Diabetic retinopathy (DR) is a major complication of diabetes mellitus. It still remains a leading cause of decreased vision and blindness globally. The pathogenesis of DR has been extensively studied for many years. Retinal microvascular dysfunction (including retinal nonperfusion, vasopermeability, and retinal neovascularization) and neurodegeneration (including neuronal apoptosis and glial dysfunction) has been implicated in the pathogenesis of DR.

Clinical DR can be identified according to retinal changes in diabetes, including nonproliferative DR without manifest visual loss and vision-threatening complications, including macular edema and proliferative retinopathy. Current methods for DR treatment mainly include vitreous surgical techniques, panretinal photocoagulation, and anti-VEGF drugs such as bevacizumab. However, the prognosis for DR patients is still poor, especially for those with advanced PDR at the proliferative stage. Thus, it is necessary to develop new diagnosis and treatment techniques based on DR pathogenesis.

Circular RNAs are a novel class of RNA transcripts characterized by the covalent bond linking the 3’ and 5’ ends. CircRNAs regulate gene expression by acting as miRNA sponges, RNA-binding protein sequestering agents, or nuclear transcriptional regulators. Increasing studies have revealed that altered circRNA expressions are found in several cancers and cardiovascular diseases. However, the expression profile and clinical significance of circRNAs in DR is unknown.

In this study, we performed circular RNA microarray to investigate differential expression profile of circRNAs between diabetic retinas and non-diabetic retinas. We identified 529 differentially expressed circRNAs, including 356 upregulated circRNAs and 173 downregulated circRNAs compared with non-diabetic controls. And then, we characterized one circular RNA derived from HAS2 gene locus (circ_0005015), which was significantly upregulated in diabetic retinas. We showed that circ_0005015 expression was significantly upregulated in the vitreous sample, plasma fraction of whole blood, preretinal fibrovascular membranes (FVMs) of DR patients. Functional assays revealed that circ_0005015 could regulate retinal endothelial cell function by acting as miRNA sponge.
Circ_0005015 may serve as a novel potential biomarker for the prognosis, diagnosis, or treatment of DR.

**MATERIAL AND METHODS**

**RNA Extraction**

The total RNA from retina, proliferative membrane, blood, or vitreous sample was extracted using TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer’s instruction. The concentration of total RNAs was determined by a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA, USA). The integrity of total RNAs was assessed by 1% agarose gel electrophoresis.

**Sample Preparation and Microarray Analysis**

Three nondiabetic and two diabetic human retinas were collected and used for circRNA expression profile by a commercial microarray (4 × 180K, Human Circular RNA Microarray; Shanghai Biotechnology Co. (SBC), Ltd., Shanghai, China) with a total of 88,371 probes. The total RNAs were treated with RNase R to remove linear RNA and enrich circRNAs. The enriched circRNAs were amplified and transcribed into fluorescent cRNA, and then purified using an RNA extraction kit (RNasey Mini Kit; Qiagen, Hilden, Germany). The Cy3-labeled cRNAs were hybridized onto the commercial Microarray (SBC, Ltd.) using a commercial kit (Gene Expression Hybridization Kit; Agilent Technologies, Santa Clara, CA, USA) for 17 hours at 65°C. After washing the slides, the arrays were scanned by a microarray scanner (Agilent Technologies).

**Circular RNA Microarray Data Analysis**

Scanned images were extracted with feature extraction software (Agilent Technologies). A series of data processing, including quantile normalization, was performed using the R software package. Differentially expressed circRNAs with statistical significance (Fold changes > 2.0; P-values < 0.05) were identified through volcano plot filtering. Hierarchical clustering was performed to display circRNA expression pattern across different samples.

**Cell Culture and Transfection**

Primary cultures of human retinal vascular endothelial cells (HRVECs) were obtained from at least three donors. They were prepared and maintained in Dulbecco’s modified Eagle’s medium (5.5 mM glucose; Gibco, Gaithersburg, MD, USA) containing 5% (vol/vol) fetal bovine serum; 1% (vol/vol) penicillin; and endothelial cell growth supplement (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C under humidified conditions of 5% CO2 and 95% air. The identity of HRVECs was validated by demonstrating endothelial cell incorporation of fluorescence-labeled acetylated LDL. HRVECs in passages 3 to 6 were used in this study. They were transfected at approximately 80% confluence using the synthesized small interfering RNAs (siRNAs) 100 nmol/L; Sigma-Aldrich Corp.) by transfection reagent (Lipofectamine RNAiMax; Life Technologies) according to the manufacturer’s protocol. The siRNA target sequence for hsa_circ_0005015 was “ATGTACAGATGCATTGTGAGA.”

**Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

Total RNAs were reversely transcribed using a reagent kit (PrimeScript RT; TaKaRa). Quantitative (q)PCR assays were performed using commercial premix (SYBR Premix Ex Taq; TaKaRa) in the sequence detection system (ABI Prism 7300; Applied Biosystems, Foster City, CA, USA). qPCRs were conducted in duplicate for each sample, and the specificity of PCR product was estimated using the dissociation curve. CircRNA quantification was conducted using the divergent primers designed based on the backsplice junction sequence of circular RNAs.

**Bioinformatics Analysis**

To predict the potential role of dysregulated CircRNAs, their host genes were input into the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) for the annotation and functional analysis, including gene set enrichment analysis and mapping gene sets to KEGG pathway. Specific binding patterns between miRNAs and circRNAs were analyzed using the bioinformatic approach defining MREs of differentially expressed circRNAs based on the miRana and TargetScan algorithm.

**Cell Proliferation Assay**

Cell proliferation was determined by ki67 immunofluorescence staining. After the required treatment for 48 hours, HRVECs were fixed in 4% formaldehyde for 10 minutes, then blocked with 5% BSA for 1 hour. They were incubated with ki67 antibody (dilution: 1:200; Abcam) overnight at 4°C. After washing, they were incubated with Cy3-conjugated secondary antibody (Life Technologies). The slides were mounted using the anti-fade medium containing DAPI.

**Cell Migration Assay**

HRVEC migration was detected by the 12-well Transwell units. The undersides of insert membranes were coated with fibronectin (10 µg/mL) for 24 hours at 4°C and blocked with 1% BSA for 1 hour at 37°C. After the required treatment, HRVECs were added to the upper compartment of Transwell unit, and allowed for migrating for 12 hours at 37°C. These non-migrated cells were scraped off. The migrated cells on the bottom surface were stained with the crystal violet.

**Tube Formation Assay**

After the required treatment, HRVECs were seeded on the growth factor-reduced Matrigel (BD Biosciences) in a 24-well plate. After a 24-hour culture, the tube-like networks were observed and calculated.

**Spheroid Sprouting Assay**

HRVECs were seeded on the growth factor-reduced Matrigel for 24 hours. In vitro angiogenesis was quantified by calculating the cumulative length of the sprouts, the number of sprouts, and the number of branch points that had grown out of each spheroid by using digital imaging software.

**Statistical analysis**

Circular RNA expression data were expressed as mean ± SEM. The significant difference was determined by Student t-test (when two groups were compared) or 1-way ANOVA to test the effect of group (when >2 groups were compared). P < 0.05 was considered statistically significant.
FIGURE 1. Identification of differentially expressed circRNAs between diabetic retina and nondiabetic retina. (A) The box plot shows the circRNA expression distribution. After normalization, the distributions of log2 ratios across different samples were shown. The box plot consists of boxes with a central line and two tails. The central line represents the median of the data, whereas the tails represent the upper and lower quartiles. (B) The volcano plot shows the differentially expressed circRNAs between nondiabetic and diabetic retinas. (C) Heatmaps were generated through hierarchical cluster analysis to show the top 10 upregulated and top 10 downregulated circRNAs between nondiabetic retinas and diabetic retinas. The color scale shows the relative expression levels of circRNAs across different samples. Red denotes the expression level greater than 0, and green denotes the expression level less than 0.
RESULTS

Identification of Differentially Expressed CircRNAs Between Diabetic Retina and Non-Diabetic Retina

High-throughput circRNA microarray was used to detect the differences of circRNA expression profiles between diabetic retinas and nondiabetic human retinas. The box plot showed the distribution of circRNA expression profiles. After normalization, the distributions of log2 ratios between nondiabetic and diabetic retinas were shown in Figure 1A. Differentially expressed circRNAs with statistical significance were identified through volcano plot filtering (Fig. 1B). We set the threshold as a fold-change greater than 2.0, and identified 529 differentially expressed circRNAs, including 356 upregulated circRNAs and 173 downregulated circRNAs (Supplementary Table S1, diabetic versus nondiabetic). We also conducted hierarchical clustering analysis to obtain systematic comparison of circRNAs expression between nondiabetic retinas and diabetic retinas using the top 10 upregulated circRNAs and top 10 downregulated circRNAs. The diabetic samples were clustered into the one branch, whereas the nondiabetic samples were clustered into the other branch (Fig. 1C).

To verify the results of microarray data, we randomly selected 16 dysregulated circRNAs, including 8 upregulated circRNAs and 8 downregulated circRNAs for expression verification using the samples for microarray analysis. We found a general consistency between qRT-PCR results and microarray results (Table 1).

GO Analysis and Pathway Analysis of the Host Genes of Differentially Expressed CircRNAs

CircRNAs are primarily generated from the exons or introns of their parental genes. Several circRNAs can regulate the expression of their host genes.13 We thus predicted the role of circRNAs through GO analysis and pathway analysis of the host genes of circRNAs. We showed that the significant enriched GO term in biologic process was ATP binding (Fig. 2A). The most significant enriched GO term in cellular component was extracellular exosome (Fig. 2B). The most significant enriched GO term in molecular function was intracellular signal transduction (Fig. 2C). Pathway analysis showed that 10 signaling pathways were potentially involved in circRNA-mediated regulatory network. Of them, PI3K-Akt signaling pathway was ranked the top 1 (Fig. 2D).

<table>
<thead>
<tr>
<th>CircRNA Name</th>
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<th>qRT-PCR</th>
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</thead>
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Table 1. Verification of Microarray Data by qRT-PCRs

(A–C) GO enrichment analysis provides a controlled vocabulary to describe the differentially expressed circRNAs. The ontology covers three domains: biologic process (A), cellular component (B), and molecular function (C). P < 0.05 is recommended. (D) The bar plot shows the result of pathway analysis, which shows the top 10 signaling pathways potentially involved in circRNAs-mediated regulatory network.
Recent studies have shown that some circRNAs could act as miRNA sponges to regulate gene expression.\textsuperscript{14} We predicted circRNA/miRNA interaction based on TargetScan and miRNA. We determined whether the top 10 upregulated circRNAs and the top 10 downregulated circRNAs could bind to miRNAs. The results showed that 17 out of 20 circRNAs could potentially bind to five different miRNAs (Table 2), suggesting that most of these circRNAs could regulate gene expression as acting as miRNA sponges.

**Table 2. Identification of Differentially Expressed CircRNAs-Binding MiRNAs**

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<th>CircRNA</th>
<th>Gene Symbol</th>
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**MiRNA Response Elements Analysis of Dysregulated circRNAs in Diabetic Retinas**

Recent studies have shown that some circRNAs could act as miRNA sponges to regulate mRNA expression.\textsuperscript{14} We predicted circRNA/miRNA interaction based on TargetScan and miRNA. We determined whether the top 10 upregulated circRNAs and the top 10 downregulated circRNAs could bind to miRNAs. The results showed that out of 20 circRNAs could potentially bind to five different miRNAs (Table 2), suggesting that most of these circRNAs could regulate gene expression as acting as miRNA sponges.

**Circ_0005015 Is Shown as a Potential Biomarker for Diabetic Retinopathy**

In this study, hsa_circ_0005015 expression was shown to be significantly upregulated in diabetic retinas. We evaluated the diagnostic value of this circRNA. Circ_0005015 expression in the FVMs of DR patients was significantly higher than that in the idiopathic epiretinal membrane of nondiabetic patients (Fig. 3A, Supplementary Table S2). We then determined whether circulating circ_0005015 is specifically upregulated in the patients with DR. We collected the whole blood from DR patients, but not in other individuals (Fig. 3B, Supplementary Table S3).

The vitreous body is a gel-like extracellular matrix that undergoes liquefaction during aging and pathologic processes.\textsuperscript{15} We collected the vitreous sample from the eyes of 20 DR patients and 15 nondiabetic patients. Vitreous level of hsa_circ_0005015 was significantly upregulated in DR patients (Fig. 3C, Supplementary Table S4). Since circ_0005015 is upregulated in diabetic retina, we investigated its role in retinal endothelial cells. Circ_0005015 siRNA transfection significantly down-regulated circ_0005015 expression at 48 h post-transfection (Fig. 4A). K67 staining showed that circ_0005015 silencing significantly decreased HRVEC proliferation at 48 hours posttransfection (Fig. 4B). Transwell and matrigel tube formation assays revealed that circ_0005015 silencing significantly inhibited the migration and tube formation of HRVECs at 24 hours posttransfection (Figs. 4C, 4D). SiRNA-mediated silencing of circ_0005015 also significantly inhibited spheroid sprouting at 24 hours posttransfection (Fig. 4E). Taken together, this evidence suggests that circ_0005015 is potentially involved in DR pathogenesis via regulating HRVEC function.

**Circ_0005015 Regulate Retinal Endothelial Cell Function by Acting as MiRNA Sponge In Vitro**

Bioinformatics analysis showed that circ_0005015 might act as a miRNA sponge to regulate gene expression. The full sequence of circ_0005015 was inserted into pGL3 luciferase reporter to generate LUC-circ_0005015 vector. Luciferase activity assays showed that miR-519d-3p—but not miR-6505–3p, miR-548an, miR-498, or miR-758—mimicked transfection and decreased the activity of LUC-circ_0005015 after 48 hours transfection (Fig. 5A). MiR-519d-3p has been reported to play important roles in cell growth, proliferation, and migration by targeting matrix metalloproteinase (MMP)-2, STAT3, or XIAP gene.\textsuperscript{17–19} MiR-519d-3p mimic transfection downregulated MMP-2, STAT3, and XIAP expression in HRVECs at 48 hours transfection (Fig. 5B). MiR-519d-3p mimic transfection significantly reduced HRVEC proliferation at 48 hours posttransfection, and inhibited the migration and tube formation of HRVECs in vitro at 24 hours posttransfection (Figs. 5C, 5D, 5E), suggesting that miR-519d-3p overexpression could mimic the effect of circ_0005015 silencing on HRVEC function.
DISCUSSION

Diabetic retinopathy is the most frequently occurring complication of diabetes mellitus. It leads to retinal microvascular dysfunction and neurodegeneration. Although great progress has been made, the precise mechanisms underlying DR pathogenesis are still not fully understood. We compared circRNA expression difference between diabetic and nondiabetic retinas by microarray analysis. A total of 529 differentially expressed circRNAs were identified in diabetic retinas. Of them, circ_0005015 was significantly upregulated in the plasma fraction, vitreous sample, and FVMs of DR patients, suggesting a promising potential for the prognosis and diagnosis of DR.

CircRNAs play their roles via multiple molecular mechanisms, such as RNA-binding protein sequestering agents or nuclear transcriptional regulators of host genes, or acting as miRNA sponges. Thus, it is possible to predict the function of some circRNAs via their host gene function. GO enrichment analysis shows that the host genes of dysregulated circRNAs are involved in the regulation of biologic process, cellular component, and molecular function. Among the GO terms in this study, ATP binding is associated with energy metabolism. DR is actually a type of metabolic disease. Thus, dysregulation of circRNA-mediated metabolic signal is involved in DR pathogenesis. Exosomes are present in nearly all body fluids, including blood, urine, and cultured medium of cell cultures. They are RNA and protein-containing small vesicles, which can transfer molecules from one cell to another cell via membrane vesicle trafficking. GO-mediated signaling mainly occurs in exosomes, which is consistent with the result of bioinformatic analysis that the most significant enriched GO term in cellular component is extracellular exosome. Pathway analysis shows that PI3K-Akt signaling pathway is potentially involved in circRNA-mediated regulatory network. Notably, PI3K-Akt signaling has been reported to be involved in the pathogenesis of DR. Based on the above-mentioned evidences, we conclude that circRNA-mediated regulatory network is involved in the pathogenesis of DR.

CircRNAs also act as miRNA sponges or competitive endogenous RNA molecules. Bioinformatics analysis shows that 17 of 20 differentially expressed circRNAs contain more than five different miRNA binding sites. CircRNAs and MiRNAs constitute competing endogenous RNA (ceRNA) regulatory network. CeRNA network is a complex posttranscriptional network mediated by miRNAs. The members, including protein-coding mRNAs and noncoding RNAs, share one or more miRNA response elements (MREs). They compete for binding to miRNAs and then adjust each other’s expression. miRNAs play important roles in many biologic processes, including developmental timing, growth control, and differentiation. The roles of miRNAs in DR have been gradually accepted. CircRNAs compete with endogenous miRNAs by direct binding, resulting in increased levels of miRNA target genes. Thus, it is not surprising that circRNA-miRNA-mRNA network is potentially involved in DR-related processes.

CircRNA/miRNA interaction prediction based on TargetScan and miRanda reveals that hsa_circ_0014796, hsa_circ_0027926, and hsa_circ_0021529 does not bind to any miRNAs. Bioinformatics analysis reveals that they potentially bind to RBP proteins, such as EIF4A3, Fus, and HuR. This evidence implies that these circRNAs may act as transcriptional regulators, and regulate the expression of host-gene via interacting RBP proteins in the promoter region of the host gene, providing another example that circular RNAs participate in the regulatory networks governing gene expression.

Preretinal FVMs form as a sequela to PDR, which are characterized by the migration and proliferation of various
FIGURE 4. Circ_0005015 regulates retinal endothelial cell function in vitro. (A) HRVECs were transfected with scrambled (Scr) siRNA, the siRNA targeting the backsplice sequence of circ_0005015, or left untreated (Ctrl) for 48 hours. qRT-PCRs were conducted to detect circ_0005015 expression ($n = 4$, $^*P < 0.05$ versus Ctrl group). (B) Cell proliferation was detected using Ki67 staining ($n = 4$, $^*P < 0.05$ versus Ctrl group). Scale bar: 20 μm. (C) Transwell assay and quantification analysis was conducted to detect HRVEC migration ($n = 4$, $^*P < 0.05$ versus Ctrl group). Scale bar: 20 μm. (D) HRVECs were seeded on the matrigel matrix. The tube-like structures were observed 24 hours after cell seeding. Average length of tube formation for each field was statistically analyzed ($n = 4$, $^*P < 0.05$ versus Ctrl group). Scale bar: 50 μm. (E) Spheroid sprouting assays were conducted by measuring the cumulative length of all sprouts of each spheroid. Ten spheroids were analyzed and averaged for each experiment. Scale bar: 20 μm ($^*P < 0.05$ versus Ctrl group). The significant difference was evaluated by 1-way ANOVA followed by post hoc Bonferroni’s test comparison test.
FIGURE 5. Circ_0005015 regulate retinal endothelial cell function by acting as miRNA sponge in vitro. (A) The entire circ_0005015 sequence was cloned into the pGL3 Luciferase reporter to construct LUC-circ_0005015 vector. HRVECs were cotransfected LUC-circ_0005015 with different miRNA mimics. Luciferase activity was detected by the dual luciferase assay at 48 hours posttransfection (n = 4, *P < 0.05 versus Ctrl group). (B) HRVECs were transfected scramble (Scr) siRNA and miR-519d-3p mimic for 48 hours. qRT-PCR assays were conducted to detect the expression of MMP-2, STAT3, and XIAP (n = 4, *P < 0.05 versus Ctrl group). (C) HRVECs were transfected with Scr mimic, miR-519d-3p mimic, or left untreated (Ctrl) for 48 hours. Cell proliferation was detected by using Ki67 staining (n = 4, *P < 0.05 versus Ctrl group). Scale bar: 20 μm. (D) Transwell assay and quantification analysis was performed to detect HRVEC migration (n = 4, *P < 0.05 versus Ctrl group). Scale bar: 20 μm. (E) HRVECs were seeded on the matrigel matrix. The tube-like structures were observed 24 hours after cell seeding. Average length of tube formation for each field was statistically analyzed (n = 4, *P < 0.05 versus Ctrl group). Scale bar: 50 μm. The significant difference was evaluated by 1-way ANOVA followed by post hoc Bonferroni’s test comparison test.
types of cells, including glial cells, macrophages, laminocytes, fibroblasts, and endothelial cells.\textsuperscript{26} Circ_0005015 is upregulated in the FVMs of DR patients. Its silencing could affect retinal endothelial cell proliferation, migration, and tube formation. Thus, circ_0005015 upregulation could facilitate endothelial angiogenic function during FVM formation.

Body fluids are the commonly used materials in the diagnosis of human diseases.\textsuperscript{27–29} CircRNAs are stable molecules and have very long half lives in cells. They are readily detectable but the corresponding linear gene products are virtually absent.\textsuperscript{30,31} Thus, blood circRNA expression may be detectable but the corresponding linear gene products may have great potentials for the diagnosis and prognosis of DR diseases.\textsuperscript{32} Circ_0005015 is significantly upregulated in the vitreous sample and plasma fraction of DR patients. Compared with other mRNA counterparts, circularity renders RNA largely resistant to exonuclease-lytic activities.\textsuperscript{5} As for the source of circRNAs in body fluids, circRNAs may be actively secreted in membrane-bounded vesicles (i.e., exosomes) or assembled in ribonucleoprotein complexes (e.g., Ago2, or other RNA binding proteins).\textsuperscript{33} They may also be passively released into the extracellular environment as by-products of dead cells have not been suitably untangled.\textsuperscript{34}

Endothelial cells are usually recognized as the major targets injured by hyperglycemia. In diabetic conditions, an unceasing and excessive proliferation and migration of endothelial cells occur in retinal vasculature. Any factors altering endothelial cell function could lead to retinal vascular dysfunction.\textsuperscript{2,32} Circ_0005015 silencing decreases retinal endothelial cell proliferation, migration, tube formation, and spheroid sprouting. Cell proliferation, migration, tube formation, and spheroid sprouting are tightly associated with the angiogenic effects of endothelial cells.\textsuperscript{16} Since circ_0005015 silencing could affect the biologic processes, it is not surprised that circ_0005015 could regulate pathological retinal vascular dysfunction.

MMP-2 is a member of the MMP gene family. It is involved in multiple pathways, including the regulation of vascularization, endometrial menstrual breakdown, nervous system, and metastasis.\textsuperscript{35} STAT3 mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis.\textsuperscript{36} XIAP encodes a protein that belongs to a family of apoptotic suppressor proteins. It is necessary for anti-apoptotic function.\textsuperscript{37} Circ_0005015 overexpression becomes a sink for miR-519d-3p, and releases the repressive effect of miR-519d-3p on STAT3, MMP-2, and XIAP. This regulatory mechanism would provide a novel insight into retinal endothelial angiogenic function.

In conclusion, we identified 529 differentially expressed circRNAs between diabetic retinas and nondiabetic retinas. Circ_0005015 is shown as a promising biomarker for the diagnosis and prognosis of DR. Moreover, circ_0005015 could facilitate retinal endothelial angiogenic function. In the future study, more studies would be required to investigate the correlations between circRNA expression and DR progression at different stages. In vivo and in vitro studies are also required to elucidate the mechanism of circRNAs-mediated DR pathogenesis.

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