The differential role of Smad2 and Smad3 in the regulation of pro-fibrotic TGFβ1 responses in human proximal-tubule epithelial cells

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In chronic renal diseases, progressive loss of renal function correlates with advancing tubulo-interstitial fibrosis. TGFβ1-Smad (transforming growth factor-β1–Sma and Mad protein) signalling plays an important role in the development of renal tubulo-interstitial fibrosis. Secretion of CTGF (connective-tissue growth factor; CCN2) by PTECs (proximal-tubule epithelial cells) and EMT (epithelial–mesenchymal transdifferentiation) of PTECs to myofibroblasts in response to TGFβ are critical Smad-dependent events in the development of tubulo-interstitial fibrosis. In the present study we have investigated the distinct contributions of Smad2 and Smad3 to expression of CTGF, E-cadherin, α-SMA (α-smooth-muscle actin) and MMP-2 (matrix-metalloproteinase-2) in response to TGFβ1 treatment in an *in vitro* culture model of HKC-8 (transformed human PTECs). RNA interference was used to achieve selective and specific knockdown of Smad2 and Smad3. Cellular E-cadherin, α-SMA as well as secreted CTGF and MMP-2 were assessed by Western immunoblotting. TGFβ1 treatment induced a fibrotic phenotype with increased expression of CTGF, MMP-2 and α-SMA, and decreased expression of E-cadherin. TGFβ1-induced increases in CTGF and decreases in E-cadherin expression were Smad3-dependent, whereas increases in MMP-2 expression were Smad2-dependent. Increases in α-SMA expression were dependent on both Smad2 and Smad3 and were abolished by combined knockdown of both Smad2 and Smad3. In conclusion, we have demonstrated distinct roles for Smad2 and Smad3 in TGFβ1-induced CTGF expression and markers of EMT in human PTECs. This can be of therapeutic value in designing targeted anti-fibrotic therapies for tubulo-interstitial fibrosis.

Key words: CTGF, connective-tissue growth factor, epithelial–mesenchymal transdifferentiation (EMT), Sma and Mad protein 2 (Smad2), Smad3, transforming growth factor-β1 (TGFβ1), tubulo-interstitial fibrosis.

INTRODUCTION

Progressive kidney diseases are characterized by tubular atrophy and tubulo-interstitial fibrosis. Irrespective of the underlying glomerular lesion, tubulo-interstitial fibrosis is a robust predictor of renal functional impairment [1]. TGFβ1 (transforming growth factor-β1) plays a key role in the development of renal fibrosis [2,3]. PTECs (proximal-tubule epithelial cells) contribute to the development of tubulo-interstitial fibrosis by secretion of CTGF [connective-tissue growth factor; also known as CCN2 (CTGF, Cyr61/Cef10 and nephroblastoma-overexpressed gene family 2)], MMP-2 (matrix metalloproteinase-2) and by transdifferentiating to myofibroblasts in response to TGFβ1 [4–6]. Transdifferentiation of tubular epithelial cells to myofibroblasts involves loss of the epithelial marker E-cadherin, up-regulation of the myofibroblast marker α-SMA (α-smooth muscle actin), disruption of tubular basement membrane and subsequent migration of transformed cells to the interstitium [5]. TGFβ1 exerts its pro-fibrotic effects by direct mechanisms and indirectly by induction of secondary mediators such as CTGF. CTGF is a downstream mediator of at least some of the pro-fibrotic effects of TGFβ [7], and CTGF expressed in the tubular epithelium plays a pivotal role in renal fibrogenesis [4]. MMP-2 secreted by the tubular epithelial cells contributes to EMT (epithelial–mesenchymal transdifferentiation) by disrupting the tubular basement membrane [5,6]. Evidence from *in vivo* and *in vitro* studies suggest that the predominant signalling pathway responsible for pro-fibrotic effects of TGFβ1 in the renal tubulo-interstitium is the Smad (Sma and Mad protein) signalling pathway, and increased activation of TGFβ1–Smad signalling pathway has been shown in a mouse model of diabetic nephropathy [8–10].

Binding of TGFβ1 to its type2 receptor results in phosphorylation of the type1 receptor, which in turn phosphorylates the two receptor-regulated Smads (Smad2 and Smad3). Activated Smad2 and Smad3 form heteromeric complexes with Smad4 and accumulate in the nucleus, where they control gene expression in a cell-type-specific manner through interaction with other transcription factors, co-activators and co-repressors [11]. Previous studies by ourselves and others have demonstrated a requirement for Smad signalling in the induction of CTGF [12] and in transdifferentiation of PTECs to myofibroblasts [5,8]. In spite of marked similarity in their structure, accumulating evidence over the last few years has suggested that Smad2 and Smad3 have distinct roles to play in TGFβ1-induced cellular responses [13,14]. Most of the evidence comes from experiments performed on fibroblasts obtained from Smad2 and Smad3 knockout mice and *in vivo* studies performed in Smad3 knockout mice [9,13,15,16]. These studies have demonstrated that TGFβ–Smad3 signalling is a key mediator of TGFβ-induced pro-fibrotic outcomes in both renal and non-renal cells. However, in these experiments it was not possible to study the role of Smad2 in TGFβ1-induced fibrotic outcomes, since the Smad2 knockout results in embryonic lethality. Furthermore, the role of Smad3 in TGFβ1-induced fibrotic responses in adult differentiated human renal cells has not previously been investigated.

Abbreviations used: CTGF (CCN2), connective-tissue growth factor; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial–mesenchymal transdifferentiation; FCS, fetal-calf serum; HKC, human kidney cells; HKC-8, transformed human PTECs (proximal-tubule epithelial cells); MMP-2, matrix metalloproteinase-2; siRNA, small interfering RNA; Smad, Sma and Mad protein; TGFβ1, transforming growth factor β1; α-SMA, α-smooth-muscle actin.

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It has been proposed that targeting Smad proteins could be a therapeutic option for the treatment of renal fibrosis [17]. In the past decade RNA interference has emerged as an effective tool for silencing gene expression [18]. However, use of this technique to achieve knockdown of Smad proteins in human PTECs has not so far been investigated. Here we have investigated the differential role played by the two TGFβ1-activated receptor-regulated Smads (Smad2 and 3) in expression of secreted CTGF and MMP-2, cellular E-cadherin and α-SMA in response to TGFβ1 in human PTECs by using the technique of RNA interference to selectively knockdown individual endogenous Smad proteins.

MATERIALS AND METHODS

Experiments were performed in transformed human PTECs (HKC-8) cells, kindly provided by Dr Lorraine Racusen of the Department of Pathology, John Hopkins Medical Institute, Baltimore, MD, U.S.A. Media and supplements for culture of HKC cells were purchased from Invitrogen (Gibco, Paisley, Renfrewshire, Scotland, U.K.) and Sigma (Sigma-Aldrich, Poole, Dorset, U.K.). Anti-CTGF antibody was purchased from Santa-Cruz Biotechnology (via Autogen-Bioclear UK Ltd, Calne, Wiltshire, U.K.). TGFβ1, anti-α-tubulin antibody and anti-α-SMA antibodies were from Sigma. Anti-E-cadherin antibody (clone 36) was purchased from BD Transduction Laboratories (Oxford, U.K.). Anti-Smad2 and -Smad3 antibodies were purchased from Zymed (Cambridge, U.K.). Anti-MMP-2 antibody was purchased from Cell Signalling Technology (New England Biolabs, Hitchin, Herts., U.K.). All the Western-blotting reagents were purchased from Invitrogen. siRNA (small interfering RNA) targeting Smad2 and Smad3 and a non-targeting control siRNA were obtained from Ambion (Huntingdon, Cambridgeshire, U.K.). The transfection reagent siPORT™ Amine was purchased from Ambion. The HKC cells were grown in uncoated plastic cultureware in DMEM (Dulbecco's modified Eagle's medium/F12 medium (1:1, v/v) supplemented with triiodothyronine (20 ng/ml), cortisol (18 ng/ml), insulin (5 μg/ml), sodium selenite (5 ng/ml), transferrin (5 μg/ml) and 5 % (v/v) FCS (fetal-calf serum) in the absence of any antibiotics and growth factors.

siRNA design

Smad3 and Smad2 siRNAs were designed using the Ambion siRNA design website. Control non-targeting siRNA was also purchased from Ambion. Sequences for Smad2 and Smad3 siRNA are as follows:

Smad2 siRNA
Sense: 5′ GUCCCAUGAAAAGACUUAAtt 3′
Anti-sense: 5′ UUAAGUCUUUUCAUGGGACtt 3′

Smad3 siRNA
Sense 5′ GGAGAAAUGGUGCGAGAAGtt 3′
Anti-sense 5′ CUUCUCGCACCAUUUCUCCtc 3′

Transfection experiments

Experimental protocol 1 (for assessment of secreted CTGF and cellular E-cadherin)

HKC-8 cells were transfected at 50–60 % confluence in 35-mm-diameter dishes. The transfection mixture was prepared by incubating 10 μl of siPORT™ Amine and 190 μl of Opti-MEM I for 20 min at room temperature. siRNA (10 μl, 20 μM) was then added to the above mixture and incubated for further 30 min at room temperature. Finally, above transfection mixture was added to a 35-mm-diameter dish containing 1 ml of DMEM/F12 (1:1, v/v) with 5 % FCS (final siRNA concn. 166 nM). Transfection was performed for 24 h, and the medium was then changed to fresh DMEM/F12 (1:1, v/v) medium containing 5 % FCS for a further 24 h. Cells were then made serum-free overnight and treated with either vehicle (0.1 % BSA) or TGFβ1 (5 ng/ml) for 24 h under serum-free conditions. Cells were about 80 % confluent at the time of TGFβ1 treatment. The total time period from start of transfection to end of the experiment was 84 h.

Experimental protocol 2 (for assessment of cellular α-SMA and secreted MMP-2)

The experimental protocol was as described above, except that TGFβ1 treatment was for 48 h under serum-free conditions. In these experiments, the total time period from start of transfection to end of the experiment was 108 h.

At the end of each experiment, knockdown of respective Smad proteins was assessed by Western immunoblotting.

Western immunoblotting

Cells were washed once with ice-cold PBS, lysed in ice-cold lysis buffer [20 mM Tris/HCl, 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 200 μM PMSF, 2 mM EDTA, 1 mM sodium orthovanadate, protease inhibitor cocktail (Sigma P-2714) and 50 mM NaF]. Lysates were cleared by centrifugation at 20000 ***g*** at 4 °C for 10 min and the supernatant recovered. After measuring the protein concentration using the BCA (bicinchoninic acid) protein assay kit (Pierce; via Perbio Science UK, Cramlington, Northumberland, U.K.), equal amounts of total cellular protein were subjected to SDS/PAGE in 10 % (w/v) Bis-Tris-containing polyacrylamide gels under reducing conditions using Invitrogen Western immunoblotting system with buffers provided by the manufacturer. Proteins were then transferred to PVDF membrane. Blots were blocked with TBS-T (Tris-buffered saline/20 mM Tris/HCl, 150 mM NaCl and 0.1 % Tween 20)/5 % (w/v) fat-free milk prior to incubation with appropriate primary antibodies in TBS-T with 5 % (w/v) BSA at 4 °C overnight to detect Smad2, Smad3, E-cadherin and α-SMA. Next, the blots were incubated with horseradish peroxidase-labelled secondary antibody for 1 h at room temperature, developed with ECL Plus® enhanced-chemiluminescence Western-blotting detection system (Amersham; Little Chalfont, Bucks., U.K.) and visualized using Hyperfilm ECL photographic film. Blots were stripped and re-probed for α-tubulin as a loading control. For assessment of secreted CTGF, supernatants were harvested at the end of the experimental period and treated with a heparin–agarose bead suspension to precipitate heparin-binding proteins. Bound proteins were extracted in Laemmli sample buffer containing mercaptoethanol. Proteins were then subjected to SDS/PAGE and immunoblotted for CTGF. The amount of eluate loaded per well was adjusted according to the protein concentration of the cell lysates to account for any differences in the total cell mass [12]. Secreted MMP-2 was assessed by Western immunoblotting. Supernatants were harvested at the end of the experimental period and subjected to SDS/PAGE, followed by immunoblotting for MMP-2. The amount of supernatant loaded per well was adjusted according to the protein concentration of the cell lysates. Bands were quantified using scanning densitometry (Genegenius instrument; Genesnap version 4 software; Syngene, Cambridge, U.K.). Results are expressed as the percentage change in the mean band density as compared with the control values (= 100 %).

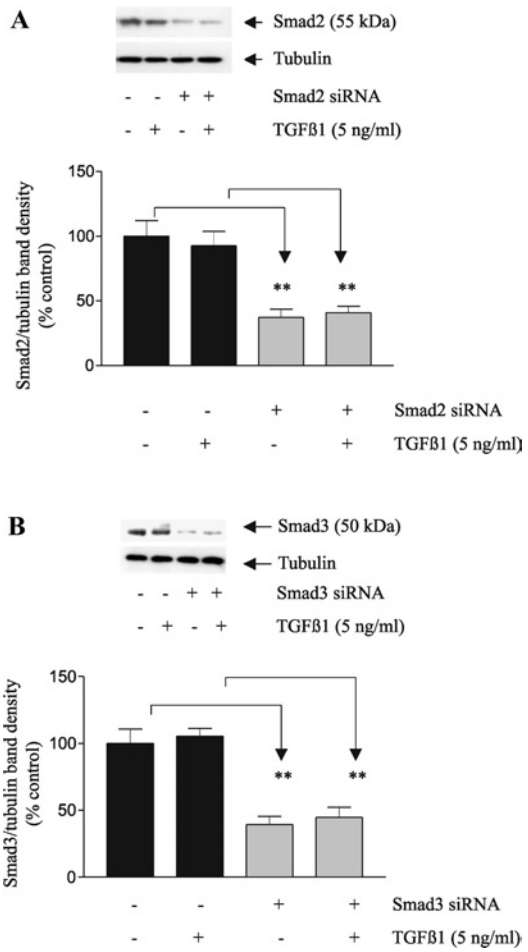

Figure 1 Smad2 and Smad3 siRNA treatment results in knockdown of respective Smad proteins in human PTECs

HKCs were transfected with Smad2 and Smad3 siRNA as described in the Materials and methods section. At the end of the experimental period, cells were lysed and Smad knockdown was assessed by Western immunoblotting. Smad2 and Smad3 siRNA treatment resulted in about a 60 % decrease in the band density of respective Smad proteins assessed 84 h after the start of transfection (**A** and **B**). This knockdown was comparable in the presence and absence of TGFβ1 treatment (**A** and **B**). Western immunoblots shown are representative blots for cellular Smad2 and Smad3 proteins. Results are means $\pm$ S.E.M. and expressed as a percentage of the control value ($n = 6$; $^{**}P < 0.01$).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. for four to six experiments and presented as percentages of the control value (= 100 %). Statistical analysis was performed using ANOVA with *post hoc t*-testing using Bonferroni–Dunn correction. All the analysis was done using INSTAT statistical software (Graphpad software version V2; University of Amsterdam, Amsterdam, The Netherlands). A P value of less than 0.05 was considered significant.

RESULTS

Smad2 and Smad3 siRNA treatment results in selective and specific knockdown of respective Smad proteins

Smad2 and Smad3 siRNA treatment resulted in approx. 60 % reduction in band density of respective Smad proteins assessed 84 h after the start of transfection. Knockdown of Smad2 and Smad3 proteins was comparable in both the presence and absence of TGFβ1 treatment (Figures 1A and 1B). TGFβ1 treatment did not alter total cellular Smad2 and Smad3 levels (Figures 1A and 1B). The knockdown of respective Smad proteins by targeting siRNA was specific, as non-targeting control siRNA did not affect either of the receptor-regulated Smads (Figures 2A and 2B). The knockdown was also selective, as Smad3 protein levels were reduced by Smad3 siRNA, but not by Smad2 siRNA (Figure 2C). Similarly, Smad2 protein levels were reduced by Smad2 siRNA and not by Smad3 siRNA (Figure 2D). Neither of the transfections had any effect on Smad4 protein levels (Figure 2E).

TGFβ1-induced secreted CTGF protein in human PTECs is dependent on Smad3

TGFβ1 treatment for 24 h resulted in significant induction (about 3-fold) of secreted CTGF protein. This induction was markedly attenuated in the presence of Smad3 knockdown. However, Smad2 knockdown had no effect on TGFβ1-induced secreted CTGF protein. Neither Smad2 nor Smad3 knockdown affected basal CTGF protein secretion. These results demonstrate that induction of secreted CTGF by TGFβ1 in human PTECs is dependent on Smad3 and not Smad2 (Figure 3).

TGFβ1-induced secreted MMP-2 in human PTECs is dependent on Smad2

TGFβ1 treatment for 48 h resulted in significant induction (about 2-fold) of secreted MMP-2 protein (Figure 4), although no

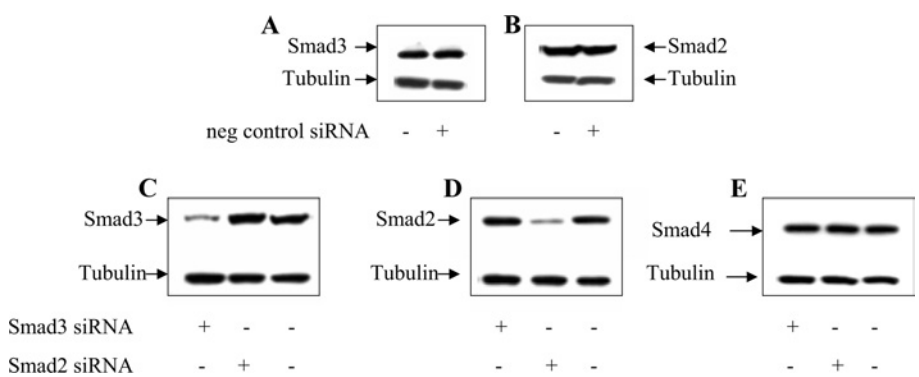

Figure 2 Smad2 and Smad3 siRNA treatment results in selective and specific knockdown of respective Smad proteins

Effect of siRNAs targeting Smad2 and Smad3 was specific as non-targeting control siRNA had no effect on either Smad2 or Smad3 protein band densities (**A** and **B**). The knockdown was also selective, as Smad3 protein levels were reduced by Smad3 siRNA, but not by Smad2 siRNA (**C**). Smad2 protein levels were decreased by Smad2 siRNA and not by Smad3 siRNA (**D**). Neither of the transfections had any effect on the level of Smad4 protein (**E**). Western immunoblots shown are representative of at least four transfection experiments.

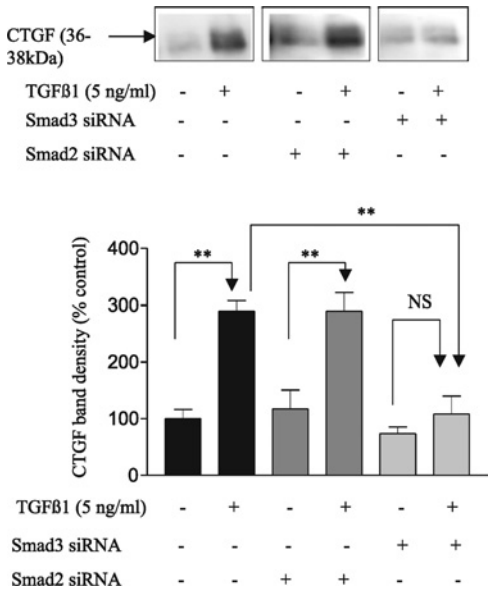

Figure 3 TGFβ1-induced secreted CTGF protein in human PTECs is Smad3-dependent and Smad2-independent

Following transfection with Smad2 and Smad3 siRNAs as described in the Materials and methods section, HKCs were treated with either vehicle (0.1 % BSA) or TGFβ1 (5 ng/ml) for 24 h under serum-free conditions. Secreted CTGF was assessed by Western immunoblotting of the cell culture supernatants. TGFβ1 (5 ng/ml) treatment for 24 h resulted in significant induction of secreted CTGF protein. This induction was markedly attenuated by Smad3 knockdown, but not by Smad2 knockdown. The Western immunoblot shown is a representative blot for CTGF in the cell culture supernatants. Results are means $\pm$ S.E.M. and expressed as a percentage of the control value ($n = 4$; $^{**}P < 0.01$).

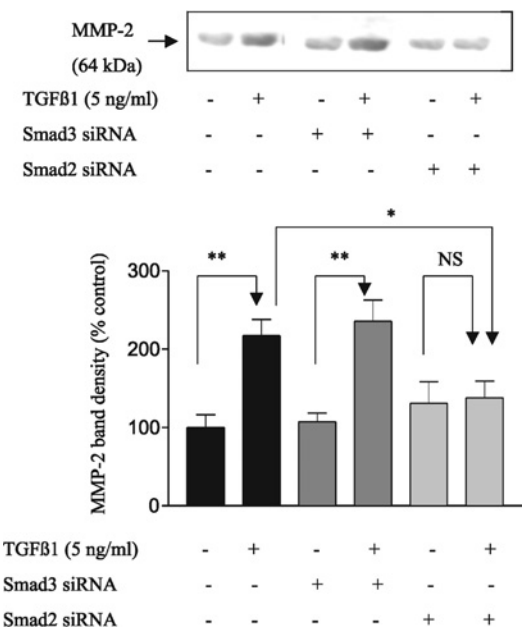

Figure 4 TGFβ1-induced secreted MMP-2 protein in human PTECs is Smad2-dependent and Smad3-independent

After transfection with Smad2 and Smad3 siRNAs as described in the Materials and methods section, HKCs were treated with either TGFβ1 (5 ng/ml) or vehicle (0.1 % BSA) for 48 h under serum-free conditions. Secreted MMP-2 was assessed by Western immunoblotting of the cell culture supernatants. TGFβ1 treatment for 48 h resulted in significant induction of secreted MMP-2 protein. This induction was markedly attenuated by Smad2 knockdown, but not by Smad3 knockdown. Western immunoblot shown is a representative blot for MMP-2 in the cell culture supernatants. Results are means $\pm$ S.E.M. and expressed as a percentage of the control value (n = 4; $^{**}P < 0.01$, $^{*}P < 0.05$).

induction was seen after 24 h of TGFβ1 treatment (results not shown). This induction was inhibited by Smad2 knockdown and not by Smad3 knockdown (Figure 4). These results confirm the efficacy of Smad2 knockdown by Smad2 siRNA and demonstrate that induction of MMP-2 by TGFβ1 in human PTECs is Smad2-dependent.

Down-regulation of E-cadherin in human PTECs in response to TGFβ1 treatment is Smad3 dependent

TGFβ1 treatment for 24 h resulted in about a 50 % decrease in E-cadherin expression. The TGFβ1-induced down-regulation of E-cadherin was prevented by Smad3 knockdown, but not by Smad2 knockdown (Figures 5A and 5B). Neither Smad2 nor Smad3 siRNAs had any effect on basal E-cadherin expression. These results demonstrate that down-regulation of E-cadherin by TGFβ1, an early event in the process of EMT in human PTECs, is critically dependent on Smad3. Similar Smad3-dependency of TGFβ1-induced down-regulation of E-cadherin was observed after 48 h of TGFβ1 treatment (results not shown).

Up-regulation of α-SMA in response to TGFβ1 in human PTECs is dependent on both Smad2 and Smad3

TGFβ1 treatment for 24 h did not result in significant up-regulation of α-SMA protein expression (results not shown). TGFβ1 treatment for 48 h resulted in 3–4-fold increases in α-SMA expression (Figure 6A). TGFβ1-induced α-SMA expression was attenuated by Smad2 or Smad3 knockdown to an equal extent (Figure 6A). However, about 2-fold induction of α-SMA was still seen in response to TGFβ1 in Smad3-siRNA-treated cells, but not in Smad2-siRNA-treated cells. Basal expression of α-SMA tended to increase in the presence of Smad2 knockdown, though this increase did not achieve statistical significance. As inhibition of either Smad2 or Smad3 resulted in partial reduction of TGFβ1-induced α-SMA expression, we next investigated the effect of simultaneous knockdown of both Smad2 and Smad3 on TGFβ1-induced α-SMA expression. Treatment with both Smad2 and Smad3 siRNA resulted in complete inhibition of TGFβ1-induced α-SMA expression (Figure 6B). We conclude from these experiments that TGFβ1-induced α-SMA expression in human PTECs involves both Smad2 and Smad3 signalling through parallel pathways.

DISCUSSION

In the present study, for the first time, we report differential roles of TGFβ1-activated receptor-associated Smads (Smad2 and Smad3) in the regulation of TGFβ1-driven key pro-fibrotic events in human PTECs, including, increased expression of CTGF, MMP-2 and α-SMA and decreased expression of E-cadherin. Our results demonstrate that Smad3 is required for induction of CTGF and down-regulation of E-cadherin, Smad2 is required for induction of MMP-2, and both Smad2 and Smad3 are involved in the induction of α-SMA in response to TGFβ1 in human PTECs.

Our group and others have previously demonstrated that induction of CTGF by TGFβ1 in various cell types including fibroblasts, mesangial cells and human PTECs requires Smad signalling [12,19,20]. In our previous work we used an HKC cell line overexpressing Smad7 to inhibit Smad signalling [12]. As this method does not allow us to differentiate between the role of Smad2 and that of Smad3, we decided in the present study to use siRNA to achieve selective knockdown of Smad2 and Smad3. This approach enabled us to study the role of endogenous Smad proteins in TGFβ1-induced cellular responses in differentiated adult human cells. This contrasts with other methods used

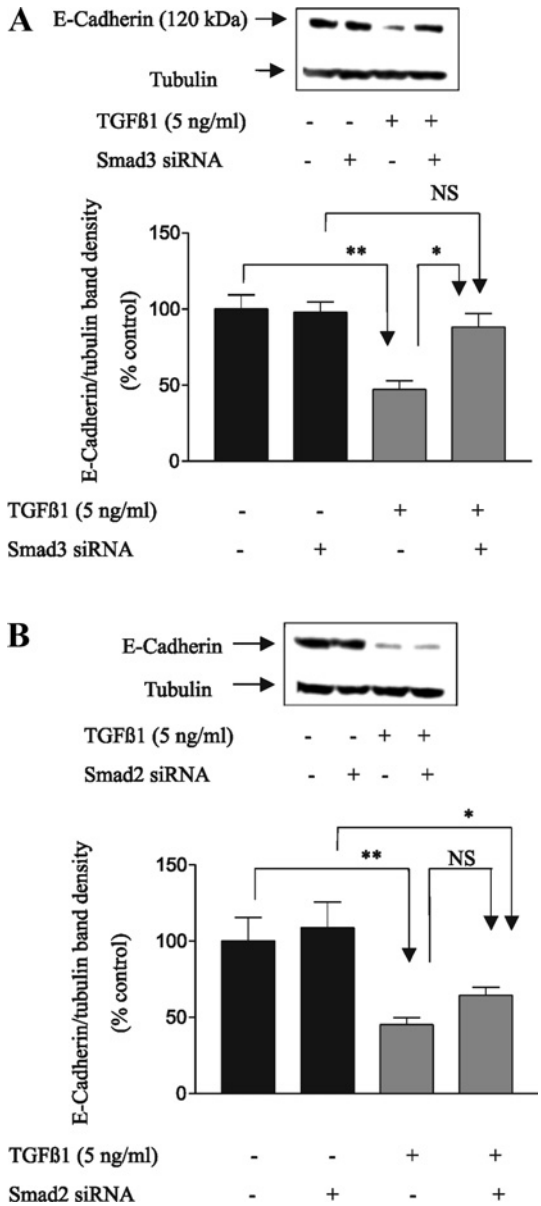

Figure 5 Decrease in E-cadherin expression in response to TGFβ1 in human PTECs is Smad3-dependent and Smad2-independent

After transfection with Smad2 and Smad3 siRNAs as described in the Materials and methods section, HKCs were treated with either TGFβ1 (5 ng/ml) or vehicle (0.1 % BSA) for 24 h under serum-free conditions. Cells were then lysed and cellular E-cadherin was assessed by Western immunoblotting. TGFβ1 treatment for 24 h resulted in an approx. 50 % decrease in cellular E-cadherin expression. This reduction was almost completely prevented by Smad3 knockdown (**A**), whereas Smad2 knockdown had a minimal, or no, effect (**B**). Results are means $\pm$ S.E.M. and expressed as a percentage of the control value ($n = 6$; $^{**}P < 0.01$, $^{*}P < 0.05$).

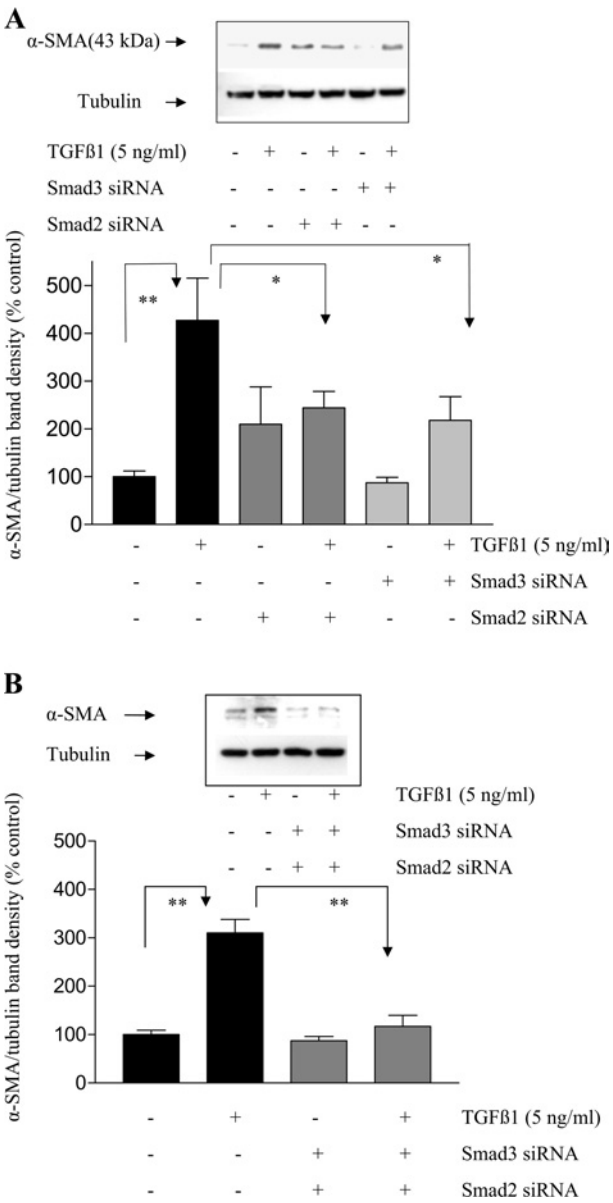

Figure 6 The TGFβ1-induced increase in α-SMA expression in human PTECs is Smad2- and Smad3-dependent

After transfection with Smad2 and Smad3 siRNAs as described in the Materials and methods section, HKCs were treated with either TGFβ1 (5 ng/ml) or vehicle (0.1 % BSA) for 48 h under serum-free conditions. Cells were then lysed and cellular α-SMA was assessed by Western immunoblotting. TGFβ1 treatment for 48 h resulted in a 4-fold increase in α-SMA expression. Either Smad2 or Smad3 knockdown resulted in a 50 % decrease in TGFβ1-induced α-SMA expression (**A**). Double knockdown of both Smad2 and Smad3 resulted in complete inhibition of TGFβ1-induced α-SMA expression (**B**). Results are means $\pm$ S.E.M. and expressed as a percentage of the control value (n = 4; $^{*}P < 0.05$, $^{**}P < 0.01$).

previously, such as overexpression systems and experiments performed in Smad2 and Smad3 knockout mice [13,21]. We first confirmed that knockdown of Smad2 and Smad3 by their respective siRNAs was selective and specific. Subsequently, we demonstrated that induction of secreted CTGF protein in response to TGFβ1 is Smad3-dependent and Smad2-independent. Lakos et al. [15] have previously reported that fibroblasts derived from Smad3 knockout mice show marked attenuation of TGFβ1-induced CTGF mRNA expression. Our results demonstrate that Smad3 is essential for the induction of CTGF protein in response to TGFβ1 in human PTECs. Smad3 knockout mice are resistant to interstitial fibrosis induced by unilateral ureteric obstruction [9], and it is suggested that CTGF expressed in tubular epithelium plays an important role in the development of tubulo-interstitial fibrosis [4]. Our observations suggest that the attenuated fibrotic response in kidneys from Smad3 knockout mice may be at least in part due to a decrease in CTGF expression. Other studies investigating the roles of Smad2 and Smad3 in induction of secondary mediators of fibrosis and tissue remodelling by TGFβ1 in fibroblasts have reported that induction of the pro-angiogenic protein vascular endothelial growth factor is Smad3-dependent

[22,23]. As CTGF is also a pro-angiogenic molecule, the results, taken together, suggest that TGFβ1–Smad3 signalling may mediate angiogenesis via multiple secondary mediators. It is likely that the TGFβ1–Smad3 signalling directly mediates transcription of the CTGF gene, as it has been shown previously that the Smad-binding element in the CTGF promoter is absolutely necessary for its induction by TGFβ [19,20].

One of the key events in the process of tubular EMT in the kidney is disruption of the tubular basement membrane mediated by MMP-2, which allows the transdifferentiating tubular epithelial cells to migrate to the interstitium [5,6]. TGFβ1 treatment for 48 h resulted in induction of MMP-2 secretion in human PTECs. In agreement with the previous experimental data from fibroblasts derived from Smad2 and Smad3 knockout mice [13], we found that TGFβ1-induced secreted MMP-2 in human PTECs was Smad2-dependent. In addition to its role in EMT, MMP-2 also contributes to the development of tubulo-interstitial fibrosis by conversion of latent TGFβ1 into active TGFβ1 by cleavage of latency-associated peptide [6]. Our results demonstrate a critical role for Smad2 in induction of MMP-2 by TGFβ1, an important pro-fibrotic event in the development of tubulo-interstitial fibrosis.

Next we investigated the regulation of two key events during the process of EMT in tubular epithelial cells, namely the reduction in E-cadherin expression and the increase in α-SMA expression in response to TGFβ1. TGFβ1 treatment for 24 h resulted in a significant (50–60 %) decrease in E-cadherin expression in human PTECs. This decrease was almost completely prevented by Smad3 knockdown. However, Smad2 knockdown had a minimal, or no, effect on down-regulation of E-cadherin by TGFβ1. These results demonstrate that Smad3 is the key mediator of E-cadherin down-regulation in response to TGFβ1 in human PTECs and are consistent with the previously published data on tubular epithelial cells obtained from Smad3 knockout mice and in other epithelial cell lines [9,24]. Furthermore, we have provided direct evidence that Smad2 is not involved in TGFβ1-induced E-cadherin loss in human PTECs. Exact mechanisms of down-regulation of E-cadherin in response to TGFβ1–Smad3 signalling in tubular epithelial cells remain unclear. As CTGF plays an important role in induction of EMT by TGFβ, it would be tempting to speculate that TGFβ1–Smad3 signalling induced CTGF at least in part contributes to the loss of E-cadherin in response to TGFβ1 [25]. In various other cell types it has been shown that TGFβ1-induced up-regulation of transcriptional repressors of E-cadherin, such as Snail, Slug and SIP-1, mediate transcriptional down-regulation of E-cadherin [26]. Whereas Snail has been shown to be an important mediator of TGFβ1-induced E-cadherin down-regulation in various cell types, including cultured tubular epithelial cells from Smad3 knockout mice [9,23], in HKCs (human kidney cells), Snail does not appear to play a role in TGFβ1-induced E-cadherin loss [8]. The mechanisms of E-cadherin loss in response to TGFβ1–Smad3 signalling in human PTECs warrant further investigation.

TGFβ1 treatment for 48 h resulted in induction of α-SMA protein in human PTECs. The roles of Smad2 and Smad3 in the regulation of α-SMA appeared more complex. Basal α-SMA protein levels increased with Smad2 knockdown, with no further induction by TGFβ1. In contrast, Smad3 knockdown did not alter basal α-SMA protein levels, but caused an approx. 50 % reduction in TGFβ1-induced α-SMA. Therefore induction of α-SMA by TGFβ1 in human PTECs shows a partial dependence on Smad3 and requires Smad2. The 2-fold increase in α-SMA protein levels in response to TGFβ1 in the presence of Smad3 knockdown could be explained by potential compensation by Smad2 for low levels of Smad3. The role of Smad3 in regulation of α-SMA is controversial. Lakos et al. [15] reported only a moderate decrease in TGFβ1-induced α-SMA expression in fibroblasts obtained from Smad3 knockout mice as compared with cells obtained from wild-type littermates. Flanders et al. [16] reported equivalent induction of α-SMA protein in Smad3 null mouse fibroblasts as compared with the wild-type fibroblasts, suggesting that Smad2 could compensate for lack of Smad3 for induction of α-SMA in response to TGFβ1 in these cells. However, induction of α-SMA in cultured tubular epithelial cells obtained from Smad3 knockout mice was dependent on Smad3 [9]. We used transient knockdowns of Smad proteins using siRNA as opposed to the knockout techniques used in previous studies. As knockout mice may have adaptive changes that could potentially modify cellular responses, results obtained from these cells may differ from those obtained from differentiated adult human cells with transient knockdowns in our experiments. The role of Smad2 in the induction of α-SMA has not been investigated previously, and our results suggest that Smad2 also plays a role in the induction of α-SMA in human PTECs. As double knockdown of both Smad2 and Smad3 resulted in complete inhibition of TGFβ1-induced α-SMA expression, we conclude that both Smad2 and Smad3 are involved in the increase in TGFβ1-induced α-SMA expression in human PTECs.

Analysis of our results as a whole suggests that early TGFβ1 fibrotic responses, such as induction of CTGF and down-regulation of E-cadherin are critically dependent on Smad3. Smad2 is involved in delayed events such as induction of MMP-2 and α-SMA, the latter requiring both Smad2 and Smad3. Although our work supports the notion that Smad3 is the key mediator of TGFβ-induced tubulo-interstitial fibrosis [17], a role for Smad2 in the induction of MMP-2 and α-SMA suggests that Smad2 may also play an important role in the development of tubulo-interstitial fibrosis. In future work we aim to extend these observations and investigate the effect of small-molecule inhibitors targeted to Smad2 and Smad3 *in vivo* using animal models of tubulo-interstitial fibrosis.

This work was supported by South West Thames Kidney fund. We thank Dr Jonathan Kwan and Dr Mike Bending of the South West Thames Institute for Renal Research, St. Helier Hospital, for their support and advice.

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Received 11 July 2005/13 September 2005; accepted 27 October 2005
Published as BJ Immediate Publication 27 October 2005, doi:10.1042/BJ20051106