Four-and-a-Half LIM Domains Protein 2 Is a Coactivator of Wnt Signaling in Diabetic Kidney Disease


Due to the number of contributing authors, the affiliations are listed at the end of this article.

ABSTRACT
Diabetic kidney disease (DKD) is a microvascular complication that leads to kidney dysfunction and ESRD, but the underlying mechanisms remain unclear. Podocyte Wnt-pathway activation has been demonstrated to be a trigger mechanism for various proteinuric diseases. Notably, four-and-a-half LIM domains protein 2 (FHL2) is highly expressed in urogenital systems and has been implicated in Wnt/β-catenin signaling. Here, we used in vitro podocyte culture experiments and a streptozotocin-induced DKD model in FHL2 gene-knockout mice to determine the possible role of FHL2 in DKD and to clarify its association with the Wnt pathway. In human and mouse kidney tissues, FHL2 protein was abundantly expressed in podocytes but not in renal tubular cells. Treatment with high glucose or diabetes-related cytokines, including angiotensin II and TGF-β1, activated FHL2 protein and Wnt/β-catenin signaling in cultured podocytes. This activation also upregulated FHL2 expression and promoted FHL2 translocation from cytosol to nucleus. Genetic deletion of the FHL2 gene mitigated the podocyte dedifferentiation caused by activated Wnt/β-catenin signaling under Wnt-On, but not under Wnt-Off, conditions. Diabetic FHL2+/+ mice developed markedly increased albuminuria and thickening of the glomerular basement membrane compared with nondiabetic FHL2+/+ mice. However, FHL2 knockout significantly attenuated these DKD-induced changes. Furthermore, kidney samples from patients with diabetes had a higher degree of FHL2 podocyte nuclear translocation, which was positively associated with albuminuria and progressive renal function deterioration. Therefore, we conclude that FHL2 has both structural and functional protein-protein interactions with β-catenin in the podocyte nucleus and that FHL2 protein inhibition can mitigate Wnt/β-catenin–induced podocytopathy.


Diabetic kidney disease (DKD) is the leading cause of ESRD, affecting 10%–40% of diabetic patients, and the cost of RRT remains a large economic burden for the health care system. Proteinuria is a major and primary clinical aspect of DKD and causes tubulointerstitial lesions that lead to renal dysfunction.1 Reducing proteinuria may therefore be a principal therapeutic target to improve renal outcome in patients with DKD. However, the pathophysiologic mechanism of DKD is multifactorial, and some patients develop treatment-resistant proteinuria that results in ESRD despite intensive BP and glycemia control. Thus, identification of major DKD mechanisms and development of new therapeutic options are needed.

Glomerular epithelial cells, also called podocytes, are predominantly responsible for maintaining the glomerular filtration barrier. Podocytes are highly specialized, terminally differentiated cells that cannot proliferate.2 Recently, hyperactive podocyte...
Wnt/β-catenin signaling has been shown to play an essential role in the development of DKD. The Wnt family of signaling proteins participates in multiple developmental events during embryogenesis and has been implicated in adult tissue homeostasis. Wnt signals modulate pleiotropic effects, such as include mitogenic stimulation and cell-fate specification.

An important component of the Wnt pathway is β-catenin, which resides in the cytoplasm in a multiprotein complex. One component of this complex, glycogen synthase kinase 3 (GSK3), phosphorylates β-catenin in the absence of Wnt signaling, and phosphorylated β-catenin is subsequently ubiquitinated and degraded via proteasomes. Upon Wnt signaling stimulation, β-catenin is stabilized and translocated to the cell nucleus. As a transcriptional activator, β-catenin binds the transcription factor of T cell–specific transcription factor (TCF) in the cell nucleus and starts target gene transcription. In clinical studies, aberrant Wnt signaling has been shown to be responsible for various malignant and metabolic diseases.

As a developmental signaling pathway, the Wnt/β-catenin system is essentially silenced in differentiated podocytes, but activated Wnt/β-catenin signaling has been found in various types of proteinuric kidney disease, particularly in DKD. Wnt/β-catenin is a key element of podocyte dysfunction by downregulating nephrin via a Snail-dependent mechanism. Target inhibition of podocyte Wnt signaling does not alter kidney microstructure and function but prevents podocytopathy in doxorubicin-induced nephropathy. On the contrary, activation of β-catenin in podocytes results in podocyte foot process effacement, glomerular basement membrane (GBM) thickening, and significant albuminuria, implying that the Wnt pathway is not necessary for normal podocyte function, but its activation during renal injury might reflect a harmful signaling.

The LIM domain is a cysteine-rich motif that has been proposed to direct protein–protein interaction. A diverse group of proteins containing LIM domains have been identified, which display various functions. A concept is emerging that the LIM domains can associate with both the actin cytoskeleton and the transcriptional machinery. The functions of LIM domain proteins in the nucleus are mainly in tissue-specific gene regulation, whereas cytoplasmic LIM domain proteins are mainly involved in cytoskeleton organization. Four-and-a-half LIM domains protein (FHL) contains four-and-a-half LIM domain–binding proteins. Proteins in this family function as adaptors or scaffolds to support the assembly of metameristic protein complexes for critical cellular processes.

The best-studied member of this family, FHL2, is highly expressed in cardiovascular and urogenital systems. The role of FHL2 has been investigated intensively in cardiovascular diseases over the past decade, but its role in kidney disease remains unknown. Interestingly, FHL2, but not FHL1 or FHL3, is involved in the Wnt/β-catenin signaling pathway. Here, we used an FHL2 gene knockout in a diabetic nephropathy mouse model, combined with in vitro experiments and clinical data, to clarify the possible role of FHL2 in DKD.

RESULTS

FHL2 Is Abundantly Expressed in Mature Podocytes

We used three different methods to identify the expression and distribution of FHL2 in kidney tissues. In human kidney samples, immunohistochemical (IHC) staining with a monoclonal antibody showed that FHL2 is highly expressed in glomerular cells (Figure 1, A and B). We further used immunofluorescence double-staining and confirmed that FHL2 is abundantly expressed in podocytes (Figure 1C, Supplemental Material). To avoid the potential antibody cross-reactivity in IHC staining, we introduced the LacZ gene in the FHL2 locus by homologous recombination, and LacZ staining confirmed that FHL2 is expressed in mouse glomeruli (Figure 1D).

A previous report demonstrated that cultured human podocytes have abundant FHL2 mRNA expression in nonpermissive conditions. Here, we used Western blot to demonstrate that FHL2 protein expression is modest in the permissive proliferating stage but becomes abundant in nonpermissive well differentiated podocytes (Figure 1E).

Diabetes Mellitus–Related Cytokines Activate the Wnt Pathway and Upregulate FHL2 Expression in Human Podocytes

To investigate the effect of high glucose conditions and diabetes-related cytokines on FHL2 regulation, differentiated human podocytes were exposed to various stimulators. As shown in Figure 2, A and B, high glucose, TGF-β, and angiotensin II stimulate Wnt/β-catenin signaling in podocytes. Western blot revealed increased active β-catenin protein level, and importantly, that these stimulators also upregulated FHL2 protein expression. The GSK-3 inhibitor, a direct Wnt pathway activator, markedly increased active β-catenin but did not affect FHL2 protein expression, which suggests FHL2 is not a Wnt targeted–protein.

Several groups have addressed the possibility that various stimuli modulate subcellular distribution of FHL2. To explore and test this hypothesis in podocytes, we used nuclear/cytoplasmic protein extraction and immunofluorescence staining to evaluate FHL2 subcellular distribution in cultured podocytes. Under normal culture conditions, FHL2 was mainly expressed in podocyte cytosol, located on the podocyte cytoskeleton, and translocated to the cell nucleus after stimulation by high glucose and diabetes mellitus (DM)–related cytokines (Figure 2C). The β-catenin was distributed in the cell-cell junction, and no protein–protein interactions were found between FHL2 and β-catenin under normal conditions. However, under high glucose conditions, both FHL2 and β-catenin simultaneously translocated to the cell nucleus and are sustained in the nucleus (Figure 2D). We further extracted cell nuclear protein for a coimmunoprecipitation experiment and confirmed that FHL2 has a structural protein–protein interaction with activated β-catenin (Figure 2E).
FHL2 Is a Coactivator of β-catenin and Knockdown of FHL2 Attenuates Wnt-Induced Podocytopathy In Vitro
To test the possibility that FHL2 facilitates translocation of β-catenin to the cell nucleus through the Wnt pathway, we analyzed the β-catenin expression in the cell nucleus with and without FHL2. As illustrated in Figure 3A, nuclear β-catenin was significantly upregulated in response to Wnt stimulation, and knockdown of FHL2 did not alter nuclear β-catenin aggregation. We then used a TCF/LEF reporter system and dual-luciferase assay to test the hypothesis that FHL2 is a cofactor of β-catenin in podocytes. After 18-hour exposure to GSK3 inhibitor, the relative luciferase units markedly increased. Administration of FHL2 small interfering RNA (siRNA), but not scrambled siRNA, significantly attenuated the TCF transduction activity (Figure 3B), suggesting FHL2 protein is a coactivator of β-catenin in podocytes.

With regard to protein levels, Wnt signaling activates β-catenin and Snail and represses the critical slit-diaphragm protein nephrin in podocytes. Under normal culture conditions, knockdown of the FHL2 protein did not affect β-catenin, Snail, or nephrin expression, but under Wnt-On culture conditions, knockdown of the FHL2 protein significantly attenuated Snail upregulation and conserved nephrin expression (Figure 3, C–F).

On the basis of these experiments, we have demonstrated that FHL2 has structural and functional protein-protein interactions with β-catenin in podocytes, and the knockdown of FHL2 protein can reduce Wnt-induced podocyte injury in vitro.

FHL2-Deficient Mice Have Attenuated Diabetic Nephropathy
To investigate the role of FHL2 in DKD in vivo, we examined the severity of kidney injury in FHL2−/− mice and their FHL2+/+ littermates after the development of diabetes by streptozotocin administration. Mice were divided into four groups: FHL2+/+ control group, FHL2+/+ DM group, FHL2−/− control group, and FHL2−/− control group. Blood glucose and hemoglobin A1c levels were similarly elevated in the two diabetic groups. Induction of diabetes produced glomerular hyperfiltration in this DM model, and the two diabetic groups had similar elevation in GFR. Urine albumin levels and GFR did not differ between the nondiabetic FHL2−/− and FHL2+/+ mice throughout the study period (Figure 4, A–C). Urinary albumin was significantly elevated in diabetic FHL2+/+ mice starting from the second month of diabetes (P<0.05), and the degree of urinary albumin progressively increased through the fourth month. However, the diabetic FHL2−/− mice had significantly less urinary albumin throughout the whole study period (Figure 4D). At the end of the study, the glomerular mesangial volume from kidney tissues of study mice was determined. Diabetic FHL2+/+ mice had glomerular hypertrophy and mesangial expansion (P<0.01) compared with nondiabetic FHL2+/+ mice, and the diabetic FHL2−/− mice had significantly less mesangial expansion compared with diabetic FHL2+/+ mice (P=0.03) (Figure 4, E and F). The thickness of GBM was further determined by electron microscopy. Induction of diabetes increased GBM thickness in FHL2+/+ mice, and deletion of FHL2 significantly attenuated GBM thickening in DKD (P<0.05) (Figure 4, G and H). In agreement with a previous study, we found that induction of diabetes in mice moderately increases podocyte apoptosis but only modestly affects podocyte number.14 The two diabetic groups had similar podocyte numbers and apoptosis ratios (Supplemental Material).

Mouse glomeruli were obtained by series sieving of kidney cortex with triple sets of sieving mesh (100 μm, 75 μm, and 53 μm), as described previously26; the sieved glomeruli were retrieved from the third mesh and the glomerular proteins were extracted for Western blotting analysis. Compared with glomeruli from nondiabetic mice, glomeruli from diabetic FHL2+/+ mice had significantly enhanced expression of active β-catenin, FHL2, Snail, TGF-β, and vascular endothelial growth factor (VEGF), and the nephrin expression was downregulated. This finding suggests podocyte dedifferentiation after induction of diabetes. Moreover, diabetic FHL2−/− mice had similarly high levels of active β-catenin, TGF-β, and VEGF but less affected Snail and nephrin expressions compared with those in diabetic FHL2+/+ mice (Figure 5). These data indicate that elimination of FHL2 protein did not affect normal renal function and glomerular microstructure in nondiabetic mice but significantly attenuated the severity of kidney injury in diabetic mice.

Figure 1. FHL2 is abundantly expressed in glomerular podocytes. (A) IHC staining shows FHL2 protein is abundantly expressed in glomeruli in normal human kidney tissue (original magnification, ×100). (B) With high-power view, the staining showed a typical podocyte distribution pattern (original magnification, ×400). (C) Double immunofluorescence staining illustrates that FHL2 colocalized with podocyte marker nephrin. (D) Lac-Z staining shows a clear glomerular distribution in the FHL2 null mouse kidney because Lac-Z is introduced in the FHL2 locus, indicating that FHL2 transcription is highly activated in glomerular cells. (E) FHL2 protein expression in cultured human podocytes under permissive and nonpermissive conditions.
FHL2 Nuclear Translocation Is Associated with Albuminuria and Predicts Renal Function Deterioration in Diabetic Patients

To compare the FHL2 distribution in nondiabetic and diabetic patients, kidney tissues from the site opposite the tumor in nephrectomized patients were stained with FHL-2 antibody with IHC, and double-stained with FHL2 and podocyte nuclear protein WT-1 by the immunofluorescence method. We excluded kidney samples from patients with eGFR < 60 ml/min per 1.73 m² after surgery or interstitial fibrosis > 25%. In total, 25 nondiabetic and 46 diabetic patients were enrolled; their demographic characters are listed in Table 1. Figure 6A illustrates FHL2 staining in control and diabetic kidneys. In control kidney samples, FHL2 is stained within the podocyte cytosol, but in diabetic kidney samples a considerable proportion of podocytes exhibit FHL2 staining in the cell nucleus in addition to the cytosol. Patients with DM had a higher proportion of podocytes with FHL2 nuclear translocation (Figure 6B), and this proportion is even higher in macroalbuminuric diabetic patients. The 46 patients with DM were followed up for renal outcome, and FHL2 nuclear translocation of > 50% was a strong predictor of renal function deterioration (Figure 6C). In mice, induction of diabetes also triggered a remarkable FHL2 cytosol–nuclear translocation as in human DKD (Supplemental Material).
Figure 3. FHL2 is a β-catenin coactivator and knockdown FHL2 that reduces Wnt-induced podocytopathy in vitro. (A) Knockdown FHL2 does not alter Wnt signaling–induced β-catenin nuclear translocation. (B) Wnt reporter system showed GSK3 inhibitor markedly increased Wnt transcription activity, and FHL2 siRNA significantly attenuated the GSK3 inhibitor–induced Wnt activation. (C) Western blot showed Wnt signaling increased active β-catenin and transcription factor Snail, and downregulated nephrin, while FHL2 siRNA
DISCUSSION

It is widely recognized that proteinuria is one of the earliest clinical markers of DKD, and the appearance of albumin in urine indicates a compromised glomerular filtration barrier. Recent studies have indicated that activation of Wnt/β-catenin signaling induces podocyte dysfunction and causes proteinuric kidney disease.10,27 The current results suggest that FHL2, an abundantly expressed protein in podocytes, is a coactivator of Wnt signaling in DKD. In cultured human podocytes, high glucose and DM-related cytokines not only upregulated FHL2 expression but also promoted FHL2 translocation from the cytosol to the nucleus. In in vitro studies, knockdown of FHL2 mitigated induced podocyte dedifferentiation by activated Wnt/β-catenin signaling and reduced albumin influx through cell-cell junctions of monolayer podocyte under Wnt-On, but not under Wnt-Off, conditions. In streptozotocin-induced diabetes, FHL2 knockout in diabetic mice significantly decreased albuminuria and attenuated the DKD pathologic changes. In human data, patients with DM had a higher proportion of podocytes with FHL2 nuclear translocation, and this proportion is even higher in macroalbuminuric diabetic patients. Of note, FHL2 nuclear translocation degree is a predictor of renal function deterioration in the future. These novel findings provide clear evidence that FHL2 plays an important role in the development of DKD, and FHL2 inhibition can reduce Wnt/β-catenin–induced podocyte abnormality.

Various mechanisms (e.g., activation of the angiotensin II pathway, the receptor for advanced glycation end products pathway, the TGF-β pathway, and an imbalance of VEGF/endothelial nitric oxide synthase expression) have been proposed to be involved in the pathophysiologic mechanism of DKD. However, glycemic control, along with currently available pharmacotherapies, cannot completely prevent the progression of DKD,28,29 Therefore, identifying the key signaling culprits of DKD to explore novel therapeutic agents demands new attention. In a microarray study performed on microdissected human DKD samples, Wnt/β-catenin signaling was one of the most consistently upregulated pathways,13 and, in parallel, overexpression of β-catenin in podocytes induces albuminuria and GBM abnormality.14 Wnt signaling is tightly controlled under normal physiologic conditions and is regulated at several levels: the Wnt ligand secretion, the ligand receptor affinity, and the target gene transcriptional activity. The transcriptional activity of the β-catenin–TCF complex can be modulated by complex coactivators and corepressors, such as CBP/p300,30,31 Chibby,32 the inhibitor of β-catenin and TCF-4 (called ICAT),33 and TNLK/Nemo.34

Wnts are a family of signaling proteins that play an essential role in embryogenesis, but their expression in the adult kidney is silenced. Unlike phylogenetically lower species, such as fish, in which Wnt activation following injury plays a key role in the repair process,35 regeneration in the mammalian kidney is highly restricted.36 Because mature podocytes are terminally differentiated cells in a state of permanent exit from the cell cycle and arrested in a postmitotic state, by the end of mitosis their cytoskeletal actin forms part of the contractile ring. Thus, when podocyte mitosis is attempted, it leads to aberrant mitosis and cell death (mitotic catastrophe).37 Meanwhile, activation of Wnt signaling induces podocyte dedifferentiation, upregulation of the transcription factor Snail, suppression of nephrin expression, and detachment of podocytes, causing glomerulosclerosis.10,36 Wnt-induced maladaptive repair and fibrosis does not seem to be restricted to glomerular podocytes. A similar process has been found in the lung, liver, and even skin, causing fibrosis and organ failure.38–40

This study showed FHL2 interacts with β-catenin and affects β-catenin–mediated transcription in a cell-specific manner. These findings are in line with previous reports showing that FHL2 and β-catenin form a protein complex in cell nucleus,21,23,41 interestingly, FHL2 could be a coactivator of β-catenin–mediated TCF/LEF transcription in HEK293 and UDC-Mel-N melanoma cells,21,41 and also a corepressor in C2C12 myoblasts and CHO cells.23 Our data indicate that in DKD, FHL2 acts as a coactivator of β-catenin in podocytes. Because FHL2 amplifies the podocyte damage caused by the reactivation of Wnt signaling, FHL2 inhibition could be a potential therapeutic target in preventing DKD progress.

Conventional β-catenin knockout is lethal in an early embryonic state, but mice with podocyte-specific β-catenin deletion have no major histologic changes or albuminuria. In addition, mice with podocyte-specific β-catenin deletion are more resistant to proteinuric kidney disease, which suggests the Wnt/β-catenin pathway is not required for podocyte maintenance.10,14,42 Several groups have reported promising results in treating podocytopathy by the inhibition of Wnt signaling. Jiang et al. reported that blocking of Wnt signaling prevents angiotensin II–induced nephropathy in mice.43 He et al. further showed that paricalcitol prevents doxorubicin-induced nephropathy by suppressing podocyte Wnt/β-catenin activation.11 Tzou et al. reported that systemic injection of monoclonal antibody against Wnt receptor attenuates kidney damage in genetically insulin-deficient Akita mice.12 However, not all of the experiments targeting Wnt–signaling inhibition were successful in treating DKD. An endogenous Wnt receptor antagonist DKK1 was thought to be a potential pharmacologic target for podocytopathy, but systemic DKK1 injection accelerated mesangial matrix accumulation in DKD.44 Kato et al. reported podocyte-specific β-catenin knockout mice have more severe podocytopenia after streptozotocin
injection, and the authors concluded that a balanced Wnt-signaling might be critical for maintaining the glomerular filtration barrier.\(^\text{14}\) In the current study, the diabetic FHL2\(^{+/−}\) mice had similar podocyte numbers and apoptosis ratio (determined by WT-1−positive cell and tissue TUNEL staining; Supplemental Material). We suggest the discrepancy might be due to the different suppression levels of Wnt-signaling. While Kato \textit{et al.} totally blocked the podocyte Wnt-signaling by knocking out β-catenin, in our study we did not manipulate the Wnt/β-catenin axis directly. We identified a coactivator of Wnt signaling which is expressed in podocytes, and genetic deletion of FHL2 did not affect kidney microstructure or albuminuria. FHL2 is known to be a cofactor, and knockout of FHL2 only partially blocks the Wnt signaling (glomeruli from diabetic FHL2\(^{+/−}\) mice still have elevated active β-catenin and Snail expression compared with nondiabetic mice). Partial inhibition of Wnt/β-catenin signaling may achieve an internal balance between podocyte survival and differentiation in proteinuric kidney disease.

To our knowledge, this study is the first to explore the role of Wnt-cofactor in proteinuric kidney disease. Because Wnt signaling is pleotropic and has various functions in maintaining organ homeostasis, systemic pharmacologic Wnt inhibition is impractical and risky. In this study, we used \textit{in vitro} experiments to demonstrate that FHL2, a protein abundantly expressed in kidney podocytes, is a Wnt-coactivator that amplifies Wnt signaling and magnifies podocytopathy. Our \textit{in vivo} experiments also confirmed that genetic deletion of FHL2 did not alter normal renal function and mesangial expansion in diabetic groups. The FHL2\(^{+/−}\) mice had less mesangial expansion than did their wild-type littermates. (F) Quantification of relative mesangial area of the four groups. (G) Electron microscopic picture of GBM in mice; induction of DM significantly increased GBM thickness in FHL2\(^{+/−}\) mice, but the severity is markedly attenuated in FHL2\(^{+/−}\) mice. Note the foot process effacement among diabetic FHL2\(^{+/−}\) mice, which is almost normal among diabetic FHL2\(^{−/−}\) mice. (H) Quantification of GBM thickness in four groups. NS, not significant.

**Figure 4.** Knockout FHL2 attenuated DKD \textit{in vivo}. (A–C) Blood glucose, hemoglobin A1c, and glomerular hyperfiltration level is comparably elevated in diabetic FHL2\(^{+/+}\) and FHL2\(^{−/−}\) mice. (D) Induction of DM significantly increased urine albumin level with time, and knockout FHL2 attenuated albuminuria. (E) Periodic acid–Schiff stain of mouse glomeruli (original magnification, ×400); note the glomeruli hypertrophy and mesangial expansion in diabetic groups. The FHL2\(^{+/−}\) mice had less mesangial expansion than did their wild-type littermates. (F) Quantification of relative mesangial area of the four groups. (G) Electron microscopic picture of GBM in mice; induction of DM significantly increased GBM thickness in FHL2\(^{+/−}\) mice, but the severity is markedly attenuated in FHL2\(^{−/−}\) mice. Note the foot process effacement among diabetic FHL2\(^{+/−}\) mice, which is almost normal among diabetic FHL2\(^{−/−}\) mice. (H) Quantification of GBM thickness in four groups. NS, not significant.
but ameliorated kidney damage caused by diabetes. This concept was further demonstrated from clinical pathology showing that patients with DM had a higher proportion of podocytes with FHL2 nuclear translocation, and increased FHL2 nuclear translocation was a strong predictor of renal function deterioration. On the basis of these findings, we propose FHL2 may be an efficient therapeutic target for the prevention of DKD progression.

Several limitations in the current study design should be noted. First, although our clinical data indicated that podocyte FHL2 nuclear translocation is strongly associated with creatinine doubling and the development of ESRD, we did not establish a causal relationship. Second, in a rodent DKD model, the major deficiency is the absence of renal failure even if unilateral nephrectomy is performed to accelerate disease progression. Rodents develop the glomerular hyperfiltration stage only after the development of diabetes.\(^{24}\) In our experiment, diabetic FHL2\(^{+/+}\) and FHL2\(^{-/-}\) mice had similar severity of hyperfiltration.

Third, although we demonstrated that genetic FHL2 inhibition attenuates the severity of DKD, we cannot prove the beneficial effect is only on podocyte pathology because FHL2 is also expressed in heart, smooth muscle, and even mesangial cells.\(^{45}\) Fourth, with regard to future potential clinical implications, the effect of FHL2 inhibition in other organ systems under DM conditions is unknown. In animals without DM, genetic FHL2 knockout has a beneficial effect on high-fat diet–induced atherosclerosis,\(^ {46}\) but it also reduced the ability for neovascularization.\(^ {20, 47}\) Future research is needed to address these unanswered questions.

In conclusion, the current study indicates that FHL2 is abundantly expressed in the podocyte cytoskeleton, and that high glucose and other DM-related cytokines upregulate FHL2 and promote its cytosol-to-nuclear shifting. In the podocyte nucleus, FHL2 acts as a coactivator to amplify Wnt/\(\beta\)-catenin signaling. This process is beneficial for nephron regeneration in lower species but is harmful for mammal kidney repair (Figure 7). We demonstrate for the first time that FHL2 plays a critical role in albuminuria and subsequent kidney damage in DM. FHL2 could be a potential therapeutic target against diabetic kidney damage.

**CONCISE METHODS**

**Histology and IHC Staining**

Paraffin-embedded mouse kidney (3 \(\mu m\) thickness) was stained with periodic acid-Schiff reagent by standard protocol. Fifteen cortical glomeruli per mouse with a 40\(\times\) lens were digitalized, and the mesangial index was calculated with MetaMorph Imaging System as (area of periodic acid-Schiff staining/total area of glomerulus) \(\times 100\).\(^ {48}\) IHC staining was performed using standard heat-induced epitope retrieval method and routine staining protocol. Human kidney specimens were obtained from diagnostic renal biopsies and nontumor kidney tissue from patients with renal malignancy and nephrectomy. Patients with GFR<60 ml/min per 1.73 m\(^2\) after surgery or kidney interstitial fibrosis >25% were excluded. To calculate the proportion of FHL2 nuclear translocation, human kidney samples were double-stained with FHL2 (green) and podocyte nuclear markers WT-1 (red), with the yellowish cell nucleus denoted as podocyte FHL2 nuclear translocation. For each sample, five non-sclerotic glomeruli were selected to obtain a mean ratio. Taipei Veterans General Hospital Institutional Review Board approved the human study, and all enrolled participants gave their informed consent.
Podocyte Cell Culture
The conditional immortalized human podocyte line was kindly provided by Dr. Morin Saleem and cultured as previously described.49 Cells were cultured at 33°C in RPMI 1640 medium supplemented with 10% FBS and 1% ITS (Sigma-Aldrich, St. Louis, MO). To induce differentiation, podocytes were moved to 37°C. All experiments were performed 14 days after thermoshift. To test the protein expression under different culture conditions, podocytes were treated with TGF-β (5 ng/ml; R&D Systems), 25 mM glucose, and angiotensin II (10^{-6} M; Sigma-Aldrich). A selective GSK3 inhibitor, SB216763 (5 μM; Sigma-Aldrich) was used to activate Wnt signaling directly in vitro. For some experiments, podocytes were transfected with Wnt TCF/LEF reporter plasmid, scrambled siRNA, or FHL2 siRNA (Santa Cruz Biotechnology) by electroporation (Neon Transfection System; Invitrogen, Carlsbad, CA).

TCF/LEF Reporter and Coimmunoprecipitation
The podocytes’ Wnt signal transcription activity was measured by a commercial TCF/LEF Dual-Luciferase Reporter Assay Kit according to manufacturer’s protocol (Qiagen). Briefly, differentiated podocytes were cotransfected with inducible Wnt transcription factor–responsive construct expressing firefly luciferase, and a 40:1 constitutively expressing Renilla luciferase construct as transfection control; at the same time, a noninducible firefly luciferase reporter and Renilla luciferase construct was cotransfected in another plate of cells to determining background reporter activity. The Wnt transcription activity was determined by relative luciferase activity between inducible/noninducible reporters. For coimmunoprecipitation experiments, podocytes were cultured under 25 mM glucose culture medium for 48 hours and the nuclear proteins were extracted; endogenous FHL2 and active β-catenin were coimmunoprecipitated by a commercial kit (Pierce Biotechnology) according to the manufacturer’s protocol.

Western Blot Analysis
Proteins of glomerular homogeneous or podocyte cell lysis were analyzed by Western blot as described previously.50 The primary antibodies used were: anti-active β-catenin (NonPhospho β-catenin, D13A1; Cell Signaling Technology), anti-nephrin (Acris, Herford, Germany), anti-snail, anti-VEGF, anti-TGF-β (Abcam, Inc., Cambridge, UK), and anti-FHL2 (MBL Life Science, Nagoya, Japan).

Immunofluorescence Staining and Confocal Microscopy
Kidney cryosections and cultured podocytes were fixed with 4% paraformaldehyde for 15 minutes and in 11% Triton X-100 for 10 minutes. After blocking with 10% bovine albumin for 30 minutes, the slides were immunostained with primary antibodies against nephrin, FHL2, total β-catenin or WT-1 (Abcam, Inc.) overnight. Slides were viewed under an Olympus FV10i confocal microscope.

Mouse Model of Diabetes
To clarify the effects of FHL2 on DKD, we created type 1 DM in FHL2^{−/−} mice and their wild-type littermates and evaluated the subsequent diabetic kidney damages. FHL2^{−/−} mice were generated in a B6 genetic background as previously described, and FHL1 and FHL3 were not increased in these FHL2 null mice.51 DM was induced by daily intraperitoneal injections of streptozotocin (Sigma-Aldrich), 50 mg/kg for 5 days, in 8-week-old male mice. Induction of DM was confirmed with fasting blood glucose level >300 mg/dl 2 weeks after streptozotocin injection. Diabetic mice received small-dose sustained-release insulin implants to reduce blood glucose level to normal levels.
nephrectomy was performed 1 week before streptozotocin injection to accelerate progression of DKD. The mice were divided into four groups: FHL2+/+ control group, FHL2+/+ DM group, FHL2−/− control group, and FHL2−/− DM group (n=12–15 for each group). All mice were housed under standard conditions with normal food and euthanized 4 months after induction of diabetes. The Institutional Animal Care Committee of Taipei Veterans General Hospital approved all experimental procedures involving animals.

GFR and Urine Albumin Measurement
Mouse GFR was assessed by the FITC-inulin clearance method as previously described. For measurement of urine albumin, mice were placed in metabolic cages. The 24-hour urine was centrifuged and albumin level was determined using a commercialized ELISA (Albuwell M kit; Exocell Inc., Philadelphia, PA). Because the Jaffe method overestimates creatinine levels in rodents, mouse urine creatinine was determined by HPLC.

Electron Microscopy
Mouse kidney cortex was fixed with 2.5% glutaraldehyde followed by 0.5% OsO4. These were then series dehydrated and embedded according to routine procedures. Thin sections were viewed and digitally recorded using a transmission electron microscope (JEM-1230; JEOL, Tokyo, Japan). The GBM thickness was determined by the orthogonal intercept method. In essence this applies a correction factor for oblique sectioning to the harmonic mean of a large number of random orthogonal intercept measurements of the GBM of several glomeruli. Glomerular peripheral capillary loops were identified under electron microscopy. To eliminate bias in the sectioning area, the capillary loops were photographed. The shortest distance between the endothelial cytoplasmic membrane and the outer lining of the lamina rara externa underneath the cytoplasmic membrane of the podocyte foot processes was measured with a logarithmic ruler. The apparent harmonic mean thickness (lh) was calculated, from which the true harmonic mean thickness (Th) was estimated by the following equation:

\[
Th = \frac{8}{3\pi} \times \left( \frac{10^6}{M} \right) lh,
\]

where M represents the final print magnification (15,000× in current study). In the current study, five glomeruli of each mouse were analyzed.
Statistical Analyses
All values are expressed as mean ± SD unless otherwise specified. Differences between multiple groups were evaluated for significance using ANOVA analysis with a Bonferroni post hoc test. Statistical analyses were performed using GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, CA). A two-tailed P value <0.05 was considered to represent a statistically significant difference.

ACKNOWLEDGMENTS
This study was supported, in part, by the following research grants: NSC 102-2633-B-075-001, NSC 102-2314-B-182A-060-MY2 and 103-2314-B-075-004, UST-UCSD International Centre of Excellence in Advanced Bio-engineering, NSC-100-2911-I-009-101-A2 from the Ministry of Science and Technology, VGH-V102B-016 and VGH-V102E-002 from Taipei Veterans General Hospital, and CMRPG3B1311 from Chang Gung Memorial Hospital.

DISCLOSURES
None.

REFERENCES

**AFFILIATIONS**

*Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital and Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan; †Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital and Institute of Clinical Medicine, and Cardiovascular Research Center, National Yang-Ming University, Taipei, Taiwan; ‡Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital, and Institute of Physiology, National Yang-Ming University, Taipei, Taiwan; ‡Division of Urology, Taipei Veterans General Hospital, Department of Urology, School of Medicine, National Yang-Ming University, Taipei, Taiwan; ‡Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital and School of Medicine, National Yang-Ming University,*

---

This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014100989/-/DCSupplemental.
Taipei, Taiwan; *Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital, and Institute of Anatomy and Cell Biology, National Yang-Ming University, Taipei, Taiwan; **Division of Hematology and Oncology, Department of Medicine, Taipei Veterans General Hospital and Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan; ††Department of Medical Research, Taipei Veterans General Hospital, Institute and Department of Pharmacology, and Cardiovascular Research Center, National Yang-Ming University, Taipei, Taiwan; ‡‡The Institute of Engineering in Medicine, University of California San Diego, La Jolla, California; §§Departments of Bioengineering, Nanoengineering, Institute of Engineering in Medicine, University of California San Diego, La Jolla, California; and ||Division of Cardiology, Department of Internal Medicine; Healthcare Center; Heart Failure Center, Chang Gung Memorial Hospital, Chang Gung University, College of Medicine, Taipei, Taiwan