Downregulation of SnoN Expression in Obstructive Nephropathy Is Mediated by an Enhanced Ubiquitin-Dependent Degradation

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Smad transcriptional co-repressor SnoN acts as an antagonist that tightly controls the trans-activation of TGF-β/Smad target genes. SnoN protein is reduced progressively in the fibrotic kidney after obstructive injury, suggesting that the loss of SnoN antagonist is a critical event that leads to an uncontrolled fibrogenic signaling. However, the mechanism underlying SnoN downregulation remains unknown. This study investigated the regulation and mechanism of renal SnoN expression in vivo. Whereas SnoN protein was markedly diminished, its mRNA levels remained relatively constant in the obstructed kidney, compared with sham controls. Smad ubiquitination regulatory factor-2, an E3 ubiquitin ligase, was induced and formed a complex with SnoN in vivo. In vitro, TGF-β1 promoted SnoN protein degradation, which was mediated by ubiquitination and a proteasome-dependent mechanism. SnoN constitutively interacted with another Smad co-repressor, Ski, and they formed ternary complex with Smad2/3 upon TGF-β1 stimulation. However, ectopic expression of Ski did not alter the degradation rate of SnoN. Blockage of SnoN degradation by proteasome inhibitor abolished TGF-β1-mediated α-smooth muscle actin and fibronectin induction, suggesting that SnoN degradation could be necessary for TGF-β1 to exert its fibrogenic action. Furthermore, knockdown of Smad ubiquitination regulatory factor-2 expression by small interfering RNA strategy led to an increase in SnoN abundance and inhibited the TGF-β1-mediated gene transcription. These results indicate that downregulation of SnoN expression in the obstructed kidney is mediated by an enhanced ubiquitin-dependent degradation. Preservation of SnoN by inhibiting its degradation may be a novel strategy for targeting hyperactive Smad signaling in renal fibrotic diseases.


TGF-β is widely recognized as a key fibrogenic cytokine that plays a critical role in the pathogenesis of renal fibrosis (1–4). Extensive investigations over the past decade have demonstrated unambiguously the importance of TGF-β in the initiation and progression of chronic kidney disease (CKD) after various injuries. Induction of TGF-β expression is found in virtually every type of CKD (1,2,5). In vitro, TGF-β1 stimulates myofibroblastic activation from glomerular mesangial cells and interstitial fibroblasts (6,7) and promotes tubular epithelial to mesenchymal induction, suggesting that SnoN was found in obstructed kidney, compared with sham controls. Smad ubiquitination regulatory factor-2, an E3 ubiquitin ligase, was induced and formed a complex with SnoN in vivo. In vitro, TGF-β1 promoted SnoN protein degradation, which was mediated by ubiquitination and a proteasome-dependent mechanism. SnoN constitutively interacted with another Smad co-repressor, Ski, and they formed ternary complex with Smad2/3 upon TGF-β1 stimulation. However, ectopic expression of Ski did not alter the degradation rate of SnoN. Blockage of SnoN degradation by proteasome inhibitor abolished TGF-β1-mediated α-smooth muscle actin and fibronectin induction, suggesting that SnoN degradation could be necessary for TGF-β1 to exert its fibrogenic action. Furthermore, knockdown of Smad ubiquitination regulatory factor-2 expression by small interfering RNA strategy led to an increase in SnoN abundance and inhibited the TGF-β1-mediated gene transcription. These results indicate that downregulation of SnoN expression in the obstructed kidney is mediated by an enhanced ubiquitin-dependent degradation. Preservation of SnoN by inhibiting its degradation may be a novel strategy for targeting hyperactive Smad signaling in renal fibrotic diseases.

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sors in a particular cellular context may determine the ultimate response of the cells to TGF-β stimulation.

Earlier studies from our laboratory demonstrated that SnoN protein is downregulated progressively in the fibrotic kidney after obstructive injury (20). Loss of Smad transcriptional corepressor seems to have a profound impact on Smad signaling, because knockdown of SnoN via small interfering RNA (siRNA) inhibition dramatically sensitizes renal tubular epithelial cells to TGF-β1 stimulation (20). Consistently, increased SnoN expression, either via transfection of SnoN expression vector (20) or through induction by hepatocyte growth factor (21), suppresses TGF-β/Smad-mediated gene transcription. These observations accentuate that the loss of Smad antagonist is a critical, pathogenic event that may lead to an uncontrolled Smad signaling. However, the mechanism underlying SnoN reduction in the diseased kidney remains unknown.

In this study, we investigated the regulation and mechanism of SnoN expression in the fibrotic kidney after obstructive injury. Our results demonstrate that downregulation of SnoN in vivo is mediated by an enhanced ubiquitin-dependent degradation.

Materials and Methods

Animal Model

Male CD-1 mice that weighed 18 to 22 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in the animal facilities of the University of Pittsburgh Medical Center, with free access to food and water. Animals were treated humanely by use of the protocols that were approved by the Institutional Animal Use and Care Committee at the University of Pittsburgh. Unilateral ureteral obstruction (UUO) was performed using an established procedure (22). Groups of mice (n = 4) were killed at days 1, 3, 7, and 14 after UUO, respectively. One group of sham-operated mice (n = 4) was killed at day 7 after surgery. One part of the kidneys was fixed in 10% phosphate-buffered formalin, followed by paraffin embedding for immunohistochemical studies. The remaining kidneys were snap-frozen in liquid nitrogen and stored at −80°C for RNA and protein extractions.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from the kidney tissue using an Ultraspec RNA isolation system according to the instructions specified by the manufacturer (Biotecx Laboratories, Houston, TX). Northern blot analysis for SnoN expression was carried out by the procedures described previously (21,23). SnoN cDNA probe was provided by R. Weinberg (MIT, Cambridge, MA). After autoradiography, membranes were stripped and rehybridized with rat glyceraldehyde-3-phosphate dehydrogenase probe to ensure equal loading of each lane.

Reverse Transcription–PCR

Reverse transcription–PCR was performed as described elsewhere (24). Briefly, the first-strand cDNA synthesis was carried out by using a Reverse Transcription System kit according to the instructions of the manufacturer (Promega, Madison, WI). PCR amplification was performed on HotStar TagMaster Mix Kit (Qiagen, Valencia, CA). The primer sequences were as follows: SnoN 5'-GATAACACATTGG-GAAATGGA-3' (sense) and 5'-AGATGTGAGCGACACATTCG-3' (antisense); α-smooth muscle actin (α-SMA) 5'-CCCCAGCAGAACGCACTGTCA-3' (sense) and 5'-TCCAGAGTCCAGACATCAG-3' (antisense); fibronectin 5'-CACCAGCGATAAGGGATAG-3' (sense) and 5'-GTAGGCGTAAAGGCAGTACTC-3' (antisense); and β-actin 5'-TCAAGATCATGCTTTCTCTGAGC-3' (sense) and 5'-TGCTGTACCTTCCACGCTG-3' (antisense). Expression levels of mRNA were calculated after normalizing with housekeeping gene β-actin.

Endogenous SnoN Degradation Assay

The endogenous SnoN degradation was assessed by an in vitro assay in tissue homogenates that were prepared from sham and UUO kidneys at 7 d after surgery, according to the method described previously (25,26). Briefly, 100 μg of the kidney homogenates from a pool of four mice per group were incubated in 100 μl of ubiquitination mixture (50 mM Tris-HCl [pH 8.3], 5 mM MgCl₂, 2 mM dithiothreitol, 5 mM ATP, and 2 mg/ml ubiquitin) for 0, 4, 8, and 16 h at 37°C, respectively. After incubation, the mixture were heated at 100°C for 5 min in SDS sample buffer, followed by immunoblotting with anti-SnoN antibody. The degradation assay also was performed in the presence of 1× proteasome inhibitor mix (0.25 mM MG132 and 0.25 mM MG115; EMD Biosciences, San Diego, CA).

In Vitro SnoN Ubiquitination Assay

SnoN ubiquitination activity in kidney tissues was detected by an in vitro assay using exogenous hemagglutinin (HA)-tagged SnoN protein as substrates, as described elsewhere (25,26). Human kidney proximal tubular epithelial cells (HKC) were transfected with HA-tagged SnoN expression vector (27). HA-tagged SnoN was immunoprecipitated by anti-HA antibody and used for in vitro ubiquitination assay. Kidney tissues from sham and UUO mice at 3, 7, and 14 d after surgery (pool from four mice per group) were lysed in Triton X-100 lysis buffer and centrifuged at 30,000 × g overnight at 4°C. The supernatants (100 μg) were incubated with 6 μl of immunopurified HA-tagged SnoN protein and 100 μl of ubiquitination mixture (50 mM Tris-HCl [pH 8.3], 5 mM MgCl₂, 2 mM dithiothreitol, 5 mM ATP, 2 mg/ml ubiquitin, 50 μg/ml ubiquitin aldehyde, 10 mM phosphocreatine, 0.28 units/ml phosphocreatine kinase, 1× protease inhibitor mix, 1× proteasome inhibitor mix, 100 nM E1, and 1 μM UbCH5b; BostonBiochem, Cambridge, MA) for 1 h at 37°C. After incubation, the reactions were boiled for 5 min in SDS sample buffer, followed by immunoblotting with anti-ubiquitin antibody.

Western Blot Analysis

Detection of protein expression by Western blot was carried out according to the established protocols described previously (22). The primary antibodies used were as follows: Anti-SnoN (sc-9595), anti-c-Ski (ss-9140), anti-Smad ubiquitination regulatory factor-2 (Smurf2; sc-25511), anti-ubiquitin (sc-8017), and anti-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA); anti-α-SMA (clone 1A4) and anti-α-tubulin (T-9026; Sigma, St. Louis, MO); anti-glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX); and anti-fibronectin (clone 10; BD Transduction Laboratories, Lexington, KY). Quantification was performed by measurement of the intensity of the signals with the use of National Institutes of Health Image analysis software.

Immunohistochemical Staining

Immunohistochemical staining was performed using established procedures as described previously (3). Briefly, paraffin-embedded kidney sections from the UUO and sham-operated mice were prepared by a routine procedure and stained with the specific primary antibody against Smurf2. As a negative control, the primary antibody was replaced with nonimmune normal goat IgG, and no staining occurred.
Cell Culture, Treatment, and Transient Transfection

HKC were cultured in DMEM and Ham’s F-12 medium (1:1) supplemented with 5% FBS (Invitrogen, Carlsbad, CA), as described previously (22). Cells typically were seeded at approximately 70% confluence in complete medium that contained 5% FBS for 24 h, and then serum-starved for 16 h. For SnoN degradation assay, HKC were transiently transfected with pHA-SnoN expression vector (27) and then treated with cycloheximide (Sigma) at the concentrations of 10 μg/ml in the absence or presence of recombinant human TGF-β1 (2 ng/ml; R&D Systems, Minneapolis, MN) for various periods of time as indicated. In some experiments, cells were pretreated with MG132 at the concentrations as indicated for 3 h, followed by various treatments. Transient transfection of HKC was carried out by using Lipofectamine 2000 according to the instructions specified by the manufacturer (Invitrogen). The empty pcDNA3 vector was used as a negative control.

For siRNA inhibition studies, HKC were transfected with control siRNA (sc-37007) or Smurf2 siRNA (sc-41675; Santa Cruz Biotechnology) at the final concentration of 120 nM by using oligofectamine reagent according to the instructions specified by the manufacturer (Invitrogen). Whole-cell lysates were collected for assessment of the expression of Smurf2 and SnoN by Western blot analyses.

Immunoprecipitation

Immunoprecipitation experiments were performed using similar methods as described previously (21). Briefly, cell or kidney tissue lysates were centrifuged at 12,000 × g for 10 min at 4°C. The resulting supernatants were collected for immunoprecipitation. After pre-clearing with normal host IgG, the lysates were immunoprecipitated overnight at 4°C with 2 μg of various primary antibodies, respectively, followed by precipitation with 30 μl of protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 3 h at 4°C. The primary antibodies used were as follows: Anti-SnoN, anti-Smurfl2, and anti-Smad2/3 (Santa Cruz Biotechnology). The precipitated complexes were boiled for 5 min in SDS sample buffer, followed by immunoblotting with various antibodies as indicated.

Reporter Constructs and Luciferase Assay

The reporter construct p3TP-Lux was provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY). HKC were co-transfected with Lipofectamine 2000 reagent with p3TP-Lux (0.5 μg), control siRNA, or Smurf2 siRNA (100 nM). A fixed amount (0.05 μg) of internal control reporter *Renilla reniformis* luciferase driven under thymidine kinase (TK) promoter (pRL-TK; Promega) also was co-transfected for normalizing the transfection efficiency. The transfected cells were incubated in serum-free medium for 16 h and then treated without or with TGF-β1 (2 ng/ml) for an additional 48 h. Luciferase assay was performed using the Dual Luciferase Assay System kit according to the manufacturer’s protocols (Promega). Relative luciferase activity (arbitrary unit) was reported as fold induction over the controls after normalization for transfection efficiency.

Immunofluorescence Staining

Indirect immunofluorescence staining was performed using an established procedure (22). Cells were incubated with the anti-fibronectin antibody, followed by staining with FITC-conjugated secondary antibody. Cells also were stained with 4’,6-diamidino-2-phenylindole, HCl to visualize the nuclei. Slides were viewed with a Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera (Melville, NY). In each experimental setting, immunofluorescence images were captured with identical light exposure times.

Statistical Analyses

Statistical analysis was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparisons between groups...
were made using one-way ANOVA, followed by the *t* test. *P* < 0.05 was considered significant.

**Results**
**SnoN Is Downregulated in Obstructive Nephropathy by a Mechanism Independent of Gene Transcription and mRNA Stability**

Figure 1A shows that SnoN was markedly downregulated in the obstructed kidney at 7 d after UUO, consistent with a previous report (20). To explore the potential mechanism by which mediates SnoN reduction, we examined the steady-state level of SnoN mRNA in the obstructed kidney at various time points after UUO. Surprisingly, SnoN mRNA abundance remained relatively constant throughout the entire experimental period, ranging from day 1 to 14 after UUO, as shown by Northern blot analysis (Figure 1B). For further confirmation of this observation, a semiquantitative reverse transcriptase–PCR approach was used to assess SnoN mRNA level. As shown in Figure 1, C and D, there was no significant alteration in the abundance of SnoN mRNA in the sham and obstructed kidneys at various time points after UUO. Therefore, it seems that the downregulation of SnoN expression in the fibrotic kidney is not caused primarily by an alteration in its gene transcription or mRNA stability; rather, it probably results from an enhanced protein degradation.

**Increased Ubiquitination and Degradation of SnoN in the Obstructed Kidney**

We then investigated the degradation of endogenous SnoN protein in the obstructed kidney after UUO. By using an *in vitro* degradation assay, we found an increased degradation of SnoN protein in the obstructed kidney, compared with the sham controls (Figure 2A). SnoN protein in the homogenates that

![Figure 2](http://example.com/figure2.png)

**Figure 2.** An enhanced ubiquitination and degradation of SnoN protein in the obstructed kidney. (A) Western blot analysis shows an enhanced degradation of SnoN protein in obstructive nephropathy. Whole-tissue homogenates were prepared from the sham and obstructed kidneys at 7 d after UUO. A pool of the homogenates from four mice per group was incubated with ubiquitination reaction mixture for various periods of time as indicated, followed by immunoblotting with antibodies against SnoN and GAPDH, respectively. A longer exposure time was used to detect SnoN protein in the samples from the obstructed kidney (right). (B) Quantitative determination of SnoN protein levels at various time points in different groups as indicated. Relative SnoN abundances are presented after comparison with the controls (100) in each group. (C) Blockade of SnoN protein degradation by the proteasome inhibitors. Whole-tissue homogenates were incubated with ubiquitination reaction mixture in the absence or presence of the proteasome inhibitors (0.25 mM MG132 and 0.25 mM MG115) at 37°C for 8 h. The reaction mixtures were immunoblotted with antibodies against SnoN and GAPDH, respectively. (D and E) Ubiquitination activity for exogenous SnoN is enhanced in the obstructed kidney. Exogenous SnoN protein was prepared by transfection of human kidney tubular epithelial cells (HKC) with hemagglutinin (HA)-tagged SnoN expression plasmid. (D) The immunoprecipitated HA-SnoN protein was incubated at 37°C for 1 h with kidney homogenates from different groups as indicated. The reaction mixtures were immunoblotted with anti-ubiquitin antibody. (E) Quantitative determination of the poly-ubiquitinated SnoN abundance shown in D.
were prepared from the obstructed kidneys at 7 d after UUO and degraded rapidly. After 16 h of incubation, >80% of SnoN protein in the homogenates vanished (Figure 2B). Under the same conditions, only 20% of SnoN protein in the homogenates that were prepared from the sham kidney disappeared (Figure 2). Moreover, treatment with proteasome inhibitors largely prevented the degradation of SnoN in the obstructed kidney (Figure 2C). These observations suggest an increased proteasome-mediated degradation of SnoN protein in the obstructed kidney.

To provide further evidence for the potential involvement of the ubiquitin-proteasome pathway in SnoN degradation, we examined the ubiquitination activity for exogenous SnoN protein in tissue homogenates from the sham and obstructed kidneys. To this end, HA-tagged exogenous SnoN protein was prepared by transient transfection of tubular epithelial HKC with pHA-SnoN expression vector, followed by immunoprecipitation with anti-HA antibody. The immunoprecipitated HA-SnoN protein was incubated with the kidney homogenates in an in vitro ubiquitination assay. As shown in Figure 2D, anti-ubiquitin antibody detected significant smeared bands of the poly-ubiquitinated SnoN protein in the reactions using the homogenates from the obstructed kidney (Figure 2D, lanes 2 through 4). However, much less smeared bands was observed in the samples from sham controls (Figure 2D, lane 1). Quantitative determination revealed a more than eight-fold induction of the polyubiquitinated SnoN abundance when the homogenates from the obstructed kidney at 7 d after UUO were used, compared with the sham controls (Figure 2E). These data suggest that ubiquitination activity for SnoN protein is enhanced in the obstructed kidney.

Smurf2 Is Induced and Physically Interacts with SnoN In Vivo

We found that Smurf2, an E3 ubiquitin ligase, was significantly induced in the obstructed kidney in a time-dependent manner (Figure 3A). Western blot analysis showed that Smurf2 induction took place in the obstructed kidneys at day 3, peaked at day 7, and was sustained at least to day 14 after UUO. Quantitative determination on renal Smurf2 abundance at various time points after UUO are presented in Figure 3B. Figure 3, C and D, exhibited the localization of Smurf2 protein in sham and obstructed kidneys at 7 d after UUO, respectively. Smurf2 staining was weak in normal kidney (Figure 3C). However, the staining for Smurf2 protein was intensified in the obstructed kidney at 7 d after UUO. Smurf2 was localized predominantly in the nuclei of renal tubular epithelial cells in the obstructed kidney. This pattern of cellular localization for Smurf2 is in harmony with the nuclear staining of SnoN (20). Of note, Smurf1 level was low

Figure 3. Smad ubiquitination regulatory factors 2 (Smurf2) is induced in the obstructed kidney. (A) Western blot analysis shows the induction of Smurf2 expression in the obstructed kidneys after UUO in a time-dependent manner. Whole-tissue homogenates from various groups as indicated were probed with antibodies against Smurf2 and GAPDH, respectively. Numbers 1, 2, 3, and 4 indicate each individual mouse in a given group. (B) Graphic representation of the relative abundance (fold induction over sham controls) of Smurf2 in different groups as indicated after normalization with GAPDH. Data are presented as mean ± SEM of four mice per group. *P < 0.001 versus sham controls. (C and D) Representative micrographs show the expression and localization of Smurf2 in the sham (C) and obstructed (D) kidneys. Smurf2 was localized mainly in the nuclei of renal tubular epithelial cells in the obstructed kidney at 7 d after UUO. Bar = 20 μm. (E) Smurf2 physically interacts with SnoN in the obstructed kidney. Whole-tissue homogenates were immunoprecipitated with specific antibody against Smurf2, followed by immunoblotting with anti-SnoN and anti-Smurf2 antibodies, respectively.
in the kidney, and it did not change significantly after UUO (data not shown).

We next investigated the potential interaction between Smurf2 and SnoN in the obstructed kidney. Physical association between Smurf2 and SnoN clearly was evident, as SnoN was readily detectable in the immunocomplexes that were precipitated by anti-Smurf2 antibody (Figure 3E). Whereas Smurf2 was increased in the obstructed kidney in a time-dependent manner, SnoN protein precipitated by Smurf2 remained relatively unchanged (Figure 3E). This suggests that SnoN protein perhaps is unstable and subjected to degradation upon binding to Smurf2.

**TGF-β1 Promotes SnoN Degradation In Vitro**

To investigate further the mechanism underlying SnoN degradation, we used renal tubular epithelial cells as an *in vitro* system. As demonstrated in Figure 4, in the absence of new protein synthesis after treatment with cycloheximide, SnoN abundance gradually reduced in a time-dependent manner. However, TGF-β1 markedly and rapidly promoted SnoN protein degradation in HKC. At 1 h after TGF-β1 treatment, SnoN level was reduced by >90%, compared with the controls. Under the identical conditions, approximately 60% of SnoN protein still was intact when the cells were incubated in the absence of TGF-β1.

To explore whether TGF-β1 promotes SnoN degradation via a ubiquitination- and proteasome-dependent pathway, we pre-treated the tubular epithelial cells with proteasome inhibitor. As shown in Figure 5A, proteasome inhibitor (MG132) was able to abolish the TGF-β1–mediated SnoN downregulation. Furthermore, TGF-β1 clearly induced the ubiquitination of SnoN protein. When the immunocomplexes that were precipitated with anti-SnoN antibody were probed with anti-ubiquitin, smeared bands of the poly-ubiquitinated SnoN were markedly increased in the presence of TGF-β1 (Figure 5B). These results suggest that TGF-β1 may be responsible for triggering or promoting SnoN degradation in kidney epithelial cells.

**SnoN Forms Complex with Ski, but Its Degradation Is Independent of Ski Abundance**

Because another Smad transcriptional co-repressor, Ski, also is downregulated in the fibrotic kidney (20), we sought to determine whether Ski interacts with SnoN in tubular epithelial cells and, if so, whether Ski abundance affects the stability of SnoN. Figure 6 shows that SnoN constitutively formed a complex with Ski at basal conditions, as Ski was detectable in the immunocomplexes that were precipitated by anti-SnoN antibody (Figure 6A, lane 7). Furthermore, the SnoN/Ski formed a ternary complex with activated Smad2/3 upon TGF-β1 stimulation (Figure 6A, lane 8).
Either SnoN or Ski alone also could form complex with activated Smad2/3 (Figure 6A, lanes 4 and 6).

Ectopic expression of Ski was found to have little effect on the stability of SnoN protein (Figure 6B). TGF-β1 was able to induce SnoN downregulation, regardless of the abundance of cellular SnoN protein (Figure 6B, lane 2 versus 4 and lane 6 versus 8). The degradation rate of SnoN essentially was identical in the HKC that were transfected with either Ski expression vector (pHA-Ski) or empty vector (pcDNA3; Figure 6, C and D). Therefore, loss of Ski is not the cause of decreased SnoN.

**SnoN Degradation Is Required for TGF-β1 to Exert Its Fibrogenic Action**

To understand the impact of SnoN degradation on TGF-β1 function, we examined the effects of blockade of SnoN degradation on TGF-β1-mediated fibrogenic response in renal epithelial cells. As shown in Figure 7, A and B, treatment with proteasome inhibitor MG132 completely abolished the α-SMA and fibronectin mRNA expression that was induced by TGF-β1 in HKC. Likewise, MG132 abrogated the TGF-β1-mediated fibronectin protein expression and its extracellular assembly in tubular epithelial cells (Figure 7, C through H).

To establish further the relevance of Smurf2 and SnoN regulation to TGF-β1 function, we knocked down Smurf2 expression in HKC by an siRNA strategy. As shown in Figure 8, A and B, knockdown of Smurf2 expression increased SnoN protein abundance in tubular epithelial cells. It is interesting that downregulation of Smurf2 significantly suppressed the TGF-β1-mediated gene transcription in a functional promoter (p3TP-Lux) reporter assay (Figure 8C). Similarly, knockdown of Smurf2 abolished the fibronectin induction in response to TGF-β1 stimulation in HKC (Figure 8, D and E). As reported previously (20), knockdown of SnoN enhanced the TGF-β1-mediated gene transcription and fibronectin induction. Together, it seems that SnoN degradation could be necessary for TGF-β1 to exert its fibrogenic action.

**Discussion**

Hyperactive TGF-β/Smad signaling is widely considered as a major pathogenic mediator in the genesis and progression of chronic renal fibrosis after various injuries. Whereas extensive studies are focused on the induction and activation of the positive components of this signal pathway in the diseased kidney, we recently demonstrated a progressive downregulation of Smad transcriptional co-repressors in the fibrotic kidney after UUO. This suggests that the loss of the negative controlling mechanism may be equally important in amplifying the fibrogenic Smad signaling. This study was carried out to address specifically the mechanism underlying SnoN suppression under pathologic conditions. Our results indicate that the downregulation of SnoN is mediated primarily by an enhanced ubiquitination/proteasome-dependent degradation, rather than by a reduced gene transcription. Functionally, SnoN deg-
radiation may be a prerequisite for TGF-β to exert its fibrogenic action, because preservation of SnoN by proteasome inhibitor blocks TGF-β1–mediated α-SMA and fibronectin expression. Furthermore, stabilization of SnoN by knockdown of Smurf2 leads to an inhibition of the TGF-β1–mediated gene transcription. Therefore, these findings establish that dysregulation of ubiquitination and proteasomal degradation pathway may be critical in rendering an uncontrolled TGF-β1–mediated fibrogenic action. Nevertheless, an increased ubiquitin-proteasomal degradation activity against SnoN is found in the obstructed kidney. Third, Smurf2, a specific E3 ubiquitin ligase that is implicated in the degradation of Smad signaling components (31,32), is induced in the obstructed kidney. Finally, TGF-β1, a fibrogenic cytokine that is markedly upregulated in the fibrotic kidney (1,2), triggers and/or promotes SnoN degradation in tubular epithelial cells, and SnoN degradation is a prerequisite for TGF-β1–mediated fibrogenic action. Nevertheless, at this stage, we cannot completely rule out the possibility that a decreased SnoN protein translation also may play a role in mediating the SnoN protein reduction in the obstructed kidney.

Protein degradation is a tightly regulated event that is essential for controlling many cellular processes. Among several proteolytic systems documented, the ubiquitin-proteasomal pathway is a complex one that requires energy and consists of a highly organized cascade of enzymatic reactions that select, tag, and execute the orderly destruction of a wide variety of physiologically important proteins (33–35). Not surprising, defects in the ubiquitin-proteasomal proteolytic pathway that render protein substrates more or less susceptible to degradation are linked to many devastating disorders, ranging from
cancer to neurodegenerative diseases and kidney failure (33–35). It has been shown that components of the Smad signaling pathway including SnoN are subjected to ubiquitin-proteasome–mediated degradation under both physiologic and pathologic circumstances (18,32,36,37). The finding that an increased ubiquitination and proteasomal degradation is directed against SnoN in the obstructed kidney suggests that SnoN is targeted specifically for ubiquitin-dependent destruction in vivo in the fibrotic state. These observations, together with previous reports on Smad2 and Smad7 degradation (25,26), suggest a general theme that alteration in the ubiquitin-proteasomal degradation of key signaling regulators may result in disturbance of vital cellular signaling, thereby leading to kidney cell malfunction and contributing to the pathogenesis of CKD.

Covalent attachment of ubiquitin to target proteins that are destined for degradation is initiated by several sequential steps that are controlled by the activity of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and a specific E3 ubiquitin ligase. It is commonly believed that the E3 ubiquitin ligase plays a decisive role in defining the substrate selectivity and the subsequent degradation by the 26S proteasomes (33,34). In this regard, the observation that Smurf2, an E3 ubiquitin ligase, is induced in the obstructed kidney provides significant insights into the mechanism of SnoN degradation. Smurf2 belongs to a family of E3 ligases that contain the so-called HECT domain that is homologous to the E6-AP COOH terminus (31,33). Unlike its functional cousin, Smurf1, that primarily targets bone morphogenetic protein signaling such as Smad1, Smad5, and Smad8, Smurf2 selectively targets Smad2 but not Smad3 for degradation (25,31,36,38). Smurf2 also interacts with Smad7 in the nucleus and induces the nuclear export of Smad7 (32,37), which in turn targets TGF-β type I receptor and enhances its degradation. In this study, we have shown that Smurf2 is localized in the nucleus and physically associates with SnoN, strongly suggesting that Smurf2 is a ubiquitin E3 ligase that targets nuclear SnoN for proteasome-dependent degradation. In accordance with this, the upregulation of Smurf2 in the nucleus of kidney cells is closely correlated with

Figure 8. Knockdown of Smurf2 expression inhibits TGF-β1–mediated gene transcription and fibronectin expression in tubular epithelial cells. (A and B) Knockdown of Smurf2 expression stabilizes the expression of SnoN. HKC were transiently transfected with control small interfering RNA (siRNA) or Smurf2 siRNA (120 nM). (A) Representative Western blots showed the expression levels of Smurf2, SnoN, and α-tubulin at 72 h after transfection. (B) Graphic representation of the relative abundance of Smurf2 and SnoN protein levels after normalization with α-tubulin. Data relative to the controls (1.0) are presented as mean ± SEM of three experiments. *P < 0.01, **P < 0.001 versus control siRNA group. (C) Knockdown of Smurf2 expression inhibits the TGF-β1–mediated gene transcription. HKC were transiently co-transfected with p3TP-Lux luciferase reporter construct and control siRNA or Smurf2 siRNA, followed by incubation without or with 2 ng/ml TGF-β1 for 48 h. Relative luciferase activities (arbitrary unit) were calculated after normalization of transfection efficiency and are presented as mean ± SEM of six experiments. **P < 0.001 versus control siRNA group in the absence of TGF-β1; ††P = 0.001 versus control siRNA group in the presence of TGF-β1. (D) Inhibition of Smurf2 expression blocks TGF-β1–mediated fibronectin expression in tubular epithelial cells. HKC were transiently transfected 120 nM control siRNA or Smurf2 siRNA for 24 h and then treated with TGF-β1 for an additional 48 h. Whole-cell lysates were immunoblotted with antibodies against fibronectin and GAPDH, respectively. (E) Immunofluorescence staining shows the fibronectin expression and deposition after various treatments as indicated. Bar = 10 μm.
reduction of SnoN after UUO (20). However, it remains to be determined whether other E3 ubiquitin ligases, such as the anaphase-promoting complex (39,40), also may be implicated in the ubiquitination and subsequent proteasome-dependent degradation of SnoN in the obstructed kidney.

Studies from an in vitro model system suggest that TGF-β1 can trigger and/or promote SnoN degradation in tubular epithelial cells (Figures 4 and 5). This is consistent with earlier reports indicating that activated Smad3 plays a crucial role in SnoN degradation in other types of cells (28,39). TGF-β1 also is shown to induce Smurf2 gene expression (41). Because TGF-β1 is induced markedly and progressively in virtually every type of CKD (1,2,8), it is plausible that TGF-β1 also plays a critical role in SnoN degradation in vivo. This raises an interesting possibility that TGF-β1 may modulate Smad signaling via dual mechanisms, with activating positive Smad signaling on the one hand and targeting Smad antagonist SnoN for ubiquitin-dependent degradation on the other hand.

The results presented herein may suggest new strategies in designing future therapeutics for CKD, in which hyperactive Smad signaling is a causative factor for the evolution of tissue scarring. It seems conceivable that preservation of SnoN by inhibiting its degradation may be a novel approach for targeting hyperactive Smad signaling in renal fibrosis. This view evidently is supported by the observation that blockade of SnoN degradation either by proteasome inhibitor or by knockdown of Smurf2 effectively prevents TGF-β1–mediated fibrogenic response. Further studies clearly are warranted to evaluate the applicability and the efficacy of this strategy in experimental animal models for the prevention and treatment of chronic renal insufficiency.

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