Sonic Hedgehog Is a Novel Tubule-Derived Growth Factor for Interstitial Fibroblasts after Kidney Injury

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ABSTRACT

Tubular epithelium constitutes the majority of the renal parenchyma and is the primary target of various kidney injuries. However, how the injured tubules drive interstitial fibroblast activation and proliferation remains poorly understood. Here, we investigated the role of sonic hedgehog (Shh), a secreted extracellular signaling protein, in fibroblast proliferation. Shh was induced in renal tubular epithelia in animal models of CKD induced by ischemia/reperfusion injury (IRI), adriamycin, or renal mass ablation, and in renal tubules of kidney biopsy specimens from CKD patients with different etiologies. Using Gli1-CreERT2 reporter mice, we identified interstitial fibroblasts as the principal targets of renal Shh signaling in vivo. In vitro, incubation with Shh promoted normal rat kidney fibroblast proliferation, which was assessed by cell counting, MTT assay, and BrdU incorporation assay, and stimulated the induction of numerous proliferation-related genes. However, Shh had no effect on the proliferation of renal tubular epithelial cells. In vivo, overexpression of Shh promoted fibroblast expansion and aggravated kidney fibrotic lesions after IRI. Correspondingly, blockade of Shh signaling by cyclopamine, a small molecule inhibitor of Smoothened, inhibited fibroblast proliferation, reduced myofibroblast accumulation, and attenuated renal fibrosis. These studies identify Shh as a novel, specific, and potent tubule-derived growth factor that promotes interstitial fibroblast proliferation and activation. Our data also suggest that blockade of Shh signaling is a plausible strategy for therapeutic intervention of renal fibrosis.


Kidney fibrosis, the final common outcome of a wide variety of CKD, is characterized by activation of the matrix-producing, α-smooth muscle actin (α-SMA)-positive myofibroblasts, relentless production and deposition of extracellular matrix, and progressive fibrous scar formation.1–3 Although myofibroblasts can originate from different sources, such as bone marrow, endothelium, and tubular epithelium, local proliferation of resident fibroblasts remains to be the major route leading to the activation and expansion of the myofibroblast population in diseased kidneys.4–6 In this context, identification of key mediators that control renal fibroblast proliferation and delineation of underlying mechanisms would be essential in unraveling the pathogenic mechanism of renal fibrosis and developing rational intervention strategies.7

In normal adult kidneys, tubular epithelium constitutes the major part of kidney parenchyma. Extensive studies have shown that tubular cells are particularly vulnerable to a wide variety of metabolic, immunologic, ischemic, and toxic insults, and they are the primary target of various injuries within the kidneys in most circumstances.8–11 These observations suggest that tubular injury often is the early and central event that triggers fibroblast expansion and interstitial fibrosis.10,12,13 However, exactly how the injured tubules drive fibroblast...
proliferation and activation in the neighboring interstitium remains poorly understood. One plausible hypothesis would be that the injured tubules produce and secrete extracellular signaling protein(s) that dictate fibroblast proliferation in a paracrine fashion. However, the identity of such a tubule-derived mitogenic factor for interstitial fibroblasts remains largely elusive.

Sonic hedgehog (Shh), the most studied member of the hedgehog signaling pathway ligands, is a secreted extracellular signal protein that plays a fundamental role in regulating a range of developmental processes in mammalian organogenesis.\(^{14,15}\) During kidney development, Shh controls the expression of a hierarchy of genes involved in tissue patterning and cell cycle regulation.\(^{16–19}\) Accordingly, mutations in the Shh gene have been associated with renal developmental defects, resulting in hypoplastic kidneys in mice.\(^{20,21}\) In normal adult kidneys, Shh expression level is extremely low and barely detectable.\(^{22}\) Whether Shh expression is altered in diseased kidneys is controversial. Although we report that Shh is induced specifically in renal tubular epithelium in obstructive nephropathy after unilateral ureteral obstruction (UUO),\(^{22}\) another study indicates that it is not changed during UUO.\(^{23}\) Nevertheless, the Gli1 transcription factor, a direct downstream target and reporter of active hedgehog signaling, is specifically induced in renal interstitial fibroblasts of fibrotic kidneys.\(^{22,23}\) We previously proposed that tubule-derived Shh mediates epithelial–mesenchymal communication (EMC) by selectively targeting interstitial fibroblasts, leading to their myofibroblastic transition.\(^{22}\) However, whether Shh also acts as a mitogen and regulates fibroblast proliferation, which could lead to the expansion of this matrix-producing cell population, remain unknown.

In this study, we investigated Shh regulation in three models of renal fibrotic diseases as well as in human kidney biopsies from CKD patients. Our results indicate that upregulation of Shh is a common finding in CKD and that Shh selectively promotes fibroblast proliferation in vitro. We also show that overexpression of Shh or blockade of its signaling with a small molecule inhibitor has a dramatic impact on the severity of renal fibrosis after kidney injury in vivo. These results establish that tubule-derived Shh is an inducible, specific, and potent fibroblast mitogen that controls renal fibroblast proliferation and kidney fibrosis.

RESULTS

Tubular Induction of Shh Is a Common Finding in Various Models of CKD

To investigate Shh regulation after kidney injury, we studied three animal models of CKD induced by renal ischemia/reperfusion, adriamycin (ADR), or subtotal renal mass ablation. These models are well established and widely used, and they represent different etiologies that lead to renal fibrosis.\(^{9,24–27}\) As shown in Figure 1A, quantitative real-time RT-PCR (qRT-PCR) analyses revealed that renal Shh mRNA was markedly induced at 10 days after ischemia/reperfusion injury (IRI) compared with sham controls. Consistently, Shh protein was also significantly induced in the injured kidneys after IRI, which was shown by Western blot analyses of whole-kidney lysates (Figure 1, B and C). Similarly, both renal Shh mRNA (Figure 1E) and protein levels (Figure 1, F and G) were significantly upregulated at 5 weeks after ADR administration.

We further examined Shh protein expression and localization in the fibrotic kidneys of various CKD models. As shown in Figure 1D, immunohistochemical staining with specific antibody revealed that little or no Shh protein was detectable in sham-operated control kidneys. However, a marked induction of Shh protein was clearly evident in the fibrotic kidneys after IRI or ADR injury. Shh protein was predominantly localized in renal tubular epithelium (Figure 1D, arrows), whereas interstitial cells in the expanded interstitium were mostly negative for Shh staining. Of note, no Shh staining occurred in the presence of Shh antigen, indicating the specificity of this staining (Supplemental Figure 1). Similarly, in a rat remnant kidney model, renal Shh protein was dramatically induced at 12 weeks after 5/6 nephrectomy compared with sham controls (Figure 1, H and I). These results suggest that tubule-specific induction of Shh is a common finding in various animal models of CKD.

Tubule-Derived Shh Targets Interstitial Fibroblasts In Vivo

Earlier studies have identified renal interstitial fibroblasts as the hedgehog-responding cells in fibrotic kidneys.\(^{22,23}\) To further address this issue, we used the Gli1-CreERT\(^2\) transgenic mice, in which the Cre-ERT2 fusion protein consisting of Cre recombinase and mutant estrogen receptor is under the control of endogenous Gli1 promoter/enhancer elements. As such, Cre is exclusively expressed in Gli1-expressing cells, thereby indicating the target cells of hedgehog signaling in these mice.\(^{28,29}\) We found that, at 10 days after IRI, Cre was expressed in renal interstitial cells but not tubular cells. Double immunostaining revealed that Cre (Figure 1J, red) was coexpressed in renal interstitial cells but not tubular cells. Of note, no Shh staining occurred in the presence of Shh antigen, indicating the specificity of this staining. The diagnosis and demographic data of the patients are presented in Supplemental Table 1. Figure 2 shows
Figure 1. Induction of Shh is a common finding in animal models of CKD. (A) qRT-PCR analyses show a significant induction of Shh mRNA expression in the fibrotic kidneys after renal IRI for 10 days. *P<0.05 versus sham controls (n=4–5). (B and C) Western blot analyses of renal expression of Shh protein in sham and injured kidneys at 10 days after IRI. Representative (B) Western blot and (C) quantitative data are presented. Numbers (1–3) indicate each individual animal in a given group. *P<0.05 versus sham controls (n=4–5). (D) Representative immunohistochemical staining shows an increased expression of Shh in mouse models of IRI (10 days) and ADR nephropathy (5 weeks). Boxed areas are enlarged in the lower panel. Arrows indicate Shh-positive tubules. (E) Renal Shh mRNA expression in ADR nephropathy. Shh mRNA expression was assessed in the kidneys at 5 weeks after ADR injection. *P<0.05 versus controls (n=5). (F and G) Western blot analyses of renal Shh protein at 5 weeks after ADR injection. Representative (F) Western blot and (G) quantitative data are presented. Numbers (1–5) indicate each individual animal in a given group. *P<0.05 versus controls (n=5). (H and I) Representative immunohistochemical staining showed induction of Shh protein in rat remnant kidney model after 5/6 nephrectomy at 12 weeks. Scale bar, 50 μm. (J) Identification of the interstitial fibroblasts as hedgehog-responding cells in fibrotic kidneys.
the representative micrographs of Shh staining in the kidney sections from patients with IgA nephropathy, membranous nephritis, and FSGS. Shh protein was predominantly localized in renal tubular epithelia in human diseased kidneys (Figure 2, arrows). Occasionally, fluids in the lumens of renal tubules also stained positively for Shh, suggesting that it could be secreted by tubular cells. Other than tubular cells, some glomerular podocytes were also positive for Shh staining (data not shown). However, renal interstitial cells were virtually negative for Shh staining. These data indicate that Shh is an inducible, tubule-derived, secreted factor that could play a role in the pathogenesis of human CKD.

**Shh Selectively Promotes Renal Fibroblast Proliferation In Vitro**

Because interstitial fibroblasts are the main targets of tubule-derived Shh in fibrotic kidneys (Figure 1I), we then investigated its potential actions in renal interstitial fibroblasts *in vitro*. To this end, normal rat kidney interstitial fibroblasts (NRK-49F) were incubated with different concentrations of recombinant human Shh protein for various periods of time as indicated. An appreciable increase in NRK-49F cell density was observed after Shh treatment, which is illustrated in the phase-contrast images in Figure 3A. Cell counting revealed that Shh substantially increased the number of NRK-49F cells in dose- (Figure 3B) and time-dependent manners (Figure 3C), suggesting that Shh acts as a potent mitogen that promotes renal fibroblast proliferation. Similar results were obtained by using a quantitative colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 3D). We further assessed the ability of Shh to promote renal fibroblasts entering cell cycle and undergoing DNA synthesis, which was reflected by bromodeoxyuridine (BrdU) incorporation. As shown in Figure 3, E and F, increased BrdU incorporation was observed in NRK-49F cells after incubation with Shh for 48 hours compared with controls. As expected, Shh was able to induce its downstream target Patched-1 (Ptc1) (Figure 3G) as well as Gli1 and α-SMA expressions in NRK-49F cells (data not shown) as previously reported.22

We also examined whether Shh affects the proliferation of renal tubular epithelial cells in which Shh is upregulated (Figures 1 and 2). To this end, human proximal tubule cell (HKC-8) and mouse inner medullary collecting duct cell (mIMCD-3) were incubated with different doses of Shh for variable durations. As shown in Figure 3, H–J, there was no effect on HKC-8 cell proliferation by Shh. Similarly, Shh also did not promote mIMCD-3 cell proliferation (Supplemental Figure 2). In fact, it exhibited a small but significant inhibition on mIMCD-3 cells as assessed by MTT assay (Supplemental Figure 2C). Therefore, it is clear that tubule-derived Shh selectively targets interstitial fibroblasts but not tubular epithelial cells and promotes fibroblast proliferation as a potent mitogen.

**Shh Activates Proliferation-Related Genes in Fibroblasts**

To delineate the mechanism underlying Shh-mediated fibroblast proliferation, we next investigated the expression of numerous proliferation-related genes in NRK-49F cells. As shown in Figure 3K, both c-Myc and proliferating cell nuclear antigen (PCNA) were rapidly induced by Shh in NRK-49F cells as early as 0.5 hour after treatment. Although the induction of c-Myc was transient, PCNA upregulation was sustained throughout the experiments. The expression of c-fos, a major component of AP-1 transcription factors, was also induced by Shh in a time-dependent fashion (Figure 3K). These results suggest that Shh can rapidly activate multiple proliferation-related genes in renal interstitial fibroblasts, leading to enhanced cell proliferation.

**Overexpression of Shh In Vivo Accelerates AKI to CKD Progression**

Given the ability of Shh in promoting fibroblast proliferation *in vitro*, we sought to examine whether overexpression of exogenous Shh *in vivo* affects fibroblast proliferation and progression of renal fibrosis after AKI. To this end, we used the mouse model of IRI, in which kidney tissues develop fibrotic lesions at late time points after ischemic AKI.9,26 As shown in Figure 4A, Shh expression vector (pFlag-Shh) or empty control vector (pcDNA3) was administered at 3 days after IRI by a hydrodynamic-based gene transfer technique, an approach that results in significant renal expression of the transgene.30,31 RT-PCR analyses revealed that mRNA for Shh and its downstream target gene Gli1 was induced at 7 days after a single injection of Shh-expressing plasmid (10 days after IRI) (Figure 4B). Shh protein was also induced, which was evidenced by Western blot analyses of whole-kidney lysates using anti-Shh or anti-Flag antibodies (Figure 4, C and D). Immunohistochemical staining showed that Shh protein was predominantly induced in renal tubular epithelium after plasmid injection (Figure 4E). Similarly, renal expression of endogenous Gli1 protein was also induced after overexpression of exogenous Shh (Figure 4F). Therefore, hydrodynamic-based gene delivery results in overexpression of Shh in renal tubular epithelium *in vivo* after IRI.

We found that overexpression of Shh *in vivo* promoted renal interstitial cell proliferation and accelerated the progression of renal fibrosis. As shown in Figure 5, A and B, exogenous Shh

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kidneys. Transgenic Gli1-CreERT2 mice were subjected to IRI for 10 days, and kidneys were subjected to double immunostaining for Cre recombinase (red) and various cell type-specific markers (green). Arrows, CD45- or CD31-positive cells; arrowheads, Cre-positive cells; wide arrows, cells with positive staining for both vementin and Cre. Scale bar, 20 μm. (K) Diagram shows that tubule-derived Shh specifically targets interstitial fibroblasts after kidney injury. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole.
exclusively promoted cell proliferation in the renal interstitial compartment but not tubular segments, which was illustrated by immunohistochemical staining for Ki67-positive cells. This increase in renal interstitial cell proliferation was closely correlated with elevated mRNA expression of interstitial matrix genes, such as types I and III collagens (Figure 5, C and D). Consistently, renal protein levels of platelet-derived growth factor receptor-β (PDGFR-β), desmin, and α-SMA, characteristic markers of active myofibroblasts,1,3 were also markedly induced (Figure 5E). Meanwhile, overexpression of Shh accelerated the accumulation and deposition of major interstitial matrix proteins, such as fibronectin, in injured kidneys (Figure 5E), which was shown by Western blot analyses of whole-kidney lysates. Consistently, Masson Trichrome staining (MTS) revealed that exogenous Shh in vivo aggravates renal fibrotic lesions after IRI (Figure 5, H and I).

Blockade of Shh Signaling Reduces Renal Fibrosis
We next examined whether blockade of Shh signaling can inhibit fibroblast proliferation and reduce renal fibrosis. To this end, we assessed the therapeutic efficacy of cyclopamine (CPN), a small molecule Smoothened (Smo) inhibitor,22,32 in established IRI-induced kidney injury. As shown in Figure 6A, CPN was administered by daily intraperitoneal injections starting at 3 days after IRI, a time point when kidney function begins to recover after AKI.33 As shown in Figure 6B, renal expression of Gli1 mRNA, the direct downstream target and reporter gene of hedgehog signaling, was substantially inhibited at 7 days after CPN administration (10 days after IRI) in vivo. Similarly, the expression of renal Gli1 protein was also reduced after CPN treatment (Figure 6C). These data indicate that CPN is effective in blocking Shh signaling transduction in vivo. However, no significant change was observed in renal Gli2 and Gli3 mRNA expression (Supplemental Figure 3).

We further assessed the severity of renal fibrosis in this model after CPN injections. As presented in Figure 6, D and E, MTS revealed fewer fibrotic lesions and reduced collagen deposition in the kidneys receiving CPN compared with vehicle controls. Consistently, renal expression of major interstitial matrix genes, such as types I and III collagens and fibronectin, was markedly reduced after CPN administration (Figure 6, F–H). The expression of α-SMA, the signature marker for active myofibroblasts, was also inhibited by CPN treatment (Figure 6I). These data indicate that blocking Shh signaling is able to ameliorate kidney injury and prevent the progression of renal fibrosis after IRI.

Blockade of Shh Signaling Selectively Inhibits Renal Fibroblast Proliferation In Vivo
To explore the mechanism underlying the beneficial effect of CPN, we examined its role in modulating renal fibroblast proliferation and expansion in vivo. As shown in Figure 7, A and B, PCNA protein expression was significantly inhibited by CPN in the fibrotic kidneys at 10 days after IRI compared with vehicle controls. To delineate the sources of renal proliferating cells, we carried out double immunostaining for Ki67 (Figure 7C, red) and laminin (Figure 7C, green), a major component of the tubular basement membrane. As shown in Figure 7C, arrows, the majority of the Ki67-positive cells was localized in the interstitial compartment, indicating a preferential expansion of the interstitial cell population at this time point (10 days) after IRI.
Figure 3. Shh selectively promotes fibroblast cell proliferation in vitro. (A) Representative micrographs show the phase-contrast images of renal interstitial fibroblasts after incubation with recombinant Shh. NRK-49Fs were incubated for 3 days with different concentrations of Shh as indicated. (B and C) Shh promotes fibroblast proliferation in a dose- and time-dependent manner. NRK-49F cells were incubated with (B) different concentrations of Shh for 3 days or (C) a fixed dose of Shh (100 ng/ml) for various periods of time as indicated. Cell numbers (×1000 per well) were counted and presented. *$P<0.05$ versus controls ($n=3$). (D) Graphic presentation shows that Shh promotes NRK-49F cell proliferation assessed by a colorimetric MTT assay. *$P<0.05$ versus controls ($n=3$). (E) Representative micrographs show that Shh promotes fibroblast DNA synthesis as shown by BrdU incorporation. NRK-49F cells were incubated with 50 and 100 ng/ml Shh for 2 days. Cells were immunostained with mouse anti-BrdU antibody (red). SYTO-Green (green) was used to visualize...
Interestingly, CPN selectively inhibited interstitial cell proliferation (Figure 7D) but did not affect the proliferation of tubular cells (Figure 7E).

To further elucidate the identity of these proliferating interstitial cells, we performed additional costaining for Ki67 (Figure 7F; red) and various cell type-specific markers (Figure 7F; green). As shown in Figure 7F, there was no costaining of Ki67 and CD31, a specific endothelial cell marker. Similarly, little costaining of Ki67 and CD45, a common leukocyte marker, was observed. However, Ki67 was largely colocalized with α-SMA, the marker of myofibroblasts (Figure 7F, arrows). Quantitative determination showed that over 80% of Ki67-positive cells were the α-SMA-positive myofibroblasts in diseased kidneys after IRI (Figure 7G), suggesting a predominant fibroblast proliferation at 10 days after IRI. Therefore, CPN largely targeted fibroblasts and selectively prevented their proliferation and expansion.

Consistent with a reduced fibroblast proliferation, CPN treatment also inhibited the expression of PDGFR-β and desmin, two signature proteins that define fibroblast activation. As illustrated by Western blot analyses, renal PDGFR-β and desmin were significantly reduced after CPN injection compared with vehicle controls (Figure 7, H and I). Immunohistochemical staining also revealed a marked suppression of PDGFR-β and desmin expressions by CPN (Figure 7K). Consistently, CPN also significantly inhibited renal expression of α-SMA and fibronectin proteins (Figure 7, H and J). These results show that blockade of Shh signaling by CPN reduces renal fibrosis through selectively inhibiting fibroblast proliferation and activation in diseased kidneys.

DISCUSSION

The proliferation and activation of interstitial fibroblasts are major and common pathologic features of CKD, which ultimately lead to an expansion of the matrix-producing cell population in diseased kidneys. However, the identity and sources of the extracellular cues that control fibroblast proliferation remained poorly characterized. The results presented in this study show that tubule-derived Shh is a novel, inducible, and potent mitogen that specifically promotes fibroblast proliferation in the neighboring interstitium. This conclusion is supported by multiple lines of in vitro and in vivo evidence. Our data illustrate a crucial role of Shh in regulating fibroblast expansion and renal fibrosis after kidney injury, primarily through mediating EMC.

Shh is the prototype of three ligands in the hedgehog signaling system. It is a cholesterol- and palmitoyl-modified secreted protein, and it functions as a classic morphogen, a substance governing tissue patterning during mammalian development.\textsuperscript{34,35} Shh exerts its action through binding to plasma membrane receptor Ptc1, which leads to Ptc1 internalization and degradation, resulting in derepression of the seven-transmembrane G protein--coupled receptor-like Smo.\textsuperscript{17,36} In normal adult kidneys, the Shh gene is relatively quiescent, and its expression is barely detectable in mice, rats, and humans (Figures 1 and 2). However, Shh expression is clearly induced predominantly in renal tubular epithelium after kidney injury. It should be pointed out that, although we have previously shown Shh induction in mouse UUO,\textsuperscript{22} another report indicates that it is not changed in the same model.\textsuperscript{23} The exact reason behind such a discrepancy remains unknown, but it could be related to the specific methodology used. Fabian et al.\textsuperscript{23} only assessed Shh expression by RT-PCR, an approach that is prone to variations in the specificity of the primers and experimental conditions. In contrast, our results on Shh induction after UUO were shown by a combination of RT-PCR, Western blotting, and immunohistochemical staining.\textsuperscript{22} Using three additional models of renal fibrotic diseases, we now have confirmed that Shh is induced in the diseased kidneys after IRI, ADR, and renal mass ablation (Figure 1). In addition, Shh is also induced in human kidney biopsies from patients with various CKD with distinct etiologies (Figure 2), suggesting the clinical relevance of Shh induction to the onset and progression of human kidney diseases as well. Taken together, our data show that Shh expression is induced in all four animal models (UUO, IRI, ADR nephropathy, and remnant kidney after 5/6 nephrectomy) tested and human CKD, indicating that Shh induction is a universal and common finding in the pathogenesis of a wide variety of CKD.

Although Shh is predominately induced in renal tubular epithelium of diseased kidneys, it selectively targets and specifically promotes interstitial fibroblast proliferation (Figures 1 and 3). This finding is consistent with earlier reports using Gli1\textsuperscript{LacZ} knockin mice, in which renal Gli1 is selectively induced in active fibroblasts in the interstitial compartment after UUO.\textsuperscript{22,23} In this study, we have revisited this issue by using Gli1-CreERT2 mice and showed that Gli1-driven Cre is specifically expressed in the vimentin-positive fibroblasts but not CD45-positive leukocytes and CD31-positive endothelial cells (Figure 1) after IRI. Therefore, using two models (UUO and IRI) and different reporter

\*P<0.05 versus controls (n=3). (O) Ptc1 is expressed in renal fibroblasts and induced by Shh. NRK-49 F cells were treated with different concentrations of Shh for 24 hours or 50 ng/ml Shh for various periods of time as indicated. (H–J) Shh does not promote renal proximal tubular cell proliferation. HKC-8 cells were incubated with (H) 100 ng/ml Shh for various periods of time or (I and J) different concentrations of Shh for 3 days. Cell proliferation was assessed by (H and I) cell number counting or (J) a colorimetric MTT assay. (K) Western blots show that Shh promotes the expression of numerous proliferation-related genes in fibroblasts. NRK-49F cells were incubated with Shh (50 ng/ml) for various periods of time as indicated. Cell lysates were subjected to Western blot analyses for c-myc, c-fos, PCNA, and actin.
mice (Gli1^lacZ and Gli-CreER^T2), we have clearly established that tubule-derived Shh selectively targets interstitial fibroblasts, leading to their proliferation and activation.22 The ability of Shh in promoting fibroblast proliferation is quite impressive, which was judged by cell counting, MTT assay, and BrdU incorporation assessment (Figure 3), suggesting that tubule-derived Shh could be the long-sought potent mitogen that controls the proliferation and expansion of fibroblasts in the adjacent interstitium. Although not tested, the possibility exists that Shh might affect fibroblast survival as well. The identification of Shh as a fibroblast growth factor is also in harmony with emerging evidence that tubular injury is an early and major event in renal fibrogenesis.9,10 Therefore, our studies have established that Shh, a soluble factor derived from the injured tubules, plays a crucial role in determining the size of the fibroblast population in diseased kidneys primarily by mediating EMC.

The process of fibroblast to myofibroblast transition during renal fibrosis is manifested by increased cell proliferation, de novo α-SMA expression, augmented production of interstitial matrix, and loss of erythropoietin-producing capabilities.1,4,37 It seems that Shh is a complete fibrogenic mitogen that not only promotes fibroblast proliferation but also induces their myofibroblastic transition, because Shh is capable of inducing α-SMA, collagen I, fibronectin, and desmin expression in cultured fibroblasts as previously reported.22 These combined effects of Shh on fibroblast proliferation and phenotypic transition would result in a dramatic expansion of the matrix-producing cell population in diseased kidneys. However, whether Shh-induced fibroblasts proliferation and their myofibroblastic activation occur in parallel or sequentially remains to be determined. Furthermore, it is also unclear whether the fibroblast proliferation and phenotypic transition controlled by Shh are mediated by common or separate intracellular signaling pathways.

The most interesting finding of this study is the confirmation of a crucial role of Shh in mediating fibroblast proliferation and renal fibrosis in vivo. Using a gain- or loss-of-function approach, we show that Shh promotes renal fibrosis during the transition from AKI to CKD after injury, a problem that is being increasingly recognized in clinical investigation.38–40 In agreement with in vitro data, overexpression of Shh through a hydrodynamic-based gene delivery approach results in significant expression of functional Shh, which was shown by renal Gli1 mRNA and protein induction (Figure 4), and markedly accelerates the progression of kidney fibrotic lesions (Figure 5). We chose to manipulate Shh expression at 3 days after IRI, because at this time point, the acute phase of kidney injury and repair has been almost completed in this model, which is judged by serum creatinine level.9,33 Consistently, inhibition of Shh signaling by the Smo inhibitor CPN also selectively blocks interstitial fibroblast proliferation and ameliorates kidney fibrosis (Figures 6 and 7). These results unambiguously show a fundamental role of Shh signaling in controlling fibroblast proliferation and renal fibrosis in vivo and suggest that blockade of Shh signaling with a small molecule inhibitor could be a plausible strategy for therapeutic intervention of CKD.

In summary, using multiple animal models and human kidney biopsies, we have shown that tubular induction of Shh is a common pathologic finding in a wide variety of CKD. These
studies also identify Shh as a novel, specific, and potent mitogen that selectively promotes fibroblast proliferation in vitro and in vivo. Therefore, Shh could be the long-sought, tubule-derived, fibroblast-specific growth factor in the injured kidneys. Our results also provide proof of principle that blockade of Shh signaling may be a novel strategy for therapeutic treatment of renal fibrosis in CKD.

CONCISE METHODS

Animal Models
Male BALB/c mice weighing about 20–25 g were obtained from Harlan Sprague–Dawley. Renal IRI was performed in BALB/c mice using an established protocol as described elsewhere. Briefly, bilateral renal pedicles were clamped for 35 minutes using microaneurysm clamps.

Figure 5. Overexpression of exogenous Shh in vivo promotes interstitial cell proliferation and accelerates the progression of renal fibrosis after AKI. (A) Representative micrographs show immunohistochemical staining for Ki67 at 7 days after plasmid injection. Boxed areas are enlarged in the right panels. Yellow arrows indicate Ki67-positive cells. Scale bar, 50 μm. (B) Quantitative determination of the Ki67-positive cells in renal tubular and interstitial compartments. *P<0.05 versus pcDNA3. HPF, high power field. (C and D) qRT-PCR analyses show that overexpression of Shh in vivo promoted renal expression of (C) collagen I and (D) collagen III after IRI. *P<0.05 versus pcDNA3 (n=4–6). (E) Western blot analyses of renal expression of various fibrosis-related genes. Kidney lysates were immunoblotted with specific antibodies against PCNA, PDGFR-β, desmin, fibronectin, α-SMA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Numbers (1–4) indicate each individual animal in a given group. (F and G) Graphic presentation of renal protein levels of various fibrosis-related genes. Data are presented as fold induction over pcDNA3 controls. *P<0.05 versus pcDNA3; **P<0.01 versus pcDNA3. (H) Representative micrographs show collagen deposition in the kidneys at 7 days after plasmid injection. Paraffin sections were subjected to MTS. Boxed areas are enlarged in the right panels. Arrows indicate collagen-deposited area with blue staining. Scale bar, 150 μm. (I) Graphical presentation shows kidney fibrotic lesions at 7 days after plasmid injection after quantitative determination. *P<0.05 versus pcDNA3.

BASIC RESEARCH

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During the ischemic period, body temperature was maintained between 35°C and 37.5°C using a temperature-controlled heating system. At 3 days after IRI, mice were subjected to either a single intravenous injection of Shh expression plasmid (Figure 4A) or daily intraperitoneal injections of CPN (Sigma-Aldrich, St. Louis, MO) at 5 mg/kg body wt for 7 days (Figure 6A) as previously reported.22,31 Mice were euthanized at 10 days after IRI, and kidney tissues were collected for various analyses. For the ADR nephropathy model, male BALB/c mice were administered with ADR (doxorubicin hydrochloride; Sigma-Aldrich) by a single intravenous injection at 10 mg/kg body wt as reported previously.24 Mice were euthanized at 5 weeks after ADR injection. In addition, the remnant kidney model was established in male Sprague–Dawley rats by

Figure 6. Blockade of Shh signaling reduces renal fibrosis after IRI. (A) Experimental design. Green arrowheads indicate the injection of CPN, whereas white arrowheads indicate vehicle injection. (B) qRT-PCR analyses show that CPN inhibited renal Gli1 mRNA expression. *P<0.05 versus sham controls; †P<0.05 versus vehicles (n=3). (C) Representative Western blots show renal Gli1 protein expression at different groups as indicated. Kidney lysates were immunoblotted with antibodies against Gli1 and actin. Veh, vehicle. (D) Representative micrographs show that CPN ameliorated renal fibrotic lesions after IRI. Kidney sections were subjected to MTS. Boxed areas are enlarged and presented in lower panels. Arrows indicate fibrotic areas with blue staining. Scale bar, 50 μm. (E) Quantitative determination of renal fibrotic lesions in different groups. Renal fibrotic lesions (defined as percentage of MTS-positive fibrotic area) were quantified by computer-aided morphometric analyses. †P<0.05 versus vehicles. (F–I) qRT-PCR analyses show that CPN inhibited renal expression of (F) collagen I, (G) collagen III, (H) fibronectin, and (I) α-SMA after IRI. *P<0.05 versus sham controls; †P<0.05 versus vehicles (n=3–5).
5/6 nephrectomy as described elsewhere. Kidneys were collected at 12 weeks and subjected to various analyses. In separate experiments, Gli1-CreERT2 transgenic mice, in which Cre recombinase is under control of the endogenous Gli1 promoter/enhancer elements, were obtained from The Jackson Laboratory (stock #007913). Mice were intraperitoneally injected with tamoxifen (T5648; Sigma-Aldrich) at 25 mg/kg body wt for 5 days and then subjected to IRI as described above. All animal experiments were performed in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee.

Figure 7. Blockade of Shh signaling selectively inhibits renal interstitial fibroblast proliferation. (A and B) Western blot analyses show that CPN inhibited renal PCNA expression at 10 days after IRI. Representative (A) Western blot and (B) quantitative data are presented. *P<0.05 versus vehicle. Numbers (1–3) indicate each individual animal in a given group. (C) Representative micrographs show that CPN inhibited renal interstitial cells proliferation after IRI. Double immunofluorescence staining for Ki67 (red) and laminin (green) shows that Ki67-positive cells were predominantly localized in the interstitial compartment. Boxed areas are enlarged and presented in right panels. Arrows indicate Ki67-positive cells in the interstitium, whereas arrowheads denote tubular cells with Ki67-positive staining. Scale bar, 50 μm. (D and E) CPN selectively inhibits interstitial but not tubular cell proliferation in vivo after IRI. Quantitative data on Ki67-positive cells in the (D) interstitial and (E) tubular compartments are presented. *P<0.05 versus vehicle. (F and G) Double immunofluorescence staining shows the costaining of Ki67 (red) and different cell markers (green) in the kidneys at 10 days after IRI. Cell type-specific markers used are CD31 (endothelial cells), CD45 (leukocytes), and α-SMA (myofibroblasts). Quantitative data on costaining for Ki67 and different cell type-specific markers are presented in G. DAPI, 4',6-diamidino-2-phenylindole. (H–J) Western blot analyses of renal PDGFR-β, desmin, fibronectin, and α-SMA expression at 10 days after IRI. Representative (H) Western blot and (I and J) quantitative data are presented. *P<0.05 versus vehicle. Numbers (1–3) indicate each individual animal in a given group. Fn, fibronectin. (K) Representative micrographs of immunohistochemical staining show that CPN inhibited renal PDGFR-β and desmin expression after IRI. Arrows indicate positive cells. Scale bar, 50 μm. HPF, high power field.
performing by procedures approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

**Construction of Shh Expression Vector and In Vivo Gene Transfer**

Plasmid vector containing human Shh cDNA was obtained from Addgene (pBBS-hShh, #13996). The Shh cDNA insert was released and subcloned into the mammalian expression vector p3xFlag (Agilent Technologies, Santa Clara, CA) using routine molecular cloning technique. The empty expression vector pcDNA3 was purchased from Invitrogen. Plasmid DNA was administered into mice by a hydrodynamic-based gene transfer technique through rapid injection of a large volume of DNA solution through the tail vein as described elsewhere. Briefly, 20 μg plasmid DNA was diluted in 1.8 ml saline and injected through the tail vein into mouse circulation within 5–10 seconds. Mice from the control group were injected with 20 μg empty vector pcDNA3 in an identical manner. Plasmids were administered at 3 days after IRI, mice were euthanized at 10 days (Figure 4A), and kidney tissue samples were collected.

**Human Kidney Biopsies Samples**

Human kidney specimens were obtained from diagnostic renal biopsies performed at the Nanfang Hospital, Southern Medical University. Nontumor kidney tissue from the patients who had renal cell carcinoma and underwent nephrectomy was used as normal controls. Paraffin-embedded human kidney biopsy sections (2.5–μm thickness) were prepared using a routine procedure. All studies involving human kidney sections were approved by the Institutional Review Board at the University of Pittsburgh and the Institutional Ethics Committee at the Southern Medical University.

**Cell Culture**

NRK-49F and mIMCD-3 cells were obtained from the American Type Culture Collection. HKC-8 was provided by L. Racusen (Johns Hopkins University, Baltimore, MD). Cells were maintained as described previously. Serum-starved NRK-49F, HKC-8, and mIMCD-3 cells were treated with recombinant human Shh protein (StemRD Inc., Burlingame, CA) at different concentrations for various periods of time as indicated. Cells were then collected and subjected to various analyses.

**Cell Proliferation Assay**

Cell proliferation was assessed by two approaches: cell counting and MTT assay. Cell numbers were counted by using a hemacytometer. Cell proliferation was also determined quantitatively by an MTT assay. Briefly, NRK-49F, HKC-8, and mIMCD-3 cells were seeded into 96-well plates at a density of 2×10^3/well. After adherence of cells, the cultures were changed to the serum-free medium and incubated for 24 hours followed by treatment with or without Shh at different concentrations for various periods of time as indicated. MTT (5 mg/ml) was added to the medium at 10 μl/well followed by incubation at 37°C for 4 hours. After the medium was removed, cells were lysed with 100 μl dimethyl sulfoxide. Absorbance of each well was measured by a microplate reader at 490-nm wavelength.

**BrdU Incorporation Assay**

The effect of Shh on fibroblast DNA synthesis was evaluated by BrdU incorporation. Briefly, cells were seeded onto 24-well plates and treated with various concentrations of Shh for 48 hours, and then, they were pulsed with BrdU (10 μM) for 24 hours. Cells were fixed with ice-cold 70% ethanol for 20 minutes, and DNA was denatured by incubation with 2.5 N HCL for 20 minutes followed by neutralization with 0.1 M boric acid. Endogenous peroxidase activity was quenched by incubating the cell with 3% H2O2 in PBS for 20 minutes, and nonspecific binding was blocked by incubating the cells with 10% donkey serum for 10 minutes at room temperature as described previously. Incorporated BrdU was detected with a mouse monoclonal anti-BrdU antibody (B2531; Sigma-Aldrich) followed by incubation with cyanine Cy3-conjugated, affinity-purified secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained cells were mounted with Vectashield antifade mounting media by using SYTO-Green to visualize the nuclei. Stained samples were viewed under an Eclipse E600 epifluorescence microscope equipped with a digital camera (Nikon, Melville, NY).

**Real-Time qRT-PCR**

Total RNA was extracted using the TRIzol RNA isolation system (Invitrogen). First-strand cDNA synthesis was carried out by using a reverse transcription system kit according to the instructions of the manufacturer (Promega, Madison, WI). qRT-PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously. The sequences of primer pairs for different genes are shown in Supplemental Table 2. PCR was run by using standard conditions. The mRNA levels of various genes were calculated after normalizing with β-actin.

**Western Blot Analysis**

Kidney tissues were lysed with radioimmunoprecipitation assay buffer containing 1% Tergitol-type NP-40, 0.1% SDS, 100 μg/ml PMSF, 1% protease inhibitor cocktail, and 1% phosphatase I and II inhibitor cocktail (Sigma-Aldrich) in PBS on ice. The supernatants were collected after centrifugation at 13,000×g at 4°C for 15 minutes. Protein expression was analyzed by Western blot analysis as described previously. The primary antibodies used were anti-Shh (sc-9024), anti–PDGFR-β (sc-432), anti-PCNA (sc-56), and anti–c-Myc (sc-40s) from Santa Cruz Biotechnology, anti-Gli1 (AF3455) from R&D Systems, anti-fibronectin (F3648), anti–α-SMA (A2547), anti-desmin (D1033), anti–Flag (F4042), and anti–α-tubulin (T9026) from Sigma-Aldrich, anti–c-fos (PC05) from Calbiochem, anti-actin (MAB1501) from Chemicon, and anti-GAPDH (#2118s) from Cell Signaling Technology.

**Histology and Immunohistochemical Staining**

Paraffin-embedded mouse kidney sections (3-μm thickness) were prepared by a routine procedure. The sections were stained with MTS reagents by standard protocol. Immunohistochemical staining was performed according to the established protocol as described previously. The antibodies against Shh (sc-9024; Santa Cruz Biotechnology), PDGFR-β (sc432; Santa Cruz Biotechnology), desmin (D1033; Sigma-Aldrich), and Ki67 (ab66155; Abcam, Inc.) were used.
Communofluorescence Staining and Confocal Microscopy

Kidney cryosections were fixed with 3.7% paraformaldehyde for 15 minutes at room temperature and immersed in 0.2% Triton X-100 for 10 minutes. After blocking with 10% donkey serum in PBS for 1 hour, slides were coimmunostained with the following antibodies: anti-Cre (MAB3120; EMD Millipore), anti-Ki67 (ab66155; Abcam, Inc.), anti-laminin (L8271; Sigma-Aldrich), anti-α-SMA (A2547; Sigma-Aldrich), anti-CD45 (ab60291; Abcam, Inc.), and anti-CD31 (550274; BD Pharmingen). To visualize the primary antibodies, slides were stained with cyanine Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Stained slides were viewed under a Leica TCS-SL confocal microscope equipped with a digital camera.

Statistical Analyses

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

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DISCLOSURES

None.

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