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PINCH-1 Promotes Tubular Epithelial-to-Mesenchymal Transition by Interacting with Integrin-Linked Kinase

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ABSTRACT

PINCH-1 is an adaptor protein that binds to the integrin-linked kinase (ILK), an intracellular serine/threonine protein kinase that plays a critical role in mediating tubular epithelial-to-mesenchymal transition (EMT). To determine whether PINCH-1 is also involved in the EMT process, we investigated its regulation and function during TGF-β1–stimulated EMT. TGF-β1 induced PINCH-1 mRNA and protein expression in human proximal tubular epithelial cells in a time-dependent fashion, an effect that was largely dependent on intracellular Smad signaling. Overexpression of PINCH-1 suppressed epithelial markers E-cadherin and ZO-1 and increased fibronectin expression and extracellular assembly, whereas knockdown of PINCH–1 *via* small interfering RNA reduced TGF–β1–mediated fibronectin expression and partially restored E–cadherin. PINCH–1 formed a ternary complex with ILK at the focal adhesion sites of tubular epithelial cells. Treatment with an ILK inhibitor or disruption of the ILK/PINCH-1 interaction by overexpressing a dominant-negative N-terminal ankyrin domain of ILK resulted in reduced fibronectin deposition, indicating that the ability of PINCH-1 to stimulate EMT is ILK-dependent. In a mouse model of obstructive nephropathy, PINCH-1 expression increased in a time-dependent manner, suggesting that it may play a role in EMT and renal fibrosis *in vivo*. We conclude that PINCH-1, through its interaction with ILK, plays an important role in regulating TGF-β1–mediated EMT and could be a potential future therapeutic target to prevent progression of renal disease.

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Tubular epithelial-to-mesenchymal transition (EMT) is increasingly recognized as one of the major pathways leading to generation of the matrix-producing effector cells in the fibrotic kidney after a diverse array of injuries.[1–4] Extensive studies indicate that TGF-β1, a potent fibrogenic cytokine whose expression is markedly induced in virtually every type of chronic kidney diseases,[5–8] is the driving force behind tubular EMT under pathologic conditions. *In vitro*, TGF-β1, as a single factor, is able to orchestrate the completion of the entire EMT course that consists of four key steps.[9,10] Blockade of TGF-β1 signaling through either up-regulating Smad transcriptional co-repressor SnoN by hepatocyte growth factor (HGF) or genetic ablation of Smad3 inhibits tubular EMT and attenuates renal interstitial fibrosis.[3,11–13]

Although the vital role of TGF-β1 in promoting tubular EMT is widely accepted, the mechanism by which TGF-β1 regulates the EMT process remains incompletely understood. We previously identified the integrin-linked kinase (ILK) as a key mediator in TGF-β1–induced EMT.[14] ILK is an intracellular serine/threonine protein kinase that interacts with the cytoplasmic domains of β integrins and mediates the integrin signaling in diverse types of cells.[15,16] Apart from its catalytic kinase activity, ILK is known as an adaptor protein that interacts with numerous intracellular proteins, including

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PINCH-1 (particularly interesting new cysteine-histidine rich protein-1),[17] the founding member of small family of proteins consisting of a tandem array of five LIN11, Isl1, and MEC-3 (LIM) domains that are involved in mediating protein–protein interactions.[18,19] Previous studies revealed that PINCH-1 and ILK form a complex in mammalian cells through an interaction between the LIM1 domain of PINCH-1 and the ankyrin (ANK) repeat domain of ILK.[20,21] Disruption of the PINCH-1/ILK complex formation has been shown to influence cell survival, proliferation, and matrix deposition in cultured cells.[20,22–25] Therefore, it seems that ILK works in concert with its interacting partner PINCH-1 to elicit its cellular function.

PINCH-1 is expressed in early embryonic development and adult tissues in a ubiquitous manner.[24,26] It primarily localizes at the focal adhesion sites of the culture cells, a pattern that overlaps with other focal adhesion proteins, including ILK. Knockout of PINCH-1 gene in mice and other model organisms such as *Caenorhabditis elegans* and *Drosophila* results in developmental defects in cell polarity, cell–matrix adhesion, cell proliferation, and apoptosis, leading to early embryonic death,[27–29] phenotypes that resemble the deletion of ILK or β1-integrin.[30–33] Despite that PINCH-1 has been shown to express in embryonic and adult kidney, relatively little is known about its regulation and function in the kidney in both physiologic and pathologic settings.

In this study, we demonstrate that PINCH-1 expression is induced in tubular epithelial cells by TGF-β1 and in the fibrotic kidney after ureteral obstruction. We further show that PINCH-1 is functionally involved in promoting tubular EMT after chronic injury, and the action of PINCH-1 is likely operated through its interaction with ILK.

RESULTS

TGF-β1 Induces PINCH-1 Expression in Tubular Epithelial Cells

Northern blot analysis demonstrated that TGF-β1 significantly induced PINCH-1 mRNA expression in human proximal tubular epithelial (HKC-8) cells. As shown in Figure 1A, the steady-state level of PINCH-1 mRNA increased at 6 h, sustained at least to 48 h after TGF-β1 treatment. PINCH-1 protein was also induced after TGF-β1 treatment in tubular epithelial cells, as demonstrated by Western blot analyses. As presented in Figure 1B, PINCH-1 protein began to increase at 12 h after TGF-β1, a time point that lagged significantly behind the mRNA induction (Figure 1A). The PINCH-1 protein induction was sustained to at least 72 h. Figure 1C shows the dose-response of PINCH-1 protein induction to TGF-β1 stimulation. Maximal induction was observed when HKC-8 cells were treated with 2 ng/ml TGF-β1. Further increase in TGF-β1 concentration did not result in additional induction of PINCH-1 in HKC-8 cells.

We next investigated the signal pathway that is responsible for the TGF-β1–mediated PINCH-1 induction. Previous studies showed that TGF-β1 activates multiple signal pathways, including Smad, Akt, and p38 mitogen-activated protein kinase in tubular epithelial cells.[14] However, blockade of Akt or p38 mitogen-activated protein kinase activation by specific chemical inhibitors did not affect TGF-β1–mediated PINCH-1 induction (Figure 2A). Of note, these inhibitors at the concentrations used were able to suppress the activation of their respective signaling in HKC-8 cells.[14,34] To examine the role of Smad signaling in PINCH-1 induction, we investigated the effect of the overexpression of a Smad transcriptional corepressor, SnoN. To this end, we established several cell lines that overexpress SnoN by stably transfecting HKC-8 cells with SnoN expression vector (Figure 2B). We found that overexpression of SnoN inhibited the TGF-β1–mediated PINCH-1 induction in tubular epithelial cells (Figure 2C). Compared with mock-transfection controls, PINCH-1 expression after TGF-β1 treatment was reduced substantially in the SnoN-overexpressing cells (Figure 2, C and D), suggesting that PINCH-1 induction by TGF-β1 is largely dependent on intracellular Smad signaling.

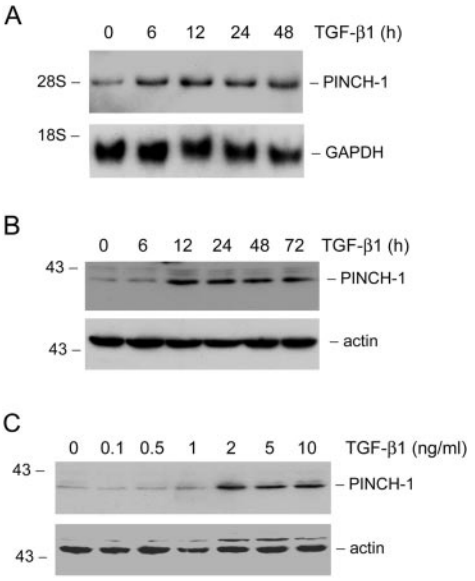

Figure 1. TGF-β1 induces PINCH-1 mRNA and protein expression in tubular epithelial cells. (A) Northern blot shows that TGF-β1 stimulated PINCH-1 mRNA expression in a time-dependent manner. HKC-8 cells were incubated with 2 ng/ml TGF-β1 for various periods as indicated. Total RNA was isolated and blotted with cDNA probes for PINCH-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. (B and C) Western blot analyses demonstrate that TGF-β1 induced PINCH-1 protein expression in a time- and dosage-dependent manner. HKC-8 cells were treated with 2 ng/ml TGF-β1 for various periods as indicated (B) or with various concentrations of TGF-β1 for 48 h (C). Total cell lysates were immunoblotted with specific antibodies against PINCH-1 and actin, respectively. Representative Western blots of three experiments.

Ectopic Expression of PINCH-1 Induces Tubular EMT

To investigate the functionality of PINCH-1 induction, we ex-

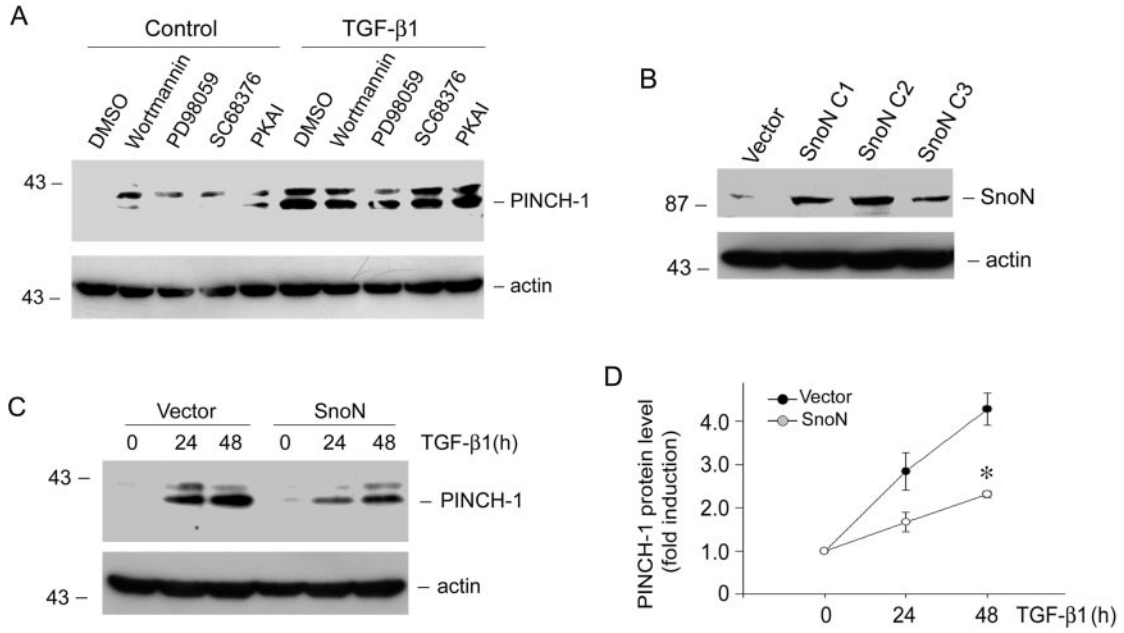

Figure 2. PINCH-1 induction by TGF-β1 is dependent on intracellular Smad signaling. (A) Pharmacologic inhibition of different signal transduction pathways did not affect PINCH-1 induction by TGF-β1. HKC-8 cells were pretreated with either various chemical inhibitors or vehicle (DMSO) for 30 min, followed by incubating in the absence or presence of 2 ng/ml TGF-β1 for 48 h. Specific inhibitors for phosphoinositide 3-kinase (10 nM wortmannin), Mek1 (10 μM PD98059), p38 mitogen-activated protein kinase (20 μM SC68376), and protein kinase A inhibitor (0.3 μM protein kinase A inhibitor) were used, respectively. (B) Establishment of stable SnoN-expressing cell lines. HKC-8 cells were stably transfected with SnoN expression plasmid (pHA-SnoN) or empty vector. Overexpression of SnoN in different cell clones was confirmed by Western blot. (C) Western blot showed that overexpression of SnoN suppressed PINCH-1 induction by TGF-β1. (D) Graphic presentation of the relative PINCH-1 abundance normalized to actin after TGF-β1 treatment. Data are means $\pm$ SEM of three experiments. *$P < 0.05$ *versus* vector controls.

amined the effect of overexpression of exogenous PINCH-1 on tubular epithelial cell phenotypes. To this end, we established several stable cell lines that overexpress PINCH-1 by transfecting with either the expression vector of the Flag-tagged PINCH-1 or empty vector. Overexpression of the Flag-tagged PINCH-1 was confirmed by Western blotting with anti-Flag antibody (Figure 3A). As shown in Figure 3B, ectopic expression of PINCH-1 suppressed epithelial cell marker E-cadherin expression in tubular epithelial cells. In addition, expression of exogenous PINCH-1 induced fibronectin expression and deposition (Figure 3C). The magnitude of E-cadherin suppression and fibronectin induction elicited by overexpressing PINCH-1 was comparable to that induced by TGF-β1.

Figure 3D shows the immunofluorescence staining of E-cadherin and fibronectin in tubular epithelial cells. Compared with the empty vector controls, overexpression of PINCH-1 resulted in disappearance of E-cadherin and zonula occludens-1 (ZO-1) staining in plasma membrane (Figure 3D), whereas extracellular matrix component fibronectin expression was markedly induced in PINCH-1–overexpressing cells. Hence, it seems that PINCH-1 triggers E-cadherin and ZO-1 suppression and fibronectin induction, the reciprocal changes associated with the phenotypic transition from epithelial to mesenchyme.

Knockdown of PINCH-1 Expression Blocks EMT

To characterize further the potential function of PINCH-1 in tubular epithelial cells, we investigated the impact of the knockdown of endogenous PINCH-1 in tubular epithelial cells. For this purpose, tubular epithelial HKC-8 cells were transfected with PINCH-1–specific small interfering RNA (siRNA) or control siRNA. As shown in Figure 4, A and B, transfection of HKC-8 cells with specific siRNA resulted in significant reduction of endogenous PINCH-1 mRNA and protein expression. Approximately 85% of HKC-8 cells were transfected with siRNA, as illustrated by a transfection efficiency assessment (Figure 4C). We found that knockdown of PINCH-1 only partially restored E-cadherin expression (Figure 4, D and E). This may reflect that another downstream effector of TGF-β1 signaling, Id1, also plays a role in conferring E-cadherin suppression by an ILK-independent mechanism.[35] However, downregulation of PINCH-1 reduced fibronectin expression and deposition in basal conditions (Figure 4F, lane 3 *versus* 1) and completely prevented fibronectin overproduction in response to TGF-β1 stimulation (Figure 4F, lane 6 *versus* 4).

PINCH-1 Forms a Complex with ILK at Focal Adhesions

PINCH-1 and ILK have been shown to interact with each

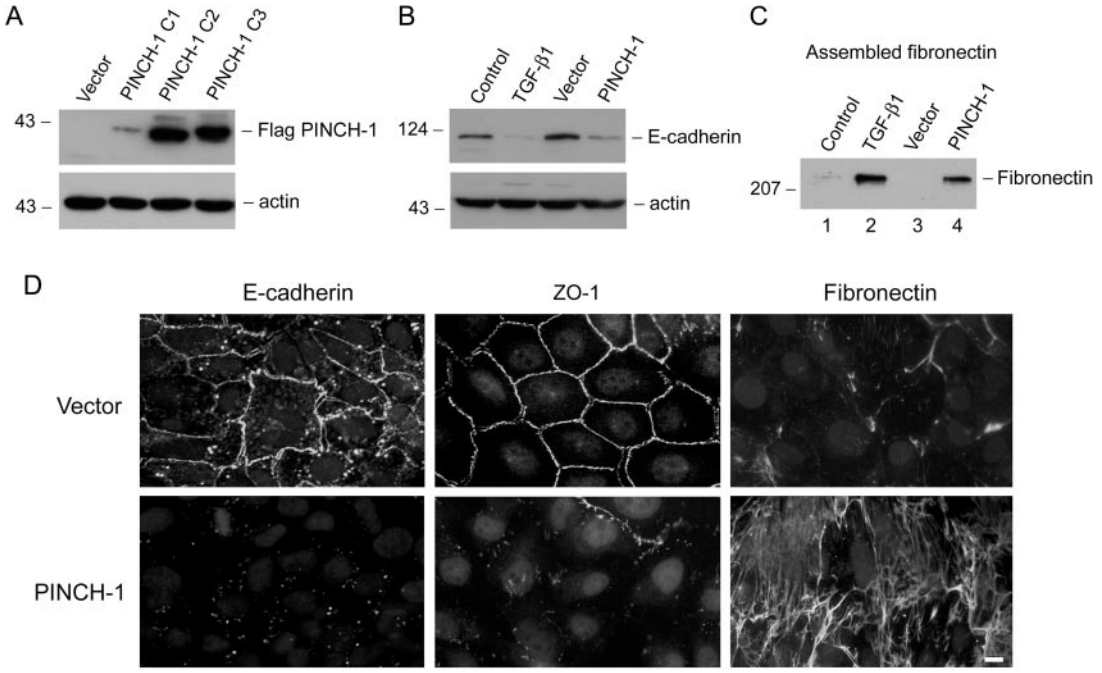

Figure 3. Ectopic expression of PINCH-1 results in loss of the epithelial markers E-cadherin and zonula occludens-1 (ZO-1) and induction of fibronectin. (A) Establishment of stable cell lines overexpressing PINCH-1. HKC-8 cells were stably transfected with either the expression vector of the Flag-tagged PINCH-1 or empty vector. The overexpression of the Flag-tagged PINCH-1 was confirmed in several stable cell lines by Western blotting with anti-Flag antibody (A). (B) Forced expression of PINCH-1 suppressed E-cadherin expression in tubular epithelial cells. TGF-β1 treatment (2 ng/ml) of HKC-8 cells was used as positive control. (C) Forced expression of PINCH-1 induced fibronectin expression and assembly. Assembled extracellular fibronectin was extracted and detected by Western blot. Representative Western blots of three experiments. (D) Immunofluorescence staining showed that overexpression of PINCH-1 suppressed epithelial markers E-cadherin and ZO-1 and induced fibronectin expression in tubular epithelial cells. Bar = 5 μm.

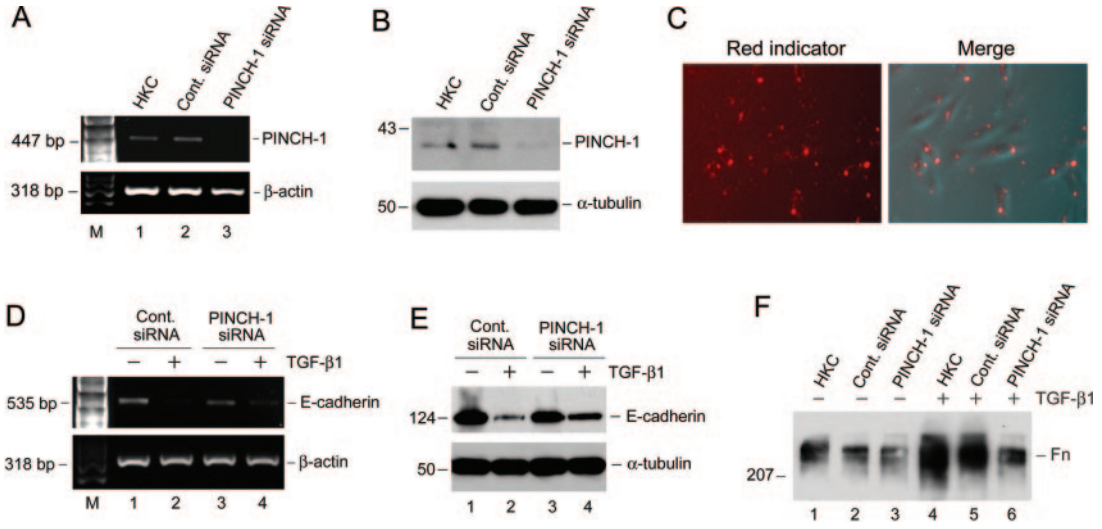

Figure 4. Knockdown of endogenous PINCH-1 expression partially restores E-cadherin expression and inhibits fibronectin induction. (A through C) Knockdown endogenous PINCH-1 expression by small interfering RNA (siRNA) strategy. Reverse transcriptase–PCR (A) and Western blot (B) demonstrated a reduced PINCH-1 mRNA and protein expression after transfection of PINCH-1–specific siRNA. (C) Micrographs showed a high efficiency of siRNA transfection in HKC-8 cells, as illustrated by siGLO transfection indicator (red). (D and E) Knockdown of endogenous PINCH-1 partially restored E-cadherin mRNA (D) and protein (E) expression repressed by TGF-β1. HKC-8 cells were transfected with either control or PINCH-1 siRNA, followed by incubation with 2 ng/ml TGF-β1 for 48 h. (F) Western blot analysis showed that knockdown of PINCH-1 expression suppressed fibronectin expression in response to TGF-β1 stimulation. Representative blots of two (D and F) or three (E) experiments.

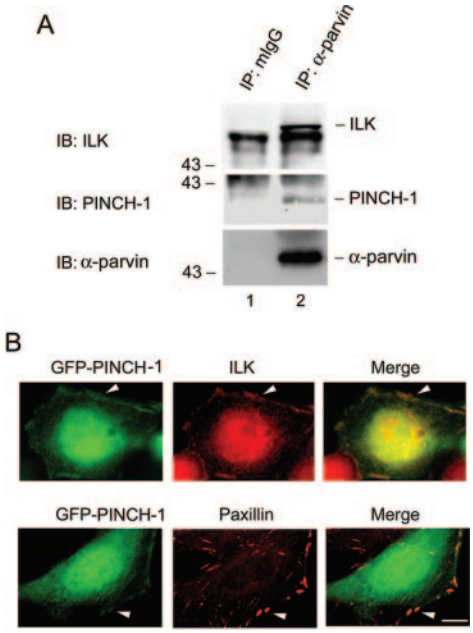

Figure 5. PINCH-1 forms a complex with integrin-linked kinase (ILK) at focal adhesion sites. (A) Co-immunoprecipitation demonstrated a ternary complex formation among PINCH-1, ILK, and α-parvin. HKC-8 cell lysates were immunoprecipitated with specific antibody against α-parvin, followed by immunoblotting with antibodies against PINCH-1, ILK, and α-parvin, respectively. mIgG, control mouse IgG. (B) Immunofluorescence staining exhibited the co-localization of PINCH-1 and ILK at the focal adhesion sites. HKC-8 cells were transiently transfected with green fluorescence protein (GFP)-tagged PINCH-1 plasmid, followed by staining with anti-ILK and anti-paxillin antibodies. Arrows indicate the co-localization sites at focal adhesions. Bar = 5 μm.

other, and together with α-parvin, they form a ternary complex that is important for ILK-mediated cellular processes in other cell type.[16,19] To investigate whether PINCH-1 forms a complex with ILK in tubular epithelial cells, we first examined the potential ILK–PINCH-1 complex formation by immunoprecipitation. As shown in Figure 5A, when HKC-8 cell lysates were immunoprecipitated with anti–α-parvin antibody, both ILK and PINCH-1 were detected in the precipitated complexes. Neither ILK nor PINCH-1 was found in the precipitates by normal control IgG. Therefore, ILK can form a ternary complex with PINCH-1 and α-parvin in tubular epithelial cells.

Immunofluorescence staining revealed a co-localization of ILK and PINCH-1 along the focal adhesion sites in tubular epithelial cells. Transfection of the HKC-8 cells with green fluorescence protein–PINCH-1 expression vector illustrated that the green fluorescence protein–tagged PINCH-1 was primarily localized in focal adhesions, the contact sites of plasma membrane to substrate (Figure 5B). Merging of PINCH-1 and ILK staining revealed a clear co-localization. The localization of PINCH-1 at the focal adhesion sites was confirmed by its co-staining with paxillin, a focal adhesion protein (Figure 5B).

Disruption of the ILK–PINCH-1 Interaction Inhibits Fibronectin Deposition

Because PINCH-1 forms a ternary complex with ILK in tubular epithelial cells (Figure 5), we sought to investigate whether PINCH-1 action depends on its interaction with ILK. To this end, we used a dominant negative approach by overexpressing the truncated form of ILK that merely contains the ankyrin domain, the binding site of ILK to PINCH-1.[22] Overexpression of Flag-ANK fragment in HKC-8 cells was confirmed after infection with recombinant adenovirus harboring Flag-tagged ankyrin domain sequence (Figure 6A). Immunostaining revealed that approximately 95% of the cells were infected and expressed exogenous protein (data not shown). Because Flag-

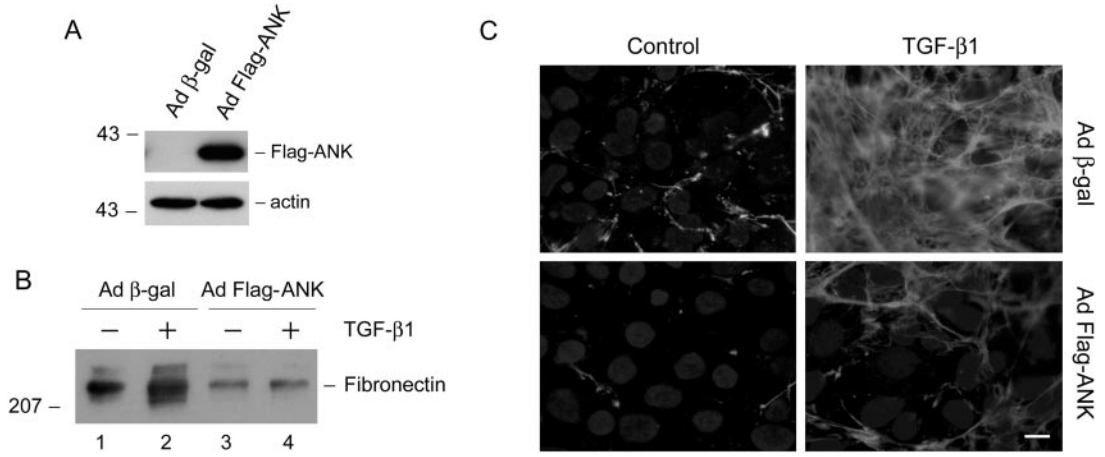

Figure 6. Disruption of the ILK–PINCH-1 interaction inhibits fibronectin expression and deposition. (A) HKC-8 cells were infected with either adenovirus harboring the Flag-tagged ILK N-terminal ANK fragment (Ad Flag-ANK) or control adenovirus containing β-galactosidase (Ad β-gal). Overexpression of Flag-ANK was confirmed by Western blot. (B) Forced expression of dominant negative Flag-ANK reduced fibronectin expression and deposition at both basal and TGF-β1–stimulated conditions. Representative Western blots of three experiments. (C) Immunofluorescence staining showed that disruption of the ILK–PINCH-1 interaction by dominant negative Flag-ANK inhibited fibronectin expression and assembly in HKC-8 cells. Bar = 5 μm.

ANK competes with endogenous ILK to bind PINCH-1,[22] overexpression of Flag-ANK presumably leads to the disruption of ILK–PINCH-1 interaction in a dominant negative manner. As shown in Figure 6B, disruption of ILK–PINCH-1 interaction significantly reduced fibronectin expression and deposition at both basal and TGF-β1–stimulated conditions. Similar results were obtained when the cells were immunostained with antifibronectin antibody (Figure 6C). These results suggest that PINCH-1 action dependent on its interaction with ILK.

Small Molecule ILK Inhibitor Abrogates PINCH-1–Mediated EMT

To establish further the involvement of ILK in mediating PINCH-1 action, we examined the effects of ILK inhibition on tubular EMT induced by PINCH-1 overexpression. As shown in Figure 7A, a highly selective small molecule ILK inhibitor, QLT0267, restored E-cadherin expression in the PINCH-1–overexpressing tubular epithelial cells. Likewise, QLT0267 inhibited fibronectin expression induced by PINCH-1 in tubular epithelial cells in a dosage-dependent manner (Figure 7B). Immunofluorescence staining also showed that QLT0267 inhibited fibronectin expression and largely restored ZO-1 expression in the PINCH-1–overexpressing cells (Figure 7C). Therefore, inhibition of ILK abolishes the phenotypic conversion of tubular epithelial cells induced by PINCH-1 overexpression.

PINCH-1 Expression Is Induced in the Fibrotic Kidney

To investigate the relevance of PINCH-1 induction to renal fibrosis *in vivo*, we examined the expression of PINCH-1 in the evolution of renal interstitial fibrosis induced by ureteral obstruction. As shown in Figure 8, Western blot analyses revealed that PINCH-1 was markedly induced in the fibrotic kidney in a time-dependent manner. PINCH-1 protein began to increase as early as day 1 after unilateral ureteral obstruction (UUO), and significant induction was observed at 3 d (Figure 8A), a time point preceding the onset of EMT in this model.[36] Quantitative determination exhibited approximately eight-fold induction of the relative abundance of PINCH-1 protein in the obstructed kidney at 14 d after UUO, when compared with the sham controls (Figure 8B). Hence, PINCH-1 induction correlates with tubular EMT and renal interstitial fibrogenesis *in vivo*.

DISCUSSION

The results presented in this study demonstrate that PINCH-1, a LIM domain-only adaptor protein that interacts with ILK, plays a critical role in mediating tubular EMT induced by TGF-β1. We have shown that PINCH-1 expression is induced during TGF-β1–mediated EMT, and such induction is dependent on, at least in part, Smad signaling, an intracellular signal pathway that is essential for EMT.[12,37] Moreover, forced expression of exogenous PINCH-1 mimics TGF-β1 action, resulting in loss of E-cadherin and ZO-1 and induction of fibronectin. Conversely, knockdown of PINCH-1 completely blocks TGF-β1–mediated fibronectin expression and partially restores E-cadherin. Furthermore, PINCH-1 co-localizes with ILK at the focal adhesion sites of tubular epithelial cells and physically interacts with ILK, and disruption of PINCH-1–ILK interaction causes a complete blockade of the TGF-β1–mediated fibronectin induction. Finally, PINCH-1 is upregulated in the obstructed kidney, and its expression is associated with tubular EMT and renal interstitial fibrosis *in vivo*. These observations, together with previous studies,[14] have established that PINCH-1–ILK complex may act as a functional element that plays a crucial role in mediating TGF-β1–triggered tubular EMT, thereby having significant implications in the pathogenesis of renal interstitial fibrosis.

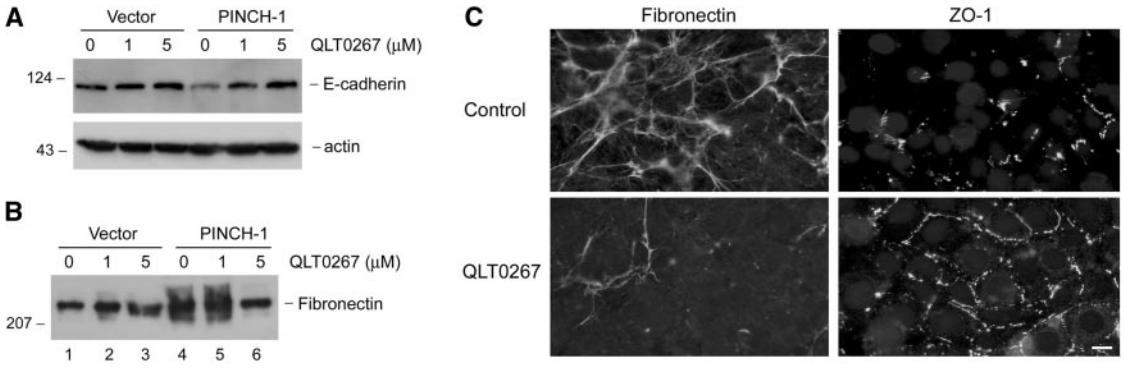

Figure 7. Small molecule ILK inhibitor QLT0267 blocks tubular epithelial-to-mesenchymal transition (EMT) induced by PINCH-1. (A) Small molecule ILK inhibitor restored E-cadherin expression in PINCH-1–overexpressing tubular epithelial cells. Stable PINCH-1–overexpressing (PINCH-1) and control (Vector) cells were treated with for 48 h at various concentrations as indicated. (B) QLT0267 inhibited fibronectin expression in the PINCH-1–overexpressing cells in a dosage-dependent manner. Representative Western blots of two experiments. (C) Immunofluorescence staining showed that small molecule ILK inhibitor QLT0267 inhibited fibronectin expression and largely restored ZO-1 expression in the PINCH-1–overexpressing cells. Bar = 5 μm.

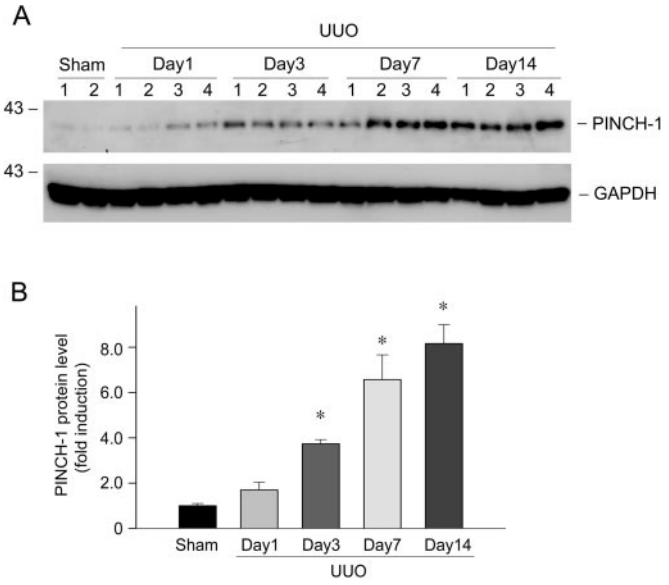

Figure 8. PINCH-1 is induced in the fibrotic kidney after unilateral ureteral obstruction (UUO). (A) Western blot analysis showed a marked induction of PINCH-1 in the fibrotic kidney induced by UUO in a time-dependent manner. Kidney tissue lysates were prepared from the obstructed kidneys at different time points after UUO as indicated. The samples were immunoblotted with specific antibodies against PINCH-1 and GAPDH, respectively. (B) Graphic presentation of the relative abundance of PINCH-1 normalized to GAPDH in the obstructed kidneys at different time points after UUO. Data are means ± SEM ($n = 4$). $P < 0.05$ *versus* sham control.

PINCH-1 is expressed early in embryonic development and in adult kidney[26]; however, little is known from the literature about its regulation and function in renal tissues. This study provides clear evidence that PINCH-1 expression at both mRNA and protein levels is induced in the process of tubular EMT triggered by TGF-β1. The intuitive association among PINCH-1, TGF-β1, and EMT is also observed in the obstructed kidney *in vivo*, in which both TGF-β1 and PINCH-1 are upregulated and tubular EMT is abundant.[9,38] PINCH-1 induction by TGF-β1 most likely occurs at the gene transcription level, because its steady-state mRNA abundance is increased after TGF-β1 stimulation (Figure 1). Consistent with this, PINCH-1 mRNA induction significantly precedes an elevation of its protein abundance (Figure 1). Therefore, it seems clear that PINCH-1 is a target gene of TGF-β1/Smad signaling. This is also corroborated by the observation that blocking Smad signaling by overexpressing SnoN reduces PINCH-1 induction by TGF-β1 (Figure 2).

The magnitude of PINCH-1 protein induction after TGF-β1 treatment (four-fold) seems significantly greater than its mRNA increase (2.3-fold) in tubular epithelial cells. This is consistent with recent studies demonstrating that the assembly of PINCH-1–ILK complex mutually stabilizes each other by preventing the proteolytic degradation of PINCH-1 and ILK.[23] It should be stressed that ILK is also induced concurrently by TGF-β1 in tubular epithelial cells during EMT.[14] Increased ILK will form complex with PINCH-1, which occurs before their localization to the cell–matrix adhesion sites,[39] thereby enhancing PINCH-1 protein stability and increasing its abundance. Accordingly, depletion of ILK *via* siRNA inhibition has been shown to reduce PINCH-1 protein, but not mRNA, levels in other cell types.[23] It therefore is conceivable that PINCH-1 and ILK depend on each other for maintenance of their protein levels in mammalian cells. Needless to say, the simultaneous induction of both PINCH-1 and ILK expression after TGF-β1 treatment will result in a substantial increase in PINCH-1–ILK complex formation during the process of tubular EMT.

One of the important observations in this study is that the functionality of PINCH-1 in tubular EMT is dependent on its interaction with ILK. This notion is supported by several observations. First, PINCH-1 physically interacts with ILK and forms a complex with it at focal adhesion sites (Figure 5). Second, disruption of PINCH-1–ILK interaction by overexpression of the ILK-ANK fragment, which results in competitive binding to endogenous PINCH-1 and reduces native PINCH-1–ILK interaction in a dominant negative manner,[22] abolishes fibronectin induction, suggesting that PINCH-1 action necessitates its interaction with ILK (Figure 6). Third, inhibition of ILK activity by selective small molecule inhibitor[40] abrogates PINCH-1–mediated E-cadherin suppression and fibronectin induction, providing a direct evidence for the involvement of ILK in PINCH-1 function in tubular epithelial cells. The dependence of PINCH-1 on ILK to promote tubular EMT is also highly consistent with several previous studies demonstrating the importance of PINCH-1–ILK complex formation in eliciting their biologic function.[22,23,39] Structurally, PINCH-1 protein merely consists of a tandem array of five LIM domains and a short C-terminal tail.[16] Such simple structural arrangement suggests that PINCH-1 may function primarily through its ability to interact with other proteins. It is plausible to postulate that PINCH-1 promotes tubular EMT mainly through binding to ILK, which leads to the recruitment of ILK to the close proximity of plasma membrane and presentation of it to the integrins. However, at this stage, we cannot completely rule out the possibility that PINCH-1 may promote tubular EMT through other mechanisms as well.

This study represents the first demonstration that PINCH-1, an adaptor protein that interacts with ILK, is dysregulated in the fibrotic kidney after obstructive injury. The concomitant induction of both PINCH-1 (as presented in this study) and ILK (as reported previously[14]) underscores that PINCH-1–ILK complex may play a fundamental role in mediating tubular EMT in the obstructed kidney, in which more than one third of the matrix-producing fibroblasts are known to derive from tubular epithelial cells through EMT.[38] In addition, the characteristic kinetic of PINCH-1 and ILK induction further strengthens this notion. Undoubtedly, further investigations on the regulation, function, and mechanism of PINCH-1 in the setting of renal fibrosis are warranted to gain

mechanistic insights into the regulation of tubular EMT and to offer new clues for designing rational strategies for the treatment of chronic kidney diseases.

CONCISE METHODS

Cell Culture and Cytokine Treatment

HKC-8 was provided by Dr. L. Racusen (Johns Hopkins University, Baltimore, MD). Cells were cultured in DMEM-Ham's F12 medium supplemented with 5% FBS. Serum-starved HKC-8 cells were treated with recombinant TGF-β1 (R & D Systems, Minneapolis, MN) for various periods of time at the concentration of 2 ng/ml except as otherwise indicated. The cells were then collected for Northern and Western blot analyses and immunofluorescence staining. In some experiments, cells were pretreated for 30 min with various chemical inhibitors at the concentrations specified, followed by incubating in the absence or presence of 2 ng/ml TGF-β1. PD98059, wortmannin, myristoylated protein kinase A inhibitor, and SC68376 were purchased from Calbiochem (La Jolla, CA). Small molecule ILK inhibitor QLT0267 was provided by QLT (Vancouver, BC, Canada).[41] 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 HA-tagged SnoN expression vector (provided by Dr. R. Weinberg, MIT, Cambridge, MA)[42] and Flag-tagged PINCH-1 expression plasmid,[23] respectively, using the Lipofectamine 2000 according to the instructions specified by the manufacturer (Invitrogen, Carlsbad, CA). The empty vector pcDNA3 (Invitrogen) or p3XFlag-CMV (Sigma) was used as a mock-transfection control. Twenty-four hours after transfection and every 3 to 4 d thereafter, the cells were refed with fresh selective medium containing G418 (Geneticin; Invitrogen) at a final concentration of 0.8 mg/ml. Neomycin-resistant clones were selected and expanded individually. Ectopic expression of SnoN and PINCH-1 in the stable cell lines was confirmed by Western blot analysis.

Northern Blot Analysis and Reverse Transcriptase–PCR

Total RNA was extracted from HKC-8 cells by using TRIzol reagent, according to the instructions specified by the manufacturer (Invitrogen). Northern blot analysis for gene expression was carried out by the procedures described previously.[36] ^{32}P-labeled DNA probes were prepared by the random primer labeling kit (Stratagene, La Jolla, CA). After autoradiography, membranes were stripped and rehybridized with glyceraldehyde-3-phosphate dehydrogenase probe to ensure equal loading of each lane.

For reverse transcriptase–PCR, the first strand of cDNA was synthesized using 2 μg of RNA in 20 μl of reaction buffer by reverse transcription using AMV-RT (Promega, Madison, WI) and random primers at 42°C for 30 min. PCR was carried out using a standard PCR kit on 1-μl aliquots of cDNA and HotStarTaq polymerase (Qiagen, Valencia, CA) with specific primer pairs designed for PINCH-1, E-cadherin, and β-actin, as described previously.[36] The sequences of primer pairs were as follows. PINCH-1 forward 5′-AAGAACGACCCCTACCATCC and reverse 5′-TGGCTTCATGTCAAACTCCA; E-cadherin forward 5′-TGGCTGAAGGTGACAGAGC and reverse 5′-CGTTAGCCTCGTTCTCAGG; and β-actin forward 5′-TCAAGATCATTGCTCCTCCTGAGC and reverse 5′-TGCTGTCACCTTCACCGTTCCAGT.

Western Blot Analysis

Western blot analysis for specific protein expression was performed essentially according to an established procedure.[11] The extracellular assembly of fibronectin in the deoxycholate-insoluble fraction was quantitatively determined by an established method as described previously.[14] The primary antibodies used were as follows: Rabbit polyclonal anti-PINCH-1 as described previously,[20] anti-ILK (05-575; Upstate Biotechnology, Charlottesville, VA), anti-SnoN (sc-9595), and anti-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA); anti–α-smooth muscle actin (α-SMA; clone 1A4; Sigma); anti–E-cadherin (clone 36) and anti-fibronectin (clone 10; BD Transduction Laboratories, Lexington, KY); anti-Flag (M2) and anti–α-tubulin (T9026; Sigma); and anti–glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX) and anti–α-parvin.[39] Quantification was performed by measurement of the intensity of the bands with the use of National Institutes of Health Image analysis software.

Immunofluorescence Staining

Indirect immunofluorescence staining was performed using an established procedure.[11] Briefly, cells cultured on coverslips were washed with cold PBS twice and fixed with cold methanol:acetone (1:1) for 10 min at −20°C. After extensive washing with PBS containing 0.5% BSA for three times, the cells were blocked with 20% normal donkey serum in PBS buffer for 30 min at room temperature and then incubated with the specific primary antibodies against E-cadherin, fibronectin, and ILK as described previously, as well as anti–ZO-1 (61–7300; Invitrogen) and anti-paxillin (610052; BD Transduction). For visualization of the primary antibodies, cells were stained with cyanine Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). As a negative control, the primary antibody was replaced with nonimmune IgG, and no staining occurred. Cells were double stained with DAPI (4′,6-diamidino-2-phenylindole, HCl) to visualize the nuclei. Stained cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and viewed under Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera (Melville, NY).

Immunoprecipitation

Immunoprecipitation experiments were performed using similar methods as described previously.[43] Briefly, HKC-8 cell lysates were centrifuged at 12,000 × g for 10 min at 4°C. The resulting supernatants were collected for immunoprecipitation. After preclearing with normal host IgG, the lysates were immunoprecipitated overnight at 4°C with 2 μg of anti–α-parvin or normal mouse IgG, followed by precipitation with 30 μl of protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 3 h at 4°C. The precipitated complexes were boiled for 5 min in SDS sample buffer, followed by immunoblotting with various antibodies as indicated.

siRNA Inhibition

The PINCH-1–specific siRNA duplex was purchased from Ambion or generated in our laboratory by using Silencer siRNA construction kit (Ambion), as described previously.[44] The oligonucleotide template for silencing PINCH-1 gene contains 21-nt target sequence 5′-AAG-GTGATGTGGTCTCTGCTC-3′ and 8-nt leader sequence 5′-CCT-GTCTC-3′ that is complementary to the T7 promoter primer. An unrelated siRNA template (with no sequence homology) was also used to generate negative control siRNA. PINCH-1–specific and control siRNA duplexes were transiently transfected into HKC-8 cells by using the siPORT NeoFX transfection reagents (Ambion). si*GLO* Red Transfection Indicator (Dharmacon, Chicago, IL) was used for transfection efficiency assessment. Whole-cell lysates were prepared and subjected to various analyses.

Animal Model

Male CD-1 mice weighing approximately 20 to 22 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). UUO was performed using an established procedure, as described previously.[45] Mice were killed at various time points as indicated after surgery, and kidneys were removed. PINCH-1 expression was examined by Western blot analysis of whole kidney lysates.

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DISCLOSURES

None.

REFERENCES

1. Strutz F, Zeisberg M: Renal fibroblasts and myofibroblasts in chronic kidney disease. *J Am Soc Nephrol* 17: 2992–2998, 2006
2. Liu Y: Epithelial to mesenchymal transition in renal fibrogenesis: Pathologic significance, molecular mechanism, and therapeutic intervention. *J Am Soc Nephrol* 15: 1–12, 2004
3. Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 112: 1776–1784, 2003
4. Neilson EG: Mechanisms of disease: Fibroblasts—A new look at an old problem. *Nat Clin Pract Nephrol* 2: 101–108, 2006
5. Bottinger EP, Bitzer M: TGF-beta signaling in renal disease. *J Am Soc Nephrol* 13: 2600–2610, 2002
6. Eddy AA: Progression in chronic kidney disease. *Adv Chronic Kidney Dis* 12: 353–365, 2005
7. Schnaper HW, Hayashida T, Hubchak SC, Poncelet AC: TGF-beta signal transduction and mesangial cell fibrogenesis. *Am J Physiol Renal Physiol* 284: F243–F252, 2003
8. Liu Y: Renal fibrosis: New insights into the pathogenesis and therapeutics. *Kidney Int* 69: 213–217, 2006
9. Yang J, Liu Y: Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. *Am J Pathol* 159: 1465–1475, 2001
10. Fan JM, Ng YY, Hill PA, Nikolic-Paterson DJ, Mu W, Atkins RC, Lan HY: Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro. *Kidney Int* 56: 1455–1467, 1999
11. Yang J, Liu Y: Blockage of tubular epithelial to myofibroblast transition by hepatocyte growth factor prevents renal interstitial fibrosis. *J Am Soc Nephrol* 13: 96–107, 2002
12. Yang J, Dai C, Liu Y: A novel mechanism by which hepatocyte growth factor blocks tubular epithelial to mesenchymal transition. *J Am Soc Nephrol* 16: 68–78, 2005
13. Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A: Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J Clin Invest* 112: 1486–1494, 2003
14. Li Y, Yang J, Dai C, Wu C, Liu Y: Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. *J Clin Invest* 112: 503–516, 2003
15. Hannigan G, Troussard AA, Dedhar S: Integrin-linked kinase: A cancer therapeutic target unique among its ILK. *Nat Rev Cancer* 5: 51–63, 2005
16. Wu C: PINCH, N(i)ck and the ILK: Network wiring at cell-matrix adhesions. *Trends Cell Biol* 15: 460–466, 2005
17. Campana WM, Myers RR, Rearden A: Identification of PINCH in Schwann cells and DRG neurons: Shuttling and signaling after nerve injury. *Glia* 41: 213–223, 2003
18. Wu C, Dedhar S: Integrin-linked kinase (ILK) and its interactors: A new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J Cell Biol* 155: 505–510, 2001
19. Wu C: The PINCH-ILK-parvin complexes: Assembly, functions and regulation. *Biochim Biophys Acta* 1692: 55–62, 2004
20. Tu Y, Li F, Goicoechea S, Wu C: The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. *Mol Cell Biol* 19: 2425–2434, 1999
21. Jung KY, Chen K, Kretzler M, Wu C: TGF-beta1 regulates the PINCH-1-integrin-linked kinase-alpha-parvin complex in glomerular cells. *J Am Soc Nephrol* 18: 66–73, 2007
22. Guo L, Wu C: Regulation of fibronectin matrix deposition and cell proliferation by the PINCH-ILK-CH-ILKBP complex. *FASEB J* 16: 1298–1300, 2002
23. Fukuda T, Chen K, Shi X, Wu C: PINCH-1 is an obligate partner of integrin-linked kinase (ILK) functioning in cell shape modulation, motility, and survival. *J Biol Chem* 278: 51324–51333, 2003
24. Yang Y, Guo L, Blattner SM, Mundel P, Kretzler M, Wu C: Formation and phosphorylation of the PINCH-1-integrin linked kinase-alpha-parvin complex are important for regulation of renal glomerular podocyte adhesion, architecture, and survival. *J Am Soc Nephrol* 16: 1966–1976, 2005
25. Legate KR, Montanez E, Kudlacek O, Fassler R: ILK, PINCH and parvin: The tIPP of integrin signalling. *Nat Rev Mol Cell Biol* 7: 20–31, 2006
26. Braun A, Bordoy R, Stanchi F, Moser M, Kostka GG, Ehler E, Brandau O, Fassler R: PINCH2 is a new five LIM domain protein, homologous to PINCH and localized to focal adhesions. *Exp Cell Res* 284: 239–250, 2003
27. Liang X, Zhou Q, Li X, Sun Y, Lu M, Dalton N, Ross J Jr, Chen J: PINCH1 plays an essential role in early murine embryonic development but is dispensable in ventricular cardiomyocytes. *Mol Cell Biol* 25: 3056–3062, 2005
28. Clark KA, McGrail M, Beckerle MC: Analysis of PINCH function in Drosophila demonstrates its requirement in integrin-dependent cellular processes. *Development* 130: 2611–2621, 2003
29. Hobert O, Moerman DG, Clark KA, Beckerle MC, Ruvkun G: A conserved LIM protein that affects muscular adherens junction integrity and mechanosensory function in *Caenorhabditis elegans*. *J Cell Biol* 144: 45–57, 1999
30. Sakai T, Li S, Docheva D, Grashoff C, Sakai K, Kostka G, Braun A, Pfeifer A, Yurchenco PD, Fassler R: Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. *Genes Dev* 17: 926–940, 2003

31. Yasunaga T, Kusakabe M, Yamanaka H, Hanafusa H, Masuyama N, Nishida E: Xenopus ILK (integrin-linked kinase) is required for morphogenetic movements during gastrulation. *Genes Cells* 10: 369–379, 2005
32. Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA, Damsky CH: Deletion of beta1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev* 9: 1883–1895, 1995
33. Fassler R, Meyer M: Consequences of lack of beta1 integrin gene expression in mice. *Genes Dev* 9: 1896–1908, 1995
34. Dai C, Yang J, Liu Y: Transforming growth factor-beta1 potentiates renal tubular epithelial cell death by a mechanism independent of Smad signaling. *J Biol Chem* 278: 12537–12545, 2003
35. Li Y, Yang J, Luo JH, Dedhar S, Liu Y: Tubular epithelial cell dedifferentiation is driven by the helix-loop-helix transcriptional inhibitor Id1. *J Am Soc Nephrol* 18: 449–460, 2007
36. Yang J, Shultz RW, Mars WM, Wegner RE, Li Y, Dai C, Nejak K, Liu Y: Disruption of tissue-type plasminogen activator gene in mice reduces renal interstitial fibrosis in obstructive nephropathy. *J Clin Invest* 110: 1525–1538, 2002
37. Li JH, Zhu HJ, Huang XR, Lai KN, Johnson RJ, Lan HY: Smad7 inhibits fibrotic effect of TGF-beta on renal tubular epithelial cells by blocking Smad2 activation. *J Am Soc Nephrol* 13: 1464–1472, 2002
38. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 110: 341–350, 2002
39. Zhang Y, Chen K, Tu Y, Velyvis A, Yang Y, Qin J, Wu C: Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localization of each component to cell-matrix adhesion sites. *J Cell Sci* 115: 4777–4786, 2002
40. Yoganathan N, Yee A, Zhang Z, Leung D, Yan J, Fazli L, Kojic DL, Costello PC, Jabali M, Dedhar S, Sanghera J: Integrin-linked kinase, a promising cancer therapeutic target: Biochemical and biological properties. *Pharmacol Ther* 93: 233–242, 2002
41. Koul D, Shen R, Bergh S, Lu Y, de Groot JF, Liu TJ, Mills GB, Yung WK: Targeting integrin-linked kinase inhibits Akt signaling pathways and decreases tumor progression of human glioblastoma. *Mol Cancer Ther* 4: 1681–1688, 2005
42. Sun Y, Liu X, Ng-Eaton E, Lodish HF, Weinberg RA: SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling. *Proc Natl Acad Sci U S A* 96: 12442–12447, 1999
43. Zhang X, Li Y, Dai C, Yang J, Mundel P, Liu Y: Sp1 and Sp3 transcription factors synergistically regulate HGF receptor gene expression in kidney. *Am J Physiol Renal Physiol* 284: F82–F94, 2003
44. Yang J, Zhang X, Li Y, Liu Y: Downregulation of Smad transcriptional corepressors SnoN and Ski in the fibrotic kidney: An amplification mechanism for TGF-beta1 signaling. *J Am Soc Nephrol* 14: 3167–3177, 2003
45. Yang J, Dai C, Liu Y: Hepatocyte growth factor gene therapy and angiotensin II blockade synergistically attenuate renal interstitial fibrosis in mice. *J Am Soc Nephrol* 13: 2464–2477, 2002