Research Article

Hsa_circRNA_0008028 Deficiency Ameliorates High Glucose-Induced Proliferation, Calcification, and Autophagy of Vascular Smooth Muscle Cells via miR-182-5p/TRIB3 Axis

Lili Shi,¹ Yuliang Li,² Meixin Shi,³ Xiaoxue Li,³ Guopeng Li,³ Jie Cen,³ Dan Liu,⁴ Can Wei,³ and Yan Lin⁵

¹Department of Cadre Ward, The First Affiliated Hospital of Harbin Medical University, Harbin 150081, China
²Department of Anesthesiology, The Fifth Hospital of Harbin, Harbin 150081, China
³Department of Pathophysiology, Harbin Medical University, Harbin 150086, China
⁴Department of Cadre Ward, The Fourth Affiliated Hospital of Harbin Medical University, Harbin 150081, China
⁵Department of Pathophysiology, Qiqihar Medical University, Qiqihar 161006, China

Correspondence should be addressed to Can Wei; canwei528@163.com and Yan Lin; yanlinqqr.aliyun.com

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1. Introduction

Diabetes, a multifactorial metabolic disorder, features hyperglycemia caused by absolute or relative lack of insulin secretion. Its related vascular diseases, especially microvascular ones, play a major influencing role in the prognosis and mortality of diabetic patients [1]. As known to all, VSMCs serve as the main component of the middle layer of the artery and show phenotypic plasticity as they can differenti-
circRNAs, a subfamily of noncoding RNAs, with multiple functions as reported, can act as the miRNA sponge or competing endogenous RNA (ceRNA) and compete for miRNA pairing with other RNAs [5–7]. It was reported that miRNAs could exert cellular functions by regulating the translation or stability of target mRNAs like cell growth, proliferation, differentiation, metabolism, immunity, and cell death [8–11]. The precise molecular and biochemical function of the majority of circRNAs and miRNAs remains unclear, but their roles in the functional regulation of VSMCs have been widely reported [2, 12, 13]. Over these years, circRNA LRP6, circRNA-0077930, and circWDR77, as well as miR-217, miR-381-3p, and miR-132 have been found to get involved in the HG-induced proliferation and migration of VSMCs [2, 14–18]. However, it remains unclear whether has_circRNA_0008028 and mir-182-5p perform biological functions in diabetes-correlated vasculopathy.

Studies showed that TRIB3 played a critical role in the induction and maintenance of contractile phenotype in VSMCs, and its expression suppression can inhibit vascular remodeling of VSMCs [19, 20]. However, the exact regulating mechanism remains unknown. Thus, this study is aimed at further exploring the underlying roles and molecular mechanisms of has_circRNA_0008028/mir-182-5p/TRIB3 in abnormal proliferation, calcification, and autophagy of VSMCs under HG exposure, and it is expected to discover potential therapeutic targets for diabetic vascular remodeling.

2. Materials and Methods

2.1. Cell Culture, Transfection, and Treatment. Human aortic vascular smooth muscle cells (ATCC, CRL-1999) were cultured in the Dulbecco’s Modified Eagle’s Medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), penicillin (100 U/mL, Beyotime, China), and streptomycin (0.1 mg/mL, Beyotime, China) in a humidified atmosphere with 5% CO₂ at 37°C. Before the subsequent experiments, all cells were kept quiescent by starvation for 24 hours and then treated with 5.5 mmol/L D-glucose (normal glucose (NG)) or 30 mmol/L D-glucose (HG) for 48 hours. All VSMCs to be tested for calcification and osteogenic differentiation were treated with HG and coincubated with 10 mmol/L β-glycerophosphate (β-GP, Sigma-Aldrich, USA) for 12 days after siRNA transfection or overexpression [21]. VSMCs exposed to 30 mmol/L mannitol (MA, Aladdin, China) was chosen as the negative control.

For cell transfection, VSMCs were transfected with has_circRNA_0008028 and TRIB3 siRNA, miR-182-5p mimic, and miR-182-5p inhibitor, while their corresponding negative control was treated using Lipofectamine 2000 (Invitrogen, USA). The siRNA targeting has_circRNA_0008028 (F: 5′-GAUU UCCCAAGAUUCCUUUGUU, R: 5′-AACAAAGGAACUG GAAAUC) was used to knockout has_circRNA_0008028, and siRNA negative control (si-NC) was used as a control. Moreover, miR-NC, miR-182-5p mimic, and miR-182-5p inhibitors were used to upregulate or downregulate its expression. All the plasmids were provided by GenePharma (Shanghai, China). The transfection effect was detected by qRT-PCR.

2.2. Cell Viability Assay. A quantitative evaluation of cell viability was performed by a Cell Counting Kit-8 assay (CCK-8, MedChemExpress, China). VSMCs were plated into 96-well plates at a density of 2 × 10⁴ cells/well and incubated with 10 μL CCK-8 solution and incubated in the dark for 2 hours at 37°C. The absorbance at 450 nm was detected spectrophotometrically.

2.3. EdU Staining. A 5-ethyl-2′-deoxyuridine (EdU) kit (RiboBio, Guangzhou, China) was used to detect cell proliferation. VSMCs were seeded in 96-well plates, incubated with EdU (50 μmol/L) for 24 hours at 37°C, then washed with PBS, fixed in 4% formaldehyde for 10 minutes, stained with Apollo staining solution (100 μL/well) for 30 minutes, and then stained with DAPI for 10 minutes at room temperature. All images were taken with a fluorescence microscope (BX61; Olympus, Tokyo, Japan). Fields were randomly...
chosen from three dishes, three for each, at least from each group to determine the number of EdU-positive cells.

2.4. Detection of Alkaline Phosphatase (ALP) Activity. After being washed with PBS twice, VSMCs’ layers were scraped into a solution. The cell lysates were homogenized and measured for ALP activity by a commercial kit (Jian Cheng Biotechnology Institute, Nanjing, China) according to the manufacturer’s instructions. As previously described, ALP activity was normalized to the total cellular protein of the cell layers by the Bradford protein assay [22].

2.5. Dual-Luciferase Reporter Assay. The sequences of hsa_circ_0008028 and TRIB3 3′UTR, harboring the miR-182-5p seed region or a mutant sequence, were subcloned into the pmirGLO luciferase vectors (Promega, USA), referred to as WT-hsa_circ_0008028, MUT-hsa_circ_0008028, WT-TRIB3 3′UTR, or MUT-TRIB3 3′UTR, respectively. VSMCs
were cotransfected with reporter plasmids and miR-182-5p mimic or miR-NC using Lipofectamine 2000. After 48 hours of transfection, the luciferase activities were tested by the Dual-Luciferase Reporter Assay System (Promega, USA).

2.6. RNA Immunoprecipitation (RIP). RIP assays were performed by RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). VMSC lysates were incubated with RIP buffer containing magnetic beads conjugated with anti-Ago2 or anti-immunoglobulin G (IgG) antibodies. After being purified, immunoprecipitated RNA was examined by qRT-PCR.

2.7. Western Blotting. The proteins in VSMCs were extracted separately and quantified using a BCA protein assay kit (Beyotime, China). Equal amounts of protein samples were separated by SDS-PAGE and electrotransferred onto a PVDF membrane (Millipore, Schwalbach, Germany). The membranes were incubated overnight with primary antibodies (at a 1:1000 dilution, 4°C) purchased from Cell Signaling Technology (Danvers, MA, USA) and Proteintech (Wuhan, China). Subsequently, the secondary antibody (ZSGB-BIO, Beijing, China) was added to the membrane and kept for 2 hours at room temperature. An enhanced chemiluminescent (ECL) kit (HaiGene, Harbin, China) and a multiplex fluorescent imaging system (ProteinSimple, California, USA) were chosen to detect these signals. The Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, USA) was used to quantify and normalize the intensities of protein bands into GAPDH.

2.8. Quantitative Real-Time PCR (qRT-PCR). Total RNAs were extracted from VSMCs using TRIzol reagent (Thermo Fisher Scientific, USA) and reversely transcribed into cDNA using a cDNA kit (Bimake, Houston, TX, USA) according to manufacturers’ instructions. qRT-PCR was performed by SYBR Green qPCR Master Mix with an ABI PRISM® 7500 Sequence Detection System. U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was regarded as the internal reference. Relative quantification was conducted

![Figure 3](image-url)
Figure 4: Continued.
3. Results

3.1. hsa_circRNA_0008028 Is Highly Expressed in HG-Treated VSMCs. To investigate the functional role of hsa_circRNA_0008028 in VSMCs under HG conditions, qRT-PCR was used to evaluate its expression pattern. First, using mannitol (MA, 30 mmol/L) as the negative control, VSMCs were exposed to different concentrations of D-glucose (5.5, 10, 20, and 30 mmol/L). The results showed that the expression level of hsa_circRNA_0008028 rose with the increase in D-glucose concentrations but was not affected by MA stimulation (Figure 1(a)). Then, the results showed an upregulation of hsa_circRNA_0008028 expression in a time-dependent manner under D-glucose (30 mmol/L) incubation (Figure 1(b)). In the subsequent experiments, D-glucose at 30 mmol/L for 48 hours was selected as an HG condition. Thereafter, RNase R digestion was performed to verify the circular nature of hsa_circRNA_0008028. Results showed that RNase R lowered the mRNA level of GAPDH and had little effect on hsa_circRNA_0008028 expression (Figure 1(c)), suggesting that hsa_circRNA_0008028 was a circular and stable transcript resistant to treatment with RNase R digestion.

3.2. Silencing hsa_circRNA_0008028 Inhibits HG-Induced Proliferation, Calcification, and Autophagy of VSMCs. Given that hsa_circRNA_0008028 was highly expressed in HG-treated VSMCs, we knocked it down. The qRT-PCR results showed that si-hsa_circRNA_0008028 transfection significantly inhibited the HG-mediated upregulation of its expression compared with the siRNA negative control (si-NC) group (Figure 2(a)). The data of CCK-8 manifested that HG treatment enhanced the viability of cells, which was then notably reversed by knockdown of hsa_circRNA_0008028 (Figure 2(b)). Meanwhile, EdU assay and western blotting verified that si-hsa_circRNA_0008028 reduced HG-evoked cell proliferation and cyclin D1 expression in VSMCs (Figures 2(c), 2(d), and 2(f)).

To further verify the effect of hsa_circRNA_0008028 on cell calcification and osteogenic differentiation, HG-treated VSMCs were coincubated with 10 mmol/L β-glycerophosphate (β-GP) for 12 days after transfection [21]. As expected, obvious calcification, characterized by increased alkaline phosphatase (ALP) activity (Figure 2(e)) can be observed in cells in the HG group and the HG and si-NC group. Western blotting showed that HG increased the protein expression of osteogenic differentiation-related indexes including runt-related factor 2 (Runx2) and osteopontin (OPN) in VSMCs together with β-GP, whereas inhibiting the expression of SM α-actin (α-SMA) (Figure 2(f)). The above changes were notably mitigated by si-hsa_circRNA_0008028 transfection in VSMCs (Figures 2(e) and 2(f)), suggesting the inhibiting role of hsa_circRNA_0008028 silencing in the calcification and osteogenetic differentiation in HG-induced VSMCs.

Substantial evidence suggests that autophagy gets involved in the regulation of VSMCs disorder [4, 23]. We found that autophagy was activated in HG-stimulated VSMCs, shown as an increased LC3B-II/LC3B-I ratio and a decreased SQSTM1/p62 expression, which was abrogated by si-hsa_circRNA_0008028 (Figure 2(g)). To sum up, the above results showed that hsa_circRNA_0008028 interference could
inhibit HG-induced proliferation, calcification, and autophagy of VSMCs.

3.3. *hsa_circRNA_0008028* Binds to *miR-182-5p*. It was reported that circRNAs could regulate gene expression by binding to miRNAs as a sponge [24]. The starBase software was used to predict the potential binding sites between *hsa_circRNA_0008028* and miR-182-5p (Figure 3(a)), which was later confirmed by dual luciferase reporter assay, as miR-182-5p overexpression markedly weakened the luciferase activity of wild-type (WT) *hsa_circ_0008028* reporter rather than the mutated (Mut) *hsa_circ_0008028* reporter (Figure 3(b)). RIP assay also verified the mutual effect of *hsa_circ_0008028* and miR-182-5p at the endogenous level. Results showed that, compared to the control anti-IgG group (Figure 3(c)), *hsa_circRNA_0008028* and miR-182-5p were enriched in the anti-Ago2 (a core component of the RNA-induced silencing complex) group. Moreover, the qRT-PCR results showed that miR-182-5p expression declined in HG-induced VSMCs compared to the NG group. The inhibitory effect of HG on the miR-182-5p expression could be reversed by si-*hsa_circRNA_0008028* transfection and further promoted by the overexpression of *hsa_circRNA_0008028* (Figure 3(d)). Altogether, *hsa_circRNA_0008028* served as a miR-182-5p sponge and negatively regulated its expression.

3.4. *miR-182-5p* Inhibition Reverses the Effect of *hsa_circRNA_0008028* Silence on HG-Stimulated VSMCs. As shown in Figure 4(a), the expression of miR-182-5p was significantly inhibited when transfected with miR-182-5p-inhibitor compared to the miR-NC group (Figure 4(a)). Subsequently, HG-stimulated VSMCs were transfected with si-*hsa_circRNA_0008028* and/or miR-182-5p inhibitor. The miR-182-5p inhibitor also eliminated the si-*hsa_circRNA_0008028* induced upregulation of miR-182-5p in HG-treated VSMCs (Figure 4(b)). Moreover, the suppressive effects of si-*hsa_circRNA_0008028* on cell viability (Figure 4(c)), cell proliferation and cell cycle regulation (Figures 4(d) and 4(e)), ALP activity (Figure 4(f)), and osteoblast differentiation (Figure 4(g)) and autophagy (Figure 4(h)) in HG-treated VSMCs were all reversed by

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miR-182-5p inhibitor. The above evidence proved that hsa_circRNA_0008028 regulated HG-induced VSMC injury via miR-182-5p.

3.5. TRIB3 Is a Downstream Target of miR-182-5p. Next, we further searched the downstream targets of miR-182-5p and predicted the binding sites between miR-182-5p and tribble 3 (TRIB3) 3′UTR region through TargetScan (Figure 5(a)). It was observed that the cotransfection of WT-TRIB3 3′UTR with miR-182-5p could significantly diminish the luciferase activity compared with the miR-NC group in VSMCs, while this inhibitory effect could be blocked in MUT-TRIB3 3′UTR transfected cells (Figure 5(b)). RIP assay also revealed that miR-182-5p and TRIB3 were specifically enriched in anti-Ago2 relative to the control anti-IgG group (Figure 5(c)), suggesting that TRIB3 served as a target of miR-182-5p. Moreover, the mRNA and protein expression of TRIB3 increased in the HG group compared with the TRIB3

**Figure 6:** TRIB3 knockdown blocks the side effects of HG on VSMCs. VSMCs were transfected with TRIB3 siRNA upon HG condition. (a) TRIB3 protein expression detected by western blotting. (b) Cell viability detection using CCK-8. (c) Cell proliferation observation by EdU staining. (d) Percentage of EdU positive cells. (e) ALP activity. (f) Western blotting detection of cell cycle and osteogenic differentiation-related protein expression. (g) Western blotting examination of autophagy-related protein expression. **p < 0.05 vs. the NG group; $^*$p < 0.05 vs. the HG+si-NC group.
Figure 7: Continued.
3.6. TRIB3 Knockdown Blocks the Side Effects of HG on VSMCs. To explore the function of TRIB3 in HG-treated VSMCs, siRNA was used to interfere with TRIB3. The data verified that the HG condition promoted TRIB3 mRNA and protein expression, which was recovered and downregulated by si-TRIB3 transfection (Figure 6(a)). Subsequent results confirmed that interfering with TRIB3 could restore the accelerating effects of HG on VSMCs, including cell viability (Figure 6(b)), cell proliferation (Figures 6(c) and 6(d)), ALP activity (Figure 6(e)), osteoblast differentiation-related protein (Runx2, OPN, and α-SMA) expressions (Figure 6(f)), and autophagy-related protein (LC3B-II/LC3B-I and SQSTM1/p62) expressions (Figure 6(g)). Taken together, the knockout of TRIB3 weakened the HG-induced proliferation, calcification, and autophagy of VSMCs.

3.7. hsa_circRNA_0008028/miR-182-5p Regulates HG-Induced VSMC Dysfunctions through TRIB3. Ultimately, to identify the interactive associations among hsa_circRNA_0008028, miR-182-5p, and TRIB3 in VSMC proliferation, calcification, and autophagy, miR-182-5p inhibitor and/or si-hsa_circRNA_0008028 were used to transfect VSMCs. The TRIB3 levels were then detected by qRT-PCR and western blotting. The data showed that, compared to the NG group, TRIB3 expression level was increased when exposed to HG condition and was further promoted by miR-182-5p inhibitor but decreased by si-hsa_circRNA_0008028. At the same time, the effect of si-hsa_circRNA_0008028 on TRIB3 expression in HG-treated VSMCs was abolished by miR-182-5p inhibitor cotransfection (Figures 7(a) and 7(b)). These results indicated that TRIB3 was regulated by hsa_circRNA_0008028/miR-182-5p axis.

Next, it was observed that miR-182-5p inhibitor enhanced cell proliferation and calcification, such as the upregulation of cell viability, ALP activity, and the protein expression of cyclin D1, Runx2, and α-SMA, and decreased SQSTM1/p62 induced by HG were strengthened by miR-182-5p inhibitor but weakened by si-hsa_circRNA_0008028 (Figure 7(h)). Moreover, the increased LC3B-II/LC3B-I and the decreased SQSTM1/p62 induced by HG were strengthened by miR-182-5p inhibitor but weakened by si-hsa_circRNA_0008028 (Figures 7(c)–7(g)). Thus, the results suggested that hsa_circRNA_0008028 knockdown attenuated HG-induced VSMC dysfunctions targeting miR-182-5p partly via TRIB3.

4. Discussion

In this study, it was revealed that HG could induce proliferation, calcification, and autophagy of VSMCs and identified that these effects were reversed by hsa_circRNA_0008028 deficiency or miR-182-5p overexpression. Mechanistically, it was first found that hsa_circRNA_0008028 could enhance the expression of TRIB3, a target gene of miR-182-5p as a miR-182-5p sponge. These findings provide new insight into noncoding RNAs in diabetic VSMC proliferation, calcification, and autophagy and indicate that modulation of the activity of noncoding RNAs, such as hsa_circRNA_0008028 and miR-182-5p, may become a novel therapeutic approach for diabetes-related vasculopathy.

Dysfunctions of VSMCs for the metabolism and phenotypic transformation are associated with the progression of
vascular diseases, such as diabetes, pulmonary hypertension, and atherosclerosis [25]. In response to vascular injury, VSMCs dedifferentiate from a “contractile” phenotype to a “synthetic” phenotype, which features proliferation, migration, extracellular matrix (ECM) component production, and reduction of the VSMC-specific markers [25–28]. It was reported that hyperglycemia plays an important role in the pathogenesis of diabetes-related vascular diseases, including vascular calcification and atherosclerosis [29]. HG could promote proliferation and migration, induce oxidative stress and inflammation of VSMCs, and speed up the formation and accumulation of advanced glycation end products (AGEs), leading to vascular calcification [30–32]. In this study, EdU staining, as well as the increased cell viability, ALP activity, cell cycle, and osteogenic differentiation-related protein expression verified that VSMCs cultured in HG occurred in serious proliferation and calcification. Moreover, the upregulated LC3B-II/LC3B-I ratio and downregulated SQSTM1/p62 expression suggested that HG promoted the autophagy process of VSMCs. All in all, the data demonstrated that HG could promote the proliferation, calcification, and autophagy of VSMCs.

circRNAs, a type of noncoding RNAs without 3’- and 5’-ends, have been suggested to be a miRNA sponge to offset miRNA-mediated mRNA degradation, or a ceRNA to network with mRNA downstream of miRNA to regulate diverse biological processes [15, 33]. Recent evidence showed that some circRNAs and miRNAs got involved in the dysfunctions of VSMCs in HG conditions. For example, circRNA LRP6 promoted HG-induced phenotypic transformation of VSMCs by regulating the miR-545-3p/HMGA1 axis [2]. CircWDR77 silencing blocked the proliferation and migration of VSMCs by downregulating FGF2 expression via sponging miR-124 [15]. Besides, previous studies called that miR-504 promoted HG-induced VSMC dysfunction, and miR-145 protected against HG-induced VSMCs by suppressing ROCK1 [34, 35]. However, there are rare reports on the effect of hsa_circRNA_0008028 and its target miR-182-5p in the regulation of VSMC functions. In this study, it was found a circRNA, hsa_circRNA_0008028, was extensively expressed in HG-treated VSMCs, while its silencing induced by special siRNAs resulted in a suppression of VSMC proliferation, calcification, and autophagy upon HG stimulation. Then, it was verified that miR-182-5p was a direct target of hsa_circRNA_0008028, and its inhibition could further promote VSMCs’ dysfunction and weaken the protective effects of si- has_circRNA_0008028 on VSMCs exposed to HG. All in all, this study verified that hsa_circRNA_0008028 could sponge miR-182-5p in HG-treated VSMCs and its function in diabetic vasculopathy was associated with the regulation of miR-182-5p expression.

As widely believed, miRNAs perform their functions by directly binding to their target mRNAs [36]. In this study, TRIB3, a 45 kDa pseudokinase identified as a target of miR-182-5p, was demonstrated to regulate metabolism and insulin signaling in diabetes-injured tissues, including liver, adipose tissue, heart, and skeletal muscle, and to be induced in a variety of cell types under different conditions of stress, including ER stress, nutrient deprivation, and oxidative stress [37–39]. Studies have shown that inhibiting the expression of TRIB3 can reduce vascular remodeling of VSMCs, but its regulating mechanism is still not very clear [19]. It was demonstrated that TRIB3 was a downstream target of miR-182-5p and significantly upregulated in VSMCs under HG conditions, and its knockdown reduced HG-induced proliferation, calcification, and autophagy of VSMCs. Moreover, its expression level and effects on HG-treated VSMCs were repressed by hsa_circRNA_0008028 knockout, which was offset by miR-182-5p inhibitor; that is, the regulatory role of hsa_circRNA_0008028/miR-182-5p in proliferation, calcification, and autophagy of VSMCs under HG condition was possibly achieved through TRIB3.

5. Conclusions
In conclusion, hsa_circRNA_0008028 was extensively expressed in HG-treated VSMCs in vitro, which can serve as the sponge of miR-182-5p and increase the expression of TRIB3, thus promoting HG-mediated proliferation, calcification, and autophagy of VSMCs. hsa_circRNA_0008028 is expected to be a molecular target for the diagnosis and treatment of diabetes-related vascular diseases.

Data Availability
The data used to support the findings of this study are included within the article.

Conflicts of Interest
The authors declare that they have no competing interests.

Authors’ Contributions
Can Wei and Lili Shi designed the research and drafted the manuscript. Yuliang Li and Yan Lin processed the data curation. Meixin Shi, Xiaoxue Li, Guopeng Li, and Jie Cen completed the experiment. All authors read and approved the final manuscript. Lili Shi, Yuliang Li, and Meixin Shi contributed equally to this work.

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Supplementary Materials
Table S1 shows the sequences of primers for qRT-PCR.

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