Diabetic kidney disease (DKD) is the leading cause of chronic kidney disease. Current therapies for DKD are insufficient. Therefore, there is an urgent need for identifying new therapies. An increasing number of micro RNAs (miRNAs) and long noncoding RNAs (lncRNAs) have been demonstrated to modulate the progression of diabetic kidney disease. Nevertheless, until now, there have been few reports evaluating the relevance of circular RNAs (circRNAs) in DKD. circRNAs have been reported to regulate the occurrence and development of multiple diseases. In this study, we intended to explore the circRNA expression profiles and determine the role of circRNA in DKD. We identified a series of dysregulated circRNAs in glucose-stressed HK-2 cells using circRNA microarray analysis. Among the candidate circRNAs, we found that circACTR2 was upregulated and may be involved in inflammation and pyroptosis. Knockdown of circACTR2 significantly decreased pyroptosis, interleukin (IL)-1β release and collagen IV and fibronectin production, indicating the effective regulation by circACTR2 of cell death and inflammation. Overall, our study identified a new circRNA, circACTR2, that regulates high glucose-induced pyroptosis, inflammation and fibrosis in proximal tubular cells. The present study preliminarily explores the role of circRNAs in pyroptosis of tubular cells, and provides novel insight into the pathogenesis of DKD and new therapeutic strategies.

**Key words**  circular RNA; pyroptosis; diabetic kidney disease; fibrosis; microarray assay

## INTRODUCTION

Diabetic kidney disease (DKD) is the leading cause of chronic kidney disease. Current therapies for DKD, including glycemic and blood pressure control, are insufficient. Therefore, the development of new treatments in diabetic kidney disease is desperately needed. DKD is characterized by mesangial matrix expansion, podocyte loss and fibrosis. In recent decades, tubular injury and interstitial fibrosis have been considered important features of DKD, which closely correlates with renal dysfunction. Emerging evidence shows that the diabetic mediators, high-glucose and advanced glycation end product (AGE), promote tubular inflammation and fibrosis. Although initially thought to be specific for immune cells, it has been recently shown that pyroptosis can be functional in nonimmune cells and multiple diseases. Our previous study indicated that pyroptosis is involved in tubular injury in diabetes, as evidenced by GSDMD p30 fragment generation and flow cytometry analysis. Understanding the molecular cascades regulating pyroptosis will yield additional targets for DKD treatment.

Circular RNAs (circRNAs) are covalently closed loops generated by back-splicing. Different from linear RNAs, circRNAs possess the distinguishing feature of splicing without a free 5′-cap or 3′-tail. For quite a long time, circRNAs were wrongly considered byproducts of genetic transcription with no function. Recently, circRNAs have been reported to participate in multiple diseases, especially in cancers. However, there are currently few reports about circRNAs in DKD. Investigating the role of circRNAs will be vital for understanding the pathogenesis of DKD and providing new therapeutic targets.

## MATERIALS AND METHODS

### Cell Culture and Treatments

The immortalized human renal proximal tubular epithelial cell line HK-2 (ATCC, CRL-2190™) was cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco) with 100 U/mL penicillin and streptomycin and 10% fetal bovine serum at 37°C with 5% CO2. HK-2 cells were treated with 30 mM glucose with or without VX-765 (30 μM) for 48 h. For silencing of hsa_circRNA_102747, until an 60–70% confluence was reached, HK-2 cells were transfected using the jetPRIME® transfection reagent (PolyPlus, France) with vectors encoding a small hairpin RNA (shRNA) targeting circRNA_102747. For transfection, the cells were exposed to 5.5 or 30 mM of glucose. CircRNAs expression was detected by reverse transcription-PCR (RT-PCR) and western blotting. The details of cell culture and treatments were described in MATERIALS AND METHODS.

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glucose and nontarget shRNA; (B) 30 mM glucose and nontarget shRNA; (C) 30 mM glucose and circRNA shRNA sequence 1; (D) 30 mM glucose and circRNA shRNA sequence 2.

Circular RNA Microarray Analysis RNA was isolated using TRIzol reagent (Invitrogen, CA, U.S.A.). NanoDrop ND-1000 was used for RNA quantification. Microarray hybridization was performed following the standard instructions. In short, total RNA was digested with ribonuclease (RNase) R (Epizentech, U.S.A.) to eliminate linear RNA. The enriched circRNAs were amplified and transcribed into cRNA employing a random priming method (Arraystar Super RNA Labeling Kit, Arraystar). The fluorescent cRNA were hybridized onto the Arraystar Human circRNA Array V2 (8 × 15K). The Agilent Scanner G2505C was used to scanned the arrays. The Agilent Feature Extraction software was used to analyze the array images. The R software limma package was utilized for data analysis. Differentially expressed circRNAs were identified through Fold Change filtering. Hierarchical clustering was performed based on distinguishable circRNA expression profiling over 2-fold change.

Quantitative Real-Time PCR RNA was isolated using TRIzol reagent and the GoScript™ Reverse Transcription System (Promega, U.S.A.) was used to reverse transcription. Then, PCR was performed on the CFX Connect™ Optics Module (Bio-Rad, U.S.A.) in triplicate using the GoTaq® qPCR and RT-qPCR Systems (Promega) following the standard protocol. All the divergent primers for circRNAs are listed in Table 1. The following primers were used for ACTR2 mRNA detection: forward 5'-GACTACACATTTGGACCA GAGA-3' and reverse 5'-CTTCTCCTGTGTTGATGGG-3'.

Immunoblot Analysis Cell lysates were extracted using RIPA buffer containing protease inhibitors. Prepared samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Immobilon-P membranes (Millipore, U.S.A.). Blots were probed with anti-fibronectin (Novus, Cat. No. NBPI-91258) or anti-collagen type IV (Proteintech, Cat. No. 55131-1-AP) antibodies. Blots were developed with the Immobilon Western HRP Substrate (Millipore) using the ChemiDoc™ Touch Imaging System (Bio-Rad).

Flow Cytometry Pyroptosis was detected using FLICA FAM-YVAD-FMK and propidium iodide (PI) staining after the indicated treatments and transfections following the manufacturer’s protocol (ImmunoChemistry Technologies, U.S.A.). Briefly, cells were incubated with FLICA, an active caspase-1 detection probe. Then, the cells were washed with wash buffer before adding PI for 10 min of incubation in the dark. The population of pyroptotic cells was evaluated by BD Accuri C6 Plus (BD Biosciences, U.S.A.). Approximately 1000–5000 cells were analyzed per experimental condition.

Detection of Secretory Interleukin (IL)-1β An enzyme-linked immunosorbent assay (ELISA) was utilized for the quantitative measurement of secretory IL-1β. Cell culture supernatants was collected after various treatments, and the Human IL-1β Instant ELISA Kit (eBioscience) was used to measure IL-1β following the manufacturer’s instructions.

Lactate Dehydrogenase (LDH) Release Assay Pyroptosis was also evaluated by assaying the LDH released into the supernatants. The CytoTox96 LDH release kit (Promega) was used to detect LDH. The LDH activity was expressed as a percentage of total LDH in the cell lysate.

Statistics Student’s t-test was used for comparisons between two groups. ANOVA and Bonferroni t-tests were used for multiple-group comparisons. Statistical values and statistical significance are also reported in the Figure Legends. Data with p < 0.05 were considered statistically significant.

RESULTS

circRNA Expression Profiles in Glucose-Stressed Proximal Tubular Cells To reveal the potential role of circRNAs in diabetic kidney disease, circRNA microarray assay was

| circRNA          | circBase ID | Primer sequence       |
|------------------|-------------|-----------------------|
| hsa_circRNA_102747 | hsa_circ_0008529 | Forward: TGTCCTTCTTGAGGATGGTCTACT |
| hsa_circRNA_104262 | hsa_circ_0078617 | Reverse: TGCCCTCACACACACACAAAG |
| hsa_circRNA_101504 | hsa_circ_0022151 | Forward: ATCCAAACTGAGATAATTTAGAG |
| hsa_circRNA_405650 | /          | Reverse: TGCAAGTCGTCACACCACAGCA |
| hsa_circRNA_100141 | hsa_circ_0007364 | Forward: AGCTGAGAGAGACTGAGAGAGG |
| hsa_circRNA_405719 | /          | Reverse: TAGTAAGTTAGAGCCACACTGC |
| hsa_circRNA_003251 | hsa_circ_0003251 | Forward: AGGACGACACTTTGCAATCAAAC |
| hsa_circRNA_001880 | hsa_circ_00001880 | Reverse: CTTCTTGTGCTGAGAAACTGAA |
| hsa_circRNA_104854 | hsa_circ_0087862 | Forward: TGTCCTTCTTGACGACAGAGAGAG |
| hsa_circRNA_105041 | hsa_circ_0092125 | Reverse: ATGTCGTCGTGACAGACGGG |

Table 1. Divergent Primers of circRNAs
performed on renal tubular HK-2 cells. Recently, several studies have indicated that VX-765, a small-molecule caspase-1 inhibitor, is able to prevent inflammasome activation and pyroptosis.\textsuperscript{15–17} We employed VX-765 in a microarray assay. In total, 11318 circRNAs were detected in our samples. The distinguishable circRNA expression profiles are demonstrated in cluster heatmaps, including 117 circRNAs between 5.5 and 30 mM glucose group as well as 157 circRNAs between 30 mM glucose and 30 mM glucose + VX-765 group (fold change ≥2.0 and \(p < 0.05\)) (Figs. 1A, B). And the scatter plots showed the variation of circRNA expression (Figs. 1C, D). The results showed 54 upregulated circRNAs and 14 downregulated circRNAs in glucose-stressed HK-2 cells with a fold change greater than 2. These expression changes were prevented after VX-765 treatment.

**circACTR2 Is Upregulated in Glucose-Stressed HK-2 Cells**

Ten significantly dysregulated circRNAs are listed in Table 2, and RT-qPCR was performed to verify their expression (Figs. 2A–J). Among these circRNAs, we screened out an aberrantly regulated circular RNA, hsa_circRNA_102747 (circBase ID: hsa_circ_0008529), that was markedly upregulated in high-glucose-treated HK-2 cells, and treatment of the cells with VX-765 markedly reduced the increased circRNA (Fig. 2A). According to the human reference genome, hsa_circRNA_102747 is derived from the ACTR2 gene (actin related protein 2). ACTR2 is located on chromosome 2p14. Accordingly, we named hsa_circRNA_102747 as “circACTR2.” Schematic structure of hsa_circRNA_102747 was shown in Fig. 2K.

**Knockdown of circACTR2 Inhibits Pyroptosis and Fibrosis**

To confirm that circACTR2 is essential for pyroptosis in tubular epithelial cells of diabetic kidney disease, we knocked down circACTR2 expression by transfection of specific shRNA sequences in cultured HK-2 cells. Effective reduction of circACTR2 expression was verified by RT-qPCR (Figs. 3A, B). Subsequent flow cytometry analysis revealed
that downregulation of circACTR2 during high-glucose stimulation reduced pyroptosis (FLICA+ and PI+ cells) in HK-2 cells (Fig. 2E). Pyroptosis was also evaluated by assaying PI uptake as well as LDH release, a hallmark of lytic cell death (Figs. 2C, F). We also found that inhibition of circACTR2 markedly attenuated the release of mature IL-1β into the medium in glucose-stressed HK-2 cells (Fig. 2D). Furthermore, western blots showed that knockdown of circACTR2 strikingly reduced collagen IV and fibronectin formation (Figs. 2G–I). Collectively, these observations suggest that aberrant expression of circACTR2 in tubular cells is an important step in the regulation of pyroptosis and cell survival in high-glucose exposure.

**DISCUSSION**

It was estimated that the prevalence of diabetes mellitus (DM) was up to 9.1%, affecting 425 million people in 2017, with 12% of global health expenditure spent on DM. DKD is currently considered an inflammatory disease, and inflammasome activation has recently been implicated in DKD. Chronic sterile inflammation and release of cytokines have been demonstrated in DKD. Our study delineated a critical role of circRNA in glucose-induced inflammation and fibrosis in proximal tubular cells.
Noncoding RNAs (ncRNAs) have attracted more attention than ever. With their extraordinary structure, circRNAs are conferred remarkable tolerance to exonuclease degradation and have a much longer half-life than other linear RNAs. Increasing investigations have revealed the biological functions of circRNAs that include acting as a miRNA or even a lncRNA sponge in the regulation of transcription, acting as an RNA binding protein (RBP) sponge, and directly regulating the transcription process by interacting with mRNAs. Although plenty of miRNAs and lncRNAs have been reported to regulate diabetic kidney disease development, the potential role of circRNAs in DKD remains unclear. In the present study, we screened out dysregulated circRNAs in HK-2 cells using circRNA microarray analysis. As VX-765 has been demonstrated to regulate pyroptosis by inhibiting caspase-1, these aberrantly expressed circRNAs may participate in DKD by regulating pyroptosis. Among the candidate circRNAs, we found that circACTR2 was upregulated in cells exposed to high glucose and may be involved in inflammation and pyroptosis.

More recently, the lytic inflammatory cell death pyroptosis has been described. Determining the molecular cascades that regulate pyroptosis will yield more novel approaches to treat diseases. Pyroptosis is also a promising therapeutic target for DKD. To confirm the involvement of circACTR2 in the development of DKD, silencing of circACTR2 was performed using shRNA transfection. Because pyroptosis is a lytic form of cell death mediated by caspase-1, -4 or -5, we demonstrated that knocking down circACTR2 inhibits the effects of high glucose on cultured HK-2 cells.

Fig. 3. Knockdown of circACTR2 Inhibits the Effects of High Glucose on Cultured HK-2 Cells
(A) ACTR2 mRNA level is not affected by circACTR2 shRNA. (B) Knockdown of circACTR2 in HK-2 cells was verified by RT-qPCR. (C) LDH release is inhibited by circACTR2 knockdown. (D) Graph representing the level of IL-1β in the supernatant of HK-2 cells. (E) A flow cytometry analysis showing that circACTR2 knockdown reduces pyroptosis in high-glucose-treated HK-2 cells. (F) Cell death was measured by PI staining. (G–I) Immunoblots showing the expression of fibronectin and collagen IV. Results are representative of at least three independent experiments. Data from at least three separate experiments are shown as the means ± S.E.M. *p<0.05, **p<0.01, ***p<0.001. NG, normal glucose; HG, high glucose; IL, interleukin; NT shRNA, nontarget shRNA sequence; sh1, shRNA sequence 1; sh2, shRNA sequence 2.
detected pyroptosis using FLICA FAM-YVAD-FMK and PI staining. circACTR2 knockdown significantly suppressed the percentage of pyroptotic cells, IL-1β release and collagen IV and fibronectin production, suggesting the effective regulation by circACTR2 of cell death and inflammation. With the high stability, evolutionary conservation, and spatial and temporal specificity of circRNA expression, circACTR2 has potential to be a promising therapy to precisely target DKD.

The competing endogenous RNA (ceRNA) hypothesis is based on abundant studies and has elucidated how RNAs regulate gene expression through competitive binding to miRNAs based on abundant studies and has elucidated how RNAs regulate gene expression through competitive binding to miRNAs. For instance, circRNA LRP6 contributes to high-glucose-induced inflammation and extracellular matrix accumulation in mesangial cells by sponging miR-205.10) circPTN regulates proliferation activity in glioma by targeting miR-145-3p/miR-330-5p.29) Accumulating evidence points to the potential role of miRNAs in DKD progression. It is reasonable to speculate that circACTR2 may regulate DKD by sponging miRNA or other targets, which encourages continued exploration of circACTR2 in the pathogenesis of DKD.

In conclusion, our study identified the new circRNA circACTR2 that regulates high glucose-induced pyroptosis, inflammation and fibrosis in renal tubular cells. These data reveal that circACTR2 plays a vital role in cell survival of diabetic tubular cells. Furthermore, this research encourages continued exploration of circACTR2 as a promising biomarker and therapeutic target for DKD.

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Conflict of Interest The authors declare no conflict of interest.

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