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Original Article

A novel lncRNA TCONS_00071187 upregulated by activated GSK3β promotes high glucose-induced mesangial cell proliferation in the diabetic nephropathy

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Abstract

Inhibiting mesangial cell proliferation is one of the strategies to control the early progression of diabetic nephropathy (DN). GSK3β is closely related to cell apoptosis as well as the development of DN, but whether it acts on the proliferation of mesangial cells is unclear. This study aimed to elucidate the role and mechanism of GSK3β-mediated lncRNA in high glucose-induced mesangial cell proliferation. HBZY-1 cells were used to establish the cell model of DN. The automatic cell counter was applied to assess cell proliferation. Flow cytometry was used to detect cell apoptosis and intracellular ROS levels. High-throughput transcriptomics sequencing was performed to detect the different expressions of long noncoding RNAs (lncRNAs) in the cell model of DN after knocking down the expression of GSK3β by the transfection of siRNA. The expression of RNA was detected by real-time PCR. In the cell model of DN using HBZY-1 cells, cell proliferation was enhanced accompanied by GSK3β activation and elevated apoptosis rate and reactive oxygen species (ROS) levels. A panel of novel lncRNAs, which were differentially expressed after GSK3β knockdown in the cell model of DN, were identified by high-throughput transcriptomics sequencing. Among them, the expression of TCONS_00071187 was upregulated under high glucose conditions while the knockdown of the GSK3β expression led to the downregulation of TCONS_00071187. The knockdown of TCONS_00071187 resulted in reduced mesangial cell proliferation, and decreased apoptosis rates and ROS levels. In conclusion, GSK3β promoted mesangial cell proliferation by upregulating TCONS_00071187, which led to enhanced ROS production under high glucose conditions in the cell model of DN. This study revealed the role of GSK3β medicated lncRNAs in the development of DN.

Keywords: Diabetic nephropathy; HBZY-1; GSK3β; LncRNAs; Mesangial cell

1. Introduction

Diabetic nephropathy (DN), also referred to as diabetic kidney disease, is a chronic kidney condition resulting from long-term diabetes. It is a grave complication affecting both type 1 and type 2 diabetes. DN stands as a leading cause of end-stage renal disease (ESRD), necessitating dialysis or kidney transplantation [1]. The characteristic pathological feature of DN is the presence of glomerular lesions, particularly in the glomeruli of the kidneys. Glomeruli serve as the tiny filtration units in the kidneys, playing a pivotal role in eliminating waste products and excess fluids from the blood to produce urine [2]. In diabetic nephropathy, these glomerular lesions are often referred to as "glomerulosclerosis" [3]. Diffuse Mesangial Sclerosis and Nodular Glomerulosclerosis (known as Kimmelstiel-Wilson Lesion) are the two main types of glomerular lesions associated with DN [4]. Both pathological changes in the glomeruli leading to reduced kidney function, impaired filtration, and eventual proteinuria (presence of excess protein in the urine). Over time, these changes can progress to ESRD.

Mesangial cell proliferation stands as a characteristic pathological feature of DN, a persistent complication of diabetes impacting the kidneys [5]. Mesangial cells are specialized cells found in the mesangium, which is the supportive tissue between the glomerular blood vessels within the kidney's filtration units (glomeruli) [2]. These cells are involved in regulating the structure and function of the glomerulus. In DN, prolonged high blood sugar levels can lead to various changes in the glomeruli [6]. Hyperglycemia in diabetes can stimulate mesangial cells to multiply and proliferate [7]. Mesangial cell proliferation can lead to an increased population of mesangial cells within the glomerulus. This process is part of the initial response to injury but can contribute to the development of glomerular lesions and structural changes in the kidney. Accompanying with mesangial cell proliferation, hyperglycemia also triggers pro-apoptotic signaling pathways in mesan-

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gial cells [8, 9]. Advanced glycation end products (AGEs), the accumulation of reactive oxygen species (ROS), and mitochondrial damage contribute to high-glucose-induced apoptosis in mesangial cells [10-13]. Interestingly, under specific conditions, apoptosis and ROS can also promote cell proliferation, although the precise mechanisms remain unclear [14-16].

Glycogen synthase kinase 3β (GSK3β), a serine/threonine protein kinase, plays a pivotal role in cell apoptosis and is also of great significance in the insulin signaling pathway by promoting glycogen synthesis [17-20]. Studies have shown that GSK3β is implicated in DN, with increased expression of both the total and activated forms of GSK3β observed in kidney biopsies from DN patients, correlating with disease severity [21]. Animal experiments further supported this finding, demonstrating increased and overactivated GSK3β expression in the kidney cells of mice with DN, leading to enhanced cell apoptosis [22, 23].

Long noncoding RNAs (lncRNAs) are RNA molecules exceeding 200 nucleotides in length that do not encode proteins. They are related to the development of various diseases, including diabetes [24-27]. Emerging evidence strongly suggests that lncRNAs also have implications for various diseases, including DN. They can affect several cellular processes relevant to kidney function and damage, such as inflammation, oxidative stress, fibrosis, and apoptosis. For instance, ENSMUST00000147869, a kind of lncRNAs, is involved in mesangial cell proliferation and fibrosis [25]. lncRNA Gm6135 was found to upregulate toll-like receptor 4 (TLR4) through competitive binding with miR-203-3p, resulting in the inhibition of mesangial cell apoptosis [28]. Dysregulation of 311 lncRNAs was observed in a mouse model of DN during microarray analysis, suggesting their close involvement in the pathogenesis of DN [29].

Currently, the potential role of GSK3β in promoting mesangial cell proliferation and apoptosis through the regulation of long noncoding RNAs (lncRNAs) remains unexplored. Hence this study aimed to explore this unknown field. The current research has unveiled that the downregulation of the expression of GSK3β inhibits mesangial cell proliferation in the cell model of DN under high glucose conditions. A panel of novel lncRNAs were identified, which are associated with the expression of GSK3β in the cell model of DN. Among them, the over-expression of TCONS_00071187 in the cell model of DN under high glucose conditions was down-regulated while the expression of GSK3β was knocked down. Interestingly, the downregulation of the expression of TCONS_00071187 was also accompanied with the inhibition of the mesangial cell proliferation in DN. All in all, this study sheds light on a novel mechanism for the role of lncRNAs in the development of DN.

2. Materials and methods

2.1. Cells and reagents

HBZY-1 cells were bought from Procell Life Science & Technology Co., Ltd., China. The cell culture medium consisted of normal glucose (glucose 5 mM, NG) DMEM, supplemented with 10% fetal bovine serum and penicillin-streptomycin. GSK3β and S9-phosphorylated GSK3β antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). PVDF membranes were obtained from Merck Chemical Co., Ltd. Horseradish peroxidase-conjugated secondary antibodies were obtained from Beyotime Biotech. Inc. (Shanghai, China). Small molecule interfering RNA (siRNA) was purchased from Shanghai Gene Pharma Co., Ltd. (Shanghai, China). Primers were purchased from China Tsingke Biological Company. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Dual-color SDS-PAGE protein loading buffer (5×), ECL luminescent solution, and RIPA lysate were purchased from Hangzhou Fude Biotechnology Co., Ltd. (Hangzhou, China).

2.2. Cell proliferation assay

Cell proliferation assay was done using the cell counter [30]. The cell number was evaluated after treatment using an automatic flow cytometer (JIMBIO FIL Flow image-coulter counter, Jiangsu Jimbio Technology Co., Ltd., Changzhou, China). HBZY-1 cells in cell culture plates or flasks were digested with trypsin, and the digestion was terminated with a complete medium. Then, an automatic flow cytometer was used for cell counting. The cell proliferation was estimated by comparing the cell number in each well of cell culture plates or flasks under treatment conditions.

2.3. Apoptosis and ROS assays

HBZY-1 cells were digested with EDTA-free trypsin. Then the cells were divided into two samples, which were stained using an apoptosis kit and a ROS kit respectively. The fluorescence signal was detected by flow cytometry.

2.4. Western blotting (WB) assay

Adherent HBZY-1 cells were lysed with RIPA lysate and 5× SDS-PAGE loading buffer. After boiling at 100°C for 5 minutes, samples were subjected to 12% SDS-PAGE electrophoresis. The proteins were transferred to PVDF membranes and blocked with a fast and high-efficiency blocking solution (Beyotime, Shanghai, China) for 0.5 hours. The primary antibody was incubated with the membranes overnight at 4°C, followed by incubation with the corresponding secondary antibody for 2 hours at room temperature. Finally, the membranes were washed and developed using ECL.

2.5. Real-time PCR

RNA was harvested with an RNA Rapid Extraction kit and reverse-transcribed. Fluorescent quantitative PCR was performed using SYBR Green qPCR Mix (Beyotime, #D7260, Shanghai, China). The internal reference gene used is β-actin, and the primers for the internal reference gene are 5'-AACCTTCTTGCAGCTCCTCC-3' and 5'-TACCCACCATCACACCCTGG-3'. The primers for GSK3β are 5'-GAGACACACCTGCCCTCTTC-3' and 5'-TGGGGCTGTTCAGGTAGAGT-3'. The primers for TCONS_00071187 are 5'- CTGGATGTTTGGCAGAACGC-3' and 5'- CAAGGGCAACTACTCGGAGC-3'.

2.6. siRNA knockdown assay

The targeted sequence of GSK3β for siRNA was CGATTACACGTCTAGTATA. The targeted sequence of TCONS_00071187 for siRNA was GGATCTCGGCCGTCAGGTA. HBZY-1 cells were inoculated on a 35 mm cell culture dish. The transfection reagent siRNA-Mate was used to transfect the cells, with a final concentration

of siRNA of 100 nM. Thirty-six hours after transfection, cells were harvested by trypsinization. RT-qPCR or WB was performed to verify the knockdown effect.

2.7. High-throughput transcriptomics sequencing

The sequencing of lncRNA was performed by Novogene Biotech Co., Ltd. China. The registered bio-project ID in NCBI is PRJNA1001262.

2.8. Differential expression analysis

Differential expression analysis between two comparison groups was conducted using DESeq2 software (version 1.20.0). DESeq2 utilizes a statistical approach based on the negative binomial distribution model to identify differential expression in numerical gene expression data. The resulting p-values were adjusted using the Benjamini and Hochberg method to control the false discovery rate. Genes with a p-value < 0.05, as determined by DESeq2, were considered to be differentially expressed genes.

2.9. Differential gene enrichment analysis

The differentially expressed genes underwent Gene Ontology (GO) enrichment analysis using clusterProfiler software (version 3.4.4). The GO enrichment analysis encompassed three categories: Molecular Function, Cellular Component, and Biological Process, with a significance threshold of adjusted p-value < 0.05. Furthermore, we conducted an enrichment analysis of the differentially expressed genes in KEGG pathways using clusterProfiler software (version 3.4.4). KEGG is a database resource utilized to comprehend the advanced functions of biological systems from high-throughput molecular databases.

2.10. Statistical Analysis

All data are presented as the mean ± SEM. The difference between the two groups was assessed using GraphPad Prism 8 software (La Jolla, CA, USA) was used to assess the difference between groups. An unpaired two-tailed t-test was generally employed. For cases with two variables, one-way ANOVA or two-way analysis of variance, along with Tukey's comparison test, was used. A P-value less than 0.05 was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

3. Results

3.1. Establishment of the diabetic nephropathy (DN) cell model

Cell proliferation was significantly increased under high glucose (glucose 30 mM, HG) conditions (Figure 1A). WB results showed that GSK3β was dephosphorylated and activated under HG conditions (Figure 1B). ROS assays and apoptosis assays also showed increased ROS and apoptosis after HG stimulation (Figure 1C and D). The above results suggest that in this study the cell model of DN closely resembled the pathological state of DN in the human body (21).

3.2. Knockdown of GSK3β inhibits HBZY-1 cell proliferation

Studies have reported that cell proliferation is inhibited after *GSK3*β knockdown (22). GSK3β was effectively silenced in HBZY-1 cells using siRNA transfection (Figure 2A). In the cell model of DN, cell proliferation was reduced in the GSK3β knockdown group (Figure 2B). Additionally, the intracellular ROS level and apoptosis rate were both reduced in the GSK3β knockdown group within the cell model (Figure 2C and D).

3.3. High-throughput sequencing analysis

HBZY-1 cells were transfected with GSK3β siRNA and subjected to high-throughput transcriptomic sequencing and differential lncRNA analysis. Using the screening criteria |log2(fold change)| > 1 and P <= 0.05, a total of 1933 differential lncRNAs were identified in the HG group compared to the NG group, with 1028 upregulated and 905 downregulated (Figure 3A). Comparing the HG group with GSK3β knockdown, 1966 differential lncRNAs were screened, with 856 upregulated and 1110 downregulated (Figure 3A). A total of 866 lncRNAs regulated by GSK3β

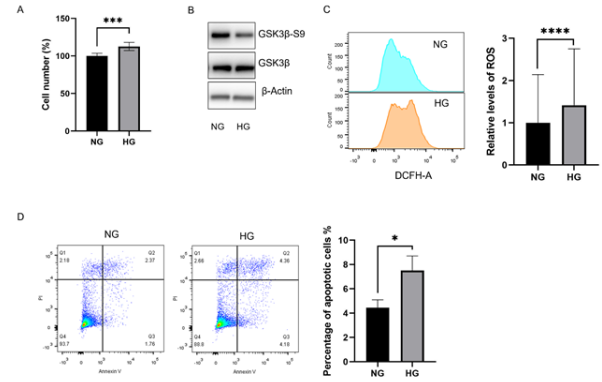

Fig. 1. Establishment of the HBZY-1 cell model of DN. (A, B) HBZY-1 cells were seeded into 12-well cell culture plates. The inoculation concentrations were 5E+5 cells/mL/well. After 24 hours of inoculation, the medium was replaced with either normal glucose (glucose 5 mM, NG) or HG medium for another 24 hours. (A)The cells were then trypsinized and counted with an automated cell counter to assess cell proliferation. (B) Following cell counting, the suspended cells were collected by centrifugation and lysed directly with an SDS loading buffer for Western blot analysis. (C&D) HBZY-1 cells were plated into 12-well cell culture plates with the inoculation concentration was 5E+5 cells/mL/well. After 24 hours, the medium was replaced with either NG or HG medium for another 24 hours, and the cells were then utilized for ROS and apoptosis assays.

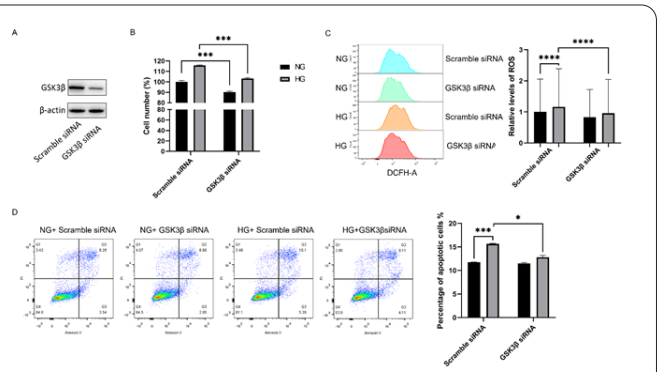

Fig. 2. GSK3β knockdown protects HBZY-1 cells in the DN cell model. GSK3β siRNA was transfected into HBZY-1 cells using a transfection reagent. (A) After 36 hours of transfection, the knockdown effect of GSK3β was confirmed by WB. (B-D) The transfected HBZY-1 cells were then seeded into 12-well cell culture plates with either NG or HG medium. Cell proliferation, intracellular ROS levels, and apoptosis rate were assessed.

were identified, with 536 upregulated and 303 downregulated (Figure 3A). The cluster analysis showed that redder colors corresponded to higher expression levels, while bluer colors indicated lower expression levels (Figure 3B).

GO and KEGG enrichment analyses were conducted on the co-located target genes of GSK3β upregulated and downregulated lncRNAs. For the upregulated lncRNAs, GO analysis revealed enrichment in three categories: biological processes related to cellular macromolecule metabolism and regulation, cellular components like intracellular parts and organelles, and molecular functions such as cyclic compound binding and nucleic acid binding (Figure 3C). KEGG analysis demonstrated enrichment in pathways like antigen processing and presentation, endocytosis, phagosome, and type I diabetes mellitus (Figure 3D). As for the downregulated lncRNAs, the GO analysis showed enrichment in cellular components like intracellular parts and organelles (Figure 3E), and KEGG analysis indicated involvement in antigen processing and presentation, phagosomes, type I diabetes mellitus, and virus infection (Figure 3F).

3.4. Knockdown of TCONS_00071187 inhibits HBZY-1 cell proliferation

The transcriptome sequencing data revealed significant upregulation of TCONS_00071187, a novel lncRNA in the HG group, and its expression was significantly downregulated after GSK3β knockdown (Figure 4A). RT-qPCR results further confirmed the downregulation of TCONS_00071187 in the HG group after GSK3β knockdown (Figure 4B). These findings indicated that GSK3β might exert its function by upregulating TCONS_00071187. To explore the role of

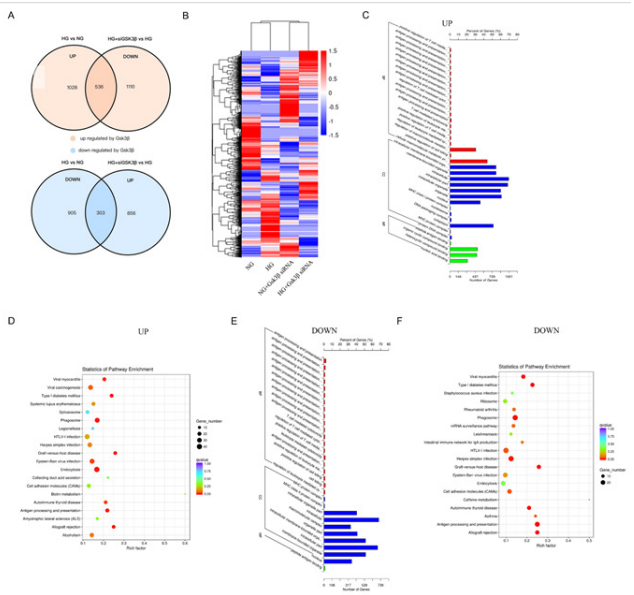

Fig. 3. Analysis of lncRNA differential expression and GO and KEGG enrichment. (A)The Venn diagram indicates the number of GSK3β-regulated lncRNAs. (B) The cluster analysis displays gene expression profiles, with colors representing relative expression levels. (C) GO analysis of GSK3β-upregulated lncRNAs and (E) GSK3β-downregulated lncRNAs includes three domains: molecular function, cellular component, and biological process. (D and F) KEGG analysis presents enriched pathways, with circle size indicating the number of genes in each pathway and color intensity representing the significance of enrichment.

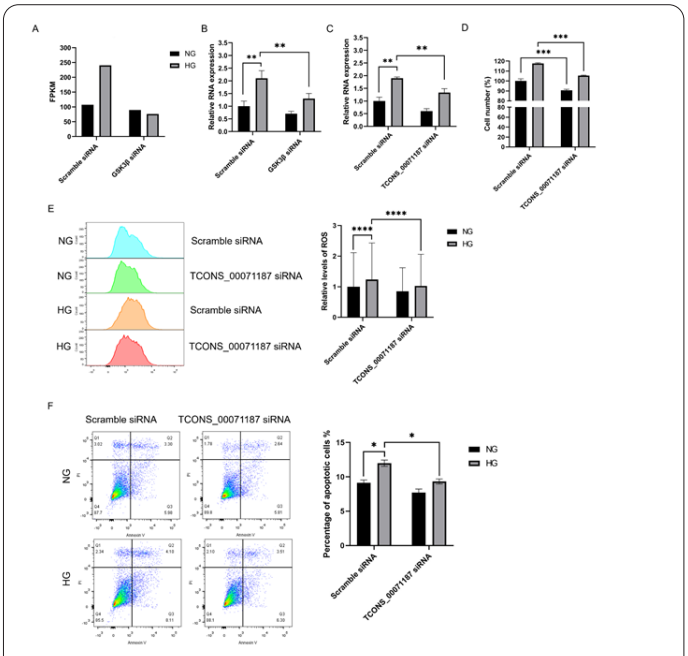

Fig. 4. Knockdown of TCONS_00071187 inhibits mesangial cell proliferation and apoptosis. (A&B) GSK3β induces high expression of TCONS_00071187. (A)Transcriptomic sequencing showed the changes in TCONS_00071187 expression. (B) GSK3β siRNA was transfected into HBZY-1 cells in the cell model of DN, and RT-qPCR was applied to detect the relative expression of TCONS_00071187. (C-I) Transfection of TCONS_00071187 siRNA into HBZY-1 cells. (C) After transfection, RT-qPCR was used to verify TCONS_00071187 knockdown. (D) Cell proliferation was measured in the TCONS_00071187 knockdown cells. (E&F) Intracellular ROS levels and apoptosis rates were measured by flow cytometry.

TCONS_00071187 in DN, HBZY-1 cells were transfected with TCONS_00071187 siRNA, and RT-qPCR confirmed successful knockdown (Figure 4C). In the cell model of DN, knocking down TCONS_00071187 resulted in decreased cell proliferation (Figure 4D), reduced intracellular ROS levels (Figure 4E) and lower cell apoptosis rate (Figure 4F). This indicates that GSK3β promotes mesangial cell proliferation, the production of ROS, and apoptosis in mesangial cells through the upregulation of TCONS_00071187.

4. Discussion

The primary objective of this study was to elucidate the role and underlying mechanism of GSK3β mediated lncRNAs in high glucose-induced mesangial cell proliferation, ultimately aiming to develop strategies to mitigate the early progression of DN [31]. Our investigation employed HBZY-1 cells as a representative cell model of DN, wherein a noteworthy enhancement in cell proliferation was observed concomitant with the activation of GSK3β, the elevated ROS, and the increased apoptosis rate. Notably, a novel lncRNA, TCONS_00071187, was identified. Its expression exhibited upregulation under conditions of high glucose, with a corresponding downregulation upon GSK3β knockdown. The significance of TCONS_00071187 in modulating mesangial cell proliferation was further underscored by the subsequent knockdown experiments, wherein reduced cell proliferation, lowered ROS levels, and a decreased rate of apoptosis were observed. Collectively, these outcomes convincingly position TCONS_00071187 as a central mediator

in the GSK3β-regulated cascade of events contributing to DN progression.

GSK3β is involved in various biological processes, including apoptosis, cancer, inflammation, immune responses, neurodevelopment, and proliferation [32,33]. PI3K/AKT/GSK-3β pathway is implicated in mesangial cell proliferation [34]. In our study, GSK3β knockdown results in decreased mesangial cell proliferation, ROS levels and apoptosis rate. The downregulation of GSK3β has been observed to inhibit cell proliferation in some cancer cells [35, 36], and dysfunction of GSK-3β led to the reduced proliferation of rat β-cells [37]. Oxidative stress could promote retinal glial cell proliferation [38]. In tissues, cell apoptosis is involved in cell growth stimulation [16]. Therefore, we hypothesize that in diabetic nephropathy, GSK3β-mediated cellular ROS and apoptosis in renal mesangial cells contribute to mesangial cell proliferation.

High through-out sequencing was applied in this study to explore the potential novel targets of GSK3β in DN. The target genes of GSK3β-regulated lncRNAs were subjected to GO and KEGG enrichment analyses to predict their functions. In this analysis, phagosomes were found to be involved. Research has indicated a close relationship between phagosomes, DN, and autophagy [39, 40]. Additionally, specific lncRNAs, such as AK044604 (Risa), have been found to inhibit autophagy, exacerbating podocyte injury in DN [41]. Autophagy plays a pivotal role in DN, and oxidative stress-induced autophagy can be protective but also lead to disruption of cellular homeostasis [42-45]. Inhibition of apoptosis in mesangial cells resulted from the induction of autophagy by TGF-β1 [46]. Another important pathway identified in our analysis is Type 1 diabetes. Type 1 diabetes is characterized by the destruction of pancreatic beta cells mediated by autoimmune mechanisms [47]. In mouse models of diabetes, macrophages accumulated and became activated in the kidney, which was associated with glomerular immune complex deposition and renal injury [48, 49].

TCONS_00071187 is a newly discovered lncRNA located on chromosome 17 (Chr17: 44758416–44794512). This study revealed that TCONS_00071187 expression increased in mesangial cells after high glucose treatment, and GSK3β knockdown significantly decreased the expression of TCONS_00071187. Furthermore, TCONS_00071187 knockdown resulted in reduced cell proliferation, decreased high glucose-induced ROS production, and inhibited mesangial cell apoptosis. These suggested that GSK3β promotes mesangial cell proliferation by upregulating TCONS_00071187. The mechanism by which TCONS_00071187 promotes mesangial cell proliferation might be similar to the mechanism where GSK3β facilitates cell proliferation, both involving an increase in ROS levels and apoptosis to drive proliferation. Interestingly, based on the analysis of the positional relationship between TCONS_00071187 and protein-coding genes, Hist1h2bo was identified as a possible transcriptionally regulated target gene of TCONS_00071187. Previous research has shown that overexpression of Hist1h1c upregulates SIRT1 and HDAC1 proteins, promoting autophagy, inflammation, and increased neuronal loss in diabetic retinopathy [50].

However, there are limitations in this study, and the roles of GSK3β-mediated-TCONS_00071187 in DN have not been verified in animal models. The specific mechanism by which TCONS_00071187 promotes mesangial cell proliferation under high glucose condition requires further exploration, and its downstream target molecules remain to be elucidated.

In conclusion, this study effectively created a cellular model of DN that closely mimics the early pathological state of diabetic kidney disease *in vivo*. The findings revealed that in the cellular model of DN GSK3β promotes HBZY-1 cell proliferation by upregulating TCONS_00071187, leading to heightened apoptosis and ROS production in response to high glucose. Our investigation sheds light on the intricate molecular interactions governing mesangial cell proliferation in the context of DN and contributes to a deeper understanding of the mechanisms that drive the early stages of DN. These insights not only expand our knowledge of diabetic nephropathy pathogenesis but also pave the way for potential therapeutic interventions aimed at modulating GSK3β-mediated pathways to mitigate disease progression. Further research in this domain holds promise for the development of innovative strategies to combat the growing burden of diabetic nephropathy.

Interest conflict
The authors declare no conflicts of interest pertaining to the publication of this paper.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
The data supporting the findings of this study will be accessible in the NCBI at [https://dataview.ncbi.nlm.nih.gov/object/PRJNA1001262] following an embargo from the date of publication.

Authors' contributions
Zhaoshou Yang, Xiaoxiao Sun and Xiaofeng Deng: Conceptualization, methodology, writing original draft preparation. Wengong Jiang, Zhihao Zhang, HaiBo Liu, Mengdan Zhong and Yanfeng Xie: Investigation, software, statistical analysis. Yongdui Ruan and Hongmei Lu: Reviewing and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

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