A Novel Mechanism by which Hepatocyte Growth Factor Blocks Tubular Epithelial to Mesenchymal Transition

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Hepatocyte growth factor (HGF) is a potent antifibrotic cytokine that blocks tubular epithelial to mesenchymal transition (EMT) induced by TGF-β1. However, the underlying mechanism remains largely unknown. This study investigated the signaling events that lead to HGF blockade of the TGF-β1-initiated EMT. Incubation of human kidney epithelial cells HKC with HGF only marginally affected the expression of TGF-β1 and its type I and type II receptors, suggesting that disruption of TGF-β1 signaling likely plays a critical role in mediating HGF inhibition of TGF-β1 action. However, HGF neither affected TGF-β1-induced Smad-2 phosphorylation and its subsequent nuclear translocation nor influenced the expression of inhibitory Smad-6 and -7 in tubular epithelial cells. HGF specifically induced the expression of Smad transcriptional co-repressor SnoN but not Ski and TG-interacting factor at both mRNA and protein levels in HKC cells. SnoN physically interacted with transcriptional events that control HGF blockade of tubular epithelial cells. HGF did not affect Smad-2 activation and its nuclear accumulation in tubular epithelium, but it restored SnoN protein abundance in the fibrotic kidney in obstructive nephropathy. Hence, HGF blocks EMT by antagonizing TGF-β1’s action via upregulating Smad transcriptional co-repressor SnoN expression. These findings not only identify a novel mode of interaction between the signals activated by HGF receptor tyrosine kinase and TGF-β receptor serine/threonine kinases but also illustrate the feasibility of confining Smad activity as an effective strategy for blocking renal fibrosis.


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indicate that HGF fails to block Smad activation and nuclear translocation. Rather, HGF specifically upregulates Smad transcriptional co-repressor SnoN expression. Such SnoN induction leads to the formation of transcriptionally inactive SnoN/Smad complex and blocks TGF-β1’s action. These findings define a novel molecular pathway by which HGF antagonizes profibrotic TGF-β1 signaling.

Materials and Methods

Cell Culture, Cytokine Treatment, and Transient Transfection

Human proximal tubular epithelial cells (HKC) were provided by Dr. L. Racusen (Johns Hopkins University, Baltimore, Maryland) (27). Cell culture and cytokine treatments were carried out according to the procedures described previously (7,28). Briefly, HKC cells were seeded in complete medium that contained 10% FBS at approximately 70% confluence. After an overnight incubation, cells were continuously incubated in serum-free medium for another 24 h, before addition of cytokines. Recombinant human TGF-β1 was purchased from R & D Systems (Minneapolis, MN). Recombinant human HGF protein was provided by Genentech Inc. (South San Francisco, CA). Chemical inhibitors PD98059, wortmannin, and SC68376 were purchased from Calbiochem (La Jolla, CA). Transient transfection of HKC cells was carried out by using the Lipofectamine 2000 according to the instructions specified by the manufacturer (Invitrogen, Carlsbad, CA). After transfection with equal amounts of HA-tagged SnoN expression plasmid (29) (provided by Dr. R. Weinberg, Massachusetts Institute of Technology, Cambridge, Massachusetts) or empty vector pcDNA3 (Invitrogen), HKC cells were incubated in the absence or presence of 2 ng/ml TGF-β1 for 2 d. Whole-cell lysates were prepared and subjected to Western blot analyses. The stable cell lines overexpressing Smad-7 (HKCpSmad7) and mock-transfection control (HKCpDNA3) were established as described previously (30).

Northern Blot Analysis

Total RNA isolation and Northern blot analysis for gene expression were carried out by the procedures described previously (31). The cDNA probe for TGF-β1 was obtained from American Type Culture collection (ATCC, Manassas, VA). The SnoN cDNA probe was provided by R. Weinberg. After autoradiography, membranes were stripped and rehybridized with rat glyceraldehyde-3-phosphate dehydrogenase probe to ensure equal loading of each lane.

Western Blot Analysis

The preparation of whole-cell lysates and kidney tissue homogenates and Western blot analysis of protein expression were performed according to the procedures described previously (7). The primary antibodies were obtained from following sources: anti-α-smooth muscle actin (anti-α-SMA) and anti-extracellular signal-regulated kinase-1 and -2 (anti-Erk-1/2; Sigma, St. Louis, MO); anti-phospho-specific Erk-1/2 (phospho-specific and total p38 mitogen-activated protein kinase (MAPK), and phospho-specific and total c-Jun N-terminal kinase (Cell Signaling Technology, Inc., Beverly, MA); anti-phospho-specific and total Smad-2 (Upstate, Charlottesville, VA); anti-TGF-βI (sc-146), anti-TGF-β II type I receptor (anti-TβRI; sc-398), anti-TGF-β type II receptor (anti-TβRII; sc-17792), anti-Smad-7 (sc-7004), anti-Smad-6 (sc-13048), anti-Smad-4 (sc-7666), anti-Smad-2/3 (sc-6032), anti-Sp1 (sc-420), anti-c-Ski (sc-9140), anti-SnoN (sc-5995), anti-TGF (sc-9826), and anti-actin (sc-1616). Santa Cruz Biotechnology, Inc., Santa Cruz, CA; and anti-fibronectin (clone 10) and anti-E-cadherin (clone 36). BD Biosciences Pharmingen, San Jose, CA). Affinity-purified secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Immunofluorescence Staining

Indirect immunofluorescence staining was performed using an established procedure (7). Cells were incubated with the specific primary antibodies described above, followed by staining with FITC- or cyanine dye Cy3-conjugated secondary antibodies. For some samples, cells were double stained with 4’,6-diamidino-2-phenylindole, HCl to visualize the nuclei. For immunostaining of kidney sections, cryosections were stained with anti-Smad-2/3 and anti-SnoN antibodies using the Vector M.O.M. immunodetection kit by the protocol specified by the manufacturer (Vector Laboratories, Burlingame, CA). The slides were then stained for the proximal tubular marker with fluorescein-conjugated lectin from Tetragonolobus purpureus (Sigma). 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.

Nuclear Protein Preparation

HKC cells were subjected to various treatments with various growth factors for 30 min except where otherwise indicated. Cell nuclei were isolated by procedures described previously (32). After being collected by centrifugation at 5000 × g for 30 min at 4°C, the nuclei were lysed with SDS sample buffer and subjected to Western blot analysis.

Immunoprecipitation

Immunoprecipitation was carried out by a procedure described elsewhere (32). HKC cell lysates were immunoprecipitated overnight at 4°C with 1 µg of anti-Smad-2/3, followed by precipitation with 20 µl of protein A/G Plus-Agarose for 3 h at 4°C. After being washed, the immunoprecipitates were boiled for 5 min in SDS sample buffer, followed by immunoblotting with specific antibodies.

Animals

Male CD-1 mice that weighed approximately 18 to 22 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Unilateral ureteral obstruction (UUO) was performed using an established procedure (6,7). Human HGF expression plasmid (pCMV-HGF) and the empty vector (pcDNA3) were administered to mice by rapid injection of naked plasmid solution through the tail vein, as described previously (6). Mice received an injection of plasmids before (day 1) and after (day 3) UUO, respectively. Groups of mice (n = 5) were killed at 7 d after UUO, and the kidneys were removed. The therapeutic effects of HGF gene delivery in this mouse model were confirmed and reported previously (6).

Statistical Analyses

Statistical analysis of the data was performed using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by Student-Newman-Kuels test. P < 0.05 was considered significant.

Results

Regulation of TGF-β1 and Its Receptors by HGF in Tubular Epithelial Cells

To gain insights into the mechanism underlying HGF blockade of TGF-β1-initiated tubular EMT, we first investigated the effect of HGF on TGF-β1 expression in tubular epithelial cells. As shown in Figure 1A, HGF did not significantly affect TGF-β1 mRNA expression at 1, 6, and 12 h after treatment in HKC cells. At 24 h, moderate inhibition of TGF-β1 mRNA level was noticeable, but it returned to the control level at 48 h. The
The size and authenticity of TGF-β with HGF (20 ng/ml), TGF-β did not alter the abundance of active TGF-β in tubular epithelial cells. Whole-cell lysates derived from the HKC cells that were treated with HGF did not inhibit the abundance of active TGF-β as measured by a specific ELISA, (Figure 1B).

Western blot analysis. In fact, a trivial increase in TGF-β protein abundance at 24 and 48 h after incubation (Figure 1B).

We further examined the potential effect of HGF on TGF-β receptor expression in tubular epithelial cells. As demonstrated in Figure 1B, treatment of HKC cells with HGF did not inhibit the abundance of TβRI and TβRII protein, as illustrated by Western blot analysis. In a fact, a trivial increase in TβRII level was noticed in HKC cells after stimulation with either HGF or TGF-β. However, it returned to the control level when the cells were simultaneously incubated with both HGF and TGF-β (Figure 1B).

level of active TGF-β1 in the supernatant of HKC cells was extremely low or undetectable as measured by a specific ELISA, and HGF did not alter the abundance of active TGF-β1 secreted by HKC cells (data not shown). Furthermore, Western blot of whole-cell lysates revealed that HGF did not affect TGF-β1 protein abundance at 24 and 48 h after incubation (Figure 1B).

We further examined whether HGF disturbs the TGF-β1–induced Smad-2 phosphorylation and activation. However, preincubation of the cells with HGF did not affect TGF-β1–induced Smad-2 phosphorylation in HKC cells (30). Hence, it seems likely that TGF-β1–induced EMT is dependent on intracellular Smad signaling.

HGF Neither Blocks Smad-2 Phosphorylation and Its Association with Smad-4 and Subsequent Nuclear Translocation nor Influences Inhibitory Smads Expression

Having established that Smad signaling is crucial in mediating TGF-β1–induced EMT, we reasoned that HGF may block EMT by impairing Smad signal transduction. To test this hypothesis, we examined the effect of HGF on major Smad signaling events in a step-wise manner. As shown in Figure 3A, treatment of HKC cells with TGF-β1 induced Smad-2 phosphorylation and activation. However, preincubation of the cells with HGF did not affect TGF-β1–induced Smad-2 phosphorylation (Figure 3A).

We next examined whether HGF disturbs the TGF-β1–induced association between Smad-2/3 and Smad-4 by immunoprecipitation. As shown in Figure 3B, after stimulation with TGF-β1, Smad-4 was clearly present in the complexes precipitated by Smad-2/3 antibody, indicating that TGF-β1 induces Smad-2/3–Smad-4 complex formation in HKC cells. However, HGF also failed to disrupt the physical association between Smad-2/3 and Smad-4 induced by TGF-β1 (Figure 3B).

We further investigated whether HGF blocks activated Smad-2 nuclear translocation, because this process has been...
shown to be tightly regulated by HGF in renal interstitial fibroblasts (33). As presented in Figure 3C, HGF did not block activated Smad-2 nuclear translocation in tubular epithelial cells, as the nuclear abundance of phospho-Smad-2 was virtually identical in HKC cells that were treated either with TGF-β1 alone or with TGF-β1 plus HGF. To confirm this result further, we used an indirect immunofluorescence staining to visualize the nuclear accumulation of activated Smad-2 in HKC cells after various treatments. Consistent with the biochemical assay, treatment of HKC cells with TGF-β1 induced marked accumulation of phospho-Smad-2 in the nuclei (Figure 3E), and HGF apparently had no effect on this TGF-β1–initiated Smad-2 nuclear translocation (Figure 3G). Collectively, this series of experiments demonstrates that HGF blocks TGF-β1–mediated EMT by a mechanism independent of disruption of Smad-2 phosphorylation, its association with Smad-4, and its nuclear translocation.

Because overexpression of inhibitory Smad-7 completely abolished TGF-β1–induced tubular EMT (Figure 2), we reasoned that HGF may antagonize TGF-β1’s action by upregulating inhibitory Smads expression. As shown in Figure 4, HGF also did not influence the protein expression of both Smad-6 and -7, two inhibitory members of the Smad protein family, at different time points in HKC cells. In addition, HGF exhibited no effects on common Smad-4 protein expression (Figure 4).

**HGF Specifically Induces Smad Transcriptional Co-repressor SnoN Expression**

We next analyzed the expression of Smad transcriptional co-repressors in HKC cells after treatment with HGF. As shown in Figure 5, HGF dramatically induced SnoN protein expression. This induction started as early as 1 h and peaked at 6 h after HGF incubation (Figure 5). Under the same conditions, HGF had little effect on the expression of Ski, a Smad transcriptional co-repressor that shares a high degree of homology with SnoN. The expression of TGIF, another Smad co-repressor that is upregulated by HGF through protein stabilization in mesangial cells (34), was extremely low or undetectable under both basal and HGF-stimulated conditions in HKC cells.

Figure 5 also shows the localization of SnoN in tubular epithelial cells after HGF treatment. In control HKC cells, immunofluorescence staining for SnoN protein was essentially negative. However, treatment with HGF dramatically induced SnoN protein expression, which was exclusively localized in
the nuclei. To study whether SnoN protein induction by HGF is due to an enhanced protein stability or increased gene expression, we examined the SnoN mRNA levels after HGF treatment in HKC cells by Northern blot analysis. As illustrated in Figure 5G, four transcripts of SnoN with the sizes of 6.2, 4.4, 3.2, and 2.1 kb were detected in tubular epithelial cells. These transcripts represent differential splicing variants (35). HGF markedly increased the steady-state mRNA levels of SnoN in HKC cells. Such induction began as early as 0.5 h and peaked at 1 h after HGF treatment, a kinetics that significantly precedes SnoN protein induction. Hence, SnoN protein induction by HGF is primarily mediated by an increased gene expression in tubular epithelial cells.

**SnoN Forms Complex with Activated Smad-2 and Overrides the Profibrotic Action of TGF-β1**

To understand how increased SnoN blocks TGF-β1’s action in tubular epithelial cells, we investigated whether SnoN can physically interact with activated Smad-2 once it has been translocated to the nuclei after TGF-β1 stimulation. As shown in Figure 6, in HKC cells that were treated with TGF-β1, phosphorylated Smad-2 (green) was translocated to the nuclei (Figure 6B). After treatment with HGF alone, SnoN (red) was induced and localized in the nuclei (Figure 6C). When HKC cells were treated with TGF-β1 plus HGF, both phosphorylated Smad-2 (green) and SnoN (red) were co-localized in the nuclei (yellow; Figure 6D). To reveal a potential interaction between activated Smad-2 and SnoN, we investigated Smad/SnoN complex formation in tubular epithelial cells by co-immunoprecipitation. As shown in Figure 6E, when cell lysates were precipitated with anti-SnoN antibody, phospho-Smad-2 was detectable in the precipitates by Western blotting. There was a profound increase in the association between Smad-2 and SnoN in the cells that were treated with TGF-β1 plus HGF, compared with TGF-β1 or HGF treatment alone. This suggests that HGF-induced SnoN physically binds to the activated Smad-2 in a TGF-β1-dependent manner, which presumably sequesters activated Smad-2 from transactivation of its target genes.

Analysis of the whole-cell lysates revealed that TGF-β1 by itself did not induce SnoN expression in HKC cells (Figure 6F). In addition, TGF-β1 did not affect HGF-induced SnoN expression. Identical magnitude of SnoN induction by HGF was observed in the absence or presence TGF-β1 (Figure 6F).

To provide direct evidence for SnoN in mediating HGF inhibition of TGF-β1 action, we ectopically transfected SnoN expression vector into tubular epithelial cells, followed by as-
sessing their responsiveness to TGF-β1 stimulation. As shown in Figure 6G, forced expression of exogenous SnoN dramatically blocked TGF-β1-induced E-cadherin suppression and α-SMA induction in a dose-dependent manner. However, transfection of empty vector in an identical manner did not affect TGF-β1-initiated tubular EMT. Similar results were obtained by using an indirect immunofluorescence staining approach (Figure 6, H through M). These results indicate that SnoN can effectively override the profibrotic action of TGF-β1.

**HGF Does not Affect Smad-2 Phosphorylation and Nuclear Accumulation in Tubular Epithelium In Vivo**

Earlier studies showed that administration of either HGF protein or its gene prevents tubular EMT and renal interstitial fibrosis induced by UUO (7). To investigate the potential mechanism underlying HGF’s beneficial effect in the fibrotic kidney, we studied the effect of HGF on Smad signaling in vivo. As shown in Figure 7, there was a dramatic activation of Smad-2, as illustrated by a marked increase in phospho-Smad-2 in the homogenates of obstructed kidney, suggesting an active TGF-β1 signaling in diseased kidneys. However, despite HGF’s preventing renal EMT and fibrosis in this model, delivery of HGF gene did not block Smad-2 phosphorylation and activation. Figure 7B shows quantitative results derived from five animals per group. This finding is consistent with in vitro data (Figure 3), suggesting that HGF blockade of renal fibrosis in vivo is not mediated by inhibiting Smad activation.

We further investigated Smad localization in normal and diseased kidney by an indirect immunofluorescence staining. As demonstrated in Figure 7C, there was abundant Smad-2/3 staining in renal tubular epithelium of sham-operated kidney, when using specific antibody against total Smad-2/3. However, Smads were apparently localized in the cytoplasm of tubular epithelial cells under normal conditions. In the obstructed kidney at 7 d after UUO, Smad-2/3 were predominantly localized in the nuclei of renal tubular epithelial cells, suggesting that Smads were activated and translocated into the nuclei. Administration of HGF did not prevent Smads activation and their nuclear accumulation in the fibrotic kidney (Figure 7E).

**HGF Restores SnoN Expression in the Fibrotic Kidney**

In view of the induction of SnoN by HGF in tubular epithelial cells in vitro, we examined the SnoN expression in the fibrotic kidney in vivo. Figure 8 shows SnoN protein expression in the
kidney of various treatment groups. In sham-operated kidney, abundant SnoN protein expression was observed by Western blot analysis of total tissue homogenates (Figure 8A). Immuno-fluorescence staining displayed that SnoN was primarily localized in the nuclei of tubular epithelium. After obstructive injury for 7 d, intrarenal SnoN protein was dramatically decreased, as described previously (26).

After administration of HGF gene, SnoN expression was markedly induced in the obstructed kidney when compared with the pcDNA3 controls (Figure 8). Whole-cell lysates derived from various groups as indicated were immunoblotted with antibodies against phospho-Smad-2 and SnoN, respectively. (F) Whole-cell lysates derived from various groups as indicated were immunoblotted with antibodies against Smad-2, SnoN, and actin, respectively. (G) Ectopic expression of SnoN overrides the profibrotic action of TGF-β1 in tubular epithelial cells. HKC cells were transiently transfected with increasing amounts of HA-tagged SnoN expression vector or empty pcDNA3 as indicated (µg/well) and followed by incubation in the absence or presence of 2 ng/ml TGF-β1 for 2 d. Whole-cell lysates were immunoblotted with antibodies against HA, E-cadherin, α-SMA, and actin, respectively. (H through M) Immunofluorescence staining demonstrates the effects of SnoN overexpression on TGF-β1–mediated E-cadherin suppression and α-SMA induction. HKC cells were transfected with either pcDNA3 (I and L) or HA-tagged SnoN expression vector (J and M; 5 µg/well), followed by incubation with 2 ng/ml TGF-β1 for 2 d. Cells were immunostained with antibodies against E-cadherin (H through J) or α-SMA (K through M), respectively. (H and K) Control HKC cells.

Discussion

Earlier studies have shown that HGF completely blocks TGF-β1–mediated tubular EMT (7), a phenotypic conversion that is thought to play an imperative role in the generation of the matrix-producing myofibroblasts (36,37). However, how exactly HGF antagonizes the TGF-β1 action in tubular epithelial cells remained unsolved. Thus, the aim of this study was to dissect the signaling events that lead to HGF blockade of TGF-β1 action. We demonstrated that HGF blocks TGF-β1–initiated EMT apparently by a mechanism independent of Smads phosphorylation and their subsequent nuclear translocation. Instead, HGF specifically induces Smad transcriptional co-repressor SnoN expression, which in turn binds to the activated Smads and consequently sequesters their ability to initiate gene transcription in tubular epithelial cells. These actions of HGF are largely recapitulated in the fibrotic kidney induced by obstructive injury in vivo. Therefore, our findings have uncovered a novel molecular pathway by which HGF specifically overrides the profibrotic action of TGF-β1.

HGF is a potent antifibrotic factor that prevents the onset and progression of chronic renal disease in a variety of animal models (6,8,14). Although the precise mechanism by which HGF inhibits tissue fibrosis is not fully understood, its interplay with TGF-β1 has been suspected to play a crucial role in mediating its antifibrotic action (7,13). TGF-β1 is shown to inhibit HGF expression in various types of cells, including renal mesangial and endothelial cells (38). However, whether HGF reciprocally suppress TGF-β1 expression remains largely elusive. In

Figure 6. SnoN binds to activated Smad-2 and overrides the profibrotic action of TGF-β1 in tubular epithelial cells. (A through D) Double immunofluorescence staining for phospho-specific Smad-2 (green) and SnoN (red) in HKC cells after various treatments for 24 h. (A) Control. (B) TGF-β1 (2 ng/ml). (C) HGF (50 ng/ml). (D) TGF-β1 plus HGF. (E) Co-immunoprecipitation demonstrates that HGF induces an association between SnoN and activated Smad-2 in tubular epithelial cells. Cell lysates derived from various groups as indicated were immunoprecipitated with anti-SnoN antibody, followed by immunoblotting with antibodies against phospho-Smad-2 and SnoN, respectively. (F) Whole-cell lysates derived from various groups as indicated were immunoblotted with antibodies against Smad-2, SnoN, and actin, respectively. (G) Ectopic expression of SnoN overrides the profibrotic action of TGF-β1 in tubular epithelial cells. HKC cells were transiently transfected with increasing amounts of HA-tagged SnoN expression vector or empty pcDNA3 as indicated (µg/well) and followed by incubation in the absence or presence of 2 ng/ml TGF-β1 for 2 d. Whole-cell lysates were immunoblotted with antibodies against HA, E-cadherin, α-SMA, and actin, respectively. (H through M) Immunofluorescence staining demonstrates the effects of SnoN overexpression on TGF-β1–mediated E-cadherin suppression and α-SMA induction. HKC cells were transfected with either pcDNA3 (I and L) or HA-tagged SnoN expression vector (J and M; 5 µg/well), followed by incubation with 2 ng/ml TGF-β1 for 2 d. Cells were immunostained with antibodies against E-cadherin (H through J) or α-SMA (K through M), respectively. (H and K) Control HKC cells.
vivo studies seem to suggest that HGF may inhibit TGF-\(\beta\)1 expression, because administration of HGF reduces TGF-\(\beta\)1 abundance in the fibrotic kidneys (7,13). However, the cause–effect relationship of this TGF-\(\beta\)1 reduction in fibrotic kidney remains ambiguous. The present study indicates that HGF only marginally affects TGF-\(\beta\)1 and its receptor expression in tubular epithelial cells, consistent with a recent report indicating that HGF fails to modulate TGF-\(\beta\)1 expression in mesangial cells at basal condition (10). Such a trivial effect of HGF on TGF-\(\beta\)1 and its receptor expression in opposite manners (Figure 1) hardly accounts for its dramatic antifibrotic action. Hence, the primary mechanism for mediating HGF activity may operate through blocking hyperactive TGF-\(\beta\)1 signaling.

Because TGF-\(\beta\)1 could induce itself and its receptor expression (39), it is conceivable that HGF blockade of TGF-\(\beta\)1 signaling would indirectly lead to an inhibition of TGF-\(\beta\)1 induction by uncoupling the TGF-\(\beta\)1–positive feedback loop under pathologic settings. In this context, we interpret the reduction of TGF-\(\beta\)1 in the diseased kidney after HGF treatment as a consequence associated with reduced fibrotic lesions.

HGF and TGF-\(\beta\)1 transduce their signals by distinct mechanisms. One would expect a cross-talk in the signaling circuits mediated by HGF receptor tyrosine kinase and TGF-\(\beta\) receptor serine-threonine kinases, to make it possible for HGF to counteract the profibrotic action of TGF-\(\beta\)1. Such cross-talk could theoretically take place at any point during the cascade of TGF-\(\beta\)1/Smad signaling events (33,40). There are at least three levels of negatively controlling mechanisms that confine TGF-\(\beta\)1 signaling along the Smad signal transduction pathway. In the extracellular compartment, active TGF-\(\beta\)1 can bind to decorin, a proteoglycan associated with the extracellular matrix, which leads to negative regulation of TGF-\(\beta\)1 activity (41). This provides a means of constraining TGF-\(\beta\)1 activity at the prereceptor stage. Smad signaling can also be negatively controlled by inhibitory Smad-7 and -6, which act as functional decoys that compete with the receptor-regulated Smads (R-Smads) for binding to the activated type I receptor, leading to an attenuated R-Smad phosphorylation and activation (17,22). This level of regulation takes place in the cytoplasm. Once inside the nuclei, Smad signaling is finally subjected to a third level of negative regulation by Smad transcriptional co-repressors. It is intriguing that HGF seems not to interfere with Smad signaling in tubular epithelial cells until the assembly of Smad transcriptional complexes. Major TGF-\(\beta\)1 signaling events are still operative upon HGF treatment (Figure 3). By upregulating transcriptional antagonist SnoN, HGF receptor signaling leads to sequestering the ability of R-Smads to initiate the transactivation of TGF-\(\beta\)-target genes. These findings underscore that HGF is capable of precisely targeting a final, decisive step in Smad signaling before the transcription of TGF-\(\beta\)-responsive genes. Our observations also establish a novel mode of interactions between the signals activated by HGF receptor tyrosine kinase and TGF-\(\beta\) receptor serine-threonine kinases ligands.

SnoN is abundantly expressed and predominantly localized in the nuclei in normal tubular epithelium (Figure 8) (26).
Under physiologic conditions, a high level of SnoN would be important in preventing the activation of transcription by Smad proteins that may find their way into the nuclei (24). In this respect, SnoN functions as a crucial gatekeeper that tightly constrains Smad signaling under normal conditions. Consistently, knockdown of SnoN by siRNA strategy dramatically sensitizes the tubular epithelial cells to respond to TGF-β1 stimulation (26). However, this confining mechanism seems not to operate in the fibrotic kidney, because SnoN protein abundance is significantly reduced (26). Consequently, Smad signal would be transduced without any restraint after TGF-β1 stimulation, leading to an amplified transcriptional activation of TGF-β1–responsive genes.

The novel finding that HGF specifically upregulates SnoN expression provides a mechanistic insight into understanding the interplay between antifibrotic HGF and profibrotic TGF-β1 signaling. It becomes clear that the opposite effects of HGF and TGF-β1 in regulating myofibrolastic activation and tissue fibrosis are coupled by SnoN expression. By inducing SnoN and restoring its level in the fibrotic kidney, HGF reinstates SnoN transcriptional repressor activity, leading to blockade of tubular EMT and renal fibrosis. Notably, administration of HGF could induce SnoN expression and block renal fibrosis even in the presence of Smad-2 phosphorylation and nuclear accumulation (Figure 7). This observation is significant in that it suggests that the levels of SnoN within the cell but not activated Smad determine an outcome of TGF-β1 response in vivo. In accordance with this, delayed administration of HGF, when the injury was already established, is still effective in reversing EMT and blocking renal fibrosis (9,10,15).

In summary, the present study identifies a unique mode of interaction between the signals activated by HGF and TGF-β1 in tubular epithelial cells. It is conceivable that HGF can precisely target the hyperactive Smad signaling through inducing Smad transcriptional antagonist SnoN expression. Such specific interception of Smad signaling by HGF may eventually override the profibrotic action of TGF-β1 in the diseased kidneys, thereby halting the progression of chronic renal fibrosis.

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References

4. Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Mor-

