Parkin ubiquitinates GATA4 and attenuates the GATA4/GAS1 signaling and detrimental effects on diabetic nephropathy

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Abstract
Renal tubular injury contributes to the progression of diabetic nephropathy (DN). This study explored the role and mechanisms of E3-ubiquitin ligase Parkin in the renal tubular injury of DN. We found that Parkin expression gradually decreased and was inversely associated with IL-6, TGF-β1, and GATA4 expression in the kidney during the progression of DN. Parkin over-expression (OE) reduced inflammation, fibrosis, premature senescence of renal tubular epithelial cells (RTECs), and improved renal function while Parkin knockout (KO) had opposite effects in DN mice. Parkin-OE decreased GATA4 protein, but not its mRNA transcripts in the kidney of DN mice and high glucose (HG)-treated RTECs. Immunoprecipitation indicated that Parkin directly interacted with GATA4 in DN kidney. Parkin-OE enhanced GATA4 ubiquitination. Furthermore, Parkin-KO upregulated growth arrest-specific gene 1 (GAS1) expression in renal tubular tissues of DN mice and GATA4-OE enhanced the HG-upregulated GAS1 expression in RTECs. Conversely, GAS1-OE mitigated the effect of Parkin-OE on HG-induced P21, IL-6, and TGF-β1 expression in RTECs. These results indicate that Parkin inhibits the progression of DN by promoting GATA4 ubiquitination and downregulating the GATA4/GAS1 signaling to inhibit premature senescence, inflammation, and fibrosis in DN mice. Thus, these findings uncover new mechanisms underlying the action of Parkin during the process of DN.

KEYWORDS
diabetic nephropathy, GATA4, Parkin, renal tubular epithelial cells, ubiquitination

Abbreviations: BUN, blood urea nitrogen; DAPI, 4’,6-diamidino-2-phenylindole; DeR2, decoy receptor 2; DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; GAS1, growth arrest-specific gene 1; HA, hemagglutinin; HbA1c, hemoglobin A1c; HG, high glucose; HRP, horseradish peroxidase; IFTA, interstitial fibrosis and tubular atrophy; IL, interleukin; KO, knockout; OE, over-expression; RTECs, renal tubular epithelial cells; SAHF, senescence-associated heterochromatin foci; SASP, senescence-associated secretory phenotype; SA-β-gal, senescence-associated cytoplasmic β-galactosidase; SCr, serum creatinine; SDs, standard deviations; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TRAF3IP2, tumor necrosis factor receptor-associated proteins; uNAG, N-acetyl-β-D-glucosaminidase; UPS, ubiquitin-proteasome system.
1 | INTRODUCTION

Diabetic nephropathy (DN) can cause end-stage renal disease. Renal tubular injury contributes to the progression of DN. Hyperglycemia leads to the accelerated senescence of renal tubular epithelial cells (RTECs), tubulointerstitial inflammation, and fibrosis. Senescent RTECs can secrete a group of pro-inflammatory and pro-fibrotic factors, as senescence-associated secretory phenotype (SASP). Furthermore, these factors can recruit inflammatory cells to drive a pro-inflammatory and pro-fibrotic cascade, leading to chronic tubular inflammation, interstitial fibrosis, and DN progression. However, little is known about the molecular mechanisms underlying RTEC premature senescence during the process of DN.

Senescent cells usually express higher levels of senescence-associated membrane-related decoy receptor 2 (DcR2), senescence-associated cytoplasmic β-galactosidase (SA-β-gal), senescence-associated heterochromatin foci (SAHF) in their nuclei, and activation of p16 and p21-related signaling. A recent study has revealed that transcription factor GATA-binding protein 4 (GATA4), a member of the zinc finger GATA family, is a regulator of senescence and secretion of pro-inflammatory and pro-fibrotic cytokines, which are involved in aging-related process in the brain, skin, and liver. Noxious stimuli can induce DNA damage responses and GATA4 protein accumulation in the senescent cells due to its decreased degradation. Subsequently, GATA4 induces the NF-κB activation by activating tumor necrosis factor receptor-associated proteins TRAF3IP2 and IL-1α, leading to cellular stress senescence and pro-inflammatory and pro-fibrotic cytokine production. The ubiquitin-proteasome system (UPS), such as USP28, is crucial for the maintenance of GATA4 protein during the process of senescence. The UPS28 deficiency dramatically reduces GATA4 accumulation during ionizing radiation-induced fibroblast cell senescence.

Disruption of the UPS can accumulate unfolded, misfolded, and damaged proteins, promoting the progression of aging and age-related diseases. During the process of protein ubiquitination, an enzymatic cascade activates the C-terminus of the ubiquitin moiety and transfers to another acceptor enzyme, which coordinates with an E3-ubiquitin ligase to specifically ubiquitinate a substrate. Parkin is an E3-ubiquitin ligase in the UPS and is expressed in various tissues and organs, such as the brain, kidney, heart, and liver. Parkin can regulate the pathophysiological processes, such as the mitochondrial quality control system, apoptosis, tumor cell growth, inflammation, and others. Mutations in Parkin have been linked to familial aging-related diseases, including Parkinson’s disease and Alzheimer’s disease. Parkin deficiency causes selective degeneration of catecholaminergic neurons in the substantia nigra and locus coeruleus of the brain, resulting in early-onset parkinsonism. Long-lived Parkin-overexpressing flies display an increase in K48-linked polyubiquitin and protein degradation during aging. Thus, Parkin inhibits the aberrant protein accumulation and aggregation in the brain, and prolongs the lifespan of Drosophila. Moreover, Parkin can regulate many stress-related signaling, and mediate proteasome-mediated degradation of selective substrates and non-degradative ubiquitination. Parkin can protect against cisplatin-induced acute kidney injury by enhancing mitophagy and inhibit podocyte damage by regulating mitochondrial homeostasis and mitophagy in diabetic mice. However, there is no information on whether Parkin can induce GATA4 protein ubiquitination and degradation and affect RTEC senescence, inflammation, and fibrosis during the process of DN.

In this study, we examined Parkin and GATA4 expression in biopsied renal specimens and the kidney of Parkin-KO and Parkin-over-expression (OE) DN mice. Furthermore, we explored the relationship between Parkin, GATA4, IL-6, TGF-β1 levels, and premature senescence of RTECs to investigate the potential mechanisms by which Parkin regulated GATA4 and downstream GAS1 expression and their effects on DN. Our data indicated that Parkin inhibited RTEC senescence, inflammation, fibrosis, and DN progression by promoting GATA4 degradation. Therefore, these findings uncover new mechanisms underlying the action of Parkin during the process of DN.

2 | MATERIALS AND METHODS

2.1 | Patients

The project was approved by the Ethical Review Committee of the Army Medical University (the approval number: 2009-40). Individual subjects signed the written informed consent form before enrollment. This study recruited 149 DN patients in the Department of Nephrology, Daping Hospital (Chongqing, China) between January 1, 2011 and January 1, 2017. All patients had a history of type 2 diabetes with hemoglobin A1c (HbA1c) levels of 7%-11% and their DN was diagnosed by histological examination of biopsied renal specimens. A procedure of renal biopsy was performed in patients with persistent albuminuria or increased serum creatinine (SCr), especially in patients with sudden onset, overt proteinuria, or rapidly progressive glomerular nephropathy. The DN patients were excluded if she/he had recent fever or infectious signs or received traditional Chinese medicines within 3 months before renal biopsy. The DN patients were regularly treated with insulin, a blocker of calcium-channel, an angiotensin receptor antagonist, an angiotensin-converting enzyme inhibitor or statin. Control kidney tissues were from 32 renal hamartoma patients, who underwent nephrectomy.
We collected peripheral venous blood and urine samples from each patient 1 day before the renal biopsies and tested for plasma blood urea nitrogen (BUN), SCr levels by the modified Jaffe method. We also calculated the estimated glomerular filtration rate (eGFR) for each patient by the Cockcroft-Gault formula and examined urinary N-acetyl-β-D-glucosaminidase (uNAG) levels using a specific kit (Bio-Quant, San Diego, USA).

2.2 Immunohistochemical analysis

The levels of Parkin expression in the kidney tissues were determined by immunohistochemical staining. In brief, the biopsied kidney tissues were paraffin-embedded. The tissue sections (2 μm) were deparaffinized, rehydrated, and undergone antigen retrieval. The sections were incubated with mouse anti-Parkin (1:400 dilution, sc-30130, Santa Cruz BioTech, Santa Cruz, USA) or control IgG overnight. Subsequently, the sections were probed with horseradish peroxidase (HRP)-conjugated anti-mouse IgG and colorized with 3,3′-diaminobenzidine tetrahydrochloride (Cat.10221, Zhongshan Golden-Bridge Biological Technology, Beijing, China). The immunohistochemical signals in the renal tubules were semi-quantified in 10 high power fields (400×) of each sample selected randomly.

2.3 Mice

Animal studies were approved by the Animal Research and Care Committee of our university. Both genders of C57BL/6 mice were purchased from Charles River (Beijing, China) and housed in a specific pathogen-free facility in our hospital. Both male and female mice at 8-10 weeks old were used in this study.

Parkin knockout (Parkin KO) C57BL/6 mice were generated by Cas9/CRISPR-mediated genome editing technology specifically to delete the exon 4 of the Parkin gene using a specific kit (Cyagen Biosciences, Guangzhou, China). To minimize potential off-target effect and maximize on-target activity of CRISPR/Cas9, we designed two different sgRNAs using the CRISPR Design to target CCT CCG AAT TTT CCA GAG TCT CG for gRNA1 and CCA TGA GAC CAT CGC TGT GCT GG for gRNA2. The Cas9 mRNA and sgRNAs were synthesized using the mMESSAGE mMACHINE T7 Ultra and MEGAscript T7 kits (Ambion), respectively. To obtain Parkin ± mice, a mixture of Cas9 mRNA (10 ng/μL) and two sgRNAs (50 ng/μL each) were microinjected into fertilized eggs. The generated mice were genotyped by PCR using primers (Parkin-F: 5′-GCA CTT GTC ATA CCC TAA GAG CTT-3′; Parkin-R: 5′-CTT CAG GCT TTA CAG GCC ATCC-3′) and sequenced, followed by intercrosses to generate homozygous Parkin-KO mice. The genotypes of Parkin-KO mice were confirmed by PCR and sequencing.

Renal Parkin over-expression (Parkin-OE) mice were established using the Tet-On advanced system and ultrasound-microbubble system, as described previously. Briefly, we cloned the DNA fragment (NM_016694.4) for Parkin expression into the Mlu I and Sal I sites of the pTRE-Tight-Luc-GFP (Cat.631059, Clontech, USA, Figure S3A) to generate the plasmid pTRE-Tight-Parkin. A mixture of 100 μL (1 μg/μL) Tet-On plasmids (rtTA-Rs-M2, Cat.630930, Clontech, Figure S3A), 200 μL Sonovue (Bracco, Milan, Italy) and 100 μL (1 μg/μL) pTRE-Tight-Luc-GFP (Control plasmid #65491, Addgene, USA) or pTRE-Tight-Parkin was injected into individual mice via their tail vein. Immediately after injection, the left and right kidney regions of mice on their back were stimulated with ultrasound at 1 MHz, 1-W power output for 1 minutes for four times with an interval of 30 seconds using an ultrasound transducer (Therasonic, Electro-Medical Supplies, Wantage, United Kingdom). Subsequently, the mice were injected intraperitoneally with 200 μg doxycycline (Sigma-Aldrich, St. Louis, MO) and provided with drinking water containing doxycycline (200 μg/mL). The mice were subjected to ultrasound-microbubble-mediated transfection every 30 days for five times during the experimental period.

To characterize the efficacy of ultrasound-microbubble-mediated gene transfer into the kidney of the mice, six wild-type (WT) C57BL/6 mice were injected intravenously with the pTRE-Tight-Luc-GFP (200 μg per mouse) and Sonovue and stimulated with or without ultrasound. Two days later, the GFP-fluorescent signals in the kidney of mice were examined by an IVIS Spectrum Imaging System (PerkinElmer, Germany) with an exposure time of 10 seconds and a standard excitation at 470 nm, and emission at 535 nm. The Parkin-OE mice were euthanized on days 2, 10, 20, 30, and 60 post one-time Parkin gene transfer with ultrasound-microbubble system. Their kidneys were dissected for the examination of Parkin expression by quantitative RT-PCR and Western blot.

2.4 Induction of diabetic mice

WT, Parkin-KO C57BL/6J mice were injected intraperitoneally with vehicle or Streptozotocin (STZ, 50 mg/kg body weight; Sigma-Aldrich) daily for five consecutive days as the WT, Parkin-KO, DN, and Parkin-KO DN groups, respectively (n = 7 per group). Similarly, WT + control vector and Parkin-OE mice were injected with the same dose of STZ 1 week after the first injection with vector plasmid or induction of Parkin-OE and continually injected with the vector plasmid or Parkin-expressing plasmid monthly as the
DN + control vector and Parkin-OE DN groups, respectively. The diabetes onset in mice was defined as two consecutive blood glucose reading ≥250 mg/dL.

### 2.5 Histologic examination

The degrees of interstitial fibrosis in the kidney tissues were examined by Masson Trichrome staining and semi-quantitatively analyzed in 10 random non-overlapped visual fields (200× magnification) in a blinded manner using the Leica QWin V3 software.

### 2.6 Cell culture and manipulation

We isolated murine primary RTECs from C57/BL6 mice (2-4 week-old), as described previously and cultured them (passage 2) in medium containing 5 mM of glucose (normal glucose, NG), 5.5 mM of glucose, and 24.5 mM of mannitol (high mannitol), or 30 mM of glucose (high glucose, HG) and changed with fresh medium daily for 2 days. The RTECs were treated with vehicle or 10 ng/mL of mouse IL-6 (406-ML, R&D systems, USA), 10 ng/mL of mouse TGF-β (7666-MB, R&D systems), 5 μM MG-132, a proteasome inhibitor (ab141003, abcam, UK) in the presence of NG or HG.

The RTECs were infected in triplicate with adenovirus (multiplicity of infection, MOI = 50) for expression of GFP, GFP-tagged Parkin, mCherry-tagged GATA4, or mCherry-tagged GAS1 (Genemine Biotechnology, Chongqing, China) for 48 hours. Furthermore, the RTECs were transfected with control siRNA (sc-36869, Santa Cruz), Parkin-specific siRNA (sc-42159, Santa Cruz) or GATA4-specific siRNA (sc-35454, Santa Cruz) using Lipofectamine 2000 (Cat. 11668019, Invitrogen). The immunofluorescent signals were observed under a confocal microscopy (SP8, Leica, Germany).

### 2.7 Immunofluorescence

The kidney sections or cultured cells were probed overnight with anti-Parkin (1:200 dilution), anti-GATA4 (1:200 dilution, sc-25310, Santa Cruz), anti-P16 (1:500 dilution, ab189034, Abcam), anti-P21 (1:500 dilution, ab109199, Abcam), anti-DeR2 (1:500 dilution, ab2019, Abcam), and anti-GAS1 (1:500 dilution, ab58653, Abcam, UK) at 4°C. The sections were stained with fluorescein isothiocyanate, Cy3-, or Cy5-labeled second antibody IgG (1:50 dilution) and nuclearly stained with 4’, 6-diamidino-2-phenylindole (DAPI). The fluorescent signals were observed under a confocal microscope.

### 2.8 Quantitative real-time polymerase chain reaction

We extracted total RNA of RTECs using TriZol (Cat. 15596026, Invitrogen) and reversely transcribed 2 μg RNA each sample into cDNA using the ImProm reverse-transcription kit (Cat. 639537, Takara Bio, Shiga, Japan), followed by quantifying target mRNA transcripts using premixed SYBR green reagents in an iCycler system (Bio-Rad, Hercules, USA). The primer sequences were sense 5’ AGGG ATTC AGAA GCAG CCAG AGG 3’ and antisense 5’ CCGG TTTG GAAT TAAG ACAT CG 3’ for Parkin; sense 5’ ACTC CCTT AGGC CAGT CAGC3’ and antisense 5’ GGAA AAGA GCAG GGAC TCG3’ for GATA4; sense 5’ CCCA TGCT CGAA TCGG TCAA3’ and antisense 5’ CTAC AAGT GTGA CCCG AGCA3’ for GAS1; sense 5’ GTTG TCGA CGAC GAGCG3’ and antisense 5’ GCAC AGAG CCTC GCCTT3’ for β-actin. The data were analyzed by the 2−ΔΔCT method.

### 2.9 Extraction of nuclei and cytoplasm of RTECs

We extracted the nuclear and cytoplasmic fractionations using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific), according to the manufacturer’s protocol. Briefly, the different groups of RTECs were harvested and suspended in 200 μL of cytoplasmic extraction reagent I, followed by incubated them on ice for 10 minutes. The cell suspension was reacted with 11 μL of the second cytoplasmic extract reagent II and centrifuged to obtain the cytoplasmic fraction (the supernatant fraction). The pallets were resuspended in 100 μL of nuclear extraction reagent and incubated on ice for 8 minutes, followed by centrifuged. The nuclear fraction in the supernatants was collected for the subsequent experiments.

### 2.10 Immunoblotting

The relative levels of target protein expression to the control β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the different groups of RTECs were quantified by immunoblotting. Briefly, RTECs in the different groups were harvested, lysed in RIPA buffer and centrifuged. After determining the protein concentrations, the cell lysates (30 μg each) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% of gels and electro-transferred onto polyvinylidene difluoride membranes. After being blocked with nonfat milk, the membranes were probed overnight with antibodies against Parkin (1:1000 dilution), P16 (1:2000 dilution), P21 (1:1000 dilution), and β-actin (1:2000 dilution) in blocking buffer (5% BSA) at room temperature.
To understand the role of Parkin in DN progression, we col-
alyzed by linear regression approach using the SPSS 13.0 soft-
comparisons and the association between two variables was an-
The significance was analyzed by repeated analysis of variance
All data are present as the means ± standard deviation (SD).
In addition, endogenous GATA4 ubiquitination in RTECs
ubiquitin (Genemine Biotechnology, Chongqing, China).
Furthermore, HEK293T cells were co-transfected with
lysates were precipitated with anti-Myc antibody (R950,
In addition, we generated kidney-specific Parkin-OE mice
IFTA scores in DN patients (Figure S1A-C). Such data
nificantly expressed in Parkin− tubules and positively associated
Inflammation and fibrosis are crucial for the pathogenesis of
Confocal microscopy analysis revealed that kidney injury
mice weighed significantly less than the WT counterparts. At 4-6 weeks, KO
slowly than their WT counterparts. At 4-6 weeks, KO mice
and β-actin (ab179467, 1:2000 dilution, abcam), or GAPDH (ab8245, 1:5000 dilution, abcam) at 4°C. Subsequently, the blots were reacted with the HRP-labeled secondary antibodies and viewed using an enhanced chemiluminescence system (Amersham Biosciences, UK). The data were analyzed using an image analyzer and Quantity One software (Bio-Rad, Hercules, USA).

2.11 Ubiquitination assays
Recombinant human Myc-GATA4 (1 mM, Genemine Biotechnology, Chongqing, China) was reacted with 100 nM recombinant human ubiquitin-activating enzyme E1 (E-304-050), 2.5 μM recombinant E2 Ubch5c (E2-802-100, R&D Systems), 2.5 μM hemagglutinin (HA)-tagged ubiquitin, and 100 nM recombinant Flag-tagged Parkin (Genemine Biotechnology, Chongqing, China) in ubiquitinylation assay buffer (Biomol, Hamburg, Germany) at 37°C for 2 hours. The samples were precipitated with anti-Myc antibody (R950, Invitrogen) using Protein A Agarose beads (#9863, Cell Signaling Technology) and analyzed by immunoblotting.

Furthermore, HEK293T cells were co-transfected with plasmids for expression of human Parkin, GATA4, and HA-ubiquitin (Genemine Biotechnology, Chongqing, China). The potential interacted protein complex was precipitated with anti-GATA4 and visualized by immunoblotting using anti-HA (ab9110, Abcam).

In addition, endogenous GATA4 ubiquitination in RTECs was also determined. Briefly, the cell lysates were precipitated with anti-GATA4. After being washed, the precipitated proteins in the beads were resolved by SDS-PAGE on Novex gels (Invitrogen), and immunoblotted with anti-HA (1:1000).

2.12 Statistics
All data are present as the means ± standard deviation (SD). The significance was analyzed by repeated analysis of variance (ANOVA), followed by the Holm-Sidak procedure for multiple comparisons and the association between two variables was analyzed by linear regression approach using the SPSS 13.0 software. A P value of <.05 was considered statistically significant.

3 RESULTS

3.1 Decreased Parkin expression is related to the severity of DN in human patients
To understand the role of Parkin in DN progression, we collected biopsied renal specimens from 149 DN patients and 32 controls with renal hamartoma. After stratification of DN patients, the demographic and clinical characteristics of all subjects are shown in Table S1. Immunohistochemistry revealed that Parkin was mainly expressed in RTECs and its expression levels were inversely associated with increased interstitial fibrosis and tubular atrophy (IFTA) scores in this population (Figure 1A,B). The percentages of Parkin+ RTECs were negatively correlated with serum Cr ($R^2 = 0.4201$, $P < .001$) and uNAG levels ($R^2 = 0.3798$, $P < .001$, Figure 1C,D), but positively correlated with eGFR values in DN patients ($R^2 = 0.3263$, $P < .001$, Figure 1E).

3.2 Parkin attenuates renal damage in DN mice
To identify the role of Parkin in the pathogenic process of DN, we generated Parkin KO mice by depleting the exon 4 of the Parkin using the CRISPR/Cas9 technology (Figure S2A). We found that Parkin KO mice developed slower than their WT counterparts. At 4-6 weeks, KO mice weighed significantly less than the WT counterparts. However, this discrepancy normalized by 8-20 weeks, with no significant difference in body weights between the groups (Figure S2B). Renal structure and function were also equivalent between the groups by 8 weeks (data not shown). In addition, we generated kidney-specific Parkin-OE mice using the ultrasound-microbubble transfection system. We found that ultrasound stimulation induced the microbubble-loaded Parkin expression in the kidney region (Figure S3B). At 2 days post-transfection, the levels of Parkin mRNA transcripts and protein expression in the kidney increased by 8.5 and 7.3 folds, respectively. The Parkin expression in the kidney gradually decreased with time and returned to a basal level at 60 days post-transfection (Figure S3C-E). The Parkin expression was mainly detected in renal distal tubular cells and some in the proximal tubules of healthy mice. There were >90% of Parkin+ cells in renal tubules and only 6.7% of Parkin+ cells in the glomerulus of Parkin-OE mice at 2 days post-transfection (Figure S3F-J). Therefore, ultrasound-microbubble transfection monthly effectively induced and maintained high levels of Parkin expression in renal tubular cells (Figure 2A).

Subsequently, control (WT, WT + control vehicle), Parkin-KO, and Parkin-OE C57BL/6 mice were injected with STZ to induce DN (Figure 2A). Longitudinal
analysis indicated that Parkin expression gradually decreased in control DN mice from 2 to 5 month post-induction (Figure 2B-D). Compared with the healthy control mice, Parkin-KO did not significantly alter plasma BUN, serum Cr, and uNAG levels in mice, while control DN mice displayed significantly elevated plasma BUN, serum Cr, and uNAG levels (Figure 2E-G). In comparison with that in the control DN mice, the plasma BUN, SCr, and uNAG levels in Parkin-OE DN mice were significantly reduced to 55%, 52%, and 70%, respectively, while the plasma BUN, SCr, and uNAG levels in Parkin-KO DN mice were significantly elevated by 1.4, 1.5, and 1.3 times, respectively (Figure 2E-G). Such results indicated that Parkin attenuated the long-term hyperglycemia-induced renal damage in DN mice.

3.3 | Parkin prevents inflammation, fibrosis, and premature senescence of RTECs in DN

Our previous studies have demonstrated that premature senescence and inflammation of RTECs were crucial for the progression of DN.22,29 To understand the mechanisms by which Parkin inhibited the process of DN, we analyzed the relationship between Parkin and P16 expression in the biopsied renal tissues from DN patients by immunofluorescence. We found that P16 was mainly expressed in Parkin-renal tubules and P16 expression was negatively associated with Parkin expression, but the percentages of P16+ RTECs were positively increased IFTA scores in DN patients (Figure 3A,B). Compared with the control DN mice, the relative P21 expression levels
Figure 2  Parkin inhibits the progression of DN in mice. A, Schematic diagram of the experimental schedule. B-D, Parkin mRNA transcripts and protein expression in renal cortex tissues of DN C57BL/6 mice induced by STZ were characterized longitudinally by qRT-PCR and immunoblotting. E-G, The levels of SCr, plasma BUN, and uNAG in healthy wild-type (WT), Parkin-KO, WT + DN, WT + DN + control vector, and Parkin-OE DN mice were measured at the indicated time points post-STZ injection. * P < .05, ** P < .01, *** P < .001 vs WT + DN. Data are representative images or expressed as the means ± SD of each group from n = 7 independent experiments. * P < .05, ** P < .01, *** P < .001.

(Figure 3C,D) in the kidney tissues and urinary IL-6, TGF-β1, and ACR levels (Figure 3E-G) were significantly elevated in the Parkin-KO DN mice, but reduced in the Parkin-OE DN mice. Meanwhile, the percentages of Collagen I+ renal tubule area and Collagen I deposition area in the glomeruli of the control DN groups were significantly higher than that in the Parkin-OE DN mice, but significantly lower than that in the Parkin-KO DN mice at 5 months post-induction (Figure 3H-K). Hence, Parkin inhibited the tubular cellular senescence, inflammation, and fibrosis in DN in vivo.

Subsequently, we stimulated primary RTECs with high glucose (30 mM, HG) for 48 hours. HG enhanced the P16, DcR2, SA-β-gal, and γ-H2AX expression and IL-6 and TGF-β1 secretion (Figure S4A-H), but significantly reduced Parkin expression in RTECs (Figure S4I-J). Furthermore, treatment with IL-6 or TGF-β1 did not significantly alter the
relative levels of Parkin expression in RTECs regardless of glucose levels (Figure S4J). Moreover, Parkin-OE mitigated the HG-induced tubular senescence, IL-6, and TGF-β1 secretion while Parkin silencing enhanced the HG-induced cellular senescence, IL-6, and TGF-β1 secretion in RTECs (Figure 4A-H, Figure S5A-B). Therefore, Parkin inhibited the long-term hyperglycemia-induced RTEC senescence, inflammation, and fibrosis in vitro.
3.4 | Parkin inhibits the senescence, IL-6, and TGF-β1 secretion of RTECs by enhancing GATA4 degradation in diabetic conditions

GATA4 is a critical regulator of SASP. We characterized the GATA4 expression in STZ-induced DN mice. And there was no significant change in the relative levels of GATA4 mRNA transcripts in the kidney of mice during the process of DN (Figure 5A). However, significantly higher levels of GATA4 protein expression were detected in the kidney of DN mice at 4 or 5 months post-induction (Figure 5B-C). In addition, while higher frequency of GATA4+P16+ RTECs was observed in the kidneys of the control DN groups of mice the percentages of GATA4+P16+ RTECs were dramatically reduced in the Parkin-OE DN mice at 5 months post-induction (Figure 5D-E). Similarly, we did not detect significant change in the GATA4 mRNA transcripts in the kidneys among different groups of mice regardless of DN induction (Figure 5F). However, induction of DN for 5 months significantly decreased Parkin, but increased GATA4 protein expression in the kidney of DN mice, which was significantly mitigated by Parkin-OE (Figure 5G-I). Consistently, HG stimulation did not affect GATA4 mRNA transcripts, but significantly increased GATA4 protein levels in RTECs, which was significantly mitigated by Parkin-OE (Figure S6A-F). Treatment with MG-132 to inhibit proteasome significantly increased the GATA4 protein levels in renal tubular cells regardless of glucose levels (Figure S6G-H). The results suggest that Parkin may directly degrade GATA4 protein in RTECs during the process of DN in mice.

Subsequently, we found that HG stimulation for 48 hours significantly increased the percentages of SA-β-gal+ RTECs, which was mitigated by Parkin-OE (Figure S7A,B). The inhibitory effect of Parkin-OE on the HG-increased senescence, pro-inflammatory, and pro-fibrotic factor secretion was completely abrogated by GATA4-OE because similar patterns of frequency of γ-H2AX+ and P16+ RTECs, as well as IL-6 and TGF-β1 levels were detected among the different groups (Figure S7A-H). Moreover, while HG significantly enhanced the relative levels of P16 expression in RTECs. Parkin silencing significantly further increased P16 expression, which was significantly mitigated by GATA4 silencing (Figure S8A,B). A similar pattern of frequency of SA-β-gal+ RTECs, and IL-6 and TGF-β1 levels was observed in the different groups of RTECs (Figure S8C-F). Such data suggest that Parkin may inhibit tubular senescence, pro-inflammatory, and pro-fibrotic factor secretion by reducing GATA4 protein levels in RTECs.

3.5 | Parkin ubiquitinates GATA4 in vivo and in vitro

To understand how Parkin regulates GATA4 protein levels, we tested whether Parkin could directly interact with GATA4 in RTECs. Compared with the cells cultured in NG, HG reduced the cytoplasmic and nuclear Parkin protein levels, but increased GATA4 in RTECs (Figure 6A). Immunoprecipitation indicated that anti-GATA4 precipitated the cytoplasmic, but not nuclear Parkin in RTECs (Figure 5B). Similarly, after induction of Myc-GATA4-OE and/or Flag-Parkin-OE, we found that anti-Myc (GATA4) precipitated Parkin while anti-Flag (Parkin) precipitated GATA4 in HEK293T cells (Figure 6C). Further immunoprecipitation revealed that anti-Parkin precipitated GATA4 in mouse RTECs (Figure 6D) and renal biopsied samples from the DN patients (Figure 6E). Together, such data clearly demonstrated that Parkin directly interacted with GATA4, predominantly in the cytoplasm of RTECs.

Given that Parkin is an E3 ligase and important for ubiquitination, we questioned whether Parkin could promote GATA4 degradation in RTECs. We incubated the purified Myc-GATA4 with hemagglutinin (HA)-ubiquitin, E1, E2 (UbchH5c) in the presence or absence of ATP or Flag-Parkin in vitro. We observed that addition of Flag-Parkin significantly enhanced GATA4 ubiquitination in an ATP-dependent manner (Figure 7A). Consistently, ubiquitination assay revealed that induction of Parkin-OE also significantly increased the GATA4 ubiquitination in HEK293T cells (Figure 7B). Moreover, we detected significantly higher levels of GATA4 ubiquitination in the kidneys of Parkin-OE mice, particularly in the Parkin-OE DN mice (Figure 7C). Therefore, Parkin effectively ubiquitinated GATA4 and reduced its protein levels in the kidney of DN mice.
FIGURE 4 Parkin inhibits the HG-stimulated DcR2, H2AX, and P16 expression in RTECs. Mouse RTECs were transfected with control or Parkin-specific siRNA or a plasmid for Parkin expression and treated with NG or HG for 48 h. A-D, The frequency of DcR2⁺ or γ-H2AX⁺ RTECs was determined by immunofluorescence and quantified. Bar = 40 µm. E and F, The relative levels of P16 expression in the different groups of RTECs were determined by immunoblotting and quantified. G and H, Enzymatic staining of SA-β-gal⁺ RTECs in the different groups of cells. Scale bar = 80 µm. Data are representative images or expressed as the means ± SD of each group from seven independent experiments. ***p < .001
FIGURE 5  Parkin reduces GATA4 protein levels in renal cortex tissues during the DN process. A-C, GATA4 mRNA and protein levels in renal cortex tissues of DN mice at the indicated time points post-STZ injection. D, Immunofluorescent analysis of GATA4 and P16 expression in the indicated groups of mice at 5th month post-STZ injection. E, Percentages of GATA4⁺P16⁺ RTECs. F and G, GATA4 mRNA transcripts and protein expression in renal cortex tissue of different groups of mice at 5th month post-STZ injection. H and I, Quantification of Parkin and GATA4 protein expression. Data are representative images or expressed as the means ± SD of each group from n = 7 independent repeats. *P < .05, ***P < .001. Scale bar = 80 μm
Parkin inhibits RTEC senescence and IL-6 and TGF-β1 secretion by attenuating the GATA4/GAS1 signaling during the DN process

Previous studies have indicated that GATA4 can bind to 16,754 gene promoters, upregulate the expression of 535 genes, and downregulate 261 genes. By comparing the results of CHIP-sequencing and RNA-sequencing, GATA4 appeared to bind to 283 genes and regulated their expression (Data are not shown). Gene Ontology (GO) analysis indicated that GATA4 upregulated the expression of growth arrest-specific gene 1 (GAS1) (Data are not shown), which is a cell cycle inhibitor for the G0 to S transition. Accordingly, we hypothesized that Parkin could promote GATA4 degradation and inhibit GAS1 expression to induce RTEC premature aging and cell cycle arrest to G0/G1 phase during the pathogenic process of DN. To address it, we characterized the distribution of GAS1 and P16 in RTECs of DN patients.
FIGURE 7  Parkin ubiquititates GATA4 in vitro and in vivo. A. Parkin promoted GATA4 ubiquitination in vitro ubiquitination system. B. Parkin enhanced GATA4 ubiquitination in HEK293T cells that had been co-transfected with plasmids for expression of HA-ubiquitin, GATA4, and/or Parkin. Cells from n = 3 independent experiments were analyzed. C. Parkin-OE enhanced GATA4 ubiquitination in renal cortex lysates from the different groups of mice. Data are representative images or expressed as the means ± SD of each group from n = 7 independent experiments. **P < .01, ***P < .001, ****P < .0001
FIGURE 8  Parkin inhibits the senescence, IL-6, and TGF-β1 secretion of RTECs by inhibiting GATA4/GAS1 during the process of DN. A, Immunofluorescent analysis of GAS1 and P16 expression in RTECs from control and DN subjects. Scale bar = 80 µm. B, Parkin-KO enhanced GAS1 expression in renal tissues of control and DN mice at 5th month post-DN induction. Scale bar = 40 µm. C, GATA4-OE enhanced the HG-stimulated GAS1 expression in RTECs. Scale bar = 40 µm. D, GAS1-OE mitigated the inhibitory effect of Parkin-OE on the HG-stimulated P21 expression in RTECs. E, ELISA determined the levels of IL-6 and TGF-β1 levels in cell supernatants. Data are representative images or expressed as the means ± SD of each group from seven independent experiments. *P < .05, **P < .01, ***P < .001
and mice. Immunofluorescence indicated that GAS1 expression was colocalized with P16 expression in RTECs of the biopsied kidneys from DN patients, but not from controls (Figure 8A). The relative levels of GAS1 mRNA transcripts and protein expression in the kidneys of Parkin-KO DN mice were 1.84 and 1.52 times higher than in the control DN mice, respectively (Figure 8B). Similarly, while GATA4-OE significantly increased the relative levels of GAS1 mRNA transcripts, but not protein expression. GATA4-OE significantly enhanced the HG-upregulated GAS1 mRNA transcripts and protein expression in RTECs (Figure 8C). Finally, GAS1-OE significantly enhanced the HG-upregulated P21, IL-6, and TGF-β1 expression and abrogated the inhibitory effect of Parkin-OE on the HG-upregulated P21, IL-6, and TGF-β1 expression in mouse RTECs (Figure 8D-E).

4 | DISCUSSION

Our results indicated that Parkin expression gradually decreased and was negatively associated with increased IFTA scores in human DN patients. Furthermore, the percentages of Parkin+ RTECs were negatively correlated with serum Cr, uNAG levels, but positively with eGFR values in DN patients. Similarly patterns of Parkin expression and its association with the severities of DN were detected in mice. Such novel data demonstrated that Parkin expression was inversely associated with the severity of DN in human patients and animal model. Hence, these findings suggest that Parkin may negatively regulate the pathogenesis of DN and the Parkin expression level in RTECs of kidney tissues may be valuable for evaluating the severity of DN.

We found >90% of Parkin+ cells in the renal tubules, but only 6.7% of Parkin+ cells in the glomeruli of the Parkin-OE mice. The beneficial effects of Parkin overexpression were due to the global expression of Parkin within the tubule system, including the proximal tubules and distal tubules (Figure S3I-J). Similarly, Parkin expression on renal tubular cells can protect from acute kidney injury, especially Contrast-induced acute kidney and sepsis-induced acute kidney injury.33 Therefore, Parkin-OE in the renal tubules may contribute to the delayed progression of DN.

Oxidative stress-related signaling can downregulate Parkin expression.34 A large number of studies have showed that HG causes obvious metabolic dysfunction in renal tubular cells, which induces high production of reactive oxygen species (ROS), contributing to the pathogenesis of DN.35 Actually, MitoQ, a mitochondria-targeted antioxidant can restore the HG-downregulated Parkin expression in RTECs.36 Therefore, it is possible that HG may enhance the degradation of Parkin protein by inducing oxidative stress, resulting in a decrease in the Parkin protein level in renal tubular cells, but further studies are needed to confirm.

Inflammation and fibrosis were crucial for the pathogenesis of DN. In this study, we found that Parkin expression was negatively associated with accelerated senescence of RTECs, and increased levels of urinary IL-6 and TGF-β1 in the DN patients. Furthermore, altered Parkin expression modulated renal interstitial fibrosis and RTEC premature senescence in DN mice. Moreover, Parkin inhibited the HG-induced RTEC senescence, IL-6, and TGF-β1 secretion, supporting the notion that HG can accelerate the senescence of RTECs and podocytes,37-39 leading to renal interstitial inflammation and fibrosis directly and indirectly (SASP) during the process of DN.38,40 Actually, recent studies have shown that Parkin can protect against the sepsis-induced kidney injury and cisplatin nephrotoxicity by enhancing mitophagy.19,41 The inhibition of Parkin on RTEC senescence, inflammation, and fibrosis provided new mechanisms by which Parkin inhibited the pathogenesis of DN.

Parkin was mainly expressed in the renal tubules of healthy mice so that the protective effect of Parkin on the glomeruli in DN mice was unclear. It is possible that the tubuloglomerular feedback may reduce single-nephron GFR when the damaged tubules fail to reabsorb a normal amount of filtrates.42,43 On the contrary, the changes in peritubular starling forces may limit the proximal reabsorption when the damaged glomeruli reduce filtration. Finally, these functional changes eventually lead to structural damages, such as tubular atrophy and glomerular shrink and sclerosis.44 Therefore, Parkin in renal tubular cells may also protect the glomeruli by the tubuloglomerular feedback regulation.

GATA4 is a critical regulator of SASP and can be activated by ATM and ATR, the DNA damage response regulators.6 The activated GATA4 can translocated into the nucleus, where it activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to initiate the SASP and inflammatory cytokine expression.6 In this study, we found that altered Parkin expression modulated GATA4 protein levels, but not its mRNA transcripts and Parkin directly bound to the cytoplasmic GATA4 and promoted the GATA4 ubiquitination and degradation in RTECs in vivo and in vitro. Such data indicated that the ubiquitination of GATA4 by Parkin may predominantly occur in the cytoplasm of RTECs. A previous study indicates that GATA4 can be degraded by p62-mediated selective autophagy.6 It is possible that p62 can bind to ubiquitinated proteins through its UBA domain.45 Given that Parkin promoted the GATA4 ubiquitination and degradation, the ubiquitination induced by Parkin may be a key upstream event in regulating the degradation of GATA4 by autophagy.

GATA4 promotes SASP and inflammation.6,46 We examined how GATA4 regulated detrimental effects on DN. Interestingly, we found that GATA4 enhanced the senescence of RTECs by upregulating the expression of GAS1, a cell cycle inhibitor for the G0 to S phase transition.31 GAS1, a
glycosyl-phosphatidylinositol (GPI)-anchored protein, can inhibit the proliferation of mesangial cells \(^{30}\) and promote apoptosis of different types of cells as well as regulate nephrogenesis.\(^{47,48}\) In this study, we found that GAS1 was expressed in P16\(^+\) RTECs in DN patients, but not the control subjects. Similarly, GAS1 expression was mainly detected in the HG-stimulated RTECs and RTECs of DN mice, particularly in the GATA4-OE RTECs and Parkin-KO DN mice. Furthermore, Parkin-OE mitigated the HG-stimulated P21 expression and IL-6 and TGF-\(\beta\)1 secretion in primarily cultured RTECs, which were abrogated by GAS1-OE because GAS1-OE enhanced the HG-stimulated P21, IL-6, and TGF-\(\beta\)1 expression in RTECs. It is well known that GAS1-OE can enhance the P38\(\text{MAPK}\) activation, which is a DNA damage response-independent regulator of the SASP.\(^{49}\) In addition, mutations in the P38\(\text{MAPK}\) gene can change the expression of pro-inflammatory and pro-fibrotic cytokines (such as IL-6, IL-1, TGF-\(\beta\)1).\(^{49}\) The opposite effects of GATA4/GAS1 and Parkin on P21, IL-6, and TGF-\(\beta\)1 expression, together with the fact that Parkin promoted GATA4 degradation, indicated that Parkin inhibited the senescence of RTECs, pro-inflammatory, and pro-fibrotic cytokine secretion by promoting the degradation of GATA4 to reduce GAS1 expression, limiting the pathogenesis of DN. Therefore, our findings uncover a new mechanism by which Parkin regulates the senescence, inflammation, fibrosis, and inhibits the progression of DN.

In conclusion, our data indicated that Parkin inhibited the progression of DN by promoting GATA4 ubiquitination and downregulating the GATA4/GAS1 signaling to inhibit premature senescence, inflammation, and fibrosis. GATA4, GATA Binding Protein 4; RTECs, Renal tubular epithelial cells; GAS1, growth arrest specific 1

**FIGURE 9** Schematic illustration of the mechanism underlying the role of Parkin during the process of DN. Parkin inhibits the progression of DN by promoting GATA4 ubiquitination and downregulating the GATA4/GAS1 signaling to inhibit premature senescence, inflammation, and fibrosis. GATA4, GATA Binding Protein 4; RTECs, Renal tubular epithelial cells; GAS1, growth arrest specific 1.
inflammation, and fibrosis during the pathogenesis of DN. Importantly, our findings suggest that Parkin may be valuable for evaluating the severity of DN and preservation of Parkin or inhibition of GATA4 and GAS1 may be new strategies for design of therapies for DN.

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CONFLICT OF INTEREST
The authors declare no conflicts of interests.

AUTHOR CONTRIBUTIONS
K. Chen and Y. He conceived of and designed the research. Experiments were performed by K. Chen, J. Chen, J. Yuan, and L. Wang. Data were analyzed and interpreted by K. Chen, J. Yang, and X. Wang. The manuscript was written by K. Chen, J. Yang, and L. Wang prepared the figures. Human clinical samples were provided by Y. He and F. Xiao. All authors contributed to discussions and revision of the manuscript.

ETHICS
Animal studies were approved by Animal Research and Care Committee of Army Medical University, Chongqing. The clinical samples were obtained upon approvals from the Ethical Review Committee of Army Medical University, Chongqing.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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