Secreted Frizzled-related Protein 1 (Sfrp1) Regulates the Progression of Renal Fibrosis in a Mouse Model of Obstructive Nephropathy*

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Background: Secreted Frizzled-related protein 1 is a secreted Wnt antagonist.
Results: The kidneys from Sfrp1 knock-out mice showed significant increase in the renal fibrosis after unilateral ureteral obstruction.
Conclusion: Deletion of Sfrp1 makes mice more susceptible to renal damage through non-canonical Wnt/PCP pathway.
Significance: The relationship between kidney damage and Wnt/non-canonical pathway definitely opens a new field to study mechanisms of renal diseases.

Renal fibrosis is responsible for progressive renal diseases that cause chronic renal failure. Sfrp1 (secreted Frizzled-related protein 1) is highly expressed in kidney, although little is known about connection between the protein and renal diseases. Here, we focused on Sfrp1 to investigate its roles in renal fibrosis using a mouse model of unilateral ureteral obstruction (UUO). In wild-type mice, the expression of Sfrp1 protein was markedly increased after UUO. The kidneys from Sfrp1 knock-out mice showed significant increase in expression of myofibrobast markers, α-smooth muscle actin (αSMA). Sfrp1 deficiency also increased protein levels of the fibroblast genes, vimentin, and decreased those of the epithelial genes, E-cadherin, indicated that enhanced epithelial-to-mesenchymal transition. There was no difference in the levels of canonical Wnt signaling; rather, the levels of phosphorylated c-Jun and JNK were more increased in the Sfrp1−/− obstructed kidney. Moreover, the apoptotic cell population was significantly elevated in the obstructed kidneys from Sfrp1−/− mice following UUO but was slightly increased in those from wild-type mice. These results indicate that Sfrp1 is required for inhibition of renal damage through the non-canonical Wnt/PCP pathway.

Renal tubulointerstitial fibrosis is regarded as a final common pathway of progressive chronic kidney diseases (1–4). Fibrotic damage is characterized by the increase of interstitial fibroblasts and myofibroblasts (5, 6). In a recent study, epithelial-to-mesenchymal transition is essential for the development of renal fibrosis, in which tubular epithelial cells are transformed into interstitial fibroblasts and myofibroblasts by activating signaling pathways, including the Wnt and TGF-β pathway (7–11).

Wnt family members secrete glycoproteins that play crucial roles in various cellular functions (12, 13) (see the Wnt Home-page, Nusse laboratory, Stanford University). Wnt proteins can signal through the canonical (Wnt/β-catenin) pathway. The Wnt/β-catenin signaling pathway is a regulator of cellular functions in embryonic development, homeostatic state, and tissue injury (14–16). The Wnt/β-catenin pathway is also activated in the process of kidney development and renal fibrosis (17–20). Wnt signals can also be transmitted through some additional pathway: non-canonical Wnt pathway, such as planar cell polarity (PCP)2 or Ca2+ pathway, which is β-catenin-independent (21, 22). The Wnt/PCP pathway transduces its signal by activating c-Jun N-terminal kinase (JNK). The disruption of the Wnt/PCP pathway is detrimental for oriented cell division and apicobasal polarity, but the function of non-canonical Wnt pathway is largely unknown in the process of kidney diseases.

The secreted Frizzled-related protein (Sfrp) is a secreted Wnt antagonist that interacts directly with Wnt ligand (23–28). Of the five Sfrp family members (Sfrp1 to Sfrp5), Sfrp1, Sfrp2, and Sfrp5 comprise Sfrp1 subfamily due to their sequence similarities (28). The phenotypes of single knock-out mice null for each Sfrp1, Sfrp2, or Sfrp5, were viable due to redundancy in their functions. The double or triple Sfrp knock-out mice were resulted in a lethal embryonic phenotype with reduction of anterior-posterior patterning (29, 30). Genetic and biochemical analyses revealed that Sfrp1/2/5 regulated Wnt/β-catenin and the Wnt/PCP pathways (30–32). Recently, some reports showed that Sfrp1 functioned as a mediator of senescence (33) and dysregulated glucose metabolism (34). However, little is still known about Sfrp1 function in the pathological events.

In the present study, we investigated the Sfrp1 function in the injured kidney. In a model of kidney injury, using the unilateral ureteral obstruction (UUO) model, the expression of Sfrp1 protein was increased. The kidneys from Sfrp1 knock-out mice showed significant increase in the renal fibrosis. Our results suggest that Sfrp1 is required for inhibition of renal tubulointerstitial fibrosis.

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**EXPERIMENTAL PROCEDURES**

*Mice*—Sfrp1-deficient mice were maintained 129 and C57BL/6 mixed genetic background (29) and backcrossed for more than five generations onto C57BL/6 background.

Renal fibrosis was induced by ligation of the left ureter in male mice (35). Sfrp1+/+ and Sfrp1−/− mice (n = 3 per group) were used at 3, 7, and 14 days after the operation. The obstructed (UUO) and non-obstructed (Sham) kidneys were collected carefully and subjected to the analyses.

**Cell Culture and Transfection**—293T cells were grown in DMEM supplemented with 10% FBS. Mammalian expression vectors for Sfrp1-FLAG was constructed by insertion into pcDNA3 vectors (Invitrogen). Transfection was performed using Lipofectamine reagent (Invitrogen).

**Preparation of Recombinant Proteins**—His-tagged (for production of mouse Sfrp1, Sfrp2, and Sfrp5 antibodies) and MBP-tagged (for immunoblotting) Sfrp1–5 were expressed in BL21-CodonPlus-RP (Agilent Technologies, Santa Clara, CA) transformed with pET-28a (Invitrogen) and pMAL (New England Biolabs), respectively. Each His or MBP fusion protein was purified through affinity chromatography with TALON metal affinity resin (Clontech) or with amylose resin (New England Biolabs), respectively.

**Antibody**—We produced mouse monoclonal Sfrp1, rat monoclonal Sfrp2, and rat monoclonal Sfrp5 antibodies, as described previously (36).

The antibodies against the following proteins: vimentin (Progen, Heidelberg, Germany); Ca2+/calmodulin-dependent kinase II (EP1829Y), phospho-Smad3 (EP823Y), actinin 4 (EPR2533, Epitomics, Burlingame, CA); E-cadherin (no. 3195), active-β-catenin (no. 8814), Cyclin D1 (DCS6; no. 2926), phospho-JNK (no. 4668), JNK (no. 9252), phospho-Ca2+/calmodulin-dependent kinase II (no. 12716), Smad3 (no. 9523) phospho-c-jun (no. 9261), c-Jun (no. 9165), phospho-p38 (no. 4511), p38 (no. 8960; Cell Signaling Technology), β-catenin (BD Transduction Laboratories), FLAG (M2), αSMA (1A4), phospho-histone H3 (Ser-10; Sigma), c-Myc (sc-764; Santa Cruz Biotechnology, Santa Cruz, CA), and MBP (New England Biolabs).

**Tissue Extract Preparation and Immunoblotting**—Mouse kidneys were homogenized directly in a SDS-PAGE sample buffer. Protein concentrations for cell extracts were determined by the Coomassie Brilliant Blue staining by SDS-PAGE gels. The lysates were loaded, transferred, and subjected to Western blotting with specific antibodies.

**Histology and Immunohistochemistry**—Mouse kidneys were fixed with 4% paraformaldehyde/PBS overnight at 4 °C, and embedded in paraffin. Three-μm-thick sections were prepared and mounted. Some slides were stained with hematoxylin and eosin. For immunohistochemistry, the slides were deparaffinized, and endogenous peroxidase was inactivated in 3% H2O2 in methanol for 30min, treated with 10 μM citrate buffer (pH 6.0) in a microwave for 15 min, and blocked in 5% serum in TBST for 1 h. Sections were incubated with primary antibodies overnight at 4 °C and then with appropriate biotinylated secondary antibodies (VECTOR Laboratories, Burlingame, CA) for 1 h at room temperature. The detection was carried out by using the VECTASTAIN ABC KIT and dianinobenzidine regent (VECTOR Laboratories).

The areas positive for αSMA, vimentin, E-cadherin, and actinin 4 were quantified by ImageJ software. Statistical significance, which was evaluated using Welch’s t test, was defined as p < 0.05. Error bars indicate S.D.

**TUNEL Assay**—Apoptosis in the sham and UUO kidneys was assayed using the ApopTag Plus peroxidase kit (Chemicon, Temecula, CA) as described previously (37).

**Ethics Statement**—All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal works were approved appropriately by the Shigei Medical Research Institute.

**RESULTS**

*Sfrp1 Protein Is Increased in the Obstructed Kidney After UUO*—To investigate the Sfrp1 function of the kidney disease, we produced monoclonal antibodies that specifically recognize Sfrp1 protein. Immunoblot analysis revealed that the monoclonal anti-Sfrp1 antibody immunoreacted specifically with a band

![FIGURE 1. Sfrp1 increases after unilateral ureteral obstruction (UUO). A, the Sfrp1 antibody reacted specifically with a band corresponding to Sfrp1 in lysisates of Sfrp1-FLAG-expressed 293T cells, but not 293T (mock) cells by Western blotting. B, characterization of an antibody specifically recognized Sfrp1, Sfrp2, and Sfrp5. Immunoreactivity was impaired specifically with Sfrp1, but not with other Sfrp protein families (Sfrp2–5). The Sfrp2 and Sfrp5 antibodies were also immunoreacted specifically with Sfrp2 and Sfrp5 proteins, respectively. C, the levels of the Sfrp1, Sfrp2, and Sfrp5 proteins were detected by Western blot analysis in the Sham and UUO kidneys in wild-type mice (Sfrp1+/+) or Sfrp1 knock-out mice (Sfrp1−/−) at different time points after UUO. Actin was evaluated as an internal control. His-Sfrp2 and His-Sfrp5 were used as a positive control to detect Sfrp2 and Sfrp5 proteins, respectively.
corresponding to the position of the similar molecular weights in 293T cell lysates expressing mouse Sfrp1 (Fig. 1A). To explore the specificity of monoclonal anti-Sfrp1 antibody, Western blotting was performed using recombinant Sfrp proteins (mouse Sfrp1–5). As shown in Fig. 1B, this antibody was only reacted with Sfrp1, but not with other Sfrp proteins.

It is previously reported that Sfrp1 was highly expressed in the kidney of the newborn (38, 39). To determine whether Sfrp1 protein was changed after kidney damage, we performed Western blot analyses in the obstructed kidneys of wild-type and Sfrp1-deficient mice. After UUO, we found that Sfrp1 protein was increased at different time points in the obstructed kidneys (Fig. 1C). There were no Sfrp1 bands detectable in the sham-operated and UUO Sfrp1/H11002/H11002 kidney lysates when using the Sfrp1 antibody (Fig. 1C).

In previous reports, Sfrp1, Sfrp2, and Sfrp5 have been suggested on the basis of the similarity of their expression patterns during embryogenesis (29, 30). To examine the extent of functional redundancy between three Sfrp members, we investigated Sfrp1−/− UOO kidneys with graded levels of Sfrp2 and Sfrp5 protein expressions. Deletion of Sfrp1 did not result in a compensatory increase of the remaining gene product, Sfrp2 and Sfrp5 proteins (Fig. 1C).

Loss of Sfrp1 Is Associated with Increased Renal Fibrosis After UUO—In the UUO injury model, Sfrp1 protein levels were significantly increased in the obstructed kidney. To analyze whether loss of Sfrp1 exacerbated the progression of fibrosis, UUO in the Sfrp1 knock-out mice was performed in the obstructed kidneys of Sfrp1+/+ and Sfrp1−/− mice. The kidney sections from sham-operated and UUO mice were stained with hematoxylin and eosin. In the wild-type (Sfrp1+/+) UUO-operated kidneys, fibrotic lesion in the cortex was observed after the surgery. In the heterozygous (Sfrp1+/−) UUO kidneys, there was no detectable difference of renal fibrosis compared with that of wild-type (data not shown). In contrast, the development of tubulointerstitial injury was progressively deteriorated in the homozygous UUO kidney (Fig. 2, A and B). Immunohistochemical analyses also revealed that the expression of αSMA, a marker of myofibroblast, was increased in the UUO kidneys (Fig. 2C). The αSMA-positive area was significantly higher in the Sfrp1-deficient kidneys compared those with wild-type kidneys at days 3 and 7 after UUO (Fig. 2, C and D).

To further explore the effects of Sfrp1 disruption in the obstructed kidney, we examined the expression of vimentin and E-cadherin after UUO. Immunohistochemical analyses showed the expression level of vimentin was increased in the damaged
kidneys from Sfrp1−/− mice when compared with those of Sfrp1+/+ littermates (Fig. 3, A and B). In contrast, the expression level of E-cadherin was decreased (Fig. 3, C and D). Actinin 4, a marker of renal glomerulus, the expression was not changed (Fig. 3, E and F). These results indicate that Sfrp1 maintain renal tubular epithelial cells during the fibrosis.

Canonical Wnt/β-catenin Pathway in the Obstructed Sfrp1−/− Kidneys—In the previous study, Sfrp regulated canonical and non-canonical Wnt pathway (30). We studied which Wnt pathway was up-regulated after UUO. As described previously (20), the canonical Wnt/β-catenin pathway was up-regulated after UUO. To investigate whether Sfrp1 protein during kidney damage-modulated canonical Wnt signaling, we performed Western blotting and immunohistochemistry of the UUO kidneys for measuring the amount of active β-catenin that is not phosphorylated on both Ser-37 and Thr-41 (40). The activity levels were not altered in the obstructed Sfrp1−/− kidneys in comparison with those in the Sfrp1+/+ kidneys (Fig. 4, A and B).

We then observed the protein expression of several target genes of the Wnt/β-catenin pathway in the obstructed kidney. Western blot analyses showed that expression levels of the target genes, c-Myc and cyclin D1, were not altered (Fig. 4A). Furthermore, we determined whether cell proliferation ratio was

**FIGURE 3.** Sfrp1-deficient mice are enhanced epithelial-to-mesenchymal transition after UUO. A, C, and E, immunohistochemical analyses of the mouse kidney sections with antibodies against vimentin (A), E-cadherin (C), and actinin 4 (E) in the sham and UUO kidneys after UUO. B, graph shows analysis of the percentage of vimentin-positive area in the UUO kidneys. D, the E-cadherin expression levels are given as a percentage of the UUO to sham-operated kidneys. F, renal glomerulus is unchanged in the obstructed Sfrp1−/− kidneys by counting actinin 4-positive glomerulus. Scale bars, 100 μm. *, p < 0.05.
changed in the Sfrp1−/− obstructed kidneys. No difference was immunohistochemically found in cell proliferation by counting phospho-histone H3-positive cells (Fig. 4, C and D). These data indicate that canonical Wnt/β-catenin pathway did not alter in the obstructed Sfrp1−/− kidneys.

**Non-canonical Wnt/PCP Pathway Modulates the Maintenance of Renal Fibrosis**—To study whether the non-canonical Wnt pathway was affected in the obstructed kidney of Sfrp1-deficient mice, we investigated activation of several putative mediators of the signaling (Fig. 5A). The JNK signaling pathway (Wnt/PCP pathway) is one branch of non-canonical Wnt pathway. We also performed Western blotting and immunohistochemistry of the UUO kidneys for measuring the levels of phospho- and total c-Jun, a downstream of JNK. We found that the levels of phospho- and total c-Jun were increased in the obstructed Sfrp1−/− kidneys (Fig. 5, A and B). In addition, we detected increased phospho-JNK levels by Western blotting (Fig. 5A). Surprisingly, the levels of total JNK were increased in Sham and UUO Sfrp1−/− kidneys (Fig. 5A).

Next, the calcium-dependent signaling pathway (Wnt/Ca2+ pathway) is a second branch of the non-canonical Wnt pathway. So, we asked whether the phosphorylation levels of Ca2+/calmodulin-dependent kinase II, which was activated in Wnt/Ca2+ pathway-mediated responses, was regulated. Western blot analyses indicated that this activity was unaltered in the obstructed Sfrp1−/− kidneys (Fig. 5A).
The activation of c-Jun was reported to regulate the diverse biological functions, including apoptosis, invasion, and metastasis, and cell polarity as well as non-canonical Wnt signaling.

To evaluate the effect of apoptosis, we performed a TUNEL assay in the Sfrp1/Sfrp1/H11002/H11002 obstructed kidneys. The numbers of TUNEL-positive apoptotic cells increased in the UUO kidneys of Sfrp1-deficient mice, compared with that of wild-type mice (Fig. 5, C and D). However, the level of phospho-p38, which regulates the apoptosis signal similar to the c-Jun pathway, showed no difference between the wild-type and homozygous mutant (Fig. 5A).

Finally, we focused the relationship between Wnt and TGF-β signaling in the Sfrp1-mediated UUO kidneys. To explore whether the TGF-β was affected in the obstructed kidney of...
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Sfrp1-deficient mice, we examined Western blotting of the UUO kidneys for measuring the levels of phospho-Smad3, which are the most critical mediators in TGF-β signaling pathway. We found that the phosphorylation levels were not altered in the obstructed Sfrp1−/− kidneys in comparison with those in the Sfrp1+/+ kidneys (Fig. 6). Thus, these observations suggest that the non-canonical Wnt/PCP pathway modulates the maintenance of renal fibrosis after kidney damage.

DISCUSSION

The present study demonstrated that Sfrp1 protein was increased in the obstructed kidney, and inactivation of Sfrp1 was associated with increased renal fibrosis during unilateral ureteral obstruction. Sfrp1 knock-out mice indicated that the renal tubules were disrupted, and the numbers of apoptotic cells were increased after UUO. Furthermore, phosphorylated c-Jun levels were significantly elevated in the obstructed Sfrp1−/− kidneys, indicating that Sfrp1 controlled renal damage through the non-canonical Wnt/PCP pathway.

It is well established that Wnt signaling is silenced in adult tissues (41, 42); however, it is activated in the injured tissues due to the progression of diseases (19, 20). The recent study showed that Wnt4 might be dispensable for myofibroblast transformation in the damaged kidney (18). In addition, Dkk-1 (Dickkopf-related protein 1), a ligand for Wnt antagonist, controls the myofibroblast progression of renal fibrosis (17). These studies indicate that Wnt/β-catenin signaling is important for the maintenance of kidney damage. Indeed, the amount of active β-catenin increases during progression of renal tubulointerstitial fibrosis (20). Dkk-1 blocks the increase of Wnt/β-catenin signaling, resulted in the reduction of kidney fibrosis (17).

In the previous reports, recombinant Sfrp4, a secreted antagonist of Wnt signaling, also reduced the number of myofibroblasts and active β-catenin, indicated that Sfrp4 was able to interfere with kidney fibrosis through Wnt/β-catenin signaling (20). In this study, we demonstrated that loss of Sfrp1 led expansion of renal fibrosis in the obstructed kidney. However, the activity of β-catenin did not alter in the obstructed Sfrp1−/− kidneys. Distinct difference in the responses of Wnt/β-catenin signaling pathway might be due to the following possibilities.

Previous data of Ren et al. (17) suggested that Dkk-1 overexpression inhibited cell proliferation in the obstructed kidney after UUO. Notably, Wnt/β-catenin pathway is implicated in regulating cell proliferation. In contrast, our results indicated that cell proliferation was not altered in the obstructed kidneys between mouse genotypes. Alternatively, the numbers of apoptotic cells were increased in the UUO kidneys of Sfrp1-deficient mice. Renal tubular apoptosis causes progressive kidney disease such as fibrosis (43). The p38 mitogen-activated protein kinase (MAPK) cascade and JNK cascade play a critical role in renal fibrosis (44). Upon kidney damage, the levels of JNK and p38 phosphorylation are increased in UUO (43, 44).

In this study, we found that the levels of phosphorylated c-Jun and JNK were increased in the obstructed Sfrp1−/− kidneys. This observation is consistent with a recent report that in small intestine of Sfrp1−/−;Sfrp2−/−;Sfrp5−/− mice, phospho-c-Jun levels were elevated significantly in the epithelium in comparison with the control small intestine epithelium (31). Moreover, the Wnt/PCP pathway does not lead to β-catenin stabilization but activates JNK/c-Jun signaling through Frizzled and Dishevelled (31). Thus, it is understandable that Sfrp1 regulation of Wnt/PCP pathway controls renal interstitial fibrosis among chronic renal diseases. In recent study, Wnt11 signaling by TGF-β was activated in the renal fibrosis through the non-canonical Wnt/PCP pathway (45). We found no evidence that the levels of phospho-Smad3 altered in the obstructed Sfrp1−/− kidneys. However, further studies are needed to draw a definite conclusion concerning contribution of Wnt/PCP cascade to kidney pathology.

In conclusion, we identified that Sfrp1 regulated the progression of renal fibrosis in mouse unilateral ureteral obstruction. The relationship between kidney damage and the Wnt/non-canonical pathway definitely opens a new field to study mechanisms of renal diseases. Sfrp1 is a reliable candidate for the anti-fibrogenesis drug; however, further studies are needed to understand the underlying molecular mechanism(s).

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