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Smad3 linker phosphorylation attenuates Smad3 transcriptional activity and TGF-β1/Smad3-induced epithelial–mesenchymal transition in renal epithelial cells

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ABSTRACT

Transforming growth factor-β1 (TGF-β1) has a distinct role in renal fibrosis associated with epithelial–mesenchymal transition (EMT) of the renal tubules and synthesis of extracellular matrix. Smad3 plays an essential role in fibrosis initiated by EMT. Phosphorylation of Smad3 in the C-terminal SSXS motif by type I TGF-β receptor kinase is essential for mediating TGF-β response. Smad3 activity is also regulated by phosphorylation in the linker region. However, the functional role of Smad3 linker phosphorylation is not well characterized. We now show that Smad3 EPSM mutant, which mutated the four phosphorylation sites in the linker region, markedly enhanced TGF-β1-induced EMT of Smad3-deficient primary renal tubular epithelial cells, whereas Smad3 3S-A mutant, which mutated the C-terminal phosphorylation sites, was unable to induce EMT in response to TGF-β1. Furthermore, immunoblotting and RT-PCR analysis showed a marked induction of fibrogenic gene expression with a significant reduction in E-cadherin in HK2 human renal epithelial cells expressing Smad3 EPSM. TGF-β1 could not induce the expression of α-SMA, vimentin, fibronectin and PAI-1 or reduce the expression of E-cadherin in HK2 cells expressing Smad3 3S-A in response to TGF-β1. Our results suggest that Smad3 linker phosphorylation has a negative regulatory role on Smad3 transcriptional activity and TGF-β1/Smad3-induced renal EMT. Elucidation of mechanism regulating the Smad3 linker phosphorylation can provide a new strategy to control renal fibrosis.

1. Introduction

TGF-β1 has been long considered as a key mediator in renal fibrosis and induces renal scarring largely by activating its downstream Smad signaling pathway [1,2]. Smad signaling has an important role in the regulation of TGF-β1-induced epithelial–mesenchymal transition (EMT) that is primarily dependent on the positive or negative regulation [3]. EMT has been long considered as a process contributing to renal fibrosis and is a process in which renal tubular epithelial cells lose their epithelial phenotype and acquire new characteristic features of mesenchyme, which includes disruption of polarized tubular epithelial cell morphology, *de novo* α-smooth muscle actin (α-SMA) expression and actin reorganization, loss of cell–cell adhesions through downregulation of E-cadherin, destruction of basement membrane, and increased cell migration and invasion [4,5].

It has been shown that Smad3 is a key molecule that mediates TGF-β1-induced renal fibrosis. Smad3 phosphorylation by TGF-β1 activates p38 MAPK signaling promoting extracellular matrix production in myofibroblast [6]. Many studies reported that a number of fibrogenic genes (collagens) and EMT markers (α-SMA and E-cadherin) are Smad3-dependent and Smad3 directly binds to the DNA sequences to regulate these target genes [7]. Knockdown of Smad3 blocks EMT and Smad3 deficiency attenuated renal fibrosis, inflammation, and apoptosis after UUO [8,9]. These results suggest that Smad3 is essential in the TGF-β1-induced EMT process during renal fibrosis, but the detailed mechanisms are not known.

Smad3 contains two conserved polypeptide segments, the MH1 and MH2 domains, joined by a less conserved linker region. The Smad3 linker region is serine/threonine-rich and contains four phosphorylation sites for proline-directed kinases. It can be phosphorylated by MAPKs, CDKs, JNK and p38, and GSK3, and can positively or negatively regulate Smad3-mediated responses in a cell context-dependent manner [10–22]. The Smad3 linker contains a transcriptional activation domain, which is necessary for Smad3 to activate transcription [23,24].

In this study, we investigated the role of Smad3 linker phosphorylation in TGF-β1-induced EMT. We found significant levels

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of constitutive linker phosphorylation for Smad3 in renal tubular epithelial cells in the absence of any TGF-β1 stimulation. Introduction of Smad3 linker phosphorylation mutant called Smad3 EPSM, which has a serine to alanine substitutions at positions 204, 208, and 213 and threonine to valine substitution at position 179 [20] into renal tubular epithelial cells resulted in the induction of EMT even in the absence of TGF-β1 stimulation. Based on our findings, we conclude that Smad3 linker phosphorylation negatively regulates TGF-β1 driven EMT in renal tubular epithelial cells and suggest a check and balance between linker and C-tail phosphorylation that regulates the distinct role of Smad3 in TGF-β1 signaling.

2. Materials and methods

2.1. Cell culture

HK-2 cells were obtained from ATCC. HK-2 cells are an immortalized proximal tubule epithelial cell line from normal adult human kidney. Cells were grown in DMEM F12 media (Gibco BRL, Grand Island, NY) supplemented with 10% FBS at 37 °C in a humidified incubator (5% CO_2, 95% air). All experiments were performed in serum-free conditions.

2.2. Primary culture of mouse renal tubular epithelial cells

Primary cell culture of mouse renal tubular epithelial cells was carried out as previously described [9]. Minced kidneys were washed with three changes of cold PBS containing 1 mM EDTA and were digested in 0.25% trypsin solution (Gibco BRL) in a shaking incubator at 37 °C for 2 h. This study was conducted under permission IACUC (110017) of CHA laboratory animal research center.

2.3. Western blot analysis

Cell extracts were separated by SDS–PAGE followed by electrotransfer to PVDF membranes and probed with: anti-E-cadherin, -Vimentin, and -fibronectin (BD Farmingen, Heidelberg); anti-α-SMA (Abcam, Cambridge); anti-PAI-1 (C-19) (Santa Cruz, CA); anti-α-tubulin (Clone B-6-1-2) (Sigma Aldrich, Beverly, MA). HRP-conjugated anti-mouse/rabbit antibodies (Millipore, Temecula, CA) were used as secondary antibodies. The reaction was visualized by WESTZOL (Intron, SeongNam). All signals were detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences).

2.4. Microscopic analysis

HK-2 cells plated on LabTek II eight-well glass slides (Nalge Nunc International) were fixed in 4% paraformaldehyde for 30 min at RT. Slides were viewed with Confocal Laser Scanning Microscope (LSM-510; Carl Zeiss, Jena) for bright-field and fluorescence applications, and were processed with ZEN software (Carl Zeiss).

2.5. RT-PCR analysis

Total RNA from cultured HK-2 cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was converted into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI).

2.6. Statistical analysis

All experiments were conducted with a minimum of three samples, and the data are presented as the mean ± S.D. Statistical analysis was performed using an unpaired parametric Student's *t*-test, unless otherwise indicated in the text.

3. Results

3.1. Smad3 linker phosphorylation negatively regulates TGF-β1-induced EMT in mouse renal primary tubular epithelial cells

Smad3 is known as a mediator in TGF-β1-induced fibrosis in kidney as well as liver and radiation-induced fibrosis. We cultured mouse renal primary tubular epithelial cells isolated from wild-type or Smad3-deficient mouse kidneys. Treatment of these cells with TGF-β1 resulted in a phenotypic change from cells exhibiting an epithelial-like cobblestone appearance to cells with spindle-shaped, fibroblastic appearance in Smad3 wild-type cells but not in Smad3 deficient cells. *In situ* immunostaining analysis also showed that TGF-β1 stimulation in the wild-type Smad3 primary renal epithelial cells increased α-SMA expression but no change in Smad3-deficient epithelial cells (Fig. 1A), suggesting that Smad3 mediates TGF-β1-induced EMT in mouse renal primary tubular epithelial cells.

To determine the contribution of Smad3 linker and C-terminal region phosphorylation in TGF-β1-induced EMT in mouse renal primary tubular epithelial cells, we infected Smad3-deficient mouse primary renal tubular epithelial cells with adenoviruses expressing wild-type Smad3, Smad3 C-terminal phosphorylation mutant called 3S-A, or Smad3 linker phosphorylation mutant called Smad3 EPSM (Fig. 1B). In the adenovirus-Smad3 infected cells, TGF-β1 induced a morphological change as shown in Fig. 1A, but Ad-Smad2 did not induce any morphological changes (Fig. 1C), suggesting that Smad3 mediates TGF-β1-induced EMT. In the Smad3 3S-A infected cells, TGF-β1 was unable to induce a morphological change. However, the expression of Smad3 EPSM was sufficient to induce a morphological change even without TGF-β1 stimulation, but TGF-β1 stimulation further induced a morphological change, suggesting that Smad3 linker phosphorylation negatively regulates TGF-β1-induced EMT, which is mediated through the Smad3 C-terminal phosphorylation in primary renal tubular epithelial cells (Fig. 1C).

The downregulation of E-cadherin expression and induction of α-SMA expression results in EMT. To determine whether Smad3 EPSM was more effective than wild-type Smad3 in suppressing the expression of E-cadherin and inducing the expression of α-SMA in the presence of TGF-β1 in mouse renal primary tubular epithelial cells, adenovirus infected cells were incubated in the absence or presence of TGF-β1 for 24 h. The expression of Smad3 EPSM induced the morphological change even without TGF-β1 stimulation (Fig. 2A). However, expression of Smad3 3S-A suppressed the TGF-β1-induced EMT, suggesting that TGF-β1-induced EMT is mediated through the Smad3 C-terminal phosphorylation. In cells infected with adenovirus-GFP, wild-type Smad3, or Smad3 EPSM, the stimulation of TGF-β1 induced α-SMA expression and suppressed E-cadherin expression. However, no reduction of E-cadherin expression or induction of α-SMA expression was detected in Smad3 3S-A infected cells in the presence of TGF-β1 by dual immunofluorescence (Fig. 2B), suggesting that Smad3 C-terminal phosphorylation is necessary for TGF-β1-induced EMT in primary renal tubular epithelial cells.

3.2. Constitutive linker phosphorylation of Smad3 in human kidney epithelial cells

As reported previously, Smad3 is also regulated by phosphorylation at its linker region. To examine whether the Smad3 linker region is phosphorylated in HK2 human kidney epithelial cells in the presence or absence of TGF-β1, we performed immunoblotting using polyclonal antibodies against specific phospho-amino acids in the linker region of Smad3. As shown in Fig. 3A, in the absence

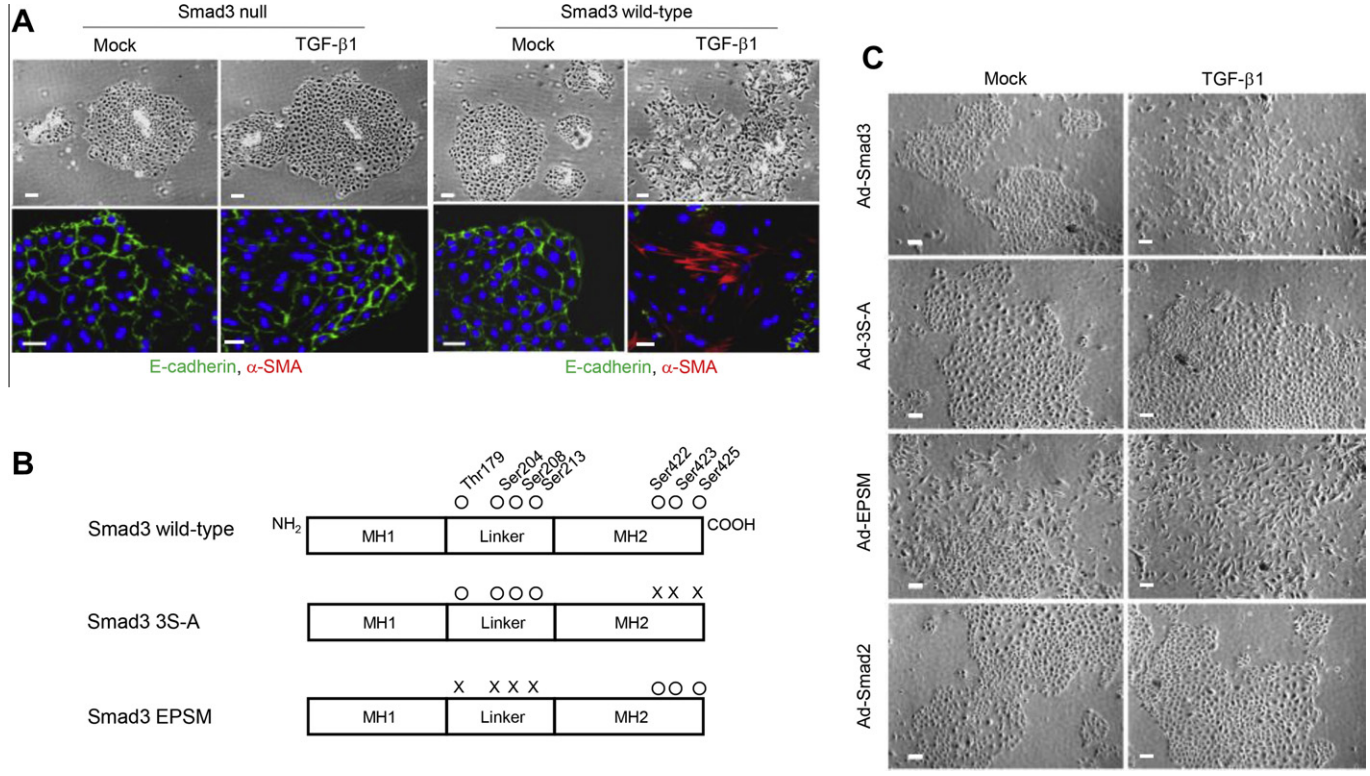

Fig. 1. Smad3 linker phosphorylation negatively regulates TGF-β1-induced EMT in Smad3-deficient mouse primary renal tubular epithelial cells. (A) Primary mouse kidney tubular epithelial cells were cultured from Smad3-null and wild-type mice and stimulated with TGF-β1 for 24 h, Scale bar: 10 μm. Immunofluorescence of E-cadherin (green) and α-SMA (red). Nuclei were visualized by staining with DAPI (blue). Scale bar: 20 μm. (B) Schematic representation of Smad3 phosphorylation site. (**C**) Phase-contrast microscopy of epithelial cells from Smad3-deficient mice were infected with adenovirus and stimulated with TGF-β1 (10 ng/ml) for 24 h. Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of TGF-β1 stimulation, HK-2 cells have a high level of Smad3 phosphorylated at Linker. The level of Smad3 linker phosphorylation was enhanced upon TGF-β1 stimulation.

We next investigated the role of Smad3 linker phosphorylation on TGF-β1-induced EMT in HK-2 cells. Treatment of HK2 cells with TGF-β1 resulted in a phenotypic change from exhibiting an epithelial-like appearance to a spindle-shaped, fibroblastic appearance (Supplementary Fig. 1). Expression of wild-type Smad3 and Smad3 EPSM induced the morphological change in the presence of TGF-β1, whereas expression of Smad3 3S-A blocked TGF-β1-induced morphological change just like the primary renal epithelial cells. To determine the contribution of Smad3 linker and C-terminal region phosphorylation in TGF-β1-induced EMT in HK2 cells, we infected HK2 cells with adenoviruses expressing wild-type Smad3, Smad3 3S-A, or Smad3 EPSM. We first examined expression of these proteins and levels of their phosphorylation after incubation of cells in the absence or presence of TGF-β1 for 60 min. Infection of HK2 cells with adenovirus expressing Smad3 3S-A and Smad3 EPSM completely blocked TGF-β1-induced Smad3 C-terminal phosphorylation and TGF-β1-induced Smad3 linker phosphorylation, respectively. TGF-β did not increase linker phosphorylation in adenovirus-Smad3 infected HK-2 cells (Fig. 3B). This may be due to too much expression of the Smad3 protein. Even constitutive C-terminal phosphorylation of endogenous Smad3 was completely inhibited by the overexpression of Smad3 3S-A, suggesting that overexpression of Smad3 3S-A may function as a dominant negative form of Smad3.

TGF-β1-induced transcriptional regulation is controlled by nuclear accumulation of Smad3. Therefore, to determine whether Smad3 linker phosphorylation influences Smad3 transcriptional activity we first examined the subcellular localization of Smad3 phosphorylated at Thr179, Ser204, and Ser208 in the absence or presence of TGF-β1 in HK2 cells. We performed confocal microscopy using polyclonal antibodies against specific phospho-amino acids in the linker region of Smad3. As reported previously, Smad3 phosphorylated at Ser423/425 is almost absent without TGF-β1 stimulation, but upon TGF-β1 stimulation, Smad3 phosphorylated at Ser423/425 is found in the nucleus of HK2 cells (Fig. 3C). Smad3 phosphorylated at Thr179 was observed in the perinuclear region without TGF-β1 treatment, whereas endogenous Smad3 phosphorylated at Ser204 and Ser208 was found in the nuclear compartment regardless of TGF-β1 treatment. After TGF-β1 treatment, Smad3 phosphorylated at Thr179 translocated to the nucleus and levels of Smad3 phosphorylated at Ser204 and Ser208 were markedly increased. These findings suggest that each phosphorylation site at Smad3 linker region may be phosphorylated by different kinases.

HK2 cells display an epithelial morphology, but TGF-β1 treatment of HK2 cells resulted in a loss of epithelial characteristics by decreasing E-cadherin expression (Fig. 4A and B). Over-expression of Smad3 EPSM resulted in a complete loss of E-cadherin mRNA as well as protein expression in the presence of TGF-β1, whereas TGF-β1-induced suppression of E-cadherin expression was completely blocked by expression of Smad3 3S-A. TGF-β1-induced mRNA and protein expression of extracellular matrix (ECM) components, including vimentin and fibronectin, was abolished in HK2 cells expressing Smad3 3S-A. However, over-expression of Smad3 EPSM markedly enhanced TGF-β1-induced mRNA and protein expression of ECM components. Interestingly, besides ECM components, the expression of plasminogen activator inhibitor 1 (PAI-1) was markedly enhanced in HK2 cells expressing Smad3 EPSM. PAI-1 is a powerful fibrogenic factor that promotes

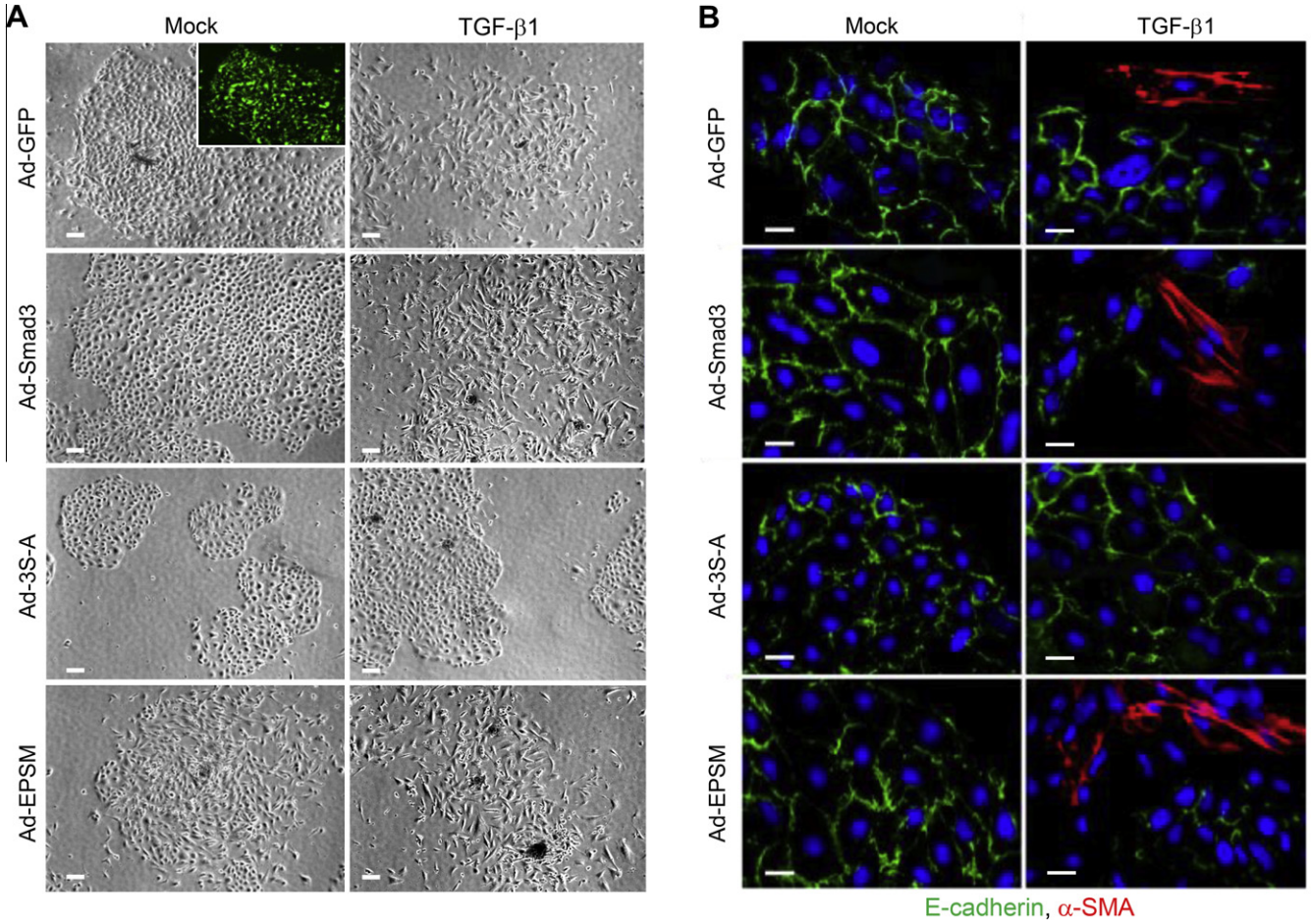

Fig. 2. Smad3 C-terminal phosphorylation is necessary for TGF-β1-induced EMT in mouse primary renal tubular epithelial cells. (A) The cells were infected Smad3 mutant adenovirus upon stimulation of TGF-β1 for 24 h. Green fluorescent protein (GFP) has been described as a means to assess gene expression and infection efficiency (small panel). Scale bar: 10 μm. (B) Dual immunofluorescence of E-cadherin (green) and α-SMA (red) and nuclei stained with DAPI (blue). Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inflammatory cell recruitment and extracellular matrix accumulation [1,25]. Consistent with the results of the PAI-1 mRNA expression, the promoter activity of PAI-1 gene was significantly increased in Smad3 EPSM expressing HK2 cells, but significantly decreased in Smad3 3S-A expressing HK2 cells (Fig. 4C). It is worth pointing out that this is not due to Ad-EPSM has higher Smad3 levels. TGF-β1 also mediated a myofibroblast phenotype in HK2 cells by inducing α-smooth muscle actin (α-SMA) gene expression. Expression of Smad3 3S-A completely inhibited TGF-β1-induced α-SMA gene and protein expression, whereas expression of Smad3 EPSM further enhanced TGF-β1-induced α-SMA expression. Immunofluorescence staining revealed an evident increase of TGF-β1-induced fibronectin and α-SMA expression after infection of adenoviruses expressing wild-type Smad3 and Smad3 EPSM compared to the control GFP expression (Supplementary Fig. 2). Expression of Smad3 3S-A resulted in a loss of the fibronectin and α-SMA signal even in the presence of TGF-β1. These results suggest that Smad3 C-terminal phosphorylation mediates TGF-β1-induced renal fibrosis, whereas Smad3 linker phosphorylation attenuates TGF-β1-induced renal fibrosis by blocking signals mediated by the Smad3 C-terminal phosphorylation.

4. Discussion

It is widely recognized that TGF-β1/Smad signaling plays an essential role in renal fibrosis by stimulating fibrogenic cells to produce ECM proteins and inducing transformation of tubular epithelial cells to myofibroblasts through EMT. Studies have reported that Smad3 is a critical mediator of TGF-β signaling in renal fibrosis. Many fibrogenic genes and tissue inhibitor of MMP-1 are known to be downstream targets of TGF-β1/Smad3 signaling [7]. We have previously demonstrated that Smad3 plays an even more essential role in fibrosis initiated by EMT, as it is also required for the generation of the fibrogenic myofibroblasts from epithelial precursors [9]. Mice lacking Smad3 are protected against tubulointerstitial fibrosis following unilateral ureteral obstruction as evidenced by blocking of EMT and abrogation of monocyte influx and collagen accumulation. Smad3 has an essential role in fibrogenesis, which is also confirmed by suppression of fibrogenesis in mice with Smad3 deletion [26]. These reports suggest that Smad3 is a key mediator in renal fibrosis. A recent study demonstrated that SIS3, a specific inhibitor of Smad3, is effective in the inhibition of endothelial−−myofibroblast transition *in vitro* and *in vivo*. SIS3 also showed renoprotective effects in streptozotocin-induced diabetic nephropathy [27–29], suggesting that inhibition of Smad3 function has therapeutic potential for diabetic renal disease.

Renal epithelial cells and kidney tissues undergoing renal fibrosis often exhibit markedly increased expression of α-SMA and interstitial matrix components, such as collagen and fibronectin. To determine whether Smad3 phosphorylation affects EMT, we investigated the TGF-β1-induced EMT marker expression in the human kidney epithelial HK-2 cells. In the TGF-β signaling pathway, C-terminal serines at the SSXS motif of Smad3 are phosphorylated by type I TGF-β receptor following TGF-β activation.

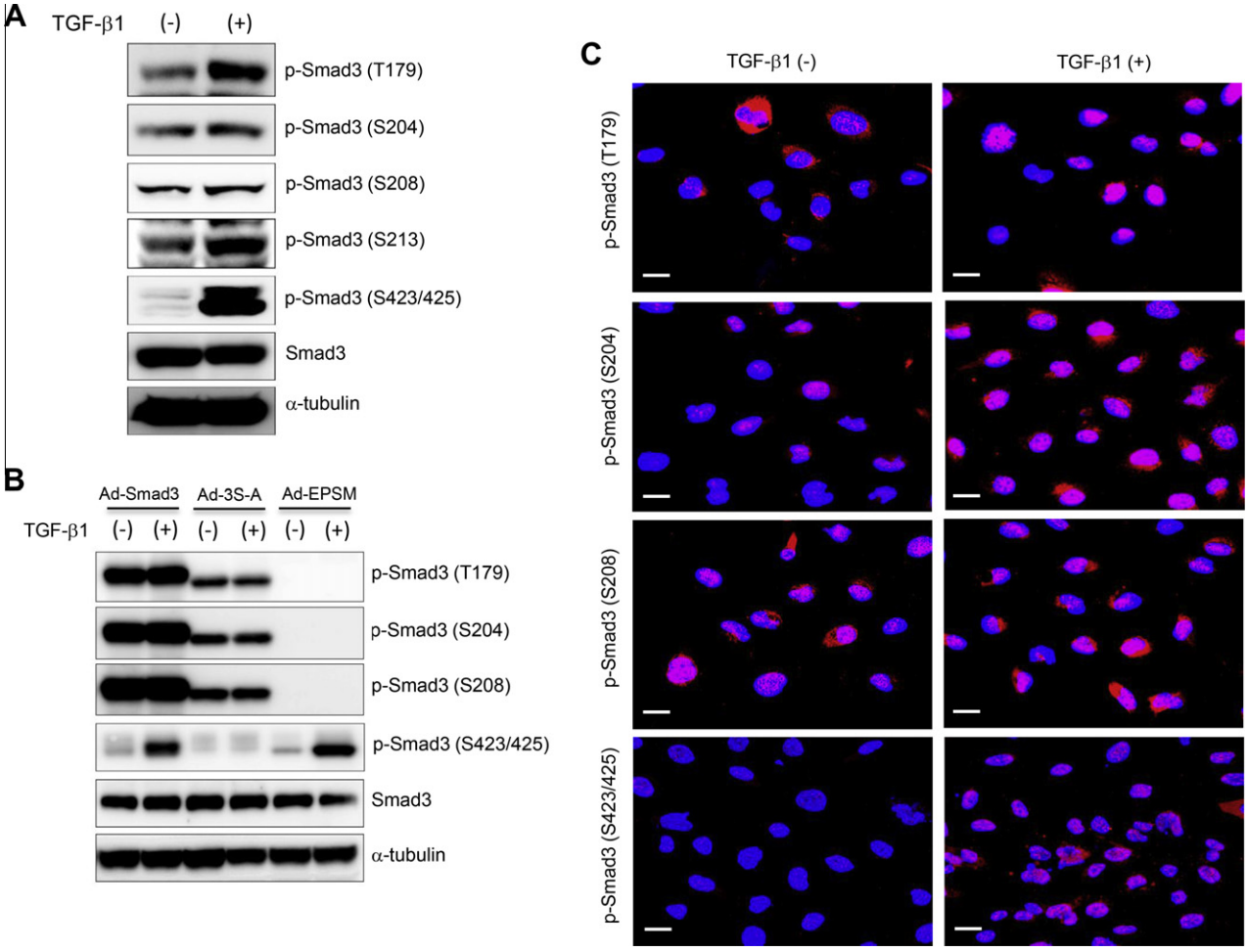

Fig. 3. The Smad3 linker region was phosphorylated constitutively. (A) Immunoblot using Smad3 phospho-antibodies with extracted proteins from HK-2 cells. The cells were stimulated with TGF-β1 (5 ng/ml) for 1 h. (B) Using Smad3 phospho-antibodies with extracted proteins from HK-2 cells and stimulated with TGF-β1 for 1 h. (C) Immunofluorescence staining upon stimulation of TGF-β1 for 1 h. Scale bar: 20 mm.

Induction of expression of mesenchymal markers by TGF-β1 was completely abrogated by expressing the C-terminal phosphorylation mutant. On the other hand, the expression of a Smad3 EPSM markedly enhanced the TGF-β1-induced EMT and induction of PAI-1 gene expression. Similarly, the Smad3 EPSM mutant showed a EMT-like feature and was further increased in response to TGF-β1 when introduced into Smad3-deficient mouse primary renal tubular epithelial cells, whereas the C-terminal phosphorylation mutant could not induce EMT in the absence or presence of TGF-β1. These results suggest that phosphorylation of the Smad3 linker region serves as an important mechanism for modulating TGF-β1-induced renal fibrosis.

Smad3 linker phosphorylation has various roles in a cell context-dependent manner as indicated in the introduction section. Our results suggest that for TGF-β1-induced EMT in renal epithelial cells, the linker phosphorylation attenuates Smad3 transcriptional activity and TGF-β1-induced EMT. A recent study shed a light into the potential underlying mechanism for our observations. It is shown that Smurf2, an E3 ubiquitin ligase, binds to the phosphorylated Smad3 linker region and induces Smad3 mono-ubiquitination. The mono-ubiquitination occurs in the C-terminal domain of Smad3 and prevents the formation of Smad3–Smad4 complex and Smad3–Smad3 homotrimer, thus inhibiting Smad3 transcriptional activity [30]. Future studies are necessary to determine whether this mechanism operates in our experimental system.

Although Smad2 and Smad3 are each activated by the TGF-β receptor, they have very different effects on gene transcription. Smad2 and Smad3 are structurally similar with over 90% homology in their amino acid sequences. These two proteins exert distinct functions in embryonic development. Smad2-deficient embryos die around day 7.5 of gestation due to failure of gastrulation and failure to establish an anterior–posterior (A–P) axis [31,32], whereas mice null for Smad3 survive with impaired immunity [33]. Smad2 knockdown in tubular epithelial cells promoted fibrosis through enhanced TGF-β1/Smad3 signaling, evidenced by increased Smad3 phosphorylation, nuclear translocation, promoter activity, and binding of Smad3 to a collagen promoter [34]. In the present study, we also demonstrated that Smad2 cannot substitute for the Smad3 to mediate TGF-β1-induced fibrosis in Smad3-null renal epithelial cells, suggesting that Smad3, but not Smad2, mediates TGF-β1-induced renal fibrosis (Fig. 2). Smad2 and Smad3 linker regions are serine/threonine-rich and contain multiple phosphorylation sites for proline-directed kinases. C-terminal phosphorylation of Smad2 and Smad3 occurs by the type I TGF-β receptor following TGF-β stimulation in almost all cell types and tissues. However, timing, extent, and functional implications of linker phosphorylation of these proteins depend on cell type and differ by the stage of disease progression. In this study, our findings suggest that constitutive Smad3 linker phosphorylation confers a selective advantage on renal epithelial cells to maintain epithelial homeostasis by blocking the Smad3 pathway, which is more characteristic of the state of flux shown by the activated mesenchymal cells.

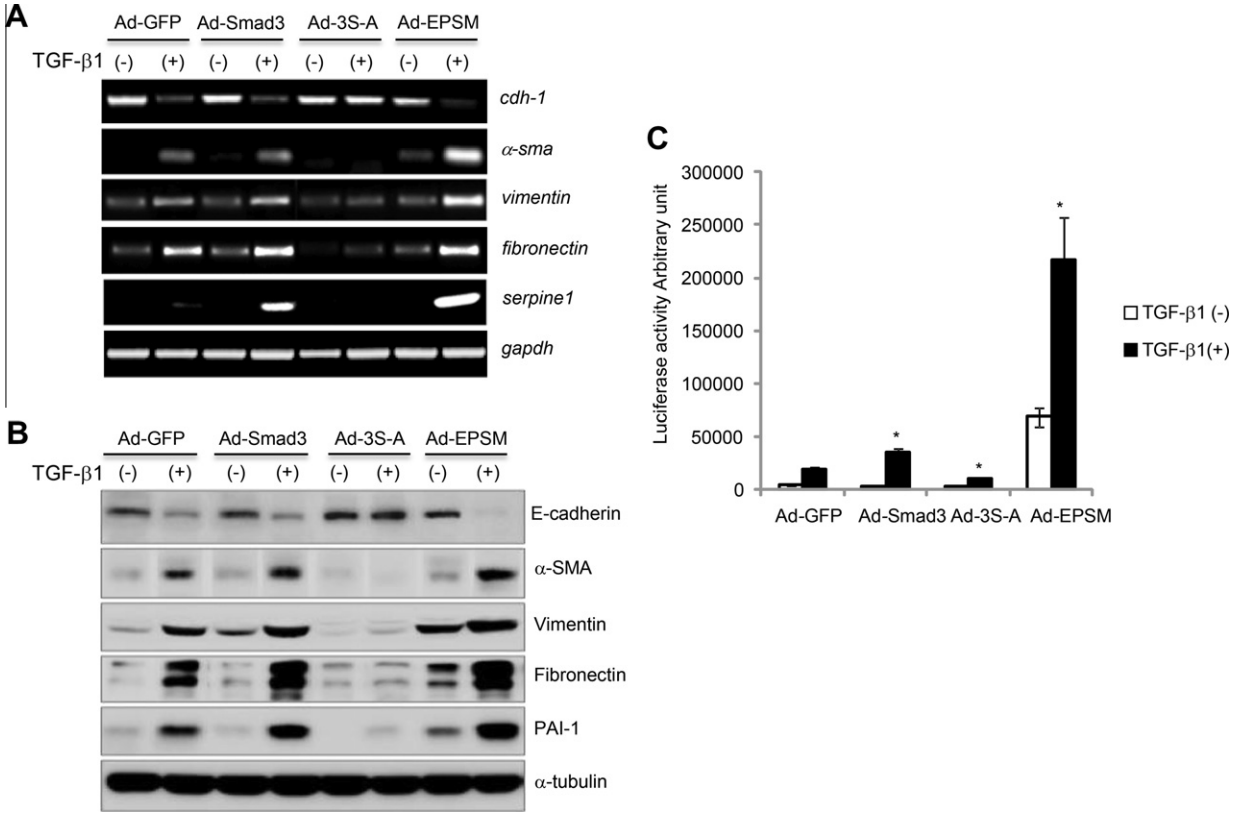

Fig. 4. Mutation of the Smad3 linker phosphorylation sites increases Smad3 transcriptional activity to regulate EMT and fibrogenic genes at the basal state and in the presence of TGF-β1. (A) The genes related to EMT were measured by RT-PCR from HK-2 cells. (B) Immunoblot with HK-2 cells which were stimulated TGF-β1 (5 ng/ml) for 48 h. (C) PAI-1 luciferase reporter was transiently transfected into HK-2 cells which stimulated with TGF-β1 for 24 h. The data shows means ± S.D. of three independent experiments.

In summary, the present results show that mutation of the C-terminal phosphorylation sites of Smad3 blocks EMT of renal tubular epithelial cells and mutation of Smad3 linker phosphorylation sites markedly enhances TGF-β1-induced EMT. This suggests a therapeutic rationale for the development of putative small molecules that activate Smad3 linker phosphorylation. Our findings indicate that elucidation of mechanisms regulating Smad3 linker phosphorylation may help to develop a new strategy to control renal fibrosis. Our data suggest that selective activators of the Smad3 pathway mediated through linker region may prove highly effective in a wide range of fibrotic disorders, not only obstructive nephropathy and chronic interstitial nephritis but also pulmonary and hepatic fibrosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.103.

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