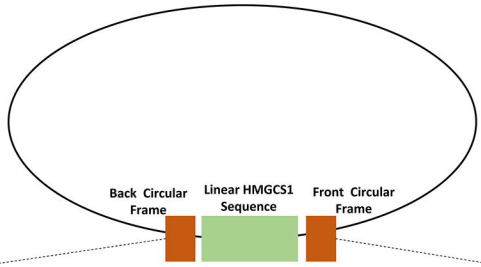
miRWalk
mirDIP
GeneCard
TargetScan
KLF6
ARG1
MAPK1
B
Control
HFHG
ARG1
β-actin
35kDa
42kDa
Relative protein expression (% of Control)
C
Blank
miR-4521 mimic
miR-4521 inhibitor
miR-NC
miR-NC inhibitor
Luciferase activity (ARG1)
ARG1-WT
ARG1-MUT
D
Relative NO production (%of pLV-NC)
pLV-NC
PAHG+pLV-NC
PAHG+pLV-ARG1
E
Relative eNOS activity (%of pLV-NC)
F
pLV-NC
PAHG+pLV-NC
PAHG+pLV-ARG1
MFI (%of pLV-NC)
G
pLV-NC
pLV-ARG1
pLV-ARG1+miR-NC
pLV-ARG1+miR-4521
ET-1
VCAM1
ICAM1
GAPDH
30kDa
100kDa
110kDa
42kDa
Relative protein expression (% of pLV-NC)
pLV-ARG1+miR-NC
pLV-ARG1+miR-4521 mimic
H
Relative mRNA expression (% of pLV-NC)
I
ARG1
Relative mRNA Expression (%)
miR-NC
miR-4521 mimic
miR-NC inhibitor
miR-4521 inhibitor
J
miR-NC
miR-4521 mimic
Relative protein expression (% of miR-NC)
K
miR-NC inhibitor
miR-4521 inhibitor
Relative protein expression (%)
L
PAHG
pLV-circNC
pLV-circHMGCS1
PAHG+pLV-circNC
PAHG+pLV-circHMGCS1
M
Relative ARG1 mRNA expression (% of pLV-circNC)
N
pLV-circHMGCS1-miR-NC
pLV-circHMGCS1-miR-4521
O
pLV-circHMGCS1 +miR-NC
pLV-circHMGCS1 +miR-4521
P
shRNA NC
circHMGCS1 shRNA
Relative protein abundance (% of shRNA NC)
Q
Relative RNA abundance (% of shRNA NC)
R
Control
PAHG
AAV9 miR-scramble
AAV9 miR-4521 sponge
AAV9 miR-4521 sponge +circHMGCS1
ns
S
DM
agomir NC
agomir miR-4521
agomir miR-4521+ circHMGCS1


A
Control
PAHG
PAHG+AAV9 shRNA NC
PAHG+AAV9 ARG1 shRNA
PAHG+AAV9 ARG1 shRNA+miR-4521 mimic
PAHG+AAV9 ARG1 shRNA+circHMGCS1
Relative NO production (% of Control)
B
Relative eNOS activity (% of Control)
C
Control
PAHG
PAHG+AAV9 shRNA NC
PAHG+AAV9 ARG1 shRNA
PAHG+AAV9 ARG1 shRNA+miR-4521 mimic
PAHG+AAV9 ARG1 shRNA+circHMGCS1
MFI (% of Control)
D
Relative mRNA expression (% of Control)
ET-1
ICAM1
VCAM1
E
PAHG
Control
PAHG
AAV9 shRNA NC
AAV9 ARG1 shRNA
AAV9 ARG1 shRNA +miR-4521 mimic
AAV9 ARG1 shRNA +circHMGCS1
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
Relative protein expression (% of Control)
F
Control
DM
DM+AAV9 ShRNA NC
DM+AAV9 ARG1 ShRNA
DM+AAV9 ARG1 shRNA+miR-4521 agomir
DM+AAV9 ARG1 ShRNA+AAV9 circHMGCS1
Weight(g)
Weeks
G
Fasting blood glucose (mmol/L)
Weeks
H
Blood glucose(mmol/L)
(min)
I
Blood glucose(mmol/L)
(min)
J
Relaxation (% of NE)
Ach Log mol/L)
K
Control
DM
DM+AAV9 shRNA NC
DM+AAV9 ARG1 shRNA
DM+AAV9 ARG1 shRNA+miR-4521 agomir
DM+AAV9 ARG1 shRNA+circHMGCS1
100μm
Relative tunica media thickness
L
SBP(mmHg)
M
Relative NO production (%of Control)
N
Relative eNOS activity (% of Control)
O
DM
Control
DM
AAV9 shRNA NC
AAV9 ARG1 shRNA
AAV9 ARG1 shRNA +miR-4521 agomir
AAV9 ARG1 shRNA+ circHMGCS1
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
Relative protein expression (% of Control)
P
MFI (% of Control)
Q
DAPI
GFP
ROS
Merge
Control
DM
DM+AAV9 shRNA NC
DM+AAV9 ARG1 shRNA
DM+AAV9 ARG1 shRNA +miR-4521 agomir
DM+AAV9 ARG1 shRNA+ circHMGCS1

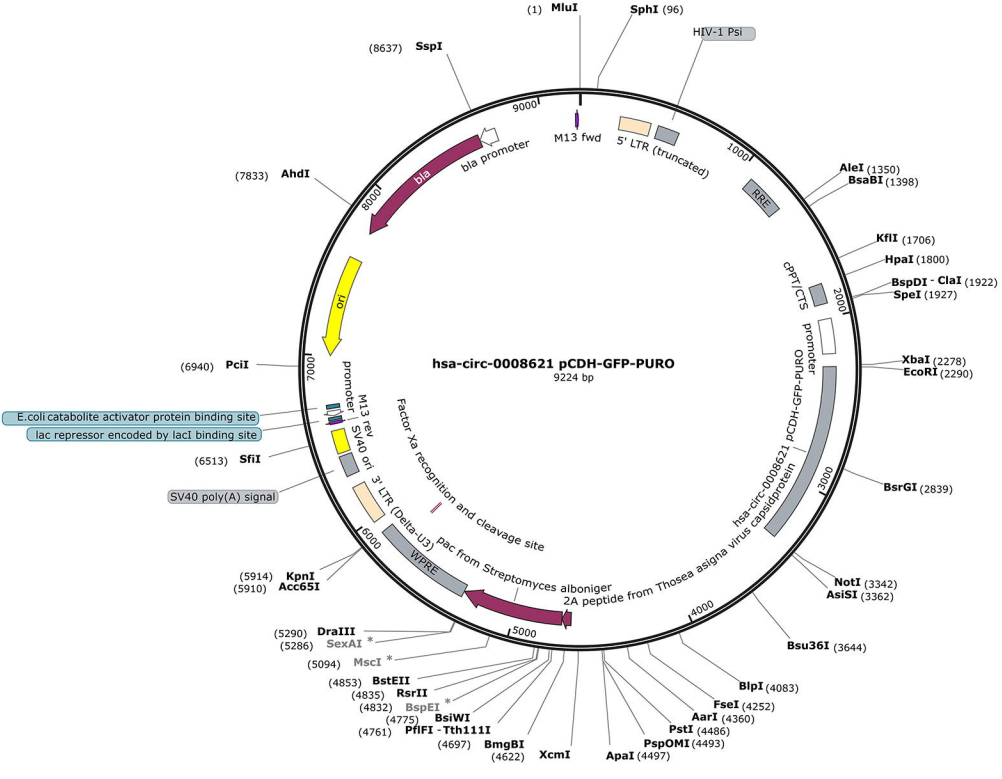

A
Relative NO production (%of Control)
Control
PAHG
B
Relative eNOS activity (%of Control)
Control
PAHG
C
Control
PAHG
Control
PAHG
ET-1
ICAM1
VCAM1
β-actin
30kDa
110kDa
100kDa
42kDa
Relative abundance (% of Control)
Control
PAHG
ET-1
ICAM1
VCAM1
D
Volcano Plot
Total Genes: 17179
hsa_circ_0008621
Significant
Sig_Up (48)
NOdif (17113)
Sig_Down (18)
-log10(p.val)
log2(FC)
E
intergenic
intron
exon
Relative abundance(%)
Control_1
Control_2
Control_3
PAHG_1
PAHG_2
PAHG_3
F
DNA marker
circHMGCS1
5000bp
3000bp
2000bp
1500bp
1000bp
750bp
500bp
250bp
100bp
G
H
Matrix: EDNAFULL
Gap penalty: 2.0
Extend penalty: 2.0
Score: 4248.0
circHMGCS1 length:899
Sanger results length:856
Alignment length: 899
Identity: 854/899 (94.99%)
Similarity: 854/899 (94.99%)
Gaps: 43/899 (4.78%)

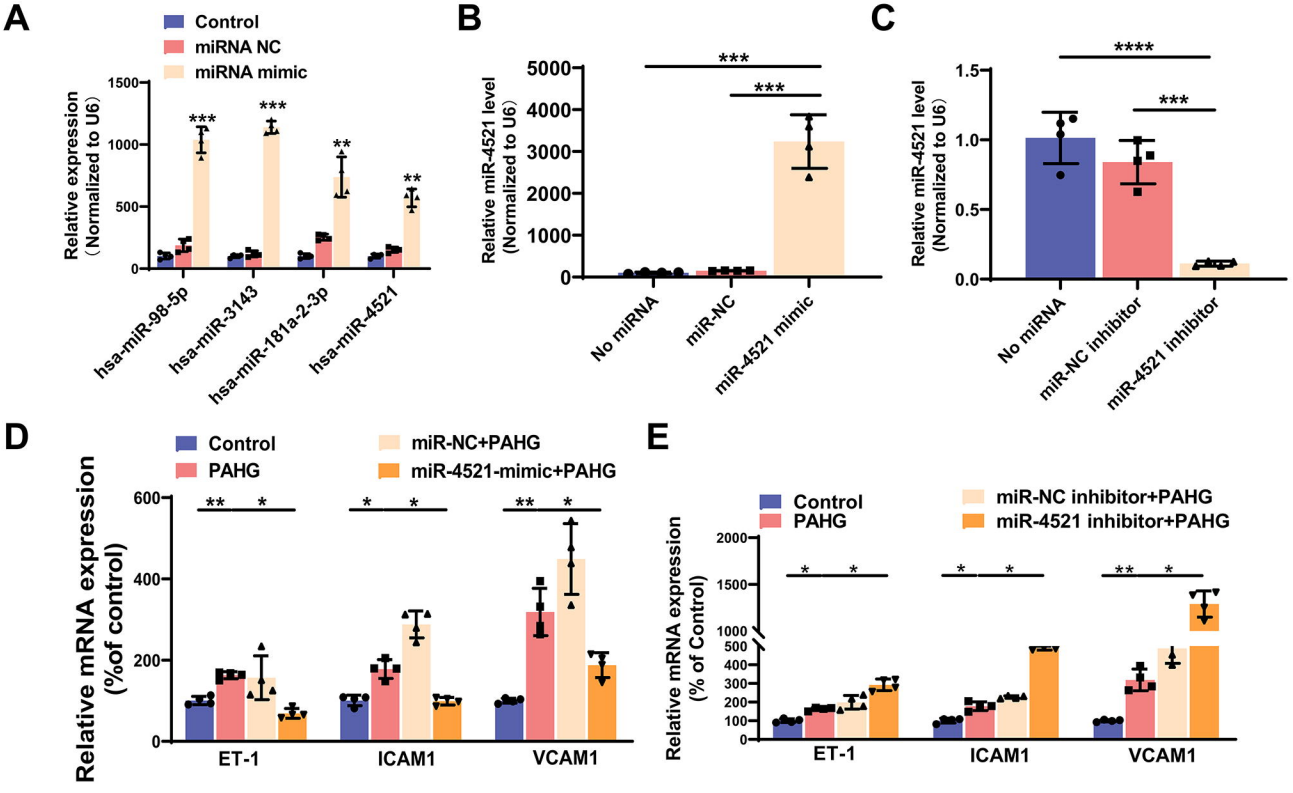


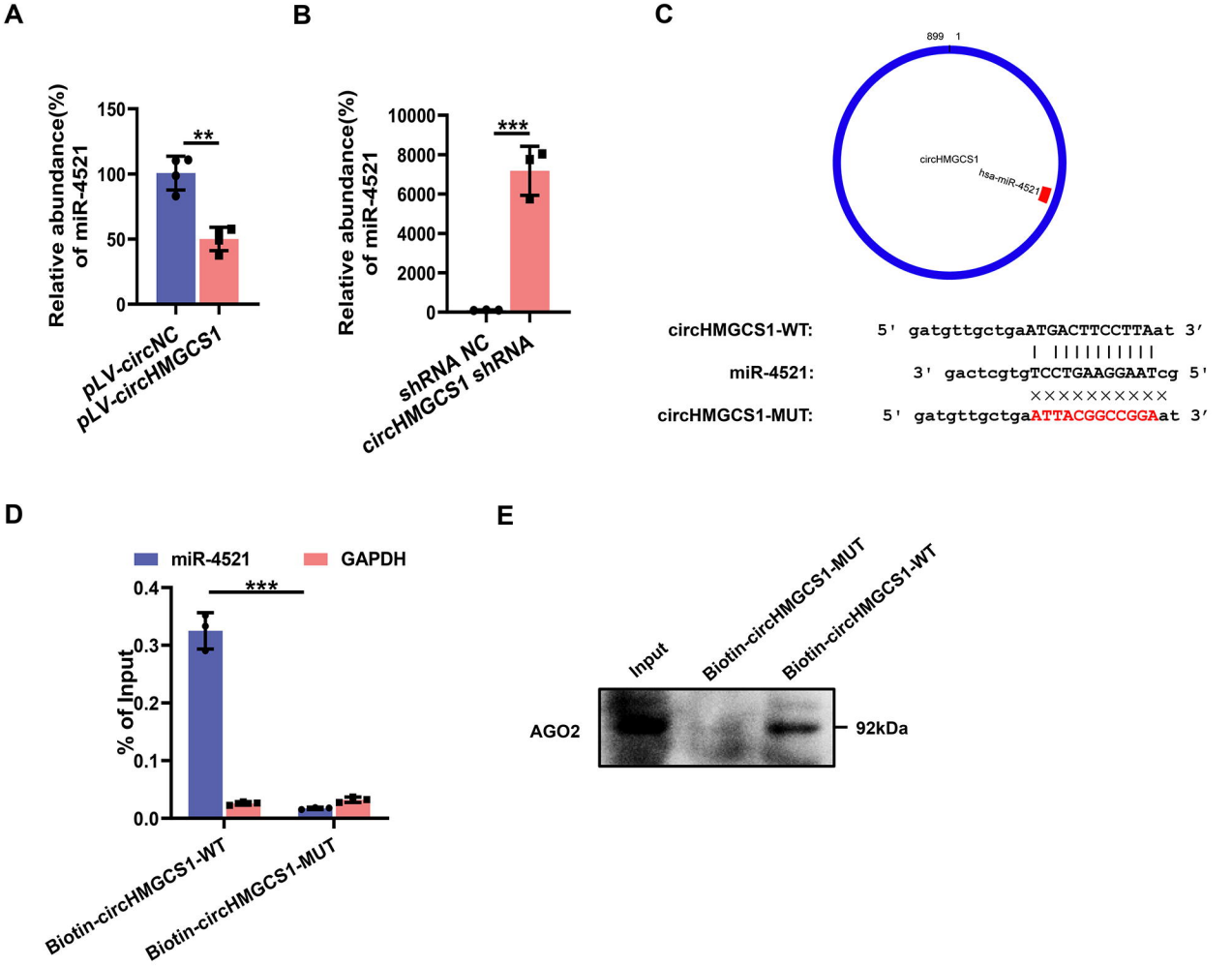
EcoRI Cyclization-mediated sequence-F

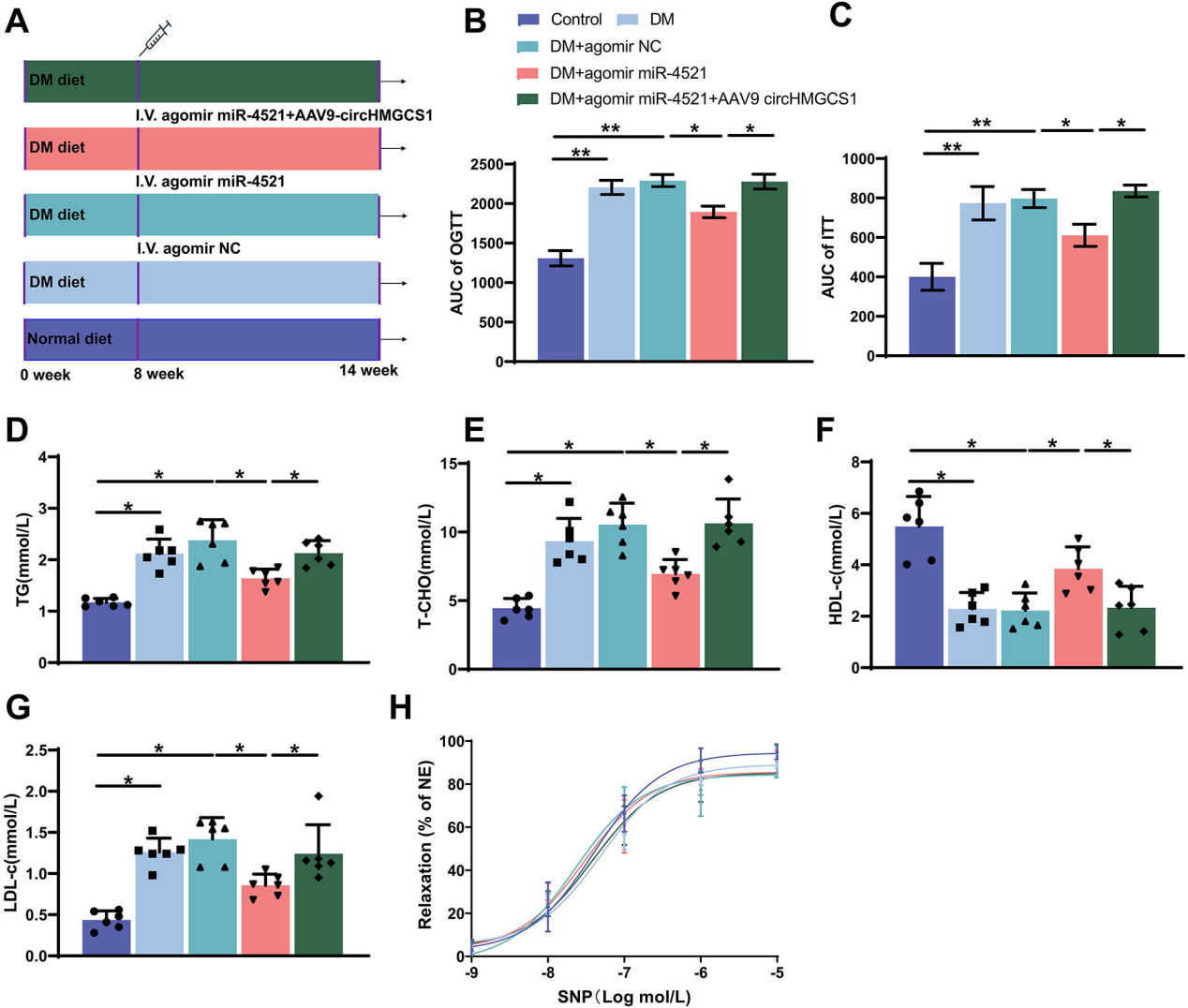
GAATTCAAAGTGCTGAGATTACAGGCGTGAGCCACCACCCCCGGCCCACTTTTTGTAAAGGTACGTACT
AATGACTTTTTTTTTATACTTCAGGGCTTCGTGGGACACATATGCAACATGCCTATGATTTTTACAAGCCT
GATATGCTATCTGAATATCCTATAGTAGATGGAAAACTCTCCATACAGTGCTACCTCAGTGCATTAGACCG
CTGCTATTCTGTCTACTGCAAAAAGATCCATGCCCAGTGGCAGAAAGAGGGAAATGATAAAGATTTTACC
TTGAATGATTTTGGCTTCATGATCTTTCACTCACCATATTGTAAACTGGTTCAGAAATCTCTAGCTCGGAT
GTTGCTGAATGACTTCCTTAATGACCAGAATAGAGATAAAAATAGTATCTATAGTGGCCTGGAAGCCTTTG
GGGATGTTAAATTAGAAGACACCTACTTTGATAGAGATGTGGAGAAGGCATTTATGAAGGCTAGCTCTGA
ACTCTTCAGTCAGAAAACAAAGGCATCTTTACTTGTATCAAATCAAAATGGAAATATGTACACATCTTCAG
TATATGGTTCCCTTGCATCTGTTCTAGCACAGTACTCACCTCAGCAATTAGCAGGGAAGAGAATTGGAGT
GTTTTCTTATGGTTCTGGTTTGGCTGCCACTCTGTACTCTCTTAAAGTCACACAAGATGCTACACCGGGG
TCTGCTCTTGATAAAATAACAGCAAGTTTATGTGATCTTAAATCAAGGCTTGATTCAAGAACTGGTGTGGC
ACCAGATGTCTTCGCTGAAAACATGAAGCTCAGAGAGGACACCCATCATTTGGTCAACTATATTCCCCAG
GGTTCAATAGATTCACTCTTTGAAGGAACGTGGTACTTAGTTAGGGTGGATGAAAAGCACAGAAGAACTT
ACGCTCGGCGTCCCACTCCAAATGATGACACTTTGGATGAAGGAGTAGGACTTGTGCATTCAAACATAG
CAACTGAGGTAAGAAGCAAGGAAAAGAATTAGGCTCGGCACGGTAGCTCACACCTGTAATCCCAGCAG
CGGCCGC

Cyclization-mediated sequence-R NotI




A
Control
miRNA NC
miRNA mimic
Relative expression
（Normalized to U6）
1500
1000
500
0
***
***
**
**
hsa-miR-98-5p
hsa-miR-3143
hsa-miR-181a-2-3p
hsa-miR-4521
B
Relative miR-4521 level
(Normalized to U6)
5000
4000
3000
2000
1000
0
***
***
No miRNA
miR-NC
miR-4521 mimic
C
Relative miR-4521 level
(Normalized to U6)
1.5
1.0
0.5
0.0
****
***
No miRNA
miR-NC inhibitor
miR-4521 inhibitor
D
Control
PAHG
miR-NC+PAHG
miR-4521-mimic+PAHG
Relative mRNA expression
(%of control)
600
400
200
0
** *
* *
** *
ET-1
ICAM1
VCAM1
E
Control
PAHG
miR-NC inhibitor+PAHG
miR-4521 inhibitor+PAHG
Relative mRNA expression
(% of Control)
2000
1500
1000
500
400
300
200
100
0
* *
* *
** *
ET-1
ICAM1
VCAM1


A
Relative abundance(%) of miR-4521
150
100
50
0
**
pLV-circNC
pLV-circHMGCS1
B
Relative abundance(%) of miR-4521
10000
8000
6000
4000
2000
0
***
shRNA NC
circHMGCS1 shRNA
C
899
1
circHMGCS1
hsa-miR-4521
circHMGCS1-WT: 5' gatgttgctgaATGACTTCCTTAat 3'
miR-4521: 3' gactcgtgTCCTGAAGGAATcg 5'
circHMGCS1-MUT: 5' gatgttgctgaATTACGGCCGGAat 3'
D
miR-4521
GAPDH
% of Input
0.4
0.3
0.2
0.1
0.0
***
Biotin-circHMGCS1-WT
Biotin-circHMGCS1-MUT
E
Input
Biotin-circHMGCS1-MUT
Biotin-circHMGCS1-WT
AGO2
92kDa


A
DM diet
I.V. agomir miR-4521+AAV9-circHMGCS1
DM diet
I.V. agomir miR-4521
DM diet
I.V. agomir NC
DM diet
Normal diet
0 week
8 week
14 week
B
Control
DM
DM+agomir NC
DM+agomir miR-4521
DM+agomir miR-4521+AAV9 circHMGCS1
AUC of OGTT
C
AUC of ITT
D
TG(mmol/L)
E
T-CHO(mmol/L)
F
HDL-c(mmol/L)
G
LDL-c(mmol/L)
H
Relaxation (% of NE)
SNP（Log mol/L)

A

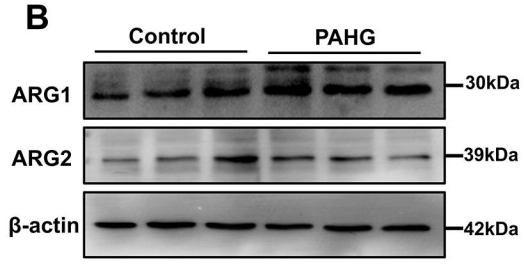
miR-4521 hARG1 3'-UTR binding site

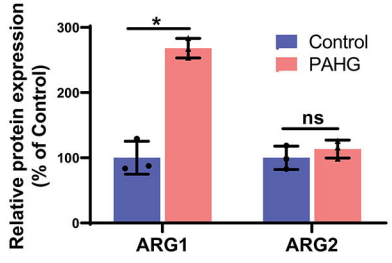
```
hsa-ARG1 wt:     5' taACTTGGCAAAAGACTTATCCTTAGa 3'
                    ||| :||| | ||||  |||||||
Hsa-miR-4521:    3' ttTGACTCGTGTCCTGA--AGGAATCg 5'
                    ××× :××× × ××××  ×××××××
hsa-ARG1 mut:    5' taTGGTGCGTATACTGGTAGGGTTTCa 3'
```

miR-4521 mARG1 3'-UTR binding site

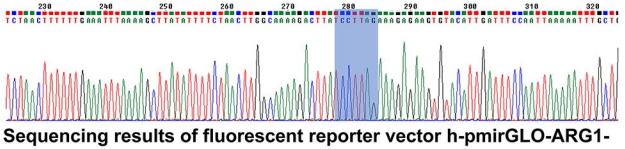
```
mmu-ARG1 wt:     5' catTTTAGCATAAGTTATCCTTAag 3'
                    | :| ||||:| | : ||||||
Hsa-miR-4521:    3' tttGACUCGUGUCCUGAAGGAAUcg 5'
                    ××× :××× × ××××  ×××××××
mmu-ARG1 mut:    5' catGGCTGGAGTCCTTGGGGAATag 3'
```

B

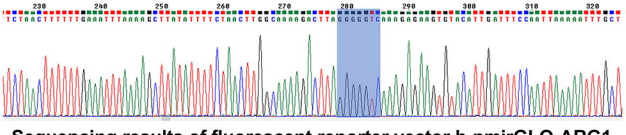




C

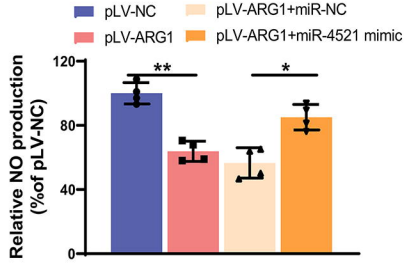


Sequencing results of fluorescent reporter vector h-pmirGLO-ARG1-3'UTR-WT

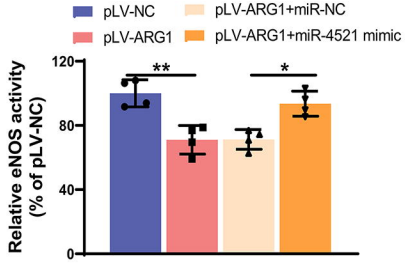


Sequencing results of fluorescent reporter vector h-pmirGLO-ARG1-3'UTR-MUT

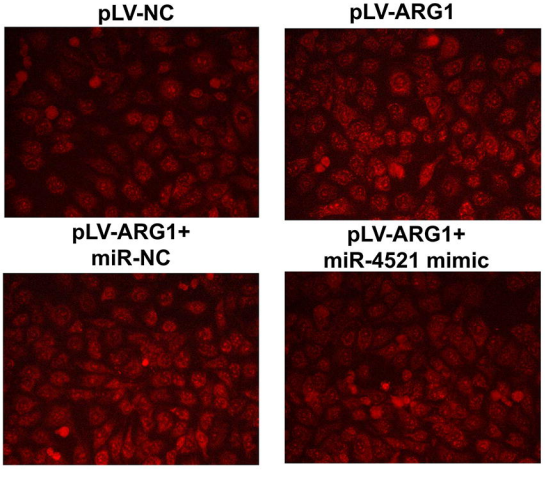
D



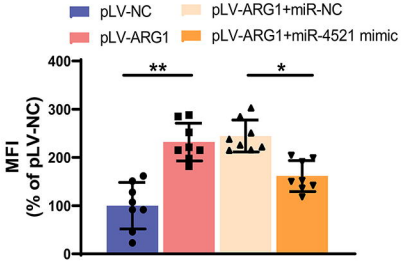
E

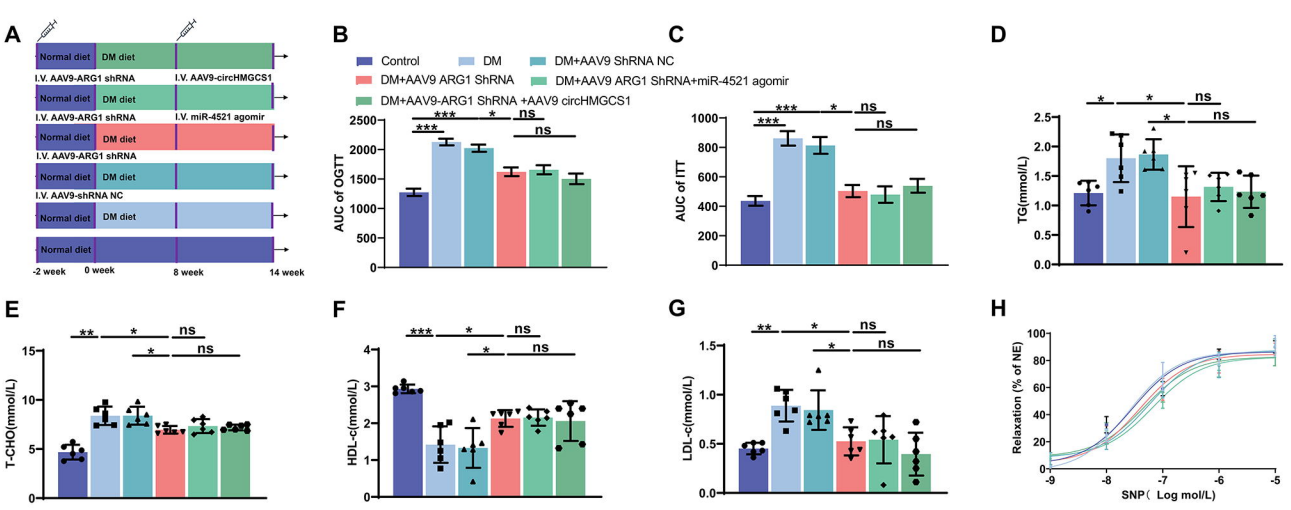


F



G




A
Normal diet
DM diet
I.V. AAV9-ARG1 shRNA
I.V. AAV9-circHMGCS1
I.V. AAV9-ARG1 shRNA
I.V. miR-4521 agomir
I.V. AAV9-ARG1 shRNA
I.V. AAV9-shRNA NC
-2 week
0 week
8 week
14 week
B
Control
DM
DM+AAV9 ShRNA NC
DM+AAV9 ARG1 ShRNA
DM+AAV9 ARG1 ShRNA+miR-4521 agomir
DM+AAV9-ARG1 ShRNA +AAV9 circHMGCS1
AUC of OGTT
C
AUC of ITT
D
TG(mmol/L)
E
T-CHO(mmol/L)
F
HDL-c(mmol/L)
G
LDL-c(mmol/L)
H
Relaxation (% of NE)
SNP（ Log mol/L)