Targeted Inhibition of β-Catenin/CBP Signaling Ameliorates Renal Interstitial Fibrosis

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ABSTRACT

Because fibrotic kidneys exhibit aberrant activation of β-catenin signaling, this pathway may be a potential target for antifibrotic therapy. In this study, we examined the effects of β-catenin activation on tubular epithelial-mesenchymal transition (EMT) in vitro and evaluated the therapeutic efficacy of the peptidomimetic small molecule ICG-001, which specifically disrupts β-catenin-mediated gene transcription, in obstructive nephropathy. In vitro, ectopic expression of stabilized β-catenin in tubular epithelial (HKC-8) cells suppressed E-cadherin and induced Snail1, fibronectin, and plasminogen activator inhibitor-1 (PAI-1) expression. ICG-001 suppressed β-catenin-driven gene transcription in a dose-dependent manner and abolished TGF-β1-induced expression of Snail1, PAI-1, collagen I, fibronectin, and α-smooth muscle actin (α-SMA). This antifibrotic effect of ICG-001 did not involve disruption of Smad signaling. In the unilateral ureteral obstruction model, ICG-001 ameliorated renal interstitial fibrosis and suppressed renal expression of fibronectin, collagen I, collagen III, α-SMA, PAI-1, fibroblast-specific protein-1, Snail1, and Snail2. Late administration of ICG-001 also effectively attenuated fibrotic lesions in obstructive nephropathy. In conclusion, inhibiting β-catenin signaling may be an effective approach to the treatment of fibrotic kidney diseases.


It is estimated that up to 13% of the adult population has some degree of chronic kidney disease (CKD), and the prevalence of patients with ESRD is increasing worldwide.1,2 Initiated by insults of diverse etiologies, CKD progressing to ESRD is a remarkably monotonous process characterized by relentless accumulation of extracellular matrix, leading to widespread tissue fibrosis and destruction of renal parenchyma.3–5 However, current treatment options in the clinical settings for this devastating condition are limited and often ineffective, except renal replacement. In this context, a better understanding of the pathogenic mechanism and key signal pathway underlying renal fibrosis is essential and necessary for ultimately developing effective strategies for the treatment of this progressive kidney disorder.

β-catenin signal cascade, a principal mediator of canonical Wnt signaling, plays a fundamental role in regulating various biologic processes such as organ development, tissue homeostasis, and pathogenesis of human diseases.6–7 Activation of β-catenin signaling is indispensible for proper nephron formation and kidney development.8–10 Although it is relatively silent in...
adult kidney, β-catenin signaling is clearly reactivated in a wide variety of fibrotic CKDs such as obstructive nephropathy, diabetic nephropathy, adriamycin nephropathy, polycystic kidney disease, and chronic allograft nephropathy. In addition to Wnt, β-catenin activation is also regulated by other signal pathways such as integrin-linked kinase and TGF-β1. In this context, it is conceivable that β-catenin signaling serves as a converging, common downstream effector of multiple key pathogenic pathways in fibrotic kidneys. Accordingly, genetic ablation of β-catenin in a podocyte-specific fashion or inhibition of its upstream Wnt and integrin-linked kinase is able to ameliorate proteinuria and kidney injury and mitigate renal fibrotic lesions after various insults. Altogether, these results suggest that β-catenin signaling could be exploited as a potential target for therapeutic intervention of fibrotic CKD.

Upon activation by its upstream signaling, β-catenin is stabilized and translocated into the nucleus, where it binds to members of the T cell factor (TCF)/lymphoid enhancer-binding factor family of transcription factors. Nuclear β-catenin/TCF then assembles a transcriptionally active complex by recruiting the transcriptional coactivators cyclic AMP response-element binding protein (CREB) binding protein (CBP) or its closely related protein p300, as well as other components of the basal transcriptional machinery, to stimulate the transcription of its target genes. Along this line, disruption of the β-catenin-mediated transcriptional complex assembly could be an effective strategy to constrain hyperactive β-catenin signaling. In that regard, recent studies have identified ICG-001, a peptidomimetic small molecule that selectively inhibits β-catenin signaling in a CBP-dependent fashion. ICG-001 is shown to repress the β-catenin-driven gene transcription in a TOPflash luciferase reporter assay and inhibit bleomycin-induced lung fibrosis in mice. Mechanistically, ICG-001 competes with β-catenin for binding to CBP and prevents β-catenin/CBP complex formation, thereby inhibiting β-catenin-mediated gene expression. However, whether targeted inhibition of β-catenin/CBP signaling by ICG-001 ameliorates renal fibrosis in a preclinical setting remains unknown.

In this study, we have examined the effects of β-catenin activation on tubular epithelial-mesenchymal transition (EMT) in vitro and evaluated the therapeutic efficacy of ICG-001 in obstructive nephropathy, a well-characterized and widely used model of renal interstitial fibrosis. Our results suggest that targeting β-catenin/CBP signaling by a small-molecule inhibitor is a novel and effective approach for the treatment of fibrotic CKD.

RESULTS

Ectopic Expression of Stabilized β-Catenin Induces Tubular EMT

Previous studies show that β-catenin is primarily upregulated in tubular epithelial cells in the fibrotic kidneys, suggesting that these cells are likely the major target of this signaling. To investigate the pathogenic action of β-catenin activation in tubular epithelia, we sought to establish stable cell lines overexpressing constitutively active β-catenin. To this end, human kidney tubular epithelial cells (HKC-8) were transfected with either Flag-tagged, N-terminally truncated, stabilized β-catenin expression vector (pDel-β-cat) or empty vector (pcDNA3). As shown in Figure 1A, several stable cell clones, designated as C1, C2, and C3, were established, in which different levels of exogenous β-catenin were evident, as shown by Western blot analysis using anti-Flag antibody. Immunofluorescence staining revealed a predominant, clear nuclear localization of β-catenin in the stable cell lines transfected with pDel-β-cat expression vector (Figure 1B, arrowheads), whereas endogenous β-catenin displayed the plasma membrane-associated staining pattern in pcDNA3 mock-transfected cells (Figure 1B, arrow). Notably, in the cells with nuclear β-catenin (Figure 1B, arrowheads), cell membrane-associated β-catenin staining was reduced, suggesting that β-catenin activation could disrupt normal cell-cell adhesions and cause cell phenotypic alteration.

We then further characterized the phenotype of the stable cells expressing constitutively active β-catenin. As shown in Figure 1C, ectopic expression of β-catenin apparently suppressed E-cadherin and induced Snail1 and fibronectin expression, rendering a phenotypic change of tubular epithelial cells that is consistent with EMT. Compared with pcDNA3 controls, the steady-state mRNA level of the E-cadherin gene was downregulated in all three stable cell clones transfected with pDel-β-cat vector. Meanwhile, induction of fibronectin and Snail1 mRNA was evident after β-catenin activation. Similarly, expression of constitutively active β-catenin also led to loss of E-cadherin protein and de novo induction of fibronectin protein in tubular epithelial cells (Figure 1, D and E). As recently reported, exogenous β-catenin also induced the expression of plasminogen activator inhibitor-1 (PAI-1) (Figure 1F), a direct transcriptional target of canonical Wnt/β-catenin signaling.

β-Catenin Potentiates the Fibrogenic Actions of TGF-β1 in Tubular Epithelial Cells

We next examined whether ectopic expression of stabilized β-catenin promotes the fibrogenic actions of TGF-β1, a potent fibrogenic cytokine that is upregulated in virtually all kinds of CKD. To test this, stable cell lines with ectopic expression of constitutively active β-catenin and pcDNA3 mock-transfected cells were treated with TGF-β1 for various periods of time. As shown in Figure 2A, ectopic expression of β-catenin augmented TGF-β1-driven suppression of E-cadherin and induction of fibronectin and Snail1, as shown by reverse-transcriptase PCR (RT-PCR) analyses. Similar results were obtained when these cells were incubated with different concentrations of TGF-β1 (data not shown). Western blot analyses also revealed that exogenous β-catenin potentiated E-cadherin suppression and fibronectin induction triggered by TGF-β1 (Figure 2, B and C). In the absence of TGF-β1, ectopic expression of exogenous β-catenin was sufficient to cause E-
cadherin suppression and fibronectin induction (Figure 2, B and C, lane 3 and 5 versus lane 1). Incubation with TGF-β1 further facilitated E-cadherin loss and fibronectin expression (Figure 2, B and C, lane 4 and 6 versus lane 2). Immunofluorescence staining for assessing fibronectin expression and extracellular assembly after various treatments produced similar results (Figure 2D). Hence, constitutive activation of β-catenin clearly potentiates the fibrogenic action of TGF-β1 in tubular epithelial cells.

ICG-001 Blocks β-Catenin-Mediated Gene Transcription
Given a critical role for β-catenin activation in mediating tubular EMT, we reasoned that targeted inhibition of this signaling might be able to mitigate the fibrogenic response of tubular cells after injury. To test this, we utilized a novel peptidomimetic small molecule (ICG-001) that selectively inhibits β-catenin-mediated gene transcription in a CBP-dependent fashion.21,23 As shown in Figure 3A, ICG-001 was able to inhibit β-catenin-driven luciferase activity in HKC-8 cells in the TOPflash reporter luciferase assay. ICG-001 not only dose-dependently inhibited the β-catenin-induced luciferase activity after transfection with pDel-β-cat expression vector, but it also reduced luciferase activity in HKC-8 cells in basal, unstimulated conditions, underscoring its ability to inhibit endogenous and exogenous β-catenin-mediated gene transcription. Consistently, ICG-001 was able to inhibit the β-catenin-mediated PAI-1 (Figure 3B) and Snail1 (Figure 3C) expression in HKC-8 cells. Similarly, ICG-001 dose-dependently inhibited PAI-1 and fibronectin expression in the stable cell line overexpressing β-catenin (Figure 3D). Of note, this inhibitory effect of ICG-001 was not relevant to its potential role in regulating tubular epithelial cell proliferation/turnover because no significant difference in cell numbers was found after ICG-001 treatment (Figure 3E).

ICG-001 Inhibits TGF-β1-Mediated Fibrogenic Action by a Smad-Independent Mechanism
We further examined whether inhibition of β-catenin/CBP signaling impedes TGF-β1-mediated fibrogenic action because TGF-β1 is recently shown to activate β-catenin in tubular epithelial cells.15 As presented in Figure 4, A through C, TGF-β1 induced PAI-1 and Snail1 mRNA expression in HKC-8 cells, and ICG-001 effectively blocked TGF-β1-induced PAI-1 and Snail1 expression in a dose-dependent fashion. Western blot analyses also showed that ICG-001 blocked PAI-1 protein expression induced by TGF-β1 (Figure 4D). Interestingly, ICG-001 did not affect TGF-β1-mediated Smad2 phosphorylation and activation in HKC-8 cells (Figure 4E). Similarly, ICG-001 was also unable to modulate Smad3 phosphorylation (data not shown). These results suggest that inhibition of β-catenin signaling by ICG-001 is able to hinder TGF-β1-mediated fibrogenic action by a mechanism independent of disruption of Smad activation.
Figure 2. Exogenous β-catenin potentiates the fibrogenic actions of TGF-β1 in tubular epithelial cells. Stable cell lines with ectopic expression of constitutively active β-catenin were treated with TGF-β1 (2 ng/ml) for various periods of time as indicated. (A) Ectopic expression of β-catenin promotes TGF-β1-mediated suppression of E-cadherin and induction of fibronectin and Snail1, as shown by RT-PCR analyses. (B, C) Exogenous β-catenin potentiates E-cadherin suppression and fibronectin induction triggered by TGF-β1. Stable cell lines were incubated with TGF-β1 (2 ng/ml) for 48 hours, followed by examining protein expression of (B) E-cadherin or (C) fibronectin by Western blot analysis. C1 and C2 indicate each individual stable cell clone. (D) Immunofluorescence staining showed fibronectin expression and extracellular assembly in various groups as indicated.

Figure 3. Small-molecule inhibitor ICG-001 blocks β-catenin-driven gene expression. (A) ICG-001 inhibited β-catenin-mediated gene transcription. HKC-8 cells were cotransfected with TOPflash reporter plasmid and pDel-β-cat or pcDNA3, followed by incubation with different concentrations of ICG-001 as indicated. Relative luciferase activity (fold-induction over the controls) was presented. *P < 0.05, **P < 0.01 versus controls; ††P < 0.01 versus pDel-β-cat alone (n = 3). (B) ICG-001 blocks the β-catenin-mediated PAI-1 induction in a dose-dependent manner. HKC-8 cells after transient transfection with pDel-β-cat or pcDNA3 were incubated with different concentrations of ICG-001 as indicated. Cell lysates were immunoblotted with antibodies against PAI-1 or α-tubulin. (C) ICG-001 inhibits β-catenin-mediated Snail1 expression. Snail1 mRNA levels in HKC-8 cells after various treatments as indicated were assessed by RT-PCR. (D) ICG-001 also inhibited PAI-1 and fibronectin expression in a stable cell line (pDel-β-cat C2) with overexpression of β-catenin. Cells were treated for 48 hours with different doses of ICG-001 as indicated. (E) ICG-001 did not significantly affect tubular epithelial cell survival. HKC-8 cells were treated with different doses of ICG-001 as indicated for 48 hours. No significant difference in cell numbers was found in various groups.
ICG-001 was also able to inhibit the expression of α-smooth muscle actin (α-SMA) and major interstitial matrix components such as collagen I and fibronectin in HKC-8 cells after TGF-β1 stimulation. Figure 5A shows that ICG-001 inhibited TGF-β1-induced collagen I and fibronectin mRNA expression in a dose-dependent manner, which was confirmed by quantitative, real-time RT-PCR analyses (Figure 5, B and C). Similarly, ICG-001 also suppressed α-SMA mRNA and protein expression in HKC-8 cells after TGF-β1 stimulation.

Figure 4. Inhibition of β-catenin signaling by ICG-001 blocks TGF-β1-mediated PAI-1 and Snail1 induction by a Smad-independent mechanism. (A) Representative RT-PCR shows that ICG-001 blocked TGF-β1-induced PAI-1 and Snail1 mRNA expression. HKC-8 cells were treated with TGF-β1 (2 ng/ml) for 48 hours in the absence or presence of different doses of ICG-001 as indicated. (B, C) Quantitative, real-time RT-PCR analyses show the relative abundances of (B) PAI-1 and (C) Snail1 mRNA after various treatments. *P < 0.05 versus controls; †P < 0.05 versus TGF-β1 alone (n = 3). (D) Western blot analyses show that ICG-001 blocked PAI-1 protein expression induced by TGF-β1. Cell lysates after various treatments as indicated were immunoblotted with antibodies against PAI-1 and α-tubulin. (E) ICG-001 does not affect TGF-β1-mediated Smad2 phosphorylation. HKC-8 cells were pretreated with ICG-001 for 0.5 hour, followed by incubation with TGF-β1 (2 ng/ml) for 1 hour. Cell lysates were immunoblotted with antibodies against phosphorylated Smad2 (p-Smad2), total Smad2, and α-tubulin.

Figure 5. Inhibition of β-catenin signaling by ICG-001 abolishes the fibrogenic action of TGF-β1. (A) Small-molecule inhibitor ICG-001 blocks TGF-β1-induced α-SMA, collagen I, and fibronectin mRNA expression in tubular epithelial cells. HKC-8 cells were treated with TGF-β1 (2 ng/ml) for 48 hours in the absence or presence of different doses of ICG-001 as indicated. (B, C) Quantitative, real-time RT-PCR analyses show the relative abundances of (B) fibronectin and (C) collagen I mRNA after various treatments. **P < 0.01 versus controls; †P < 0.05 versus TGF-β1 alone. (D) Western blot analyses show that ICG-001 blocked α-SMA protein expression induced by TGF-β1. Cell lysates after various treatments as indicated were immunoblotted with antibodies against α-SMA and GAPDH. (E) Immunofluorescence staining revealed that ICG-001 inhibited the TGF-β1-induced fibronectin and α-SMA expression. HKC-8 cells were incubated with TGF-β1 (2 ng/ml) for 48 hours in the absence or presence of different concentrations (5 or 10 μM) of ICG-001.
pression induced by TGF-β1 in HKC-8 cells (Figure 5, A and D). Comparable results were obtained when fibronectin and α-SMA were detected by immunofluorescence staining (Figure 5E).

**ICG-001 Attenuates Renal Interstitial Fibrosis in Obstructive Nephropathy**

Because ICG-001 blocks the fibrogenic action induced by β-catenin or TGF-β1 in vitro, we sought to test its therapeutic efficacy in mitigating renal fibrosis after injury in vivo. To this end, ICG-001 was administrated into mice after unilateral ureteral obstruction (UUO) via intraperitoneal injection at the dosages of 2 and 5 mg/kg body weight, respectively. Figure 6A shows the representative micrographs of kidney sections after Masson trichrome staining (MTS) from various groups at 7 days after UUO. Quantitative determination by computer-aided morphometric analyses revealed that ICG-001 dose-dependently reduced renal fibrotic lesions in obstructive nephropathy (Figure 6B). We further examined the mRNA expression of major interstitial matrix genes by quantitative, real-time RT-PCR. As shown in Figure 6, C through E, ICG-001 inhibited renal expression of collagen I, collagen III, and fibronectin in obstructive nephropathy. *P < 0.05, **P < 0.01 versus sham controls; †P < 0.05, ††P < 0.01 versus UUO alone (n = 5). (F) Immunofluorescence staining shows that ICG-001 reduced collagen I and fibronectin deposition in obstructed kidney at 7 days after UUO. Numbers in the brackets (2 and 5) indicate the dosages of ICG-001 used (in mg/kg body weight). Scale bar, 50 μm.

**ICG-001 Inhibits Fibrogenic Gene Expression in Obstructive Nephropathy**

We further investigated the effects of ICG-001 on the expression of several fibrogenic genes in obstructive nephropathy. As shown in Figure 7, A through C, renal expression of α-SMA, the molecular signature of myofibroblasts, was significantly reduced by ICG-001 as demonstrated by immunofluorescence staining, real-time RT-PCR, and Western blot analyses, respectively. Similarly, ICG-001 also suppressed PAI-1 expression in obstructive nephropathy (Figure 7C).

We also assessed the effects of ICG-001 on the expression of several key EMT regulatory genes such as Snail and fibroblast-specific protein-1 (Fsp1) in obstructive nephropathy. As shown in Figure 7, D and E, ICG-001 was able to inhibit the
expression of Snail1 and Snail2, also known as Slug, two transcription factors that play a critical role in regulating EMT and renal fibrosis.30,31 Consistently, ICG-001 also inhibited renal expression of Fsp1 (also known as S100A4) mRNA in a dose-dependent manner at 7 days after UUO (Figure 7F). Immunohistochemical staining revealed a marked induction of Fsp1 protein not only in the interstitium (Figure 7G, arrows), but also in the tubular epithelia (Figure 7G, arrowhead). However, administration of ICG-001 predominantly inhibited tubular expression of Fsp1, suggesting a potential role of ICG-001 in blocking tubular EMT in this model.

We further examined the effects of ICG-001 on β-catenin expression in the obstructed kidneys. As shown in Figure 8A, ureteral obstruction caused marked induction of β-catenin, primarily in renal tubular epithelia. However, nuclear staining for β-catenin, albeit weak, was also observed in the interstitial cells (Figure 8B, arrows). Interestingly, ICG-001 appeared not to significantly affect β-catenin induction in the obstructed kidneys (Figure 8A). This is not surprising because ICG-001 targets β-catenin/CBP interaction,25 an event that is downstream of β-catenin induction (Figure 8C). Similarly, ICG-001 also exhibited little effect on Smad2 phosphorylation and activation in the obstructed kidney 7 days after UUO (Figure 8D), suggesting that ICG-001 also exerts its antifibrotic action by a mechanism independent of disruption of Smad activation in vivo.

Delayed Administration of ICG-001 Also Attenuates Renal Fibrosis

We finally tested whether delayed administration of ICG-001 is also effective in reducing renal fibrosis, a scenario that is clearly relevant to the clinical settings. As shown in Figure 9A, ICG-001 at different doses was administrated to mice starting 3 days after UUO, a time point when significant kidney injury is already established in this model.32 Interestingly, delayed administration of ICG-001 at 3 days after UUO also reduced α-SMA expression in the obstructed kidneys (Figure 9, B and C). Likewise, late administration of ICG-001 inhibited collagen I, collagen III, and fibronectin mRNA expression in the injured kidneys (Figure 9, D through F). Similar results were obtained when kidney morphologic injury and fibrotic lesions were assessed by MTS (Figure 9, G and H). Notably, renal expression levels of α-SMA, collagen I, collagen III, and fibronectin after late administration of ICG-001 were compara-
ble to that before treatment. These data suggest that delayed administration of ICG-001 virtually hinders, albeit does not reverse, the progression of renal fibrotic lesions in this aggressive model of renal fibrosis.

DISCUSSION

Renal fibrosis is a devastating, common final outcome of many different progressive CKDs. Because current therapies only have limited efficacy, identifying new agents that target key molecular pathways involved in the pathogenesis of renal fibrosis to halt progression remains an unmet daunting task. As aberrant activation of β-catenin signaling occurs in the fibrotic kidneys, it is conceivable that this pathway could be exploited as a potential target for antifibrosis therapy. In the study presented here, we have tested this hypothesis by targeting β-catenin signaling for therapeutic intervention of renal interstitial fibrosis using ICG-001, a novel β-catenin-selective inhibitor that selectively inhibits β-catenin-mediated gene transcription in a CBP-dependent fashion. Our results indicate that targeted inhibition of β-catenin/CBP signaling is able to attenuate matrix gene expression, hamper myofibroblast activation, and ameliorate renal fibrotic lesions. These findings are in line with a recent report that blockade of Wnt/β-catenin/CBP signaling by ICG-001 prevents, and even reverses, bleomycin-induced pulmonary fibrosis in mice, in which aberrant activation of β-catenin signaling is also evident. Therefore, specific targeting of hyperactive β-catenin signaling with a small-molecule inhibitor could represent a novel and effective strategy for therapeutic intervention of tissue fibrosis in many organs.

Despite a predominantly tubular localization of β-catenin activation in the fibrotic kidneys, the pathogenic role of this signaling in renal tubular epithelia remained ambiguous. By establishing cell lines expressing stabilized β-catenin, we have clearly demonstrated a pivotal role of this signaling in inducing tubular EMT and damaging tubular epithelial integrity. Ectopic expression of β-catenin in tubular epithelial cells repressed E-cadherin and induced Snail1 and fibronectin (Figure 1), consistent with the characteristic feature of EMT under pathologic conditions. An increased Snail1 could, in turn, mediate the suppression of E-cadherin expression because it is well known that Snail1 directly binds to the E-boxes in the E-cadherin promoter and represses its transcription. The importance of β-catenin in promoting EMT is also demonstrated in many tumor cells with metastatic and invasive capacity. Therefore, by virtue of its ability to induce Snail1 and repress E-cadherin expression, β-catenin activation likely plays a crucial role in mediating tubular EMT, a profibrotic process in diseased kidneys. Intriguingly, EMT is often considered an adaptive response of epithelial cells after injury in a desperate attempt to escape from apoptosis, and this notion is compatible with an earlier report that constitutively active β-catenin improves proximal tubular epithelial cell survival by inhibiting apoptosis after metabolic stress. It should be noted that β-catenin activation is not only sufficient to induce EMT by itself, but it also potentiates the tubular EMT triggered by TGF-β1 (Figure 2). Because Wnt/β-catenin and TGF-β1 signals are activated after kidney injury, they would constitute a combination...
fibrogenic niche in which two fibrogenic signals work in concert to promote EMT and renal fibrosis in diseased kidneys.

Considering an imperative role of β-catenin in mediating tubular EMT, it is predictable that inhibition of this signaling by ICG-001 leads to the preservation of tubular epithelial cell integrity. Indeed, ICG-001 not only prevents β-catenin-mediated gene expression in tubular epithelial cells, but it also abolishes the fibrogenic action of TGF-β1 by a mechanism independent of disruption of Smad activation (Figures 4 and 5). Mechanistically, the action of ICG-001 in disrupting β-catenin-mediated gene transcription is quite unique in that it selectively blocks the β-catenin/CBP interaction by binding to CBP rather than β-catenin itself.22,24 As a β-turn bicyclic peptidomimetic small molecule, ICG-001 competes with β-catenin for binding to the N-terminal region of CBP, thereby preventing β-catenin/CBP interaction. ICG-001 does so without interfering with the β-catenin/p300 interaction.24 Although CBP and p300 are generally considered to be indistinguishable in terms of promoting their downstream gene expression, recent studies indicate that differential coactivator usage in fact determines the selective expression of subsets of Wnt/β-catenin target genes.22,23 Although p300/β-catenin signaling is instrumental in initiating normal cellular differentiation, CBP/β-catenin-driven gene transcription is shown to be critical for inducing a dedifferentiated/proliferative state.38 Notably, the expression of Snail1, fibronectin, and PAI-1 genes in tubular epithelial cells is apparently dependent on the CBP pathway arm of β-catenin signaling because selective disruption of CBP/β-catenin interaction by ICG-001 abrogates their induction. These results are extended by earlier reports that β-catenin controls survivin, Fsp1, and collagen I and III expression in a CBP-dependent manner.21,22 Therefore, the CBP/β-catenin pathway constitutes a powerful profibrotic/prodedifferentiation signaling that directly controls the expression of a battery of fibrogenic genes such as Snail1, fibronectin, PAI-1, and Fsp1. By selectively targeting CBP/β-catenin signaling, ICG-001 effectively inhibits the expression of these fibrogenic genes in vitro and in vivo, thereby leading to amelioration of renal fibrotic lesions.

Our in vivo data indicate that targeted inhibition of β-catenin/CBP signaling by ICG-001 results in impressive renal protection after obstrusive injury, supporting the notion that hyperactive β-catenin plays an important role in the pathogenesis of renal fibrosis.36 Importantly, late administration of ICG-001 3 days after UUO is also effective when kidney injury is already established in this model.32 These findings are obviously of clinical significance and are consistent with the fact that it targets β-catenin/CBP-driven gene transcription.
rather than β-catenin expression (Figure 8). Of note, there is no clear reversal/remission of renal fibrosis after late administration of ICG-001. This could be due to the extremely aggressive nature of UUO model because complete ureteral obstruction is persistently present. Several potential mechanisms may account for the efficacy of ICG-001. First and foremost, because β-catenin activation is sufficient to induce Snail1 and drive tubular EMT in vitro, inhibition of β-catenin/CBP interaction by ICG-001 would block tubular EMT in the fibrotic kidneys. This notion is corroborated by the observations that ICG-001 inhibited Snail1 and Snail2 expression in the obstructed kidney after injury (Figure 8). Second, Fsp1/S100A4, a cytokine-related calcium binding protein that is instrumental in mediating cell migration and invasion, is a novel transcriptional target of β-catenin. Therefore, ICG-001 could directly repress β-catenin/CBP-mediated Fsp1 expression in tubular epithelial cells and block their migration, one of the key steps in the course of EMT. Third, ICG-001 could exert its renoprotective actions by repressing the expression of PAI-1, a multifunctional glycoprotein that plays a critical role in the pathogenesis of chronic kidney and cardiovascular diseases. Because PAI-1 is also a direct transcriptional target of β-catenin, as recently reported, inhibition of its expression by ICG-001 would certainly diminish its fibrogenic effects. Finally, ICG-001 could also elicit its renoprotection by directly affecting interstitial fibroblast expansion and matrix production. Because earlier studies have identified fibronectin as a direct transcriptional target of β-catenin in fibroblasts, it is therefore conceivable to speculate that β-catenin activation is also important for interstitial fibroblast activation and matrix expression.

In summary, we have herein demonstrated that β-catenin activation is sufficient to initiate tubular EMT in vitro, possibly by inducing key EMT regulatory transcription factor Snail1. Targeted inhibition of β-catenin/CBP signaling by a novel small molecule, ICG-001, is able to inhibit the expression of a battery of fibrogenic genes, block tubular EMT, and ameliorate renal fibrotic lesions after obstructive injury. Interestingly, these antifibrotic actions of ICG-001 are operated at the stage of post-β-catenin induction and by a mechanism independent of any disruption of Smad activation. Although more investigations are clearly needed, our current studies provide a proof of principle that selective blockade of β-catenin/CBP signaling by a small-molecule inhibitor may hold up the promise as a novel therapeutic strategy for the treatment of fibrotic kidney diseases.

CONCISE METHODS

Cell Culture and Treatment
Human proximal tubular epithelial cells (HKC-8) were cultured in DMEM–Ham’s F12 medium supplemented with 5% calf serum, as described previously. HKC-8 cells were serum starved for 16 hours and then treated without or with recombinant TGF-β1 (R & D Systems, Minneapolis, MN) at 2 ng/ml for various periods of time, except otherwise indicated. A novel small molecule, ICG-001, which selectively inhibits TCF/β-catenin-mediated gene transcription, was described previously. The chemical structure of ICG-001 and its detailed characterization were reported elsewhere. HKC-8 cells were typically pretreated with ICG-001 for 0.5 hour, followed by incubation with TGF-β1 for 48 hours. The cells were then collected for RT-PCR, Western blot analysis, and immunofluorescence staining. All other chemicals were of analytic grade and were obtained from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA) unless otherwise indicated.

Establishment of Stable Cell Lines
HKC-8 cells were transfected with Flag-tagged, N-terminally truncated, stabilized β-catenin expression vector (pDEL-β-cat) using the Lipofectamine 2000 reagent according to the instructions specified by the manufacturer (Invitrogen). The empty vector pcDNA3 (Invitrogen) was used as a mock transfection control.Twenty-four hours after transfection and every 3 to 4 days thereafter, the cells again fed with fresh selective medium containing G418 (Geneticin; Invitrogen) at a final concentration of 0.8 mg/ml. Neomycin-resistant clones were selected and individually expanded. Ectopic expression of exogenous β-catenin in the stable cell lines was confirmed by Western blot analysis using anti-Flag antibody.

Animal Model
Male CD-1 mice weighing approximately 20 to 22 g were obtained from Harlan Sprague Dawley (Indianapolis, IN). Four groups of mice (n = 5) were used: (1) sham control, (2) UUO + vehicle control, (3) UUO + ICG-001 (2 mg/kg body weight), and (4) UUO + ICG-001 (5 mg/kg body weight). UUO was performed using an established protocol, as described previously. ICG-001-phosphate was dissolved in PBS and administered into mice by daily intraperitoneal injection at the dosages of 2 and 5 mg/kg body weight. Mice were killed 7 days after UUO, and the kidney tissues were removed for various analyses. To assess the therapeutic effects of ICG-001 on an established kidney injury, another set of animal experiments was performed in which ICG-001 was administrated starting 3 days after UUO. The detailed experimental design is presented in Figure 9A. The Institutional Animal Care and Use Committee at the University of Pittsburgh approved all animal protocols.

Western Blot Analysis
Western blot analysis for specific protein expression was performed essentially according to an established procedure. The primary antibodies used were as follows: anti-E-cadherin (clone 36) and anti-fibronectin (clone 10) (BD Transduction Laboratories, San Jose, CA), anti-PAI-1 (SC-5297) and anti-actin (SC-1616) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-SMA (clone 1A4), anti-Flag (clone M2) and anti-α-tubulin (Sigma, St. Louis, MO), anti-phospho-Smad2 (Ser465/467, catalog number 3101), anti-phospho-Smad3 (catalog number 9514; Cell Signaling Technology, Inc., Beverly, MA), and anti-Smad2 (catalog number 51-1300; Invitrogen), and anti-glyceraldehyde 3-phosphate dehydrogenase (Ambion, Austin, TX).
Immunofluorescence Staining
Indirect immunofluorescence staining was performed using an established procedure. Briefly, cells cultured on coverslips were fixed with cold methanol for 10 minutes at −20°C and blocked with 10% normal donkey serum in PBS buffer for 30 minutes. Cells were then incubated with the specific primary antibodies against β-catenin, fibronectin, and α-SMA. Kidney cryosections were prepared at 5-μm thickness and fixed for 10 minutes with 3.7% paraformaldehyde and block with 10% normal donkey serum in PBS for 1 hour. Sections were incubated with anti-α-SMA (ab5694; Abcam, Cambridge, MA), anti-fibronectin (SC-9068), or anti-collagen I. To visualize the primary antibodies, cells and cryosections were stained with cyanine Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). For some samples, cells were double stained with 4',6-diamidino-2-phenylindole–HCl to visualize the nuclei. Stained cells and cryosections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and viewed with a Nikon Eclipse E600 microscope equipped with a digital camera (Melville, NY).

MTS and Morphometric Analysis
To evaluate the collagen deposition, 4-μm sections of paraffin-embedded tissue were subjected to MTS by routine procedures. Stained sections were examined by a Nikon Eclipse E600 epifluorescence microscope equipped with a digital camera (Melville, NY). To estimate fibrotic area, computer-aided morphometric analysis on MTS sections was performed as described previously. Briefly, a grid containing 117 (13 × 9) sampling points was superimposed on images of a cortical high-power field (400×). The number of grid points overlapping the MTS-positive area was counted and expressed as a percentage of all sampling points. For each kidney, five randomly selected non-overlapping fields were analyzed.

Immunohistochemical Staining
Immunohistochemical staining of kidney sections for Fsp1 using a specific antibody (polyclonal rabbit anti-S100A4; catalog number A5114; DakoCytomation, Glostrup, Denmark) was performed using the Vector M.O.M. immunodetection kit according to the protocol specified by the manufacturer (Vector Laboratories, Burlingame, CA). As a negative control, the primary antibody was replaced with nonimmune IgG, and no staining occurred. Immunohistochemical staining for β-catenin was carried out as described previously. The sequences of the primer pairs are given in Supplementary Table 1. The PCR reaction was run using standard conditions. The mRNA levels of various genes were calculated after normalizing with β-actin.

TOPflash Luciferase Assay
Transcriptional activity of TCF/β-catenin was evaluated by TOPflash luciferase assay (Upstate Biotechnology). This method utilized a β-catenin-responsive luciferase reporter plasmid under the control of three copies of the TCF binding sites. HKC-8 cells were cotransfected using Lipofectamine 2000 reagent with the TOPflash luciferase reporter plasmid (1.0 μg) plus pDel-β-cat (2.0 μg) expression vector or empty pcDNA3 vector. A fixed amount of 0.1 μg of internal control reporter Renilla reniformis luciferase driven under a thymidine kinase promoter (pRL-TK, Promega) was also cotransfected for normalizing the transfection efficiency. After transfection, cells were treated with different concentrations of ICG-001 as indicated. 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 controls after normalizing for transfection efficiency.

Statistical Analyses
All data examined are expressed as mean ± SEM. Statistical analyses of the data were performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by the Student-Newman-Keuls test. P < 0.05 was considered statistically significant.

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DISCLOSURES
None.

REFERENCES