FOXO1 Overexpression Attenuates Tubulointerstitial Fibrosis and Apoptosis in Diabetic Kidneys by Ameliorating Oxidative Injury via TXNIP-TRX

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Objective. The generation of hyperglycemia-induced reactive oxygen species (ROS) is a key event in diabetic nephropathy (DN) development. Since forkhead box class O1 (FOXO1) is associated with oxidative stress and shows a positive effect on DN, its role on renal function and the underlying mechanism is still unclear.

Methods. We examined the role of FOXO1 in vivo (in a transgenic diabetic mouse model overexpressing Foxo1) and in vitro (in human HK-2 cells with FOXO1 knockin (KI) and knockout (KO) cultured under high glucose).

Results. Renal proximal tubular cells of kidney biopsies from patients with DN showed tubulointerstitial fibrosis and apoptosis. Accordingly, these proximal tubular injuries were accompanied by the increase of ROS generation in diabetic mice. Tissue-specific Foxo1 overexpression in transgenic mice had a protective effect on the renal function and partially reversed tubular injuries by attenuating the diabetes-induced increase in TXNIP and decrease in the TRX levels. FOXO1 knockin and knockout HK-2 cells were constructed to identify the associations between FoxO1 and TXNIP-TRX using CRISPR/CAS9. Similarly, the effects of FOXO1 KI and KO under high glucose were significantly modulated by the treatment of TRX inhibitor PX-12 and TXNIP small interfering RNA. In addition, TXNIP and TXN were identified as the direct FOXO1 transcriptional targets by chromatin immunoprecipitation.

Conclusion. The regulatory role of FOXO1/TXNIP-TRX activation in DN can protect against the high glucose-induced renal proximal tubular cell injury by attenuating cellular ROS production. Modulating the FOXO1/TXNIP-TRX pathway may be a new therapeutic target in DN.

1. Introduction

Reactive oxygen species (ROS) are increasingly recognized as the most important factors regulating altered metabolic pathways in differentiated cells, ultimate contributing to inflammation, fibrosis, dysfunction, and apoptosis [1, 2]. Diabetic nephropathy (DN) is an important cause of end-stage renal disease [3], and accumulating evidence implicates renal proximal tubular cells (RPTCs) as drivers of the structural and functional changes in diabetic kidneys [4, 5]. Given that ROS generation in RPTCs is increased under diabetic conditions [6], ROS accumulation might accelerate the irreparable progression of DN.

Thioredoxin-interacting protein (TXNIP) is a negative regulator of thioredoxin (TRX). TXNIP-TRX appear to be important contributors among the enzymatic systems implicated in ROS generation and renal oxidative stress [7]. Chronic exposure of hyperglycemia increases the production of ROS, resulting in the serious damage by promoting DNA damage, lipid peroxidation, and protein modification [8]. ROS also plays a critical role in stimulating the growth factors and cytokines like transforming growth factor-beta 1 and connective tissue growth factor [9]. These ROS-induced profibrotic factors lead to an excessive buildup of extracellular matrix, which further exacerbates kidney injury [10].
Forkhead transcription factor O1 (FOXO1) is a member of the forkhead box-containing transcription factor O family. Activation of the PI3K/Akt pathway under a high-glucose condition phosphorylates three threonine sites of FOXO1 and promotes its translocation from the nucleus to the cytoplasm, resulting in the loss of its function [11]. FOXOs have been reported to function together with TXNIP [12] and TRX [13] to mediate oxidative stress in pancreatic β cells and endothelial cells. However, the understanding of the involvement of FOXO1 in TXNIP-TRX mediated ROS accumulation in RPTCs, and their relationship are still limited.

Therefore, the present study aims at elucidating how RPTCs respond to diabetes-induced ROS accumulation under the condition of FOXO1 overexpression, contributing to interstitial fibrosis and apoptosis, and at determining whether the effects occur downstream of the TXNIP-TRX. Toward this end, we compared the RPTC damage in kidney biopsy samples from patients with and without DN. To determine the role of FOXO1 in this damage and the underlying mechanisms, we established a tissue-specific transgenic (Tg) diabetic mouse model overexpressing Foxo1 and determined the effects on physiological parameters, extent of apoptosis and interstitial fibrosis, and production of ROS. Moreover, we examined the interaction between FOXO1 and TXNIP-TRX in mediating these effects using high glucose-treated human RPTCs in vitro. These results should contribute new insight into the molecular mechanisms underlying the development of DN and highlight potential therapeutic targets for treatment and prevention.

2. Materials and Methods

2.1. Kidney Biopsy Specimens. Ten human subjects with suspected kidney pathology underwent a standard punch kidney biopsy at the First Affiliated Hospital of Zhengzhou University. Five patients were diagnosed with type 2 diabetes for more than 10 years accompanied by microalbuminuria (30-300 mg/g Cre) as well as retinopathy. The other five individuals were classified as normal glucose-tolerant accompanied by IgA nephropathy with minimal proteinuria.

All patients provided written informed consent in human physiological parameters, extent of apoptosis and interstitial fibrosis, and production of ROS. Moreover, we examined the interaction between FOXO1 and TXNIP-TRX in mediating these effects using high glucose-treated human RPTCs in vitro. These results should contribute new insight into the molecular mechanisms underlying the development of DN and highlight potential therapeutic targets for treatment and prevention.

2.2. Generation of CA-Foxo1 Tg Mice. The CA-Foxo1 construct included a specific cDNA fragment encoding full-length Foxo1 with mutated positions in Thr24, Ser256, and Ser319 to prevent phosphorylation when fused with the GFP-tag. Tg mice (C57BL/6 background) overexpressing CA-Foxo1 in the kidney were established by embryo microinjection at ViewSolid Biotech (Beijing, China) and then crossed with wild-type mice (C57BL/6 background) purchased from Charles River (Beijing, China). CA-Foxo1 Tg mice were identified by PCR amplification of the genomic DNA sequence for the transgene using the following primers: CA-Foxo1 sense primer 5′- TTACAAGTGCTCCTCCTGCCAC-3′ and the GFP (a tag sequence encoding amino acid residues) antisense primer 5′- ACTTCAGGCTACGCTTGCCCTAG-3′.

2.3. Animal Experimental Protocol. Nine Tg male mice and their wild-type littermates at the age of 8 weeks were used in the present study. All animals received standard mouse diet and water ad libitum. The Animal Care and Use Committee of Zhengzhou University approved all animal experiments. After 12 h of fasting, diabetes was induced in wild-type and Tg mice by one intraperitoneal injection of a 130 mg/kg streptozotocin (Sigma; St. Louis, MO, USA) solution in 0.05 M citrate buffer (pH 4.5), serving as the diabetes group and Foxo1 Tg diabetes group. Wild-type mice injected with citrate buffer served as the normal control group. Blood glucose was measured to confirm the successful establishment of the diabetes model at 72 h after streptozotocin injection, which was defined as a blood glucose level higher than 16.7 mmol/L. Body weight was recorded and blood glucose was monitored every 2 weeks for a period of 12 weeks as described in our previous study [14]. A 24 h urine sample was collected to determined 24 h UTP, 8-OH DG, and creatinine levels 12 weeks after injection. The mice were sacrificed with chloral hydrate, and blood was collected form the orbital vein to measure Scr and urea. The kidney weight/body weight ratios were subsequently determined. Kidney proximal tubules were collected using Precoll as described previously [15]. Separated cortices were fixed in either 4% paraformaldehyde for staining or 4% precooled glutaraldehyde for transmission electron microscopy. The remaining kidney cortex was stored in liquid nitrogen for subsequent experiments.

2.4. Establishment of FOXO1 Knockin (KI) and Knockout (KO) Cell Lines through CRISPR/Cas9. FOXO1 KI and KO HK-2 cell lines were established using the CRISPR/Cas9 system according to a standard protocol. The AAVS1 safe harbor gene knockin kit was provided by GeneCopoeia (Rockville, MD, USA), and the donor plasmid was a constitutively active form of FOXO1 with mutated positions in Thr24, Ser256, and Ser319 (NM_002015.3). The two plasmids were cotransfected into HK-2 cells for 24 h by EndoFectin Max (GeneCopoeia). Subsequently, the cells were selected by puromycin for another 24 h to eliminate nontransfected cells. DNA was extracted for the verification of the recombination sites by PCR. Successfully edited HK-2 cells were then seeded onto 96-well plates for monoclonalization. FOXO1 expression of cell clones was detected by western blotting and sequencing. HK-2 cells were transfected with the all-in-one sgRNA clone for the human FOXO1 gene (HC205747-CG01-3-10a, c; GeneCopoeia) by EndoFectin Max for 72 h. The target sites of HCP205747-CG01-3-10a and c were GGAGTGGCTAAAACCTCCCGGCC and GGCTGC CAACCCCGACGGCG, respectively. After that, the cells were selected by G418 for another 48 h. DNA was extracted for screening using the T7 endonuclease I assay kit (GeneCopoeia). Subsequently, limiting dilution was performed to isolate single cells. Single clones were allowed to grow for about 3 weeks prior to testing via immunoblotting and sequencing to identify FOXO1 KO clones. Cells with successful genotyping were used for subsequent experiments.

2.5. Cell Culture and Treatments. HK-2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in
Table 1: Primer sequences used for quantitative reverse transcription polymerase chain reaction.

| Target gene | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| ACTB (h)    | CTTGCCACCGCCAGAAAT | GGGGCGGACCTGTGATCAC |
| ACTB (m)    | GGTCTATGGTGCTCTAGATCCG | ATGCACAGATGGCTCATAAC |
| FOXO1 (h/m) | CAGCAAAACGTTATGGGAGGA | TATCATTGTTGGGAGAGAGTC |
| TXNIP (h/m) | CGGCACTAATTACCTGCAAATG | GCTCTTTGACGCGATATG |
| TXN (h/m)   | TTTCTCTGTACGTTGGTG | AGCAACATCTGCGCAGTCAT |

h, human; m, mouse.

Dulbecco’s modified Eagle’s medium (low glucose, 1 g/L) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin solution at 37°C in a 5% CO₂ atmosphere. The TRX1 inhibitor PX-12 was purchased from SelleckChem and used at a final working concentration of 10 μM for 16 h. TXNIP expression was silenced in FOXO1 KO cells using siRNAs specifically targeting TXNIP (RiboBio Co., Guangzhou, China). 24 h prior to transfection, FOXO1 KO cells were plated into a 6-well plate. Then cells were transfected with 50 nM si-TXNIP or siNC (negative control) by EndoFectin Max according to the manufacturer’s protocol for 4-6 h. The medium was replaced with fresh culture medium after treatments, and cells were harvested for stimulation in high glucose (HG, 4.5 g/L) at 48 h.

2.6. Quantitative Real-Time PCR. Total RNA was extracted from RPTCs and cultured HK-2 cells using TRIzol reagent (TaKaRa Bio, Shiga, Japan). cDNA was synthesized from RPTCs and cultured HK-2 cells using TRIzol reagent solution at 37°C in a 5% CO₂ atmosphere. The reactions were performed on an ABI Fast 7500 cycler (Applied Biosystems, Foster City, CA, USA). Relative expression standardized to GAPDH was calculated by the comparative cycle threshold method (2^ΔΔCt).

2.7. Western Blot Analysis. After different treatments, total protein from HK-2 cells and RPTCs was extracted and western blotting was performed as described previously [14] using the following primary antibodies: rabbit anti-FOXO1 and TXNIP (Abcam, Cambridge, UK); rabbit anti-p-FOXO1 Ser256 (Cell Signaling Technology, Danvers, MA, USA); rabbit anti-TRX, anti-FN, anti-Col IV, anti-BAX (Proteintech, Chicago, IL, USA), and anti-ß-actin (Sangon Biotech, Shanghai, China). Signals were visualized in the samples by enhanced chemiluminescent substrate (Life Technology) after incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sangon Biotech). The relative optical density of each band was quantitated by ImageJ software (National Institutes of Health, Bethesda, MD, USA), and ß-actin was probed to ensure equal protein loading.

2.8. Immunohistochemistry Analysis. Paraaffin sections of the mouse kidneys were prepared by a conventional method and treated with the immunohistochemistry staining protocol as described previously [14]. The working concentrations of the FOXO1 and TXNIP antibodies were 1:100 and 1:25, respectively. The distribution and subcellular localization of the target proteins were examined by light microscopy (Olympus, Tokyo, Japan). Densitometric analysis was performed using Image-Pro Plus software version 6.0 (Media Cybernetics, Rockville, MD, USA).

2.9. ChIP Assays. ChIP assays were conducted using the EZ-ChIP Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions as described previously [14]. De-cross-linked DNA samples were subjected to PCR amplification using forward (5’- AGCCGAAACACCTATTTC C-3’) and reverse (5’- TTGGTTACAGGAGGCCCAC-3’) primers targeting the TXNIP promoter and forward (5’- GCGTGCTGCCCATTGTAAA-3’) and reverse (5’- CTTGCAAAGACGTGCTTG-3’) primers targeting the TRX promoter. Precipitated DNA fragments were analyzed by quantitative PCR.

2.10. Measurement of Intracellular ROS Accumulation. The intracellular ROS accumulation was detected using the 2’7-dichlorodihydrofluorescein diacetate (DCFH-DA) probe (Beyotime, Shanghai, China) by flow cytometry as described previously [16]. In brief, after treatments for the indicated time intervals, cells were harvested and washed twice with phosphate-buffered saline (PBS), incubated with DCFH-DA at a final concentration of 10 mmol/L in PBS at 37°C for 20 min, washed twice with PBS, and analyzed by flow cytometry. Fluorescence intensity was analyzed by flow cytometry using excitation/emission wavelengths of 488/525 nm.

2.11. Measurement of 8-OHDG and MDA. Urinary samples were centrifuged at 4000 rpm, and then each sample was assessed for 8-OHDG using an enzyme-linked immunosorbent assay kit from Cusabio Biotech. All the reagents were of analytical grade. Concentrations of 8-OHDG were estimated by a spectrophotometer and calculated by measuring the optical density of 575 nm. Renal cortex MDA levels were quantified using the lipid peroxidation MDA assay kit (Beyotime Institute of Biotechnology, Jiangsu, China), according to the manufacturer’s protocol and as described in our previous report [16].

2.12. Apoptosis Assay. A TdT-mediated dUTP nick-end labeling (TUNEL) staining kit (Roche, Basel, Switzerland) was used to detect apoptosis according to the manufacturer’s instructions. Numbers of TUNEL-positive tubular cells were quantified by counting 10 randomly chosen nonoverlapping fields per slide. All slides were observed independently by two blinded investigators.
Treated cells were washed with PBS, resuspended in binding buffer, and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min according to the kit protocol from BD Biosciences. Annexin V-FITC and PI fluorescence were estimated by the use of a flow cytometer (Beckman), according to the manufacturer’s instructions.

2.13. Light Microscopy and Electron Microscopy. Kidney pathology in hematoxylin and eosin, periodic-acid Schiff, and Masson trichrome sections was examined by light microscopy. Renal cortices were sectioned into 1 mm³ and 1 cm³ cubes with a cold blade and fixed with 4% glutaraldehyde. The ultrastructure of the renal cortex was observed with a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan). The investigators were blinded to the treatment conditions when performing the electron microscopy observations.

2.14. Statistical Analysis. The data were analyzed using SPSS 17.0 software (IBM SPSS, Watson, NY, USA) and are expressed as the mean ± standard error of the mean. The differences among the experimental groups were assessed using the one-way analysis of variance followed by the Bonferroni test for multiple comparisons and the multiple range test. P value < 0.05 was considered statistically significant.

3. Result

3.1. DN Kidneys Show Signs of Nuclear Damage and Apoptosis. To examine the extent of RPTC damage in DN, kidney biopsy samples taken from patients with and without DN were analyzed. Atrophy of proximal tubular cells and thickening of the tubular basement membrane were observed in the kidneys of patient with DN based on hematoxylin and eosin and periodic-acid Schiff staining (Figures 1(a), 1(b), and 1(d)). Likewise, Masson’s trichrome staining indicated enhanced expression of collagen compounds in DN kidneys (Figures 1(c) and 1(e)).

Transmission electron microscopy showed that the RPTCs of non-DN patients had normal round nucleus with no apoptotic morphology and normal collagen fibers, whereas those of patients with DN showed morphological features of apoptosis. Moreover, a reduction in volume and formation of membrane protrusion were observed in the nucleus, along with the reorganization of chromatin aggregation and edge accumulation, potentially indicating the early stages of nuclear envelope rupture. Substantial interstitial collagen fibers were also detected in the kidneys from DN patients, resulting in a thickened tubular basement membrane (Figure 1(f)).

3.2. Successful Generation of Tg Mice with Kidney-Specific Foxo1 Expression. Tg mice were generated to produce specific and inducible expression of constitutively active- (CA-
Foxo1 in the kidney using a Pax2-Foxo1 construct (Figure 2(a)). Expression of the green fluorescent protein-(GFP-) tagged CA-Foxo1-GFP transgene directed by Pax2 was confirmed in the progeny of the CA-Foxo1 line 381 crossbred with C57BL/6 mice using polymerase chain reaction (PCR) (Figure 2(b)). Animals displaying the 490-bp Foxo1-GFP fragment were used in subsequent experiments. Western blot analysis further confirmed that FOXO1 protein expression was increased in the RPTCs of Foxo1-Tg mice compared with that of the wild type (Figure 2(c)), and enhanced FOXO1 expression was detected in the RPTCs of Foxo1-Tg mice by immunohistochemistry (Figure 2(d)). Taken together, these results demonstrated that Pax2 directs CA-Foxo1 transgene expression in the RPTCs of Foxo1-Tg mice.

3.3. Foxo1 Overexpression Has a Protective Effect in Diabetic Mice. The body weights of male wild-type diabetic and Tg diabetic mice were significantly lower, whereas the blood glucose levels were significantly higher than those of normal mice. There was no significant difference in the body weight and blood glucose level between diabetic and Tg diabetic mice. These results indicate that Foxo1 overexpression in
the kidney alone is ineffective in preventing weight loss and hyperglycemia in diabetic mice.

Prominent increases in the kidney weight/body weight ratio, 24 h urinary total protein (UTP), serum creatinine (Scr), and urea levels were detectable in both diabetic and Tg diabetic mice after week 12 compared with those of normal mice. However, these parameters were significantly reduced in Tg diabetic mice compared with diabetic mice, demonstrating that Foxo1 overexpression had a protective effect on the renal function in the diabetic mice (Table 2).

### 3.4. Foxo1 Overexpression Prevents Interstitial Fibrosis and Apoptosis in the RPTCs of Diabetes Mice

We next examined the effect of Foxo1 overexpression on the morphological change characteristic of interstitial fibrosis and apoptosis. Hematoxylin and eosin and periodic-acid Schiff staining revealed the atrophy of proximal tubular cells and thickening of the tubular basement membrane in the kidneys of diabetic mice, whereas the control mice showed a normal kidney morphology. However, these changes were attenuated in Foxo1 Tg diabetic mice (Figures 3(a), 3(b), and 3(d)).

Likewise, Masson’s trichrome staining showed enhanced expression of collagen in the kidneys of diabetic mice compared with that of the normal mice, although collagen components were normalized in the Tg diabetic mouse kidneys (Figures 3(c) and 3(e)). Similarly, transmission electron microscopy revealed a significant apoptosis in RPTCs and a substantial amount of interstitial collagen fibers in diabetic mice. In contrast, Foxo1 overexpression prevents these injuries to some extent, indicating a protective effect (Figure 3(f)).

We also performed TUNEL assays on the kidney sections to determine apoptosis. In comparison to normal mice, RPTCs from diabetic mice resulted in elevated apoptosis, and Foxo1 overexpression could significantly decrease tubular cell apoptosis in diabetic mice (Figures 3(g) and 3(h)).

We further investigated whether Foxo1 overexpression could attenuate the protein indicators of apoptosis and interstitial fibrosis induced by hyperglycemia in mouse RPTCs. Although there was no change detected in the mRNA and protein levels of FOXO1 between normal and diabetic mice, the phosphorylated (p)-FOXO1/total-FOXO1 ratio was significantly higher in the diabetic conditions. Importantly, the expression level of FOXO1 increased and the phosphorylated (p)-FOXO1/total-FOXO1 ratio was markedly elevated expression of fibronectin (FN) and collagen IV (Col IV) was detected in RPTCs from diabetic mice compared to that in normal mice. Moreover, the expression of BAX was also enhanced in RPTCs from diabetic mice. Importantly, the expression of these indicators was significantly attenuated in Foxo1 Tg mice. These observations demonstrated that RPTC apoptosis and interstitial fibrosis in diabetic mice can be attenuated by Foxo1 overexpression (Figure 4).

### 3.5. Oxidative Damage Products and TXNIP-TRX Expression Are Reduced in Tg Diabetic Mice

Since FOXO1 plays an important role in regulating oxidative stress, we assessed the effect of Foxo1 overexpression on oxidative stress in the kidneys of diabetic mice by detecting the expression of the DNA oxidative damage product urinary 8-oxo-2′-deoxyguanosine (8-OHdG) and the lipid peroxidation product malondialdehyde (MDA). As shown in Figures 5(a) and 5(b), the generation of urinary 8-OHdG and MDA was significantly reduced in Tg diabetic mice compared with that of diabetic mice.

Moreover, Foxo1 overexpression significantly increased the expression level of TXNIP in the RPTCs of the diabetic mice compared to that of normal mice as determined by reverse transcription quantitative PCR (RT-PCR), western blot, and immunohistochemistry analysis. The mRNA and protein levels of TRX were also significantly reduced in diabetic mice as expected. Importantly, TXNIP expression was downregulated while TRX expression was upregulated in the RPTCs of Tg diabetic mice (Figures 5(c)–5(g)). These results demonstrated that *in vivo* Foxo1 upregulation expression led to increased TRX expression accompanied by reduced oxidative stress.

### 3.6. FOXO1 Overexpression Changed the Expression of TXNIP and TRX in Human RPTCs under a High-Glucose Condition In Vitro

To verify the relationship between FOXO1 activity and TXNIP-TRX expression in RPTCs, we analyzed the expression levels of the three factors in FOXO1 knockin (KI) and knockout (KO) human proximal epithelial tubule HK-2 cells cultured under a high-glucose (HG, 4.5 g/L glucose) condition *in vitro*.

Although there was no difference in the mRNA and protein levels of FOXO1 between the normal glucose (NG, 1 g/L) and high-glucose groups, the p-FOXO1/total-FOXO1 ratio increased significantly in the HG group compared to that of the NG group. Importantly, FOXO1 KI increased the expression of FOXO1 and decreased the p-FOXO1/total-FoxO1

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**Table 2: Body weight, kidney weight/body weight ratio, and biochemical indicators in the normal, diabetic (DM), and Foxo1 transgenic diabetic (Tg DM) groups at week 12 after injection.**

| Group   | n  | BW (g)       | KI (x10-3) | BG (mmol/L) | UTP (mg) | Scr (μmol/L) | Urea (mmol/L) |
|---------|----|--------------|------------|-------------|----------|--------------|---------------|
| NG      | 9  | 28.0 ± 0.9   | 5.3 ± 0.5  | 9.1 ± 1.1   | 2.0 ± 0.3 | 11.9 ± 1.0   | 9.0 ± 0.8     |
| DM      | 9  | 22.2 ± 1.1*  | 9.2 ± 0.3* | 28.5 ± 1.6* | 16.7 ± 0.9* | 28.9 ± 2.4* | 17.3 ± 1.2*   |
| Tg DM   | 9  | 22.2 ± 1.3*  | 7.2 ± 0.3*# | 27.0 ± 2.1* | 12.5 ± 1.4*# | 20.3 ± 2.3*# | 13.5 ± 1.0*# |

The data are displayed as the means ± standard errors. BW, body weight; KI, kidney weight/body weight ratio; BG, blood glucose; UTP, 24 h urinary total protein; Scr, serum creatinine. *P, 0.05 vs. the normal group. #P, 0.05 vs. the DM group.
ratio in the HG-treated cells. Likewise, the mRNA and protein levels of TXNIP were significantly increased in HG-treated HK-2 cells compared with the NG group, while the TRX expression level was markedly reduced. However, the modulation of TXNIP-TRX reversed in the HG-treated KI cells. In contrast, TXNIP expression was increased, and TRX expression was decreased in the cells with FOXO1 KO cells under the HG condition. When the HG-treated FOXO1 KO cells were infected with TXNIP small interfering (si-TX), the mRNA and protein expression levels of TXNIP were reduced and the protein level of TRX was increased, whereas transfection with control siRNA (si-NC) did not induce these changes. Treatment with TRX inhibitor PX-12 also significantly reduced the protein level of TRX. Taken together,
these observations indicated that FOXO1 overexpression prevented the HG-induced upregulation of TXNIP and down-regulation of TRX in cultured HK-2 cells under high glucose (Figures 6(a)–6(h)).

3.7. FOXO1 Binds to the TXNIP and TRX Promoters In Vitro. To determine the potential role of FOXO1 in TXNIP and Txn gene transcription in HK-2 cells, the interaction between FOXO1 and the TXNIP and Txn promoter elements was examined by chromatin immunoprecipitation (ChIP) assays, using an antibody specific for FOXO1. As shown in Figures 6(i)–6(o), FOXO1 clearly bound to the TXNIP and Txn promoter regions in cultured HK-2 cells, and FOXO1 overexpression strongly promoted these bindings.

3.8. FOXO1 Regulates the Accumulation of ROS via TXNIP-TRX. To verify whether FOXO1 attenuate ROS accumulation through the action of TXNIP-TRX, we measured the cellular ROS production using flow cytometry. HG-induced ROS production was decreased in FOXO1 KO cells compared with that detected in the HG group. However, this effect was reversed by treatment with the TRX inhibitor PX-12. FOXO1 KO increased the ROS accumulation under HG condition, whereas this effect was greatly reduced by infection with si-TX. There was no statistical difference in the ROS levels between the FOXO1 KO and si-NC groups, indicating that si-NC did not affect the production of ROS. Taken together, these results demonstrated that regulating FOXO1 alters TXNIP-TRX induced cellular ROS accumulation under high-glucose condition (Figure 6(p)).

3.9. Overexpression of FOXO1 Protects HK-2 Cells from HG-Induced Apoptosis and Fibrosis via TXNIP-TRX Regulation. Figures 6(b) and 6(i)–6(k) show that the protein levels of BAX, FN, and Col IV were markedly increased in the HG group compared with those of the NG group. As expected, FOXO1 KO suppressed and promoted the upregulation of these apoptosis and fibrosis indicators, respectively. In addition, the PX-12 induced downregulation of TRX expression and reversed the suppressing effect in HG-treated FOXO1 KO cells. However, infections of HG-treated FOXO1 KO cells with si-TX greatly reduced the levels of apoptosis and fibrosis indicators compared with these infected with si-NC.

As shown in Figure 7, the apoptosis ratio detected by flow cytometry was 3.1% in the NG group and was 22.8% in the HG group, suggesting that the HG treatment markedly increased apoptosis. In contrast, the apoptosis ratio of FOXO1 KO cells cultured under HG was apparently reduced (7.5%), and treatment with PX-12 inhibited this protective effect of FOXO1 overexpression. The effect of FOXO1 KO was also largely reduced when HG-treated FOXO1 KO cells were infected with TXNIP siRNA. Thus, PX-12 and TXNIP siRNA affect not only the expression of apoptosis-related proteins but also the overall apoptosis ratio.

4. Discussion

Oxidative stress plays an important role in diabetic nephropathy (DN); however, the underlying mechanism is unclear. Thioredoxin-interacting protein (TXNIP) and thioredoxin (TRX) maintain oxidative stress balance. Here, we shed new light on the regulation of TXNIP-TRX by FOXO1 in renal proximal tubular cells. FOXO1 overexpression attenuated the high glucose-induced enhancement of TXNIP expression and impairment of TRX expression via direct binding to the promoter. Thus, by inhibiting TXNIP and promoting TRX,
FOXO1 may have additional beneficial effects in protecting against oxidative damage in RPTCs, suggesting a new target for the treatment of DN.

FOXO1 regulates the expression of several genes [17] that play roles in the development and progression of diabetes mellitus [18] and DN [19]. We previously reported that forced FOXO1 activation in glomerular cells by infection with lentiviral vectors protected the mesangial cells and podocytes in the kidneys of streptozotocin-induced diabetic animals [16, 20]. However, there has been minimal research focused

**Figure 5:** Effect of FOXO1 on oxidative damage products and TXNIP-TRX expression in diabetic mice. (a) Effect of overexpressing Foxo1 on urinary 8-OHdG levels (ng/mg Cr) in each animal group (n = 9). Urinary 8-OHdG levels were measured by enzyme-linked immunosorbent assay and adjusted using urinary creatine. (b) Malondialdehyde (MDA) concentrations in the kidney tissues of various groups. (c) Relative Txnip and Trx mRNA levels measured by RT-PCR. (d, e) Protein levels of TXNIP-TRX detected by western blot analysis. (f, g) Immunohistochemistry and quantitative analysis of TXNIP and TRX expression in normal, diabetic, and transgenic (Tg) diabetic mice. Data are means ± SEM, n = 6 (*P < 0.05 vs. the normal group and #P < 0.05 vs. the diabetic group).
Figure 6: Continued.
on the mechanisms underlying the diabetic-induced injury of the renal tubules. In this study, staining and transmission electron microscopy revealed severe tubular damage in patients with DN. Histological examinations confirmed the characteristics of renal tubular injury in diabetic mice. Moreover, Foxo1 Tg diabetic mice (specifically overexpressing CA-Foxo1 in the RPTCs) exhibited similar renal histology to that of normal mice, and Foxo1 overexpression effectively attenuated the progression of albuminuria in diabetic mice. These data indicated a potentially protective role of FOXO1 in the development of DN.

As glucose-induced ROS cause injury to the podocytes and RPTCs at the onset of DN, protecting cells against oxidative stress is important. For example, overexpressing catalase...
in RPTCs was shown to attenuate interstitial fibrosis and tubular apoptosis in db/db mice [15]. Given the critical role of FOXO family in ROS homeostasis in diabetes mellitus [21, 22], we hypothesized that FOXO1 would be effective in inhibiting HG-induced ROS production. In support of this hypothesis, we found that oxidative stress indicators 8-OHDG and MDA were normalized in Foxo1 Tg diabetic mice compared with those of wild-type diabetic mice in vivo. Similarly, overexpressing FOXO1 attenuated the increased ROS level in vitro in HG-treated HK-2, while FOXO1 knockdownregulation increased the accumulation of cellular ROS under HG. These findings point to a relationship between FOXO1 and ROS generation, although more experiments are warranted to verify these effects and understand the mechanism.

As an important redox regulatory mechanism of intercellular ROS generation, the TXNIP-TRX interaction goes hand in hand with glomerular [23, 24] and tubular [25, 26] oxidative stress and also plays a critical role in the progression of DN. Moreover, Foxo3 was reported to reduce ROS levels by inducing TRX expression [27]. Based on these findings, we speculated that the transcription factor FOXO1 can regulate TXNIP and TRX expression in RPTCs, thereby preventing ROS-related apoptosis and fibrosis. As expected, we detected TXNIP upregulation and TRX downregulation in the RPTCs of diabetic mice, which were significantly ameliorated in Tg diabetic mice. Further supporting these effects, treatment with a TRX inhibitor and TXNIP siRNA could partially block the effects of FOXO1 overexpression and KO in human RPTCs.

Nevertheless, the understanding of the mechanism(s) by which FOXO1 induces the observed changes of TXNIP and TRX remains incomplete. Previous studies have shown that FOXO regulates TXNIP and TRX transcription by binding to their promoters and regulating their transcriptions in various cell types. For example, FOXO3 was found to bind to the TRX promoter and recruit the histone acetylase p300 to form a transcription activator complex in human aortic endothelial cells, resulting in a reduction in ROS levels [13, 27]. Similarly, through ChIP assay, we found a direct interaction of FOXO1 with the TRX promoter in HK-2 cells grown under the HG condition, and overexpression of FOXO1 could increase the recruitment on the TRX promoter and prevent the HG-induced reduction of TRX expression.

TXNIP regulation is known to be tissue- and disease-specific. The FOXO family has previously been shown to differentially regulate TXNIP expression in hepatoma carcinoma cells, glucose-treated endothelial cells, and beta cells. FOXO1 bound to the TXNIP promoter, leading to an increase of TXNIP in HepG2 cells [28, 29]. However, in the normal liver, TXNIP expression was directly repressed by FOXO1A [30]. The specific role of FOXO1 on TXNIP regulation may result from the heterogeneous nature of hepatoma carcinoma cells and other proteins in the transcription complex. In beta cells, FOXO1 inhibited TXNIP transcription, probably by interfering with the DNA binding of ChREBP in the target gene promoters [12]. However, until now nothing has been known about the regulation of TXNIP by FOXO1 in renal proximal tubular cells. Our date indicated that FOXO1 bound to the TXNIP promoter in HK-2 treated with HG and increased the recruitment of FOXO1 on TXNIP promoter in FOXO1 KO resulting in a decrease of HG-induced TXNIP enhancement, which is consistent with its effects in the beta cells and liver.

5. Conclusions
In summary, the present study suggested a critical role of FOXO1 in attenuating ROS production, albuminuria, RPTC apoptosis, and interstitial fibrosis both in vivo and in vitro. FoxO1 bound to the TXNIP and TXN promoter and regulates the oxidative stress balance maintained by TXNIP-TRX. These findings indicate that modulating the FOXO1/TXNIP-TRX pathway may have therapeutic utility in the treatment of DN.

Abbreviations

DCFH-DA: 2′,7-Dichlorodihydrofluorescein diacetate
DN: Diabetic nephropathy
FITC: Fluorescein isothiocyanate
FN: Fibronectin
FOXO1: Forkhead transcription factor O1
HG: High glucose
KI: Knockin
KO: Knockout
MDA: Malondialdehyde
NG: Normal glucose
PBS: Phosphate-buffered saline
PI: Propidium iodide
ROS: Reactive oxygen species
RPTCs: Renal proximal tubular cells
SCr: Serum creatinine
Tg: Transgenic
TRX: Thioredoxin
TXNIP: Thioredoxin-interacting protein
UTP: Urinary total protein.

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

Conflicts of Interest
All authors declare that there is no conflict of interest associated with this manuscript.

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