Dickkopf-1 Promotes Hyperglycemia−Induced Accumulation of Mesangial Matrix and Renal Dysfunction

Chun-Liang Lin,*†‡ Jeng-Yi Wang,§ Jih-Yang Ko,¶ Yu-Ting Huang,* Yu-Hsia Kuo,* and Feng-Sheng Wang**

Departments of *Nephrology and §Colorectal Surgery, Chang Gung Memorial Hospital, Chiayi, Taiwan; †Kidney Research Center, Department of Nephrology, Chang Gung Memorial Hospital, Taipei, Taiwan; ‡School of Traditional Chinese Medicine and ¶Graduate Institute of Clinical Medical Science, Chang Gung University College of Medicine, Taipei, Taiwan; and Departments of §Orthopedic Surgery and **Medical Research, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Kaohsiung, Taiwan

ABSTRACT

Wnt/β-catenin signaling mediates renal fibrosis in several model systems including diabetic nephropathy. Dickkopf-1 (DKK-1) is an endogenous inhibitor of Wnt/β-catenin signaling, but whether DKK-1 modulates diabetic nephropathy is unknown. Here, we studied whether DKK-1 participates in high glucose (HG)-induced expression of profibrotic factors and renal damage. In vitro, HG increased expression of DKK1, receptor Kremen-2, TGF-β1, and fibronectin in mesangial cells. Loss and gain of DKK1 function modulated HG-mediated c-Jun, TGF-β1, and fibronectin expression. DKK1 mediated HG-induced phosphorylation of Ser45-β-catenin and reduction of nuclear β-catenin levels, but not phosphorylation of ERK kinase. Wnt3a protein and the β-catenin (Δ45) mutation increased nuclear β-catenin but abrogated HG-induced DKK1 and fibronectin expression. Exogenous DKK1 antisense oligonucleotide attenuated the increase in both serum DKK1 and urinary protein excretion in streptozotocin-induced diabetic rats. Knocking down DKK1 inhibited mesangial expression of TGF-β1 and fibronectin and reduced both the glomerular volume and deposition of mesangial matrix in diabetic kidneys. Taken together, DKK1 mediates HG-induced destabilization of β-catenin and matrix accumulation in mesangial cells. Knocking down DKK1 prevents diabetes-induced renal dysfunction and microstructure deterioration, suggesting that inhibition of DKK1 offers therapeutic potential for diabetic nephropathy.


Received October 9, 2008. Accepted September 9, 2009. Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Feng-Sheng Wang, Department of Medical Research, Kaohsiung Chang Gung Memorial Hospital, 123 Ta-Pei Road, Niao-Sung, Kaohsiung 833, Taiwan. Phone: +886-7-731-7123, ext. 8876; Fax: +886-7-7338456; E-mail: wangfs@ms33.hinet.net, linchunliang@adm.cgmh.org.tw

BASIC RESEARCH www.jasn.org

Diabetes-mediated renal fibrosis is one of the leading causes of ESRD. High glucose (HG) concentration increases remodeling of glomerular microarchitecture by promoting accumulation of extracellular matrix (ECM) and expression of fibrotic factor in mesangial cells, which leads to renal dysfunction and subsequent diabetic renal injury. Induction of fibrotic matrix deposition in mesangial cells by TGF-β1 is an important pathogenic reaction in diabetic renal injury.

Wnt proteins interact with receptor Frizzled and co-receptor LDL receptor–related protein 5 (LRP5) and stabilize downstream transcription regulator β-catenin by inhibiting β-catenin phosphorylation toward proteasome degradation, which reportedly participates in nephrogenesis and renal disorders. Modulation of Wnt protein secretion, β-catenin stabilization, and glycogen synthesis kinase-3β activation...
tion induces tubule formation, epithelial differentiation,\textsuperscript{7} polycystic kidney disease,\textsuperscript{8} and renal cell carcinoma.\textsuperscript{9} Wnt signaling components reportedly also act as HG-responsive regulators for cardiovascular calcification\textsuperscript{10} and cardiac function in diabetic animals.\textsuperscript{11}

Dickkopf-1 (DKK1), by binding to transmembrane receptors Kremen-1 and Kremen-2, has been found to induce endocytosis of LRP5; disturb the formation of a complex by Wnt, Frizzled, and LRP5; and inhibit Wnt protein-responsive biologic activities.\textsuperscript{12} This Wnt inhibitor reportedly controls the development of multiple myeloma–induced osteolysis,\textsuperscript{13} esophageal carcinomas,\textsuperscript{14} arthritic joints,\textsuperscript{15} and the neural differentiation of embryonic stem cells.\textsuperscript{16} DKK1 mutant mice show earlier nephron development in the metanephric mesenchyme.\textsuperscript{17} Impairment of Wnt signaling is found to modulate renal tissue remodeling,\textsuperscript{18} proteinuric nephropathy,\textsuperscript{19} and diabetes-induced renal injury;\textsuperscript{20} however, the biologic role of DKK1 in homeostasis of diabetic kidney tissue has not been verified.

Regulation of Wnt/\beta
d-catenin signaling by Wnt and the Wnt inhibitor is one of the emerging therapeutic approaches for controlling tissue remodeling and regeneration.\textsuperscript{21,22} Inactivated glycogen synthesis kinase-3\beta promotes nephron differentiation.\textsuperscript{7} Secreted Frizzled-related protein 1 knockout mice display increased renal glomeruli,\textsuperscript{23} suggesting that attenuated Wnt inhibitor action is beneficial for the development of renal tissue. Modulation of DKK1 action reportedly controls tumor formation in breast cancer cells,\textsuperscript{24} development of basolaid follicular hamartoma,\textsuperscript{25} skin pigmentation in dermal fibroblasts,\textsuperscript{26} and formation of fibrous histiocytoma in human mesenchymal stem cells.\textsuperscript{27}

We hypothesized that control of DKK1 actions on mesangial cells and the renal tissue microenvironment may alter HG-mediated fibrotic matrix deposition and renal dysfunction.

In this study, we investigated whether DKK1 participates in HG-induced matrix accumulation in renal mesangial cells and examined whether \textit{in vivo} knockdown of DKK1 expression by end-capped phosphorothioate DKK1 antisense oligonucleotide (DKK1-AS) can alleviate renal injury in a diabetic animal model.

RESULTS

HG-Induced Expression of DKK1 and Profibrotic Factor in Mesangial Cells

We investigated whether high concentrations of \textit{d}-glucose altered expression of profibrotic factor or DKK1 in renal mesangial cells. \textit{d}-Glucose at 35 mM significantly increased expression of TGF-\beta1 and fibronectin (Figure 1A) in association with increased expression of DKK1 and Kremen-2 in cell cultures. \textit{d}-Glucose at 35 mM increased TGF-\beta1, fibronectin, DKK1, and Kremen-2 expression by 24 hours. Increased \textit{d}-glucose did not significantly affect Kremen-1 or LRP5 mRNA expression throughout the study period. Cells (1 \times 10\textsuperscript{6} cell/well, in a six-well plate) were cultured in medium containing 15 to 35 mM \textit{d}-glucose or the osmolarity control 35 mM mannitol for 24, 48, and 72 hours. The graphed results represent the relative abundance of mRNAs determined by quantitative RT-PCR and normalized to the housekeeping gene \(\beta\)-actin. Experimental results are presented as means \pm SEs calculated from at least three experiments. *Significant difference (\(P < 0.05\)) from the vehicle groups. Veh, vehicle; M, 35 mM mannitol.
**DKK1- and Kremen-2–Mediated HG-Induced Expression of Profibrotic Factor**

We investigated whether loss or gain of function of DKK1 could change expression of profibrotic factor in mesangial cells. DKK1 small interfering RNA (siRNA) significantly attenuated HG-induced promotion of DKK1 protein (Figure 2A), DKK1 mRNA (Figure 2B), TGF-β1 (Figure 2C), and fibronectin expression (Figure 2D) in cell cultures. The β-actin levels were not affected by the treatment, indicating that knocking down DKK1 by RNA interference (RNAi) did not cause general suppression of gene expression.

Transfection of DKK1 cDNA increased expression of DKK1 protein and mRNA (Figure 3A) and significantly increased expression of TGF-β1 and fibronectin in cell cultures (Figure 3B). Moreover, treatment with 400 ng/ml recombinant DKK1 protein significantly promoted expression of TGF-β1 and fibronectin in cell cultures. Treatment with 200 ng/ml recombinant DKK1 protein significantly promoted expression only of fibronectin in mesangial cells (Figure 3C).

Kremen-2 siRNA significantly attenuated HG-induced promotion of Kremen-2 (Figure 4A), TGF-β1, and fibronectin expression (Figure 4B) in mesangial cell cultures. Compared with the vehicle group, HG- and DKK1-treated mesangial cells displayed evident fibronectin expression. The cell cultures transfected with DKK1 siRNA and Kremen-2 siRNA showed weak immunostaining for fibronectin (Figure 4C).

**β-Catenin–Dependent Pathway Regulated DKK1 Modulation of Profibrotic Factor Expression**

We investigated whether a β-catenin–dependent or –independent pathway participated in the promotion of profibrotic factor expression by DKK1. Treatments with HG, DKK1 cDNA, and recombinant DKK1 protein increased the levels of phosphorylated Ser45–β-catenin and nuclear c-Jun but attenuated the abundance of nuclear β-catenin in mesangial cells. DKK1 siRNA and Kremen-2 siRNA attenuated the increase in levels of c-Jun and phosphorylated Ser45–β-catenin induced by HG but restored the expression of nuclear β-catenin in cell cultures (Figure 5A). HG, DKK1, and Kremen-2 did not markedly alter total β-catenin expression in cell cultures (Figure 5A). HG increased the levels of phosphorylated extracellular signal–regulated kinase (ERK) but not phosphorylated p38 or phosphorylated JNK in cell cultures. Modulation of DKK1 did not obviously alter the phosphorylation of ERK, p38, or JNK mediated by HG in mesangial cells (Figure 5B).

Cell cultures were transfected with the β-catenin (Δ45) mutant to suppress phosphorylation of β-catenin at the Ser45 residue. Recombinant Wnt3a protein and the β-catenin (Δ45) mutation suppressed the increased expression of phosphorylated Ser45–β-catenin, DKK1, c-Jun (Figure 5C), TGF-β1, and fibronectin (Figure 5D) induced by HG levels but increased nuclear β-catenin abundance (Figure 5C) in cell cultures. Pretreatment with the ERK inhibitor PD98059 did not significantly affect HG-induced alteration of phosphorylated Ser45–β-catenin, nuclear β-catenin, or DKK1 abundance (Figure 5C) but inhibited expression of TGF-β1 and fibronectin (Figure 5D) in cell cultures. Recombinant Wnt3a protein, the β-catenin (Δ45) mutation, and treatment with PD98059 did not significantly affect total β-catenin expression in cell cultures.

**Treatment with DKK1-AS Alleviated Diabetes-Induced Urinary Protein Excretion**

We verified whether in vivo knockdown of DKK1 could reduce diabetes-induced renal injury. Blood glucose concentrations were detected in each rat before and after overnight fasting (Table 1). Compared with the normal control group, the diabetic rats had significantly reduced body weight but increased postfasting concentrations of blood glucose (Figure 6A), glycosylated hemoglobin (HbA1c), serum DKK1 (Figure 6B), urinary protein and albumin (Figure 6C), and
kidney weight (Supplemental Data 2). Exogenous treatment with DKK1-AS significantly reduced the serum concentration of DKK1 and excretion of urinary protein and albumin in the diabetic rats but did not significantly alter their body weight, blood glucose concentrations, HbA1c levels, or kidney weight (Figure 6). Sense control DKK1-S did not significantly affect the diabetes-induced changes in body weight, blood biochemistry, or renal function throughout the study period.

Figure 3. Effects of DKK1 cDNA and recombinant DKK1 protein on expression of profibrotic factor in mesangial cells. (A and B) DKK1 cDNA increased DKK1 protein and mRNA expression (A) and promoted TGF-β1 and fibronectin mRNA expression (B) in cell cultures. (C) Treatment with recombinant DKK1 protein induced TGF-β1 and fibronectin expression in mesangial cells. Mesangial cells transfected with DKK1 cDNA and empty vector were cultured in basal medium for 72 hours. Cell cultures were treated with 200 and 400 ng/ml recombinant DKK1 protein for 72 hours. Experimental results are presented as means ± SEs calculated from at least three experiments. *Significant differences (P < 0.05) from the vehicle groups. rDKK1, recombinant DKK1 protein.

Figure 4. Effect of Kremen-2 RNAi on expression of fibrotic factor in mesangial cells. (A and B) Kremen-2 RNAi attenuated expression of Kremen-2 (A) and TGF-β1 and fibronectin (B) induced by HG in mesangial cell cultures. (C) Representative immunocytochemical photographs of mesangial cells. In the HG and DKK1 cDNA groups, mesangial cells displayed intense fibronectin expression. Cell cultures treated with DKK1 RNAi and Kremen-2 RNAi showed weak fibronectin expression. Expression of fibronectin in cell cultures was determined immunocytochemically with anti-fibronectin antibody and horseradish peroxidase-3'-3'-diaminobenzidine. Specimens were observed under ×400 magnification. Experimental results are presented as means ± SEs calculated from at least three experiments. *, #Significant differences (P < 0.05) from the vehicle- and HG-treated groups, respectively. SC, scramble control; rDKK1, recombinant DKK1 protein.
Treatment with DKK1-AS Alleviated Fibrosis in Renal Mesangial Cells

The glomerular mesangium in renal tissue was harvested using laser-captured microdissection to extract the total RNA and perform quantitative reverse transcriptase–PCR (RT-PCR; Figure 7A). Diabetes significantly increased expression of DKK1, TGF-β1, and fibronectin in the whole renal tissue (Figure 7B) and glomerular mesangium (Figure 7C), whereas treatment with DKK1-AS significantly alleviated the diabetes-induced increase in expression of DKK1, TGF-β1, and fibronectin in whole renal tissue and glomerular mesangium. DKK1-S did not significantly alter expression of DKK1, TGF-β1, or fibronectin in diabetic renal tissue.

DISCUSSION

Induction of DKK1 expression by high concentrations of glucose is associated with increased accumulation of ECM in renal mesangial cells. Inhibition of DKK1 stabilized β-catenin and attenuated HG-induced promotion of TGF-β1 and fibronectin expression in mesangial cells. Numerous reports have demonstrated the associations between Wnt antagonist and renal fibrosis induced by unilateral ureteral obstruction in experimental animals26 and
Table 1. Effect of treatments with DKK1-AS and DKK1-S on blood glucose level and immunostaining of glomerular mesangium in diabetic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>Vehicle</th>
<th>DKK1-AS</th>
<th>DKK1-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose before (mg/dl)</td>
<td>113.3 ± 2.5</td>
<td>418.8 ± 9.8a</td>
<td>427.9 ± 8.6b</td>
<td>443.3 ± 9.0a</td>
</tr>
<tr>
<td>Blood glucose after (mg/dl)</td>
<td>72.6 ± 3.2</td>
<td>284.8 ± 15.0a</td>
<td>289.7 ± 11.8a</td>
<td>263.6 ± 10.4a</td>
</tr>
<tr>
<td>GV (µg/10^2)</td>
<td>3.3 ± 0.2</td>
<td>4.6 ± 0.2a</td>
<td>3.3 ± 0.4b</td>
<td>4.2 ± 0.1a</td>
</tr>
<tr>
<td>Mesangial matrix index (%)</td>
<td>8.1 ± 0.1</td>
<td>14.9 ± 0.2a</td>
<td>7.5 ± 0.4b</td>
<td>14.4 ± 0.2a</td>
</tr>
<tr>
<td>DKK1 expression (%)</td>
<td>16.8 ± 0.4</td>
<td>37.0 ± 1.3a</td>
<td>19.2 ± 1.0b</td>
<td>34.9 ± 1.3a</td>
</tr>
<tr>
<td>β-catenin expression (%)</td>
<td>31.5 ± 0.9</td>
<td>11.9 ± 0.7a</td>
<td>30.7 ± 0.8b</td>
<td>11.1 ± 0.5a</td>
</tr>
<tr>
<td>c-Jun expression (%)</td>
<td>10.5 ± 0.6</td>
<td>28.2 ± 0.9a</td>
<td>16.6 ± 0.5b</td>
<td>28.3 ± 0.9a</td>
</tr>
<tr>
<td>TGF-β1 expression (%)</td>
<td>17.6 ± 0.8</td>
<td>34.1 ± 1.1a</td>
<td>22.4 ± 1.0b</td>
<td>32.2 ± 0.6a</td>
</tr>
<tr>
<td>Fibronectin expression (%)</td>
<td>24.5 ± 1.0</td>
<td>49.6 ± 2.1a</td>
<td>28.0 ± 0.4b</td>
<td>50.1 ± 0.9a</td>
</tr>
</tbody>
</table>

Blood glucose levels were detected 6 weeks after treatment with STZ or vehicle. Six regions within the renal glomeruli from three sections obtained from three rats were microscopically studied. Three random images of 0.75 mm² from each selected region were analyzed under ×400 magnification by image analysis software.

*Significant difference (P < 0.05) from the vehicle-treated group.

#Significant difference (P < 0.05) from the HG-treated group.

Figure 6. Effect of DKK1 antisense oligonucleotide on renal function in diabetic rats. (A) Diabetes attenuated body weight but increased blood glucose levels in rats. Diabetes increased levels of HbA1c and serum DKK1 (B) and urinary protein and albumin (C) in experimental animals. Treatment with exogenous DKK1-AS attenuated levels of serum DKK1 and urinary protein and albumin in diabetic rats. Rats were given STZ to induce diabetes. Two weeks after injection, diabetic rats were given 20 µg/kg per d DKK1-AS (n = 6), DKK1-S (n = 6), and vehicle (n = 6) for 4 consecutive weeks. Experimental results are presented as means ± SEs calculated from six rats in each group. *, #Significant differences (P < 0.05) from the normal control and diabetic groups, respectively. DM, diabetes.

the diagnosis of renal cell carcinoma progression in human patients; however, little research has been conducted to define the biologic significance of DKK1 in diabetic nephropathy. Our findings are the first indication that in vivo reduction of DKK1 expression abrogates diabetes-induced glomerular injury, fibrotic matrix deposition in renal microenvironments, and urinary protein excretion. We suggest that interruption of DKK1 action alleviated the renal tissue deterioration induced by HG concentrations.

Reduced expression of DKK1 and receptor Kremen-2 attenuated HG-induced profibrotic factor expression in renal mesangial cells. This is the first study to characterize the deleterious action of DKK1 on the kidney. DKK1 reportedly controls fibronectin expression in embryonic mouse lung tissue. Kremen antagonizes Wnt-responsive biologic actions in skeletal tissue. The upregulation of fibrotic matrix accumulation by DKK1 highlights a new spectrum of deleterious reactions in renal cells induced by HG.

HG increased phosphorylation of β-catenin at the Ser45 residue but reduced nuclear β-catenin levels. Destabilization of β-catenin correlated with increased expression of c-Jun and profibrotic factor in mesangial cells. Translocation of β-catenin toward the nucleus is an important reaction of Wnt-mediated tissue remodeling, and β-catenin is found to interact with c-Jun in tissue morphogenesis and tumorigenesis. Suppression of β-catenin promotes TGF-β1-mediated proliferation of proximal tubular epithelial cells and colon carcinoma cells. In this study, restoration of nuclear β-catenin abundance by recombinant Wnt3a protein and DKK1 siRNA attenuated the increase in expression of profibrotic factor induced by HG in mesangial cells, suggesting that impaired β-catenin signaling is one prominent pathologic reaction responsible for ECM metabolism in mesangial cells. Wnt4 promotion of β-catenin signaling participates in renal tissue development and HG-induced mesangial cell apoptosis. We suggest that Wnt3a and Wnt4 communicate with similar pathways to regulate HG-induced impairment of mesangial cell metabolism. Stabilized β-catenin reportedly promotes renal tissue development, and active β-catenin induces polycystic...
kidney disorder, renal cell carcinoma, and obstructive nephropathy.\textsuperscript{8,9,28} The experimental results of this study indicate that β-catenin is essential for preventing HG-induced mesangial cell dysfunction. We speculate that the discrepant biologic functions of β-catenin may result from the varying physiologic and pathologic conditions of renal tissue.

Stable β-catenin alleviated the expression of DKK1 induced by HG in mesangial cells, indicating a reciprocal interaction between β-catenin and DKK1 in mesangial cells. The β-catenin signaling pathway reportedly controls DKK1 expression in colon cancer cells.\textsuperscript{36} We suggest that DKK1 and β-catenin have distinct actions on the homeostasis of mesangial cells under HG conditions. Previous studies demonstrated that β-catenin–independent pathways, including JNK and p38 kinases, actively respond to DKK1-mediated tissue remodeling.\textsuperscript{37,38} The LRP5 gene is associated with type 1 diabetes in human patients,\textsuperscript{39} and TGF-β1 reportedly attenuates DKK1 expression in endometrial stromal cells.\textsuperscript{40} Our experimental results revealed that control of the β-catenin–dependent pathway by DKK1 is involved in expression of profibrotic factor in mesangial cells. HG did not significantly affect LRP5 expression in mesangial cell cultures. We speculate that the varying biologic reactions of LRP5 and DKK1 may depend on the type of cell cultures and extracellular stress. Moreover, inhibition of ERK by β-catenin–independent signaling alleviated HG-induced profibrotic factor expression in mesangial cells, indicating that β-catenin–dependent and –independent pathways contribute to the accumulation of ECM induced by HG.

Notably, treatment with DKK1-AS alleviated diabetes-induced expression of DKK1 and renal dysfunction. Alleviation of renal fibrosis by controlling the Wnt inhibitor in the diabetic kidney microenvironment has not, to our knowledge, been previously reported. We provided the first molecular evidence that treatment with DKK1-AS abrogated expression of TGF-β1, fibronectin, and c-Jun in the glomerular mesangium of the diabetic kidney. Cytoplasmic and nuclear expression of c-Jun in renal and lung tissues has been reported.\textsuperscript{41,42} The experimental results of DKK1-induced profibrotic factor expression observed in an in vivo model are consistent with those detected in cell culture models, indicating that renal microenvironments actively responded to the treatment. The decreased GV and accumulation of mesangial matrix in diabetic kidneys also indicate that DKK1-AS induces a protective reaction against tissue damage induced by diabetes.

In this study, diabetic rats had increased levels of serum DKK1. Glomerular mesangial cells, podocytes, and tubular cells in diabetic kidneys displayed strong expression of DKK1, indicating that multiple cell populations in renal tissue contribute to DKK1 expression. The level of circulating DKK1 reflects the changes in DKK1 expression in diabetic renal microenvironments, and decreased serum DKK levels correlated with attenuation of DKK1 expression and diabetic renal injury by DKK1-AS. Our experimental results are consistent with the concept that control of secreted DKK1 potentially attenuates tissue deterioration.\textsuperscript{13,15} DKK1 reportedly induces apoptosis of various cell types.\textsuperscript{43,44} HG impairs Wnt/β-catenin–dependent survival of mesangial cells.\textsuperscript{20} We speculate that DKK1 may be a potent Wnt inhibitor that promotes apoptosis of mesangial cells induced by HG. We cannot exclude the possibility that DKK1 can perturb the biologic activity of podocytes and tubular cells. The pathologic role of DKK1 in diabetes-stressed podocytes and tubular cells requires further exploration. Wnt inhibitors secreted Frizzled-related protein 4\textsuperscript{48} and DKK1\textsuperscript{45} have been reported to attenuate β-catenin signaling and renal fibrosis promoted by unilateral ureteral obstruction, respectively. In contrast, although we have confirmed that suppression of DKK1 restored expression of β-catenin, we found that...
It attenuated accumulation of fibrotic matrix in renal cells under conditions of HG. We speculate that these differences in the biologic role of DKK1 in fibrotic matrix deposition in renal tissue may result from variations in the pathologic reactions in renal tissue and experimental animal models.

In this study, diabetic rats (6 weeks after administration of 50 mg/kg streptozotocin [STZ]) were found to have reduced body weight and elevated postfasting blood glucose levels. All animals remained healthy and survived throughout the study period. Although the in vitro HG stress cannot be extrapolated to the blood glucose level in the STZ-induced diabetic rats, increased expression of DKK1 and profibrotic factors also add to the evidence supporting the deleterious effect of HG (blood glucose) on renal cells in vitro and in vivo. We speculate that the discrepant blood glucose levels in diabetic rats may depend on the overnight fasting used for experimental animals. The deleterious effect of STZ observed in the animals is consistent with previous studies reporting that STZ reduces body weight but increases blood glucose and kidney mass in rats.46,47 Diabetic rats (4 weeks

**Figure 8.** Representative histologic photographs of glomeruli in diabetic kidneys. In the diabetes and DKK1-S groups, glomeruli displayed intense PAS staining and DKK1 expression. In the DKK1-AS and normal groups, glomeruli displayed weak PAS staining. Mesangial cells and podocytes in glomeruli and tubular cells expressed DKK1 weakly. Cells positively immunostained for DKK1 showed brown color. Specimens were observed under ×400 and ×1000 magnification. DM, diabetes.

**Figure 9.** Representative photographs of β-catenin and c-Jun immunostaining in glomeruli from diabetic kidneys. In the DKK1-S and diabetes groups, mesangial cells, podocytes, and tubular cells expressed β-catenin weakly but showed intense immunostaining for c-Jun. In the DKK1-AS and normal groups, the cells displayed weak c-Jun immunoreactivity but strong β-catenin expression. Cells positively immunostained for c-Jun and β-catenin showed brown color. Specimens were observed under ×400 and ×1000 magnification. DM, diabetes.
after treatment with 55 mg/kg STZ) were noted to have increased postfasting blood glucose levels (approximately 207 mg/dl) and significantly increased kidney weight. Modulating the action of DKK1 in tissue remodeling is a potential therapeutic approach for controlling osteoporosis, sacroiliac joints, and multiple myeloma. Renal mesangial cells responded to HG stress by inducing β-catenin–dependent and –independent (ERK) pathways that promote ECM accumulation. DKK1 participates in the β-catenin destabilization and mesangial cell dysfunction induced by HG. Thus, control of DKK in renal tissue could be an alternative option for attenuating the renal dysfunction induced by diabetes.

CONCISE METHODS

In Vitro HG Stress

Rat mesangial cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM containing 10% FBS (Life Technologies, Carlsbad, CA) in 5% CO₂ at 37°C until subconfluent and then harvested by trypsinization for subsequent studies. Cells (1 × 10⁶ cells/well, six-well plate) were cultured in basal medium containing 15, 25, or 35 mM-glucose for 72 h. Cell cultures treated with 35 mM mannitol were used as the osmolarity control. In some experiments, cells were cultured in basal medium containing 200 or 400 ng/ml recombinant DKK1 protein and 200 ng/ml recombinant Wnt3a protein (R&D Systems, Minneapolis, MN) for 72 h, respectively.

Real-Time PCR

Total RNA was extracted from cell cultures, whole renal tissues, and glomerular mesangium using Tri reagent (Sigma Chemical Inc., St. Louis, MO). One microgram of total RNA was reverse-transcribed into cDNA. Twenty-five microliters of PCR mixture containing cDNA template (equivalent to 20 ng of total RNA), 2.5 μM each of forward and reverse primers, and 2 × q SYBR Green Supermix were amplified in an iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with an initial melt at 95°C for 5 minutes followed by 40 cycles at 94°C for 15 seconds, 52°C for 20 seconds, and 72°C for 30 seconds. The following gene-specific primers were used: TGF-β1 (forward 5'-TGA GTG GCT GCC TTC AAC TTC TC-3'; reverse 5'-AGT CCT TTA GGG CGG TCA AT-3'), DKK1 (forward 5'-GGG CGG TCA AT-3'), LRP5 (forward 5'-CCA AAG CCT GAG CCC AGA-3'; reverse 5'-GCA CCA CTC CCA TGG CAT-3'), Kremen-1 (forward 5'-GAG GAC AGT GGG AAG GAA GG-3'; reverse 5'-AGG AAG GGA GGG ACA TAG GG-3'), Kremen-2 (forward 5'-GAG GCC TTC TTC AAG TTC TG-3'; reverse 5'-GGC GCC TCT GCC TCT GC-3'); β-actin (forward 5'-GGG CGG TCA AT-3'). The number of amplification steps required to reach an arbitrary intensity threshold (Ct) was computed. The relative gene expression was represented as $2^{-ΔΔCt}$, where $ΔCt = Ct_{target} - Ct_{β-actin}$. The fold change after treatment was calculated as $2^{-ΔCt_{vehicle}}$, where $ΔCt = ΔCt_{treatment} - ΔCt_{vehicle}$.

cDNA Transfection

The cDNA encoding DKK1, stable β-catenin (∆45), and wild-type β-catenin (∆52) were ligated and cloned into the pcDNA3.1 vectors (Invitrogen) and pC1-neo vectors, respectively. Cells (5 × 10⁵ cells/well, six-well plate) were plated to reach 80% confluence and then trans-
fected with 1 and 2 μg of plasmids using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Cells stably transfected with the plasmids were selected in a medium containing 600 μg/ml G418 (Life Technologies, Gaithersburg, MD).

**RNAi Transfection**

Plasmids (pLKO-1-puro) encoding DKK1 RNAi (TRCN0000055154) and Kremen-2 RNAi (TRCN0000071704), which targeted DKK1 mRNA and Kremen-2 mRNA, respectively, were obtained from the National RNAi Core Facility Academia (Sinica, Taiwan). The RNAi sequences were submitted to NCBI blast and aligned with DKK1 mRNA (NM_010051.3) and Kremen-2 mRNA (NM_028416). Subconfluent cell cultures were transiently transfected with siRNA and scramble controls using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

**Western Blotting**

Cytosolic and nuclear extracts of cell cultures were prepared as described previously.20 Proteins in cell lysates (100 μg) were separated by SDS-PAGE, then analyzed by Western blot. The designated proteins on the blots were probed with mAbs against DKK1 (Abcam, Cambridge, UK), β-catenin, phosphorylated Ser45–β-catenin, c-Jun, phosphorylated ERK, phosphorylated p38, phosphorylated JNK, and actin (Cell Signaling Technology Inc., Beverly, MA), followed by horseradish peroxidase–conjugated IgG as the secondary antibody, and visualized by chemiluminescence. Nuclear extracts were immunoprecipitated with anti–β-catenin antibody and Protein A (Sigma-Aldrich Inc., St. Louis, MO). The immunocomplexes were detected by Western blot probed with anti–β-catenin antibody. Lysates of HeLa cells were used as the positive control for immunoblotting of DKK1.53

**STZ-Induced Diabetes**

The experimental animal studies were approved by the Institutional Animal Care and Use Committee of the hospital. Twenty male Wistar rats (4 months of age) were given a single intraperitoneal injection of 50 mg/kg STZ (Sigma-Aldrich Inc.) to induce diabetes as described previously.20 Blood glucose levels in tail blood samples were measured before and after the rats fasted overnight. For equalization of blood glucose levels in tail blood samples were measured by Western blot probed with anti–β-catenin antibody. Lysates of HeLa cells were used as the positive control for immunoblotting of DKK1.53

**STZ-Induced Diabetes**

The experimental animal studies were approved by the Institutional Animal Care and Use Committee of the hospital. Twenty male Wistar rats (4 months of age) were given a single intraperitoneal injection of 50 mg/kg STZ (Sigma-Aldrich Inc.) to induce diabetes as described previously.20 Blood glucose levels in tail blood samples were measured before and after the rats fasted overnight. For equalization of blood glucose levels, intermittent-acting insulin (Montard; Novo Nordisk A/S, Bagsvaerd, Denmark), 1 to 2 U/kg, was administered subcutaneously once a day until the animals were killed. Eighteen rats with high postfasting blood glucose (200 to 300 mg/dl), defined as those with previously once a day until the animals were killed. Eighteen rats with high postfasting blood glucose (200 to 300 mg/dl), defined as those with previously.

**Preparation and Microdissection of Kidney Tissue**

Kidneys were dissected, weighed, and perfused with PBS. Fresh kidney tissues were fixed in 4% PBS-buffered formaldehyde and embedded in paraffin under RNase-free conditions. Specimens were sliced longitudinally into 4-μm-thick sections. Fresh kidney tissues were also ground with a mortar and pestle under liquid nitrogen under RNase-free conditions to harvest total RNA for quantitative RT-PCR assessment. In some experiments, the glomerular mesangium in the formaldehyde-fixed renal sections was harvested by a laser-capture microdissector (VERITAS; Arcturus Bioscience Inc., Sunnyvale, CA) according to the manufacturer’s instructions. Two hundred glomerular mesangium from six sections of each rat in each group were dissected to extract the total RNA and perform quantitative RT-PCR.

**Histomorphometry**

Sections were dewaxed for staining with hematoxylin–eosin and PAS stains according to the manufacturer’s instructions (Sigma-Aldrich Inc.). Ten glomeruli in each section were randomly selected for microscopy under ×200 magnification (Zeiss Axioskop 2 Plus; Carl Zeiss, Gottingen, Germany). Images were captured by a cool charge-coupled device camera. Areas of glomeruli were counted by the Image-Pro Plus image analysis software (SNAP-Pro c.f. Digital kit; Media Cybernetics Inc., Silver Spring, MD). Mean GV (μ²/10⁶) was calculated according to the Weibel and Gomez formula.24 The areas that stained positively with PAS within the glomerular tuft were counted. The mesangial matrix index (%) was expressed as mesangial matrix area/tuft area × 100%.48 Immunoreactivity in the sections was detected with a horseradish peroxidase-3′,3′-diaminobenzidine kit (BioGenex, San Ramon, CA) and antibodies against DKK1, TGF-β1, fibronectin, c-Jun, and β-catenin, followed by counterstaining with hematoxylin. Sections without primary antibodies were used as negative controls for immunostaining. Six regions within the renal glomeruli from three sections obtained from three rats were microscopically studied. Three random images of 0.75 mm² from each selected region were then taken, captured, and analyzed under ×400 magnification. Mesangial cells, podocytes, and tubular cells were identified morphologically. The number of positively immunolabeled and total cells per high-power field in each section were counted, and the percentage of positively labeled cells was calculated.

**Urinary Protein and Serum HbA1c and DKK1 Levels**

Urine was collected using metabolic cage systems. The levels of creatinine, protein (Formosa Biomedical Technology Corp., Taipei, Taiwan), and albumin (Dade Behring Inc., Newark, NJ) in the urine were measured by the respective assay kits. Blood was harvested by an intracardiac needle and processed to collect serum for measuring levels of HbA1c (PRIMUS Diagnostics, Trinity Biotech Co., Kansas City, MO) and DKK1 (DuoSet ELISA Development Kit; R&D Systems) according to the manufacturers’ instructions.

**Treatment with DKK1-AS**

End-capped phosphorothioate DKK1-AS (5′-TAC AGA TCT TGG ACC AGA-3′) complementary to nucleotides 4 through 21 of the DKK1 mRNA coding region and DKK1-S (5′-TCT GGT CCA AGA TCT GAT-3′) were custom synthesized (Bio Basic Inc., Markham, Ontario, Canada). Diabetic rats were given intraperitoneal injections of vehicle (n = 6), DKK1-AS (20 μg/kg per d; n = 6), and sense control DKK1-S (20 μg/kg per d; n = 6) for 4 consecutive weeks. Rats without STZ injections (n = 6) were used as normal controls. All animals remained healthy throughout the study period. At week 4, all of the rats in each group were killed by an overdose of pentobarbital sodium, and their kidneys were harvested for study.

**Histomorphometry**

Sections were dewaxed for staining with hematoxylin–eosin and PAS stains according to the manufacturer’s instructions (Sigma-Aldrich Inc.). Ten glomeruli in each section were randomly selected for microscopy under ×200 magnification (Zeiss Axioskop 2 Plus; Carl Zeiss, Gottingen, Germany). Images were captured by a cool charge-coupled device camera. Areas of glomeruli were counted by the Image-Pro Plus image analysis software (SNAP-Pro c.f. Digital kit; Media Cybernetics Inc., Silver Spring, MD). Mean GV (μ²/10⁶) was calculated according to the Weibel and Gomez formula.24 The areas that stained positively with PAS within the glomerular tuft were counted. The mesangial matrix index (%) was expressed as mesangial matrix area/tuft area × 100%.48 Immunoreactivity in the sections was detected with a horseradish peroxidase-3′,3′-diaminobenzidine kit (BioGenex, San Ramon, CA) and antibodies against DKK1, TGF-β1, fibronectin, c-Jun, and β-catenin, followed by counterstaining with hematoxylin. Sections without primary antibodies were used as negative controls for immunostaining. Six regions within the renal glomeruli from three sections obtained from three rats were microscopically studied. Three random images of 0.75 mm² from each selected region were then taken, captured, and analyzed under ×400 magnification. Mesangial cells, podocytes, and tubular cells were identified morphologically. The number of positively immunolabeled and total cells per high-power field in each section were counted, and the percentage of positively labeled cells was calculated.
**Statistical Analysis**

All values were expressed as means ± SEs. *In vitro* experimental data were collected from at least three repeated experiments. A parametric ANOVA test and Bonferroni post hoc test were used to evaluate the differences among the groups. P < 0.05 was considered statistically significant. Power values of the HBA1c, urinary albumin, and urinary protein levels ranged from 0.9721 to 0.9984 under α = 0.05.

**ACKNOWLEDGMENTS**

This work was supported in part by grants (NSC94-2314-B-182A-127) from the National Science Council, Taiwan, NHRI (NHRI-Ex99-9942SI and NHRI-Ex98-9834EI), and Chang Gung Memorial Hospital (CMRPG630042), Taiwan.

We thank Dr. B. Vogelstein (Johns Hopkins Medical Institute and Howard Hughes Medical Institutes) and Dr. A. Munoz (Instituto de Investigaciones Biomedicas “Alberto Sols,” Consejo Superior de Investigaciones Cientificas-Universidad Autonoma de Madrid) for the generous gifts of stable β-catenin (A45) and DKK1 constructs, respectively. We also thank the Core Laboratory for Clinical Proteomics and Genomics (CMRPG83038) and the Center for Laboratory Animals (CMRPG86053), Chang Gung Memorial Hospital-Kaohsiung Medical Center, for the use of facilities and Dr. Nyuk-Kong Chang for technical assistance with the experimental animal model.

**DISCLOSURES**

None.

**REFERENCES**


www.jasn.org BASIC RESEARCH