FoxO1 Promotes Mitophagy in the Podocytes of Diabetic Male Mice via the PINK1/Parkin Pathway

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We recently showed that forkhead-box class O1 (FoxO1) activation protects against high glucose–induced injury by preventing mitochondrial dysfunction in the rat kidney cortex. In addition, FoxO1 has been reported to promote putative kinase 1 (PINK1) transcription and promote autophagy in response to mitochondrial oxidative stress in murine cardiomyocytes. In this study, we ascertained whether overexpressing FoxO1 in the kidney cortex reverses preestablished diabetic nephropathy in animal models. The effect of FoxO1 on mitophagy signaling pathways was evaluated in mouse podocytes. In vivo experiments were performed in male KM mice. A mouse model of streptozotocin (STZ)-induced type 1 diabetes (T1D) was used, and lentiviral vectors were injected into the kidney cortex to overexpress FoxO1. A mouse podocyte cell line was treated with high concentrations of glucose and genetically modified using lentiviral vectors. We found aberrant mitochondrial morphology and reduced adenosine triphosphate production. These mitochondrial abnormalities were due to decreased mitophagy via reduced phosphatase/tensin homolog on chromosome 10–induced PINK1/Parkin-dependent signaling. FoxO1 upregulation and PINK1/Parkin pathway activation can individually restore injured podocytes in STZ-induced T1D mice. Our results link the antioxidative activity of FoxO1 with PINK1/Parkin-induced mitophagy, indicating a novel role of FoxO1 in diabetic nephropathy. (Endocrinology 158: 2155–2167, 2017)

Autophagy is a highly regulated lysosomal pathway (1) that is particularly important in quiescent and terminally differentiated cells (2). Mitophagy refers to autophagy of mitochondria and is a type of macroautophagy that occurs selectively. Imbalances in mitochondrial dynamics contribute to oxidative stress– and hyperglycemia-induced alterations in mitochondrial morphology and function (3). Podocytes, a type of highly differentiated neuronlike epithelial cell in the kidney (4), have a limited capacity for cell replenishment. The autophagy of podocytes is inhibited under diabetic conditions, and defective autophagy might accelerate the irreparable progression of diabetic nephropathy (5).

Forkhead transcription factor O1 is a member of the forkhead box-containing transcription factor O family (6). High glucose stimulation can activate the PI3K/Akt pathway. Activation of Akt phosphorylates three threonine sites of forkhead-box class O1 (FoxO1) (Thr24, Ser256, and Ser319) to phosphorylate the FoxO1 protein (7). The phosphorylated FoxO1 protein binds to the 14-3-3 protein to cover the nuclear localization sequence, facilitating the binding of the nuclear export sequence to the modified protein, leading to the translocation of FoxO1 protein from the nucleus to the cytoplasm, followed by the loss of its function (8). Forkhead-box class O3 has been reported to stimulate lysosomal proteolysis.

*These authors contributed equally to this study.
Abbreviations: ATP, adenosine triphosphate; BUN, blood urea nitrogen; CA, constitutively active form; CIMP, cultured mouse podocyte cell; DB, diabetic mice; EM, electron microscopy; FoxO1, forkhead-box class O1; GFP, green fluorescent protein; HG, high glucose; ILK, integrin-linked kinase; LM, light microscopy; LV, lentivirus; mRNA, messenger RNA; NG, normal control group; PCR, polymerase chain reaction; PINK1, putative kinase 1; ROS, reactive oxygen species; Scr, serum creatinine; shPINK1, short hairpin RNA targeting PINK1; STZ, streptozotocin; UAlb, urinary albumin; UPro, urinary protein.
by activating autophagy via a transcriptional mechanism in muscle cells (9). FoxO1 acetylation occurs in autophagy mediated by benzylo isothiocyanate and curcumin (10). In addition, FoxOs have been reported to function together with putative kinase 1 (PINK1) to mediate mitochondrial oxidative stress in vivo (11). The accumulation of PINK1 on the mitochondrial outer membrane triggers the recruitment of Parkin to the mitochondria, leading to autophagic degradation of the dysfunctional mitochondrion (12-14). However, whether FoxO1 is also involved in PINK1-mediated autophagy in podocytes under diabetic conditions is not well understood.

The current study sought to elucidate how mitochondria respond to diabetes-induced mitophagy under the condition of FoxO1 overexpression, which results in podocyte injury, and to determine whether the effects occur downstream of the PINK1/Parkin pathway.

Methods

Podocyte culture and transfections

The immortalized mouse podocyte cell line [cultured mouse podocyte cells (CIMPs)] was a kind gift from Professor Peter Mundel (Harvard Medical School, Charlestown, MA) and was cultured as previously described (15). Positions Thr24, Ser256, and Ser319 in the FoxO1 coding sequence (NM_019739.3) were mutated to Ala to prevent phosphorylation and generate a constitutively active form of FoxO1 (CA-FoxO1). CIMPs were then transfected with a lentivirus (LV) containing CA-FoxO1 (LV-CA-FoxO1) or short hairpin RNA targeting FoxO1 (LV-shFoxO1) or PINK1 (LV-shPINK1) or empty control vector (NC) as mentioned in the "Methods" section. The investigators were blinded to the treatment conditions when performing the electron microscopy (EM) and immunohistochemistry experiments.

Specimen collection and preservation

Twelve weeks after the injection, urine was collected to determine 24-hour urinary protein (UPro) and urinary albumin (UAlb). Mice were anesthetized with chloral hydrate, and blood was collected from the orbital vein to measure serum creatinine (Scr) and blood urea nitrogen (BUN). The ratio of kidney weight/ body weight was measured after euthanization. The renal cortex at the injection sites was obtained and separated; the glomeruli were collected using a series of nylon sieves, as described previously (17). Tissues were sectioned at a thickness of 6 μm at −20°C to prepare frozen sections. Cortices were fixed in either 4% precooled glutaraldehyde for EM or in 4% paraformaldehyde for LM.

Immunoblotting assays

Total protein from the glomeruli or podocytes was extracted in RIPA with 1% protease inhibitor cocktail. The protein concentration was measured with the Bicinchoninic Acid Assay Kit (Sangon Biotech, Shanghai, People’s Republic of China). Total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were blocked with 5% bovine serum albumin for 2 hours. Rabbit anti-FoxO1 and antinephrin (Abcam, Cambridge, UK); rabbit antiphospho-FoxO1 (Ser256), anti-Drp-1, anti-LC3, and antidesmin (Cell Signaling Technology, Danvers, MA); rabbit anti-PINK1, anti-Parkin, antipodocalyxin, and anti-Mfn1 (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit anti-integrin-linked kinase (anti-ILK), anti-p62, and anti-β-actin (Biobasic, Inc., ON, Canada) primary antibodies were used. Binding sites of the primary antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Life Technology). The relative optical density of each band was determined using National Institutes of Health (Bethesda, MD) ImageJ software (public domain) and normalized to β-actin levels.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from glomeruli or podocytes using TRIzol reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed according to the manufacturer’s protocol (TOYOBO, Osaka, Japan). Amplification reactions were performed with a

<table>
<thead>
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<th>Table 1. Sequences of Primers Used for Quantitative Reverse Transcription PCR Analysis</th>
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</tr>
<tr>
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</tr>
<tr>
<td>β-Actin</td>
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<td>PINK1 promoter</td>
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KOD SYBR Green Master Mix Kit (TOYOBO) and specific primers (Table 1) on an ABI Fast 7500 cycler (Applied Biosystems, Foster City, CA). Quantification of gene expression standardized to β-actin messenger RNA (mRNA) levels was performed using the 2^(-ΔΔCt) method.

**Immunohistochemistry and immunofluorescence analysis**

Paraffin sections of mice kidneys were prepared by a conventional method and treated as described previously (16). The working concentrations of the ILK, podocalyxin, nephrin, and desmin antibodies were 1:500, 1:100, 1:100, and 1:200, respectively. The immunofluorescence staining protocol was performed as described previously (16). The anti-FoxO1 antibody and Cy3-labeled secondary antibody were diluted at 1:250 and 1:800, respectively. The distribution and subcellular localization of target proteins were examined under an IX71 fluorescence microscope (Olympus, Tokyo, Japan). Densitometric analysis was performed using Image-Pro Plus software, version 6.0 (Media Cybernetics, Rockville, MD).

Figure 1. FoxO1 and PINK1 expression in cultured mouse podocyte cells (CIMPs) and glomeruli of diabetic nephropathy mice. (A) FoxO1 and (B) PINK1 mRNA levels were detected by quantitative reverse transcription PCR analysis of total RNA. CIMPs were infected with a lentiviral-vector expressing CA-FoxO1 or small interfering RNA against FoxO1 (shFoxO1) under HG (25 mM glucose) or NG (5.6 mM) conditions. Cells infected with lentivirus expressing empty vector were used as a negative control (data not shown). (C–E) FoxO1, p-FoxO1, and PINK1 were detected by western blot analysis. (C) Ratio of p-FoxO1/total FoxO1 as determined by densitometric analysis. (D) Representative immunoblots. (E) PINK1 protein expression. (F) FoxO1 and (G) PINK1 mRNA levels were detected by quantitative reverse transcription PCR analysis of total RNA. (I) FoxO1, (J) p-FoxO1, and (K) PINK1 were detected by western blot analysis. (H) Representative immunoblots. (L) Ratio of p-FoxO1/total FoxO1 as determined by densitometric analysis. (M–P) Immunofluorescence images of FoxO1 labeled with Cy3 and 4',6-diamidino-2-phenylindole. (M) Quantitative analysis based on FoxO1 fluorescence intensity. (N) CIMPs cultured in NG. (O) CIMPs cultured in HG. (P) CIMPs cultured in HG and infected with short hairpin FoxO1. The data are presented as the means ± standard errors (n = 3). *P < 0.05 vs NG; †P < 0.05 vs HG/DM; ‡P < 0.05 vs HG + CA-FoxO1/CA. DM, diabetes mellitus; NC, diabetic mouse group administered lentivirus expressing empty vector.
Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were conducted using the EZ-ChIP Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Chromatin immunoprecipitation was performed as described previously.16 De-cross-linked DNA samples were subjected to polymerase chain reaction (PCR) amplification using forward (5′-GCTACCCCTCCACGGGGTTC-3′) and reverse primers (5′-GAGGCCCCAGGGGTACGC-3′) targeting the mouse promoter. Precipitated DNA fragments were analyzed by quantitative PCR.

Luciferase assays

The FoxO1 binding site of the PINK1 promoters was cloned into the vector pGL4.10 (Promega, Madison, WI) using primers described in Table 1. A T-to-A substitution of the sequence was described previously (16). De-cross-linked DNA samples were subjected to polymerase chain reaction (PCR) amplification using forward (5′-GCTACCCCTCCACGGGGTTC-3′) and reverse primers (5′-GAGGCCCCAGGGGTACGC-3′) targeting the mouse promoter. Precipitated DNA fragments were analyzed by quantitative PCR.

MitoTracker staining and GFP-LC3 infection

CIMPs infected with different lentiviral vectors were plated on coverslips and infected with an adenovirus vector containing GFP-LC3B (HANBIO). After high-glucose (HG) treatment, cells were treated with 250 nM MitoTracker Red (Invitrogen) in culture medium for 30 minutes at 37°C. The cells were fixed, washed, and then observed using a laser scanning confocal microscope (Olympus).

Mitochondrial membrane potential

The mitochondrial membrane potential of CIMPs was monitored using JC-1. Briefly, cells were trypsinized, resuspended, and incubated in the dark with JC-1 for 20 minutes at 37°C. The cells were then washed with JC-1 washing buffer, and the fluorescence was observed with an IX71 microscope (Olympus).

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) production was measured using 2′,7′-dichlorofluorescein diacetate (Beyotime, Shanghai, People’s Republic of China). The cells were trypsinized, resuspended, and incubated at 37°C for 20 minutes with 2′,7′-dichlorofluorescein diacetate at a final concentration of 10 mmol/L. Fluorescence intensity was analyzed by flow cytometry using excitation/emission wavelengths of 488/525 nm.

Adenosine triphosphate measurement

In accordance with the standard experimental protocol of Beyotime, cultured cells were homogenized and sonicated, and the adenosine triphosphate (ATP) content in CIMPs was determined using an ATP Bioluminescence Assay Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions.
with a luminescence plate reader (Molecular Devices, Sunnyvale, CA) and an integration time of 10 seconds.

Data analysis
All data are expressed as the mean ± standard error. The significance of differences among experimental groups was determined by one-way analysis of variance followed by the Bonferroni test for multiple comparisons and the multiple range test. A *P* value, 0.05 was considered statistically significant. Statistical analyses were conducted using SPSS 17.0 software (IBM SPSS, Watson, NY).

Results

**HG inhibited FoxO1 transcriptional activity, and overexpressing FoxO1 reversed HG-dependent PINK1 downregulation in vivo and in vitro**

FoxO1 and PINK1 play important roles in regulating mitophagy. To determine the relationship between FoxO1 transcriptional activity and PINK1 expression in CIMP, we analyzed the expression of FoxO1 and PINK1 in cultured CIMP [Fig. 1(A–E)] and mouse glomeruli [Fig. 1(F–K)]. FoxO1 mRNA and protein expression did not differ between the glomeruli from the NG and DM groups, whereas p-FoxO1 levels and the p-FoxO1/FoxO1 ratio increased in the glomeruli of mice from the DM group and in HG-cultured CIMP, indicating a relative increase in FoxO1 transcriptional activity. Similarly, PINK1 mRNA and protein levels were significantly decreased in the DM group compared with the NG group. In STZ-induced mice, CA-FoxO1 overexpression increased FoxO1 mRNA levels and decreased the p-FoxO1/FoxO1 ratio. Interestingly, the downregulation of PINK1 was reversed in the CA group. This phenomenon was confirmed by immunofluorescence. A notable decrease in FoxO1 nuclear translocation was observed in CIMP exposed to HG. FoxO1 nuclear translocation increased after CA-FoxO1 overexpression. Infection with the short interfering RNA-FoxO1 lentiviral vector caused the opposite effect [Fig. 1(L–P)].

**FoxO1 bound to and activated the PINK1 promoter in CIMP**

The interaction between FoxO1 and the PINK1 promoter element was examined by chromatin immunoprecipitation...

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Figure 3. Effects of FoxO1 on mitophagy-related protein expression in CIMP and glomeruli of diabetic nephropathy mice. (A–E) FoxO1 overexpression prevented the HG-dependent decrease in the expression of the mitophagy-related proteins (A) PINK1, (B) Parkin, (C) Drp-1, and (D) Mfn1 in cultured CIMP. Cells infected with lentivirus expressing empty vector, CA-Foxo1, or shPINK1 were analyzed under HG conditions (25 mM glucose). (E) Representative immunoblots. (F–L) The mitophagy-related proteins (H) Parkin, (I) Drp-1, (J) Mfn1, (K) p62, and (L) LC3 were detected by western blot analysis. (F–G) Representative immunoblots. The data are presented as means ± standard errors (n = 3). *P < 0.05 vs NG; #P < 0.05 vs HG/DM; &P < 0.05 vs HG + CA-FoxO1/CA. DM, diabetes mellitus; NC, diabetic mouse group administered lentivirus expressing empty vector.
Figure 4. Overexpression of FoxO1 protected mitochondrial function and mitochondrial membrane potential in HG-treated CIMPs. (A–G) Intracellular ROS production as quantified by (G) flow cytometry analysis using 2',7'-dichlorofluorescein diacetate. (A) CIMPs cultured in NG. (B) CIMPs cultured in HG. (C) CIMPs cultured in HG and infected with CA-FoxO1. (D) CIMPs cultured in HG and infected with both CA-FoxO1 and shPINK1. (E) CIMPs cultured in HG and infected with lentivirus expressing empty vector. (F) CIMPs without 2',7'-dichlorofluorescein diacetate staining. (H) CIMPs seeded into 6-well plates were treated for 72 hours as indicated. The cells were then coincubated with the fluorescence probe JC-1 for 20 minutes at 37°C; images (not shown) were scanned with a fluorescence microscope, and the red/green fluorescence intensity values were calculated. (I–O) Transmission EM images of cultured CIMPs (top: ×5000, bottom: ×15,000). (I) Mitochondrial area and (J) number were calculated; at least 30 images were quantified. (K) CIMPs cultured in NG. (L) CIMPs cultured in HG. (M) CIMPs cultured in HG and infected with CA-FoxO1. (N) CIMPs cultured in HG and infected with both CA-FoxO1 and shPINK1. (O) CIMPs cultured in HG and infected with lentivirus expressing empty vector. (P) ATP levels. The data are presented as the means ± standard errors (n = 3). *P < 0.05 vs NG; #P < 0.05 vs HG; &P < 0.05 vs HG + CA-FoxO1. NC, diabetic mouse group administered lentivirus expressing empty vector.
assays [Fig. 2(A) and 2(B)] using an antibody specific for FoxO1. The promoter region of mouse PINK1 contains a conserved FoxO consensus DNA-binding site “GGTGTTGT.” FoxO1 bound to the PINK1 promoter region in CIMPs and HG treatment strongly suppressed this binding. To further explore the mechanism of regulation of PINK1 by FoxO1, we inserted the human PINK1 (with the normal FoxO1 binding site or a mutated sequence) into the pGL4.10 vector, which was then transfected into CIMPs; no promoter vector was used as a negative control. The promoter activity of PINK1 was increased 5.3-fold by FoxO1. Moreover, a point mutation of the main FoxO1 binding site of the PINK1 promoter region, as determined by analysis with Genomatix (Munich, Germany) software, reduced its transcriptional activation capacity by >50%, indicating that FoxO1 acts mainly through this PINK1-binding site [Fig. 2(C)].

FoxO1 overexpression increased HG-induced mitophagy and mitophagy-related protein deficiency by activating the PINK1/Parkin pathway

We determined the basal level of mitophagy using GFP-LC3B and MitoTracker Red [Fig. 2(D–G)]. Immunofluorescence microscopy imaging of cells expressing GFP-LC3B showed that LC3B was mainly cytosolic in CIMPs grown in HG but was localized to punctate structures in CIMPs grown in NG. The image analysis indicated that the CIMPs grown in NG showed a threefold increase in the number of overlapping vesicles labeled by GFP-LC3B and MitoTracker Red relative to that in CIMPs grown in HG. However, the number of dual-labeled vesicles was twofold higher in CIMPs overexpressing FoxO1 than in HG-cultured CIMPs.

To verify the role of FoxO1 in mitophagy, we measured the protein levels of PINK1, Parkin, Drp-1, and Mfn1 in cultured CIMPs [Fig. 3(A–E)]. Western blot analysis showed that HG treatment decreased PINK1, Parkin, and Mfn1 expression and increased Drp-1 expression. However, these effects were reversed by FoxO1 overexpression. In contrast, the effects of overexpressing FoxO1 were largely suppressed when HG-treated CIMPs were infected with both LV-CA-FoxO1 and LV-shPINK1.

In the DM and the lentivirus-expressing empty vector groups compared with the NG group, Parkin, LC3/II, and Mfn1 expression decreased, and Drp-1 and p62 expression increased [Fig. 3(F–L)], indicating a deficiency in autophagy under HG conditions. In addition, overexpressing FoxO1 restored the levels of Parkin, LC3/II, and Mfn1 and inhibited Drp-1 and p62 expression.

Overexpression of FoxO1 protected mitochondrial function in HG-treated CIMPs

To determine whether FoxO1 alters the mitochondrial oxidative damage of podocytes, we examined cellular ROS production in CIMPs [Fig. 4(A–G)]. ROS production was increased in HG-treated CIMPs compared with the NG-treated group. ROS generation in CA-FoxO1–infected CIMPs dramatically decreased compared with CIMPs in the HG group. In contrast, ROS generation was significantly increased in short hairpin FoxO1-infected CIMPs compared with the CIMPs in the HG group. In addition, the effect of FoxO1 was largely decreased when HG-treated CIMPs were infected with both CA-FoxO1 and shPINK1.

JC-1 fluorescence detection showed a decrease in the red fluorescence that accumulated in the mitochondria and an increase in the green fluorescence distributed in the cytoplasm in CIMPs incubated with HG, suggesting that HG treatment reduced the mitochondrial membrane potential [Fig. 4(H)]. In contrast, FoxO1 overexpression resulted in an apparent improvement in the mitochondrial membrane potential compared with that observed following incubation with HG. However, the effect of FoxO1 was largely decreased when HG-treated CIMPs were infected with CA-FoxO1 and shPINK1.

Transmission EM confirmed that FoxO1 upregulation significantly preserved mitochondrial morphology by maintaining the mitochondrial area, number, and shape [Fig. 4(I–O)]. Treatment with HG and CA-FoxO1 resulted in significantly increased ATP levels [Fig. 4(P)] in CIMPs

Table 2. Body Weight, Kidney Weight–to–Body Weight Ratio, and Biochemical Indicators in the NG and DM Groups and Mice Infected With CA or NC After 12 Weeks

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>BW, g</th>
<th>BG, mmol/L</th>
<th>UPro, mg/24 h</th>
<th>UAlb, μg/mL</th>
<th>Scr, μmol/L</th>
<th>BUN, μmol/L</th>
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<td>NG</td>
<td>9</td>
<td>53 ± 5</td>
<td>5.3 ± 0.7</td>
<td>15.2 ± 2.3</td>
<td>23.5 ± 1.4</td>
<td>9.0 ± 0.5</td>
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<td>9</td>
<td>30 ± 3a</td>
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<td>40.4 ± 5.6b</td>
<td>43.6 ± 1.4b</td>
<td>12.6 ± 0.3b</td>
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<td>9</td>
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<td>26.1 ± 0.9a</td>
<td>90.6 ± 6.8</td>
<td>85.3 ± 2.4</td>
<td>25.8 ± 0.5</td>
<td>20.2 ± 0.6</td>
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The data are displayed as the means ± standard errors. BG, blood glucose; BW, body weight; NC, diabetic mouse group administered lentivirus-expressing empty vector.

aP < 0.05 vs the NG group.

bP < 0.05 vs the DM group.
compared with those treated only with HG. When shPINK1 treatment was administered to the former group, ATP levels significantly declined.

FoxO1 overexpression protected against podocyte injury and ameliorated diabetic nephropathy progress in vivo

We found that blood glucose levels, the kidney weight/body weight ratio, and the UPro/24-hour, UALb, Scr, and BUN levels significantly increased in the diabetic mice (Table 2) compared with the normal mice. FoxO1 overexpression had a protective effect on renal function in the DM mice. However, FoxO1 overexpression failed to decrease blood glucose levels.

We examined the podocyte slit diaphragm proteins nephrin and desmin, the adhesion molecule ILK, and the sialoglycoprotein podocalyxin (Fig. 5). Under HG conditions, nephrin and podocalyxin expression decreased, and desmin and ILK expression increased. These expression levels changed in the opposite direction in the CA group compared with the DM group.

We also histopathologically examined the kidneys of these mice. Hematoxylin and eosin staining revealed a larger renal glomeruli volume, mesangial cell expansion,
and thickening of the glomerular basement membrane in the kidneys of the diabetic mice [Fig. 6(A–D)]. Periodic acid–Schiff staining showed the accumulation of mesangial matrix [Fig. 6(E–H)]. Conversely, the renal pathology was ameliorated in mice from the CA-FoxO1 group. Moreover, by EM, we found serious podocyte injury in diabetic mice. In contrast, FoxO1 overexpression somewhat promoted the recovery of injured podocytes [Fig. 6(I–P)].

**Discussion**

In the current study, we report what, to our knowledge, is an organ-specific novel role of FoxO1 in diabetes-induced mitophagy. Our data show a PINK/Parkin-dependent connection between mitophagy in diabetic podocytes and FoxO1. Mitophagy has been mainly regarded as an acute degradative process that is triggered by local and severe mitochondrial damage or as part of a developmental process for removing excess mitochondria (18–20). Podocytes are enriched with mitochondria and strongly rely on oxidative phosphorylation for energy production. A growing body of evidence indicates that mitochondrial dysfunction may be pathologic in diabetic kidney disease (21, 22). An inability to remove aged mitochondria by mitophagy would be a considerable problem. We observed that in the presence of HG, CIMPs strongly repressed mitochondrial activity,
and ATP accumulated. Conversely, when these cells were infected with CA-FoxO1, mitochondrial energy metabolism was highly activated, and cellular ATP was produced via oxidative phosphorylation. It was reported that hematopoietic stem cells in triple-mutant mice deficient for FoxO1, FoxO3a, and FoxO4 exhibit an aberrant increase in ROS (23). This result agrees with our observation in CIMP5S: cells that overexpressed FoxO1 showed a marked reduction in ROS content under HG conditions. Mitochondrial function is strongly dependent on mitochondrial morphology (24), and the changes in mitochondrial morphology that were observed by transmission EM were consistent with previous analyses of mitochondrial metabolism markers. Furthermore, our findings demonstrate that FoxO1 overexpression protected against the decrease in HG-induced mitophagy, both in cultured CIMP5S and in the glomeruli of STZ-induced mice.

Briefly, these data indicate that FoxO1 expression is essential for promoting a normal level of mitophagy under HG conditions.

PINK1 is a cytosolic serine/threonine kinase that is localized to mitochondria via an N-terminal mitochondrial-targeting sequence (25). Sengupta et al. (26) reported that in cultured cardiomyocytes, increased FoxO function leads to decreased ROS production, increased antioxidant levels, and increased expression of autophagy-related genes and that FoxO directly binds to the PINK1 promoter regions in neonatal cardiomyocytes. Our data described herein showed that FoxO1 could interact with the PINK1 promoter in cultured CIMP5S (Fig. 7). PINK1 transcription has been shown to be induced by FoxO (27), and forkhead-box class O3a was identified as a key transcription factor directing the expression of PINK1 in cells deprived of growth factors, a culture condition that is known to
activate FoxOs (28). We have provided several lines of evidence suggesting that mitophagy is accelerated when the transcriptional activity of FoxO1 increases. First, we demonstrated that an increased rate of mitochondrial energetic activity accompanies an improvement in mitochondrial morphology. Second, we showed that these phenomena occur through the activation of PINK1, thus linking FoxO1 and mitochondria. Finally, the inhibition of PINK1 impaired mitochondrial metabolism by counter-acting the FoxO1 stimulation signal.

Thus, the low FoxO activity observed under conditions of oxidative stress may contribute to PINK1

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<th>Name of Antibody</th>
<th>Manufacturer, Catalog No.</th>
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<td>ILK</td>
<td>Anti-ILK rabbit polyclonal antibody, Sangon Biotech (Shanghai) Co., Ltd., D120857</td>
<td>Anti-ILK rabbit polyclonal antibody, Sangon Biotech (Shanghai) Co., Ltd., D120857</td>
<td>Rabbit; polyclonal</td>
<td>1:800 (WB); 1:500 (IH)</td>
<td>AB_2631340</td>
<td></td>
</tr>
<tr>
<td>PINK1</td>
<td>Amino acids 61 to 360 mapping within an internal region of Parkin of human origin</td>
<td>Parkin antibody (H-300), Santa Cruz, sc-33796</td>
<td>Rabbit; polyclonal</td>
<td>1:1000</td>
<td>AB_2164259</td>
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<tr>
<td>Parkin</td>
<td>Amino acids 10 to 74 mapping within an N-terminal cytoplasmic domain of Mfn1 of human origin</td>
<td>Mfn1 antibody (H-65), Santa Cruz, sc-50330</td>
<td>Rabbit; polyclonal</td>
<td>1:500</td>
<td>AB_2250540</td>
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<tr>
<td>Mfn1</td>
<td>Amino acids 23 to 322 mapping within an N-terminal extracellular domain of podocalyxin-like 1 of human origin</td>
<td>Podocalyxin-like 1 antibody (H-300), Santa Cruz, sc-33138</td>
<td>Rabbit; polyclonal</td>
<td>1:500 (WB); 1:100 (IH)</td>
<td>AB_2166002</td>
<td></td>
</tr>
<tr>
<td>PCX</td>
<td>Amino acids 23 to 322 mapping within an N-terminal extracellular domain of podocalyxin-like 1 of human origin</td>
<td>Podocalyxin-like 1 antibody (H-300), Santa Cruz, sc-33138</td>
<td>Rabbit; polyclonal</td>
<td>1:500 (WB); 1:100 (IH)</td>
<td>AB_2166002</td>
<td></td>
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<tr>
<td>Goat anti-rabbit (HRP)</td>
<td>Rabbit IgG, whole molecule</td>
<td>Goat anti-rabbit IgG H&amp;L (HRP), Abcam, ab6721</td>
<td>Goat; polyclonal</td>
<td>1:10,000</td>
<td>AB_955447</td>
<td></td>
</tr>
<tr>
<td>Donkey anti-rabbit (Cy3)</td>
<td>Donkey anti-rabbit IgG Cy3 conjugate, Millipore, AP182C</td>
<td>Donkey anti-rabbit IgG Cy3 conjugate, Millipore, AP182C</td>
<td>Donkey; polyclonal</td>
<td>1:800</td>
<td>AB_11210062</td>
<td></td>
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Abbreviations: aa, amino acids; HRP, horseradish peroxidase; ICF, immune cell fluorescence; IgG, immunoglobulin G; IH, immunohistochemistry; RRID, research resource identifier; WB, western blot.
suppression in HG states. Notably, we observed that the pathological changes in kidneys were improved in diabetic rats treated with CA-FoxO1. For example, the UPro/24-hour, UAlb, Scr, and BUN levels were significantly decreased, and the podocyte injury was partly reversed. In addition, diabetic rats with FoxO1 overexpression showed an improvement in the glomerular lesions in the kidney. EM examination of the glomeruli at 12 weeks showed typical changes of podocytopathy with extensive effacement of podocyte processes along the glomerular basement membrane. The new appreciation for the role of FoxO1 in mitochondrial self-renewal highlights the importance of mitochondria-targeted treatment in diabetic nephropathy.

In conclusion, our findings demonstrated that FoxO1 is sufficient to protect mitochondrial dysfunction and podocyte injury under HG conditions. In addition, this renal-protective effect is associated with an increased level of PINK1, which was shown to be a direct downstream target of FoxO1 and other mitophagy-related genes in cultured CIMPs and diabetic nephropathy mice. Together, these findings support a vital role for FoxO1 in limiting ROS production and maintaining mitochondrial morphology and stability in response to HG-induced injury in the kidney. Thus, it remains to be seen whether manipulating FoxO1 function or specific FoxO1 downstream target genes, such as PINK1, in the kidney will be an effective therapeutic approach for diabetic nephropathy.

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Author contributions: W.L. and M.D. conceived and designed the study, performed the experiments, analyzed the results, and wrote the manuscript. Q.W. collected clinical samples and revised the manuscript. L.W. performed the statistical analyses and participated in the study design. F.G. participated in the manuscript preparation and immunohistochemistry experiments. X.M. helped with sample collection and writing. F.H. helped with the statistical analyses. H.J. helped to build the animal model. G.Q. is the guarantor of this work, provided financial support and supervised the laboratory processes, had full access to all the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

Disclosure Summary: The authors have nothing to disclose.

References


