

**CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY****Glypican-5 Increases Susceptibility to Nephrotic Damage in Diabetic Kidney**

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Type 2 diabetes mellitus is a leading health issue worldwide. Among cases of diabetes mellitus nephropathy (DN), the major complication of type 2 diabetes mellitus, the nephrotic phenotype is often intractable to clinical intervention and demonstrates the rapid decline of renal function to end-stage renal disease. We recently identified the gene for glypican-5 (*GPC5*), a cell-surface heparan sulfate proteoglycan, as conferring susceptibility for acquired nephrotic syndrome and additionally identified an association through a genome-wide association study between a variant in *GPC5* and DN of type 2 diabetes mellitus. *In vivo* and *in vitro* data showed a progressive increase of *GPC5* in type 2 DN along with severity; the excess was derived from glomerular mesangial cells. In this study, diabetic kidney showed that accumulation of fibroblast growth factor (Fgf)2 strikingly induced progressive proteinuria that was avoided in *Gpc5* knockdown mice. The efficacy of *Gpc5* inhibition was exerted through expression of the Fgf receptors 3 and 4 provoked in the diabetic kidney attributively. Extraglomerular Fgf2 was pathogenic in DN, and the deterrence of *Gpc5* effectively inhibited the glomerular accumulation of Fgf2, the subsequent increase of mesangial extracellular matrix, and the podocytes' small GTPase activity. These findings elucidate the pivotal role of *GPC5*, identified as a susceptible gene in the genome-wide association study, in hyperglycemia-induced glomerulopathy. (*Am J Pathol* 2015, 185: 1889–1898; <http://dx.doi.org/10.1016/j.ajpath.2015.03.025>)

Among diabetes mellitus nephropathy (DN) cases, intractable dysfunction rapidly propagating to end-stage renal disease often accompanies the nephrotic proteinuria phenotype. Our recent study implicated glypican-5 (*GPC5*) as a susceptible gene for acquired nephrotic syndrome and demonstrated its functional significance by using podocyte-specific knockout mice; a genome-wide association study of DN satisfying the criterion of nephrotic syndrome also clarified *GPC5* as the candidate gene [odds ratio 1.45 (95% CI, 1.18–1.79)].<sup>1</sup> Glypicans share 14 conserved and unique cysteine residues and covalently link to the cell membrane via a glycosylphosphatidylinositol anchor.<sup>2</sup> Glypican is a heparan sulfate proteoglycan, which facilitates selective protein–protein interactions establishing transient cell-signal platforms in concert with lipid rafts; glypican plays a critical role in regulating the signaling of Wnt; hedgehogs; bone morphogenetic protein; and, especially, of fibroblast growth factor (FGF)2.<sup>3</sup>

FGF2 is a highly conserved 18-kDa cationic protein released from the cytosolic storage site through plasma membrane disruptions.<sup>4,5</sup> In the kidney, the release of FGF2 is probable from mesangial cells<sup>6</sup> and podocytes.<sup>7</sup> It stimulates *in vitro*

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Disclosures: None declared.

**Table 1** Basic Characteristics of Human Subjects

Characteristic	Control group	DN group
<i>n</i>	2	5
Sex (F/M)*	1/1	1/4
Age, years <sup>†</sup>	28.5 ± 3.5	62.0 ± 17.1
Proteinuria, grams per 24 hours <sup>†</sup>	0.1 ± 0.1	5.1 ± 3.5
Serum creatinine, mg/dL <sup>†</sup>	0.9 ± 0.5	1.5 ± 0.2

\*Data are expressed as numbers of subjects.

<sup>†</sup>Data are expressed as means ± SEM.

F, female; M, male; DN, diabetes mellitus nephropathy.

proliferation of mesangial cells,<sup>8</sup> endothelial cells,<sup>9</sup> and podocytes.<sup>7</sup> An excess of FGF2 clearly injures podocytes *in vivo* and induces proteinuria.<sup>10,11</sup> Based on the information from the genome-wide association study that *GPC5* is a susceptible gene, this study was undertaken to clarify the functional role of *GPC5* in DN and related pathways.

## Materials and Methods

All the chemical compounds are purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and culture medium and related supplements were purchased from Life Technologies (Carlsbad, CA) unless otherwise specified.

### Human Specimens

For human glomerulus staining, human tissues were obtained from Asterand (Detroit, MI) and used with written informed consent and institutional review board approval. Human kidney tissues were obtained from diabetic patients and nondiabetic controls during surgery (Table 1).

### Animals

We obtained 8- to 12-week-old C57BL/6 and C57BL/6Cr *Ins2<sup>+/-C96Y</sup>* (Akita)<sup>12</sup> mice (Japan SLC, Tokyo). Podocyte-specific *Gpc5* knockdown mice and *Gpc5* systemic knockdown mice were produced as reported previously.<sup>1</sup> All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals<sup>13</sup> and were approved by The University of Tokyo Institutional Review Board.

### Cell Culture

MES13 mouse mesangial cell line (ATCC, Manassas, VA) was cultured in a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium. This mixture was supplemented with 5% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Conditionally immortalized mouse podocytes were described previously<sup>14</sup> and were characterized by positive staining for Wilms tumor 1, synaptopodin, podocin, CD-associated protein, nephrin, ezrin, and podocalyxin. These cells were grown in RPMI 1640 medium containing 10%

fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES buffer, and 0.075% sodium bicarbonate. For passaging the cells, podocytes were grown under growth-permissive conditions, which involves growing cells at 33°C in the presence of 50 U/mL interferon-γ. For podocytes to acquire a differentiated and quiescent phenotype, cells were grown under restrictive conditions at 37°C without interferon-γ for 10 days.

A rat mesangial cell line had been established by our group and was reported previously.<sup>12,15</sup> Characterization of rat cultured mesangial cells was performed using antibodies of Thy-1.1. Cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum, 0.66 U/mL insulin, 100 U/mL penicillin, and 100 µg/mL streptomycin.

### Immunofluorescence Staining

Frozen sections cut in 8-µm pieces were dried and fixed with ice-cold acetone at -20°C for 20 minutes. Primary antibodies were FGF receptor (FGFR)1, FGFR2, FGFR3, FGFR4, FGF2 (Abcam, Cambridge, UK), *GPC5* (R&D Systems, Minneapolis, MN), and nephrin (Acris Antibodies, Herford, Germany). After washing them with phosphate-buffered saline (PBS), we applied fluorescent-conjugated (Alexa488, Alexa555, or Alexa633) secondary antibody (Thermo Fisher Scientific, South San Francisco, CA) for 60 minutes. The sections were then examined using confocal microscopy (LSM 510 META NLO imaging system; Carl Zeiss, Oberkochen, Germany).<sup>1,16</sup>

### Immunoelectron Microscopy Analysis

Immunotransmission electron microscopic analysis was performed using the pre-embedding method. Briefly, 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) was used for the fixation of sliced C57BL/6 kidney, and the overnight and dehydration procedure used was the sucrose gradient method from 10% to 30%. The kidney was embedded in Tissue-Tek (Sakura Finetek, Tokyo, Japan) and sliced to 8 µm. After blocking processes, the first antibody [*GPC5* antibody (R&D Systems) described in *Immunofluorescence Staining*] was applied for 2 days. After a rigorous wash with PBS, the second biotinylated antibody was applied for 1 hour. Then avidin-conjugated horseradish peroxidase was applied after a rigorous wash with PBS. After the colorimetric reaction, the sample was fixed again in osmium tetroxide and transferred to Epon (Miller-Stephenson, Fairfield, CT). Ultrathin sections were examined by means of transmission electron microscopy (JEOL, Tokyo, Japan).

### *In Vitro* Analysis of FGFRs, FGF2, and *GPC5* Expression

Cells were incubated in each glucose concentration for 72 hours, and each osmolality was adjusted using D-mannitol (Sigma-Aldrich, St. Louis, MO). Because glucose-free F12 medium is not available, Dulbecco's modified Eagle's medium

**Table 2** Quantitative Real-Time PCR Primer Sequences

Target gene	Primer sequence
Mouse <i>Gpc5</i>	F: 5'-CTAGTGGAGACTGTGATGACGAA-3' R: 5'-TTCATGCTGTCTGGCATCC-3' Probe#76 (Roche)
Mouse <i>Fgfr3</i>	F: 5'-GGAGGACGTGGCTGAAGA-3' R: 5'-CGGAAGCGGACAGTGTTC-3' Probe#76 (Roche)
Mouse <i>Fgfr4</i>	F: 5'-ATGACCGTCGTACACAATCTTAC-3' R: 5'-TGCCAGTAGGGTGCTTGC-3'
Mouse <i>Fgf2</i>	F: 5'-CGGCTCTACTGCAAGAACG-3' R: 5'-TGCTTGGAGTTGTAGTTTGACG-3'
Rat <i>Fn1</i>	F: 5'-CCGGTGGCTGTCAGTCAGA-3' R: 5'-CCGTTCCCCTGCTGATTTATC-3'
Rat <i>Col4a2</i>	F: 5'-TGATGGAGAAAAAGGAGTCTCAG-3' R: 5'-GCCTTGGAAACCATCCAG-3'
Mouse <i>Actb</i>	F: 5'-CGCACCCTGGCATTGTCAT-3' R: 5'-TTCTCCTTGATGTCACGCAC-3'
Rat <i>Gapdh</i>	F: 5'-CAAGGTCATCCATGACAACCTTTG-3' R: 5'-GGCCATCCACAGTCTTCTGG-3'

F, forward primer; R, reverse primer.

supplemented with 5% fetal bovine serum was alternatively used for the MES13. Total RNA was isolated using TRIzol (Life Technologies). After performing deoxyribonuclease I treatment, we performed reverse transcription (ImProm-II; Promega, Madison, WI) and quantitative real-time PCR by using the SYBR Green system (Life Technologies) or the Universal ProbeLibrary system (Roche, Basel, Switzerland) (mouse *Gpc5* and mouse *Fgfr3* only) and normalized for the relative expression of the genes of interest by using  $\beta$ -actin. The quantitative real-time PCR primer sequences are presented in Table 2. All samples were analyzed in triplicate.

### In Situ Hybridization

The mouse kidney was dissected after perfusion, fixed with tissue fixative (Genostaff, Tokyo, Japan), embedded in paraffin, and sectioned at 6  $\mu$ m. The standard *in situ* hybridization protocol was used.<sup>17</sup> Tissue sections were dewaxed and rehydrated. The sections were fixed in 4% paraformaldehyde for 15 minutes, treated with 8  $\mu$ g/mL proteinase K (Merck, Whitehouse Station, NJ) for 30 minutes at 37°C, refixed with 4% paraformaldehyde (Sigma-Aldrich), and placed in 0.2 N HCl for 10 minutes. The sections were acetylated by means of incubation in 0.1 mol/L triethanolamine HCl (Sigma-Aldrich), pH 8.0, and 0.25% acetic anhydride for 10 minutes. The sections were then dehydrated again. Hybridization was performed with probes at concentrations of 300 ng/mL in Probe Diluent-1 (Genostaff) at 60°C for 16 hours (GenBank; <http://www.ncbi.nlm.nih.gov/nucleotide>; Accession number NM\_008006.2, position 444 to 691, size 248). After hybridization, the sections were washed in 5 $\times$  HybriWash (Genostaff), equal to 5 $\times$  standard saline citrate, at 60°C for 20 minutes and then in 50% formamide, 2 $\times$  HybriWash at 60°C for 20 minutes followed by ribonuclease treatment in 50  $\mu$ g/mL ribonuclease A (Qiagen, Hilden, Germany)

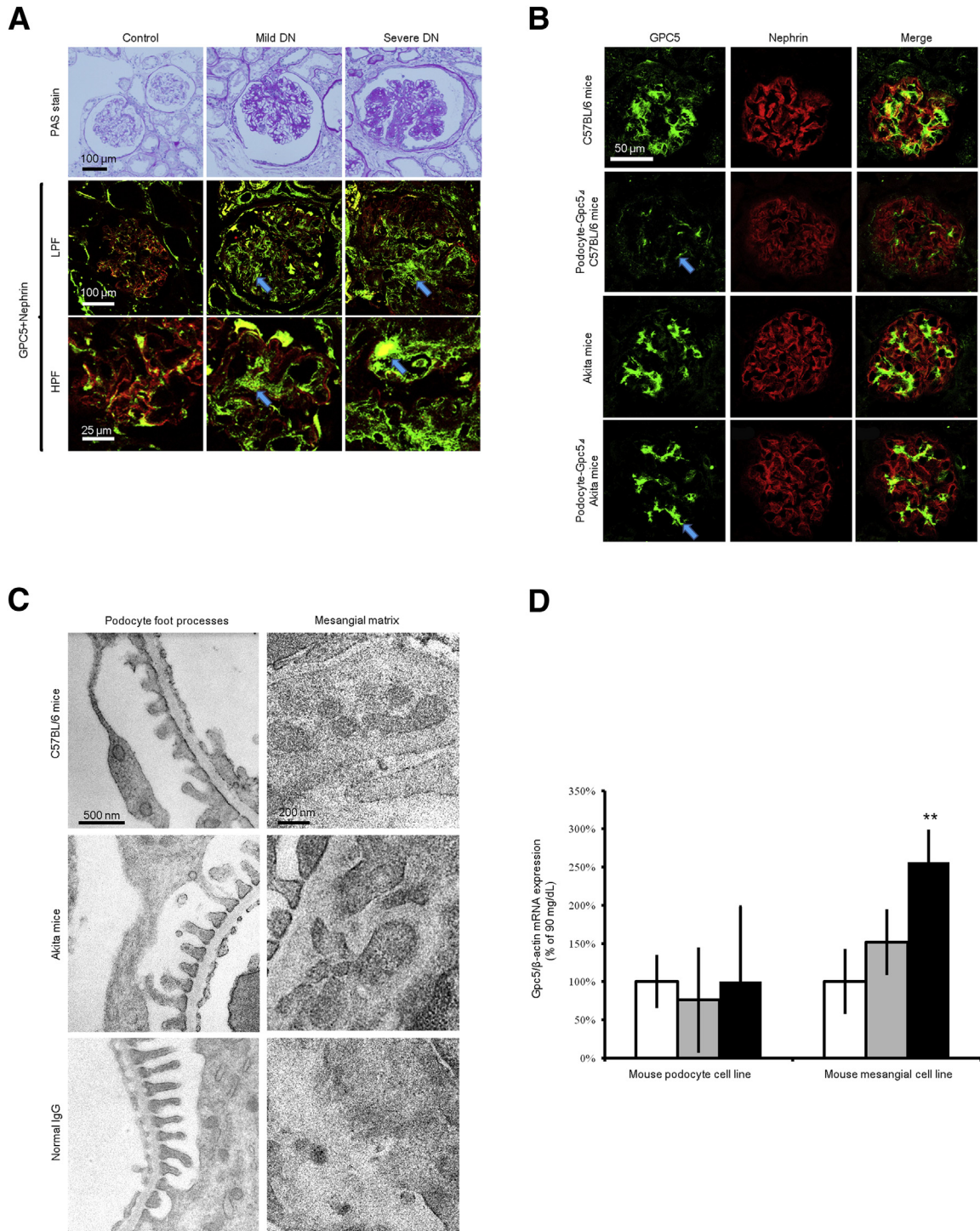
in 10 mmol/L Tris-HCl, pH 8.0, 1 mol/L NaCl, and 1 mmol/L EDTA for 30 minutes at 37°C. Then the sections were washed twice with 2 $\times$  HybriWash (Genostaff) at 60°C for 20 minutes, twice with 0.2 $\times$  HybriWash at 60°C for 20 minutes, and once with Tris-buffered saline with 0.1% Tween 20. After treatment with 0.5% blocking reagent (Roche) in Tris-buffered saline with 0.1% Tween 20 for 30 minutes, the sections were incubated with antidigoxigenin alkaline phosphatase conjugate (Roche) diluted 1:1000 with Tris-buffered saline with 0.1% Tween 20 for 2 hours at room temperature. The sections were washed twice with Tris-buffered saline with 0.1% Tween 20 and then incubated in 100 mmol/L NaCl, 50 mmol/L MgCl<sub>2</sub>, 0.1% Tween 20, and 100 mmol/L Tris-HCl, pH 9.5. Coloring reactions were performed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate solution (Sigma-Aldrich) overnight and then washed with PBS. The sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan) and mounted with CC/Mount (Diagnostic BioSystems, Pleasanton, CA).

### Measurement for FGF2 *in Vivo*

Endogenous FGF2 protein was detected using a mouse basic FGF enzyme-linked immunosorbent assay kit (Ray Biotech, Norcross, GA). To elucidate the accumulation of systemic circulating FGF2 *in vivo*, we biotinylated recombinant human FGF2 (Kaken Pharmaceutical, Tokyo, Japan) by using a Pierce EZ-Link Micro Sulfo-NHS-LC biotinylation kit (Thermo Fisher Scientific, Waltham, MA) according to the protocol and purified it using a G-25 column (GE Healthcare, Little Chalfont, UK). Biotinylated FGF2 (10  $\mu$ g per mouse) was injected intravenously, and the kidneys were harvested 1 hour later. Frozen sections (8  $\mu$ m pieces) were dried and fixed with ice-cold acetone at -20°C for 20 minutes. After conjugation with Alexa-555 conjugated streptavidin (Life Technologies), sections were examined using confocal microscopy. We quantified at least five glomeruli in the outer cortex selected from each animal. The signal intensity was measured and standardized with the interstitial background.

### Animal Experiment Protocol for Extrarenal FGF2 Infusion

We assigned the animals to one of three groups: C57BL/6, Akita, and systemic *Gpc5* knockdown mice. FGF2 mice were injected intravenously on days 0, 1, 2, and 3 (5  $\mu$ g per animal). Systemic *Gpc5* knockdown was undertaken 2 days before the FGF2 injection as reported previously.<sup>1,18</sup> Briefly, the mouse *Gpc5* siRNA (5'-GCAGGCGCUUA-AUCUGGGCAUUGAA-3', 5'-UUCAAUGCCCAGAAUUAAGCGCCUGC-3') and scrambled siRNA (5'-CCUAC-AUGACGACGAUGUACCGUGA-3', 5'-UCACGGUAC-AUCGUCGUCAUGUAGG-3') were obtained from Life Technologies as annealed *in vivo*-grade siRNA. Synthetic



**Figure 1** Expression pattern of GPC5 in developing diabetic albuminuria. **A:** Immunofluorescence staining of human kidney shows diabetes mellitus nephropathy (DN). Representative images in periodic acid-Schiff (PAS) stain, low-power field (LPF) and high-power field (HPF), are shown. GPC5 (green) was stained with Alexa 488 fluorescence. Nephrin (red) was counterstained with Alexa 633; images from the same sections were merged to form an overlapping image (yellow). **Arrows** depict GPC5 expression in nodular glomerular sclerosis. **B:** Immunofluorescence staining of kidney from C57BL/6 mice, podocyte-specific *Gpc5* knockdown ( $\Delta$ ) C57BL/6 mice, Akita mice, and podocyte-specific *Gpc5* knockdown ( $\Delta$ ) mice that were inbred with Akita mice. Gpc5 (green) was stained with Alexa 488. Nephrin (red) was counterstained with Alexa 633. An **arrow** depicts total absence of intraglomerular Gpc5 staining on podocyte in Podocyte-Gpc5 $\Delta$  C57BL/6 whereas intraglomerular Gpc5 staining is seen in Podocyte-Gpc5 $\Delta$  Akita, and this **arrow** indicates mesangial region. Images from the same sections were merged to form an overlapping image. **C:** Localization of Gpc5 in kidney sections by means of immunoelectron microscopy from 10-week-old C57BL/6 mice, Akita mice, and normal IgG. Electron micrographs show localization of Gpc5 by using a pre-embedding method. **D:** Gpc5 mRNA expression levels in cultured mouse podocyte and mesangial cell lines after three different administrations of the 72-hour glucose challenge [90 (white bars), 270 (gray bars), or 450 mg/dL (black bars)]. Osmolality was controlled with D-mannitol. Data are expressed as means  $\pm$  SEM.  $n = 5$  (D). \*\*Post hoc  $P < 0.01$  versus 90 mg/dL.

siRNA 400  $\mu\text{g}$  dissolved in 0.8 mL PBS was rapidly injected intravenously following the hydrodynamic method.

### Analyses of Urinary Albumin and Urinary Creatinine

We determined the urinary albumin levels with an enzyme-linked immunosorbent assay by using a murine microalbuminuria enzyme-linked immunosorbent assay kit (Albuwell M; Exocell, Philadelphia, PA) and measured the urine creatinine concentration with Nescotat VLII CRE (Alfresa Pharma, Osaka, Japan). Albuminuria was determined at the ratio of urinary albumin to urinary creatinine (mg/g Cre). All procedures were performed in accordance with the manufacturer's protocols.

### Extracellular Matrix Expression by FGF2 Signal Transduction in the Mesangial Cell Line

Rat mesangial cells were transfected either with Stealth RNAi Negative Control Med GC (Life Technologies) or with pre-designed *Gpc5* siRNA targeted against *Gpc5* (NM\_001107285.1; 5'-GCAGGCGCUAAUCUGGGCAUUGAA-3', 5'-UUCAUGCCCAGAUUAAGCGCCUGC-3'; Life Technologies) by using Lipofectamine 2000 (Life Technologies). After transfection, cells were subjected to different concentrations of glucose (300 or 600 mg/dL) for 48 hours with subsequent incubation by using different concentrations of FGF2 for 24 hours. Then mRNA was harvested to examine fibronectin 1 (*Fn1*) and collagen type IV (*Col4a2*) expression.

### Rac1 Activation Assay in the Mouse Podocyte Cell Line

Mouse podocyte cells were subjected to different concentrations of glucose (300 or 600 mg/dL) for 96 hours with subsequent incubation with 25 nmol/L FGFR antagonist PD173074 (Sigma-Aldrich) or dimethyl sulfoxide for 3 hours. Then the protein lysate was harvested to examine active Rac1 levels (Rac1 G-LISA Activation Assay Biochem Kit; Cytoskeleton, Denver, CO).

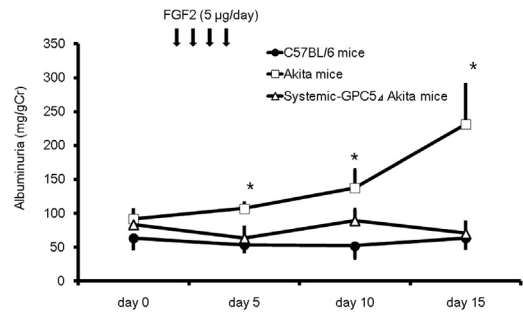
### Statistical Analysis

Data are expressed as means  $\pm$  SEM. Unless otherwise specified, differences in each group were tested using an unpaired *t*-test for the two-group comparison. Statistical significance was inferred for  $P < 0.05$ . One-way analysis of variance followed by Bonferroni *post hoc* comparison tests were performed in statistical analyses for comparison of the three groups (Dr. SPSS II version 11.01; IBM, Armonk, NY).

## Results

### GPC5 Expression Increases in Diabetes

Immunofluorescence analysis of diabetic glomeruli showed that higher expressions of GPC5 were proportional to diabetes



**Figure 2** Pathogenetic role of *Gpc5* and fibroblast growth factor 2 in developing diabetic albuminuria in mice. Albuminuria (milligrams of urinary albumin to grams of creatinine) was increased significantly in Akita mice after FGF2 injection; days 5, 10, and 15, respectively, compared with that of day 0. This increase was repressed significantly to the level of nondiabetic C57BL/6 by the knockdown of *Gpc5*. Data are expressed as means  $\pm$  SEM.  $n = 6$ . \* $P < 0.05$  versus systemic *Gpc5* knockdown.

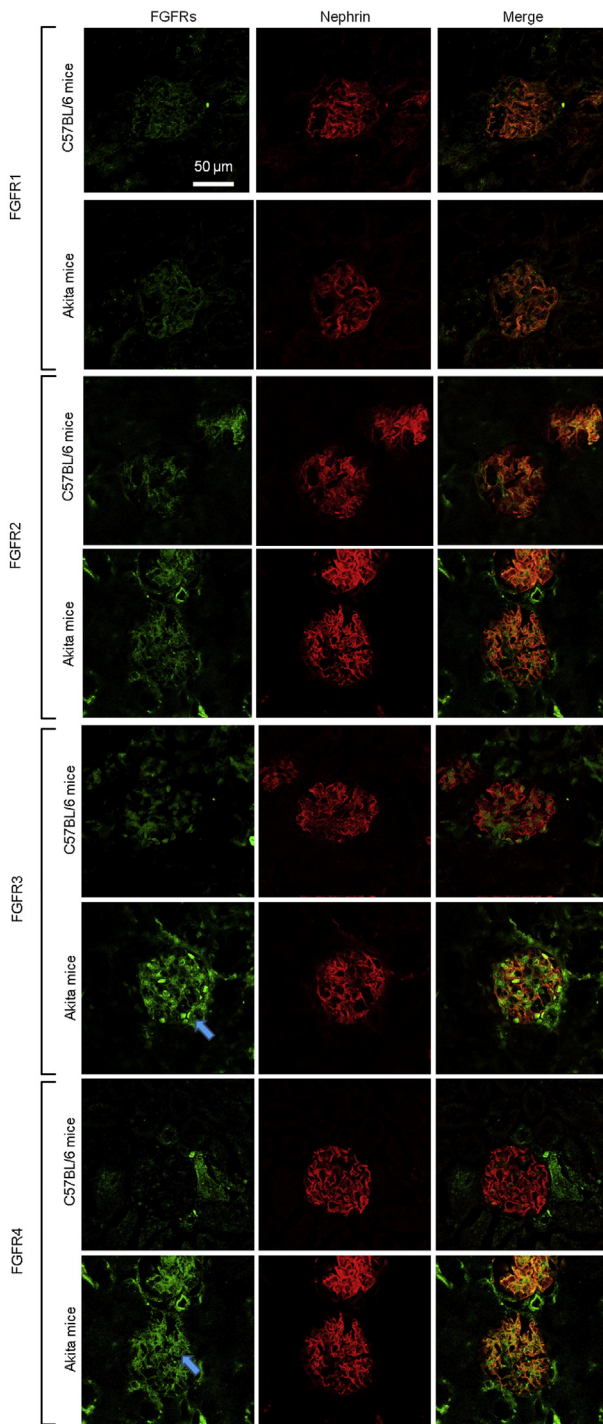
severity (Figure 1A). The expression pattern of GPC5 in DN was not limited to the glomerular capillary region but was also seen in the mesangial area along the progression to nodular glomerular sclerosis. The signal intensity was increased in mild DN cases (204%  $\pm$  37%) and severe DN cases (271%  $\pm$  43%) compared with the control (100%  $\pm$  32%).

### GPC5 Expression in Podocytes and Mesangial Cells

The glomerular localization of *Gpc5* was examined in C57BL/6 and in C57BL/6Cr *Ins2*<sup>+/<sup>C96Y</sup> (Akita) mice<sup>19</sup>; localization of *Gpc5* appeared in podocytes and in the paramesangial matrix (Figure 1, B and C). Consistent with previous findings,<sup>1</sup> podocyte-specific *Gpc5* knockdown mice (Figure 1B) revealed that most expression in the glomerulus was podocyte derived. *Gpc5* expression of nonpodocyte origin was dramatically increased (182%  $\pm$  32%) in podocyte-GPC5 $\Delta$  Akita mice compared with that in podocyte-GPC5 $\Delta$  control mice (Figure 1B). In Akita mice, *Gpc5* localization was more accentuated at the paramesangial area (Figure 1C). *In vitro* analysis further revealed that a high glucose level affected the expression level of *Gpc5* in the MES13 mouse mesangial cell line and mouse podocyte cell line.<sup>14</sup> In response to a 72-hour exposure of the cells to elevated glucose concentrations, there was a dose-dependent increase in *Gpc5* mRNA expression in the mesangial cell line but not in the podocyte cell line (Figure 1D). This increase was significant at the concentration of 450 mg/dL glucose compared with 90 mg/dL glucose. These results suggest that GPC5 is mainly produced in the podocyte, and it is located on the cell surface of podocytes and secreted into the paramesangial matrix in nondiabetic states. In a high-glucose state, not only podocytes but also mesangial cells conspicuously express GPC5.</sup>

### FGF2 and Proteinuria in Diabetic Mice and *Gpc5* Knockdown Mice

Next we examined whether a challenge with exogenous FGF2 would induce progressive proteinuria in diabetic mice,



**Figure 3** Expressions of fibroblast growth factor receptor (FGFR)3 and FGFR4 increase in diabetic mice. Immunofluorescence staining of kidney from C57BL/6 mice and Akita mice is shown. Fgfr1, Fgfr2, Fgfr3, and Fgfr4 (green) were stained with Alexa 488 fluorescence. Nephrin (red) was counterstained with Alexa 633. Images from the same sections were merged to form an overlapping image (yellow; right column). Arrows depict increased FGFR3 and FGFR4 expression in the glomerulus.

and conversely, whether inbreeding podocyte-specific *Gpc5* knockdown mice<sup>1</sup> with the Akita strain could mitigate the proteinuria. Mice without diabetes (C57BL/6) showed no proteinuria after an injection of recombinant human FGF2, as

reported previously.<sup>1</sup> However, the same protocol of FGF2 injection induced proteinuria in Akita diabetic mice (Figure 2). Systemic *Gpc5* siRNA knockdown mice had virtually no induction of proteinuria.

### Expression of Fgfr3 and Fgfr4 Increases in Diabetes

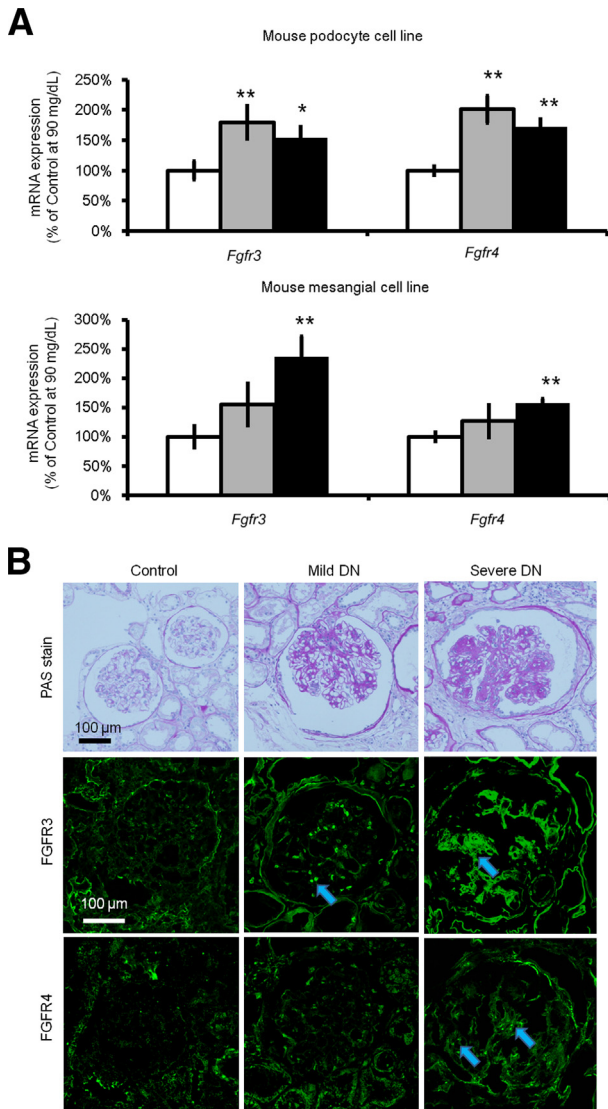
The expression levels of FGFRs in nondiabetic mice and diabetic mice were examined using Akita mice and C57BL/6 mice as controls. Immunofluorescence staining revealed the increase of Fgfr3 and Fgfr4 in Akita mice, which was not seen in C57BL/6 nondiabetic mice (Figure 3). The expression levels of FGFRs under various concentrations of glucose (90, 270, and 450 mg/dL) were examined using the mouse podocyte cell line and mouse mesangial cell line. Fgfr3 and Fgfr4 expression increased significantly under high-glucose conditions in both cell lines (in the podocyte cell line at 270 mg/dL and in the mesangial cell line at 450 mg/dL) (Figure 4A). Moreover, human diabetic glomeruli showed higher expressions of FGFR3 and FGFR4 in proportion to diabetes severity (Figure 4B).

### Accumulation of Systemic Circulating FGF2 in Diabetic Mice

Immunofluorescence staining revealed that the protein level of FGF2 in the Akita glomerulus was more prominent than that in the C57BL/6 nondiabetic mice (Figure 5A). However, *in situ* hybridization showed no evident induction of the *Fgf2* gene transcript in the diabetic kidney (Figure 5B). *In vitro* assays in which the mouse podocyte cell line and mesangial cell line were used showed no increase in mRNA (Figure 5C). These results meant that the Fgf2 protein found in the glomeruli would derive from an extracellular origin other than podocytes and mesangial cells in these settings. For clarification, we biotinylated human recombinant FGF2 (labeled FGF2) and injected it systemically into both Akita and C57BL/6 mice. A greater accumulation of signal intensity at the glomerulus was remarkably detectable in Akita mice (Figure 5D). When labeled FGF2 was administered to systemic *Gpc5* siRNA knockdown Akita mice, a minimal accumulation of labeled FGF2 was found in the glomeruli (Figure 5D). This observation supports our inference that extraglomerular FGF2 accumulates GPC5 dependently in the glomerulus during the progression of DN.

### Pathological Effect of FGF2 on the Glomerular Cells

In DN, the mesangial cell proliferation and mesangial matrix expansion was reportedly observed with the increase of fibronectin and collagen type IV in mesangial cells.<sup>20–22</sup> Similarly in the rat mesangial cell line,<sup>12</sup> high glucose concentrations (600 mg/dL) markedly induced fibronectin (Figure 5E) and collagen type IV (Figure 5F) expression co-incubated with FGF2. This induction was blocked



**Figure 4** Expression of *Fgfr3* and *Fgfr4* increases in a high-glucose medium *in vitro* and in a human specimen. **A:** *Fgfr3* and *Fgfr4* mRNA expression levels in a cultured mouse podocyte cell line and mesangial cell line after three different administrations of a 72-hour glucose challenge [90 (white bars), 270 (gray bars), or 450 mg/dL (black bars)] are shown. Osmolality was controlled using D-mannitol. **B:** Immunofluorescence staining of the human kidney is shown. Lanes from the left: Nondiabetic control, patient with mild diabetes mellitus nephropathy (DN), and patient with severe DN. Top panels are representative images in periodic acid-Schiff (PAS) stain. FGFR3 and FGFR4 (green) were stained with Alexa 488 fluorescence. **Arrows** depict increased expression of FGFR in the glomerulus. Data are expressed as means  $\pm$  SEM (**A**).  $n = 6$  (**A**). Post hoc \* $P < 0.05$ , \*\* $P < 0.01$  versus 90 mg/dL.

remarkably by *Gpc5* knockdown mice. In podocytes, diabetes induces hypertrophy, apoptosis, structural changes, and extracellular matrix synthesis.<sup>23</sup> Rac1 is reportedly necessary for the modulation of cell adhesion and motility by fibronectin. We examined Rac1 activation in mouse podocytes. High glucose concentrations (600 mg/dL) markedly activated Rac1 (Figure 5G), and this induction was blocked by an FGFR antagonist.<sup>24</sup>

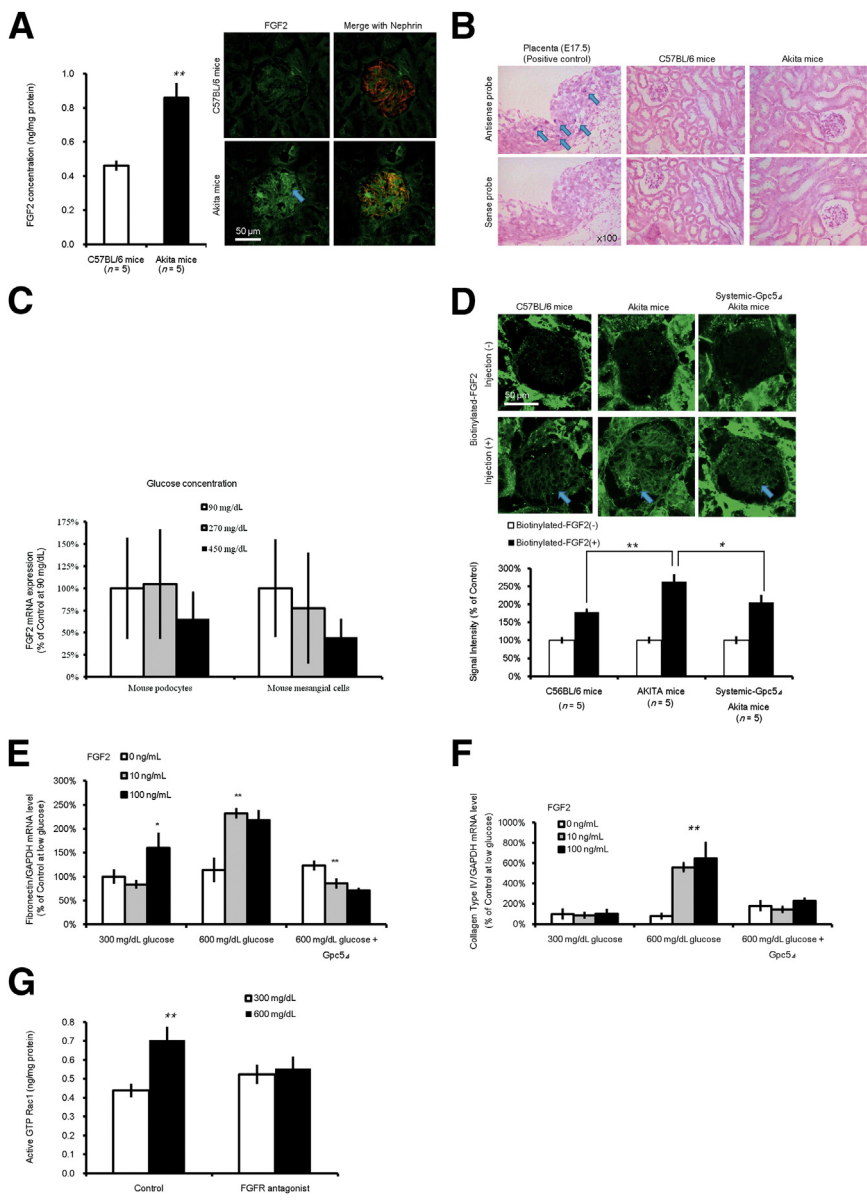
## Discussion

Nephrotic proteinuria in DN is an ominous sign of progression to end-stage renal disease accompanied with intractable dropsy. Here, the glomerulus in cases of DN shows increased *Gpc5* expression. The glomerular distribution of *Gpc5* in DN was not limited to the capillary podocyte area but became more evident in the paramesangial area as DN progressed. This finding provided a unique clue about *Gpc5* distribution in DN; therefore, further analysis was conducted. *Gpc5* was found mostly in the glomerular capillary, more properly the glomerular endothelial cells and the basal side of the foot process (Figure 1C), in C57BL/6, as reported in previous research.<sup>1</sup> This finding was again clarified by using *Gpc5* knockdown mice. In contrast, the glomerular localization of *Gpc5* in Akita mice was not abrogated completely in *Gpc5* knockdown Akita mice but was rather clearly seen in the paramesangial area. This observation was consistent with findings in human DN and the *in vitro* analysis, leading us to the interpretation that the increase of glomerular GPC5 is mostly derived from the mesangial region.

In physiological conditions, heparan sulfate proteoglycans, including GPC5, contribute negative charge in the slit membrane. Once glucose concentration is increased, mesangial cells also start to express GPC5 and contribute to keep a reservoir for detrimental growth factors such as FGF2. Several different factors are well known to induce mesangial cell proliferation and matrix expansion, but the potential involvement of FGF2 was examined in the diabetic kidney because FGF2 is the best studied pathway in relation to GPC5.

A previous study in rodents demonstrated clearly the characteristics of FGF2 to unveil the potential malicious pathopoiesis in experimental membranous nephropathy.<sup>10</sup> More specifically, FGF2 might enhance the disease process of nephropathies. Therefore, we next administered FGF2 to Akita diabetic mice and found the emergence and acceleration of proteinuria.<sup>25</sup> This treatment did not develop proteinuria in nondiabetic control mice, C57BL/6, in our previous report,<sup>1</sup> and the current findings again confirmed the impact of FGF2 on diabetes. Systemic *Gpc5* knockdown by siRNA injection prevented the emergence of proteinuria in diabetic mice. Therefore, some type of Fgf receptor is participating in this pathogenicity. Results of *in vitro* and *in vivo* analyses suggest that *Fgfr3* and *Fgfr4* are candidate receptors, and similar results have been confirmed in human DN glomeruli as well. We discovered for the first time that human glomeruli in DN were receptive to FGF2 by using FGFR3 and FGFR4. We found that the kidneys obtained from diabetic Akita mice showed concentrations of Fgf2 that were twice as high as those from the kidneys of nondiabetic C57BL/6 mice. This sign of an underlying disorder accounts for the topping effect of Fgf2 to develop proteinuria in diabetic mice, as shown in Figure 2.

FGF2 has been reported *in vitro* in glomerular apparatus such as endothelial and mesangial cells.<sup>6,26–29</sup> In previous reports, intraglomerular FGF2 proteins were increased in



**Figure 5** Accumulation of extraglomerular fibroblast growth factor (FGF2) in diabetic glomerulus and efficacy of *Gpc5* deletion. **A:** FGF2 protein levels in whole lysates of the kidney of C57BL/6 and Akita mice are shown (left). Immunofluorescence staining of kidney from C57BL/6 and Akita mice (right). FGF2 (green) was stained with Alexa 488 fluorescence, nephrin (red) was costained with Alexa 633, and images from the same sections were merged to form an overlapping image (yellow). **Arrow** depicts increased FGF2 expression in the glomerulus. **B:** *In situ* hybridization for *FGF2* mRNA in kidney from C57BL/6 and Akita mice. Placenta (E17.5) was used for positive control (arrows); images with sense probe were used for negative control. **C:** *FGF2* mRNA expression levels in a cultured mouse podocyte cell line and mesangial cell line after three different administrations of a 72-hour glucose challenge (90, 270, or 450 mg/dL). Osmolality was controlled using D-mannitol. **D:** Human recombinant biotinylated FGF2 was injected intravenously in C57BL/6, Akita, and systemic *Gpc5* knockdown ( $\Delta$ ) Akita mice. The prebiotinylated FGF2 was detected with the biotin-avidin reaction. Although a strong background signal of biotin was detected in the tubular area (upper panels), the signal from injected FGF2 was detected clearly in the glomerulus (arrows; lower panel), especially in diabetic Akita mice. Average fluorescent (Alexa 555) signals in glomeruli were calculated. **E** and **F:** Fibronectin (**E**) and collagen type IV (**F**) mRNA expression levels in a cultured rat mesangial cell line subjected to different concentrations of glucose for 48 hours with subsequent incubation with different concentrations of FGF2 for 24 hours. Osmolality was controlled with D-mannitol. **G:** Mouse podocyte cells were subjected to different concentrations of glucose for 96 hours with subsequent incubation using FGF receptor (FGFR) antagonist or dimethyl sulfoxide for 3 hours. Then protein lysate was harvested to examine active Rac1. Osmolality was controlled with D-mannitol. Data are expressed as means  $\pm$  SEM (**A, C–F**).  $n = 5$  (**A, D–F**);  $n = 6$  (**C**). \* $P < 0.05$ , \*\* $P < 0.01$ .

DN kidney specimens.<sup>27,30</sup> Therefore, we sought to detect intraglomerular gene expression of *FGF2*. However, our glomerular analysis using *in situ* hybridization of *FGF2* and immortalized cell lines showed virtually undetectable increased expression in the diabetic kidney. In addition, labeled FGF2 showed remarkable accumulation in the glomerular region in a human diabetes model. Only a limited number of studies previously reported FGF2 mRNA transcript in end-stage renal disease.<sup>29</sup> According to a report of normal rat kidney, a weak mRNA signal was detected only in parietal glomerular epithelial cells, although FGF2 protein was found in the glomerular capillary.<sup>28</sup> Therefore, we concluded the extraglomerular FGF2 was recruited as the causal etiology toward escalation of glomerulopathy in early DN without obvious light microscopic changes.

The FGF2 level in the systemic blood circulation is negligible under normal conditions because of a lack of an amino

terminal signal sequence necessary for efficient release of FGF2 from cells. However, an increase in plasma FGF2 levels is expected in the pathophysiological condition of endothelial injury. Regarding diabetes, Stephan et al<sup>31</sup> showed that FGF2 is closely involved in glucose-induced vascular dysfunction. Hamed et al<sup>32</sup> also reported a marked increase in serum levels of FGF2 in T2DM compared with those of healthy controls. Zimering et al<sup>33</sup> measured plasma FGF2 in type 2 diabetes mellitus and reported a significant increase in a subset of diabetic patients with persistent microalbuminuria or overt proteinuria. The pathogenicity that increases FGF2 levels in type 2 diabetes mellitus remains unknown, but macrovascular and microvascular endothelial injuries in type 2 diabetes mellitus are a potential source. In addition, patients with DN not only displayed an elevated serum FGF2 level but also showed increased expression of FGF2 in the tubulointerstitium, as well as a good correlation with the degree of



tubulointerstitial injury.<sup>30</sup> Both systemic circulating FGF2 and renal tubulointerstitial FGF2 levels are thought to be the source of FGF2 accumulation in glomeruli.

There is a limitation that the FGF2 injection *per se* resulted in higher concentrations of FGF2 in plasma than that seen in patients, but our results suggest both cell types, podocytes and mesangial cells, are affected by increased levels of FGF2. A higher FGF2 level can induce podocytopathy through the induction of caspase-3<sup>1</sup> and can simultaneously stimulate cell proliferation and matrix increase in mesangial cells.<sup>6,26</sup> In general, Rho regulates the formation of stress fibers, whereas Cdc42 and Rac1 regulate that of filopodia and lamellipodia, respectively. In previous reports, increased active Rac1 signaling in podocytes produced albuminuria.<sup>34,35</sup> Induction of Rac1 is reportedly crucial for cell adhesion and motility on the extracellular matrix.<sup>24</sup> Intraglomerular imbalance can manifest as a proliferation of mesangial cells and a change of adhesion and motility in podocytes. This pathway will further accelerate the vicious cycle of DN in a nephrotic phenotype.

In diabetic glomerular injury, our findings explain how GPC5 and FGF signals influence podocytes and mesangial cells. Given that glomerular GPC5 is the key molecule for developing proteinuria encompassing both acquired nephrotic syndrome and DN, what will engender a difference in pathological findings apart from the structural change induced by comorbidities? It is noteworthy that mesangial GPC5 expression is virtually absent in the basal condition, but it is increased considerably under hyperglycemic conditions. This response shows a stark contrast to that of podocytes.

However, our study has a few limitations. GPC5 in DN must be evaluated with a large clinical cohort, and this was a small experimental study. The efficacy of the FGFR antagonist in the mouse DN model was not examined in this study, although a previous report indicated that the complete deletion of FGF2 showed an impairment of blood pressure regulation.<sup>36</sup> GPC5 is an escort protein, apart from its effects on FGF2, which potentially interacts with other pathways such as those of Wnts and bone morphogenetic proteins. The role of GPC5 in other pathways awaits further investigation in human DN.

## Conclusions

In summary, GPC5, extraglomerular circulating FGF2, and intraglomerular FGF receptors are involved in progressive DN. The induction of FGFRs under hyperglycemic conditions is crucial for the progression of DN, but this process can be controlled by the deletion of GPC5. Our results suggest that the urinary GPC5 level might be a useful biomarker for forecasting the acceleration of nephrotic deterioration of DN during clinical follow-up.

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