GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Opposing Roles for Smad2 and Smad3 in Peritoneal Fibrosis in Vivo and in Vitro

Wen-Juan Duan,* Xueqing Yu,* Xiao-Ru Huang,1 Jian-wen Yu,1 and Hui Yao Lan1

From the Department of Nephrology,* The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou; and the Department of Medicine and Therapeutics,1 Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, China

Accepted for publication April 28, 2014.

Address correspondence to Hui Yao Lan, M.D., Ph.D., Department of Medicine and Therapeutics, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong; or Xueqing Yu, M.D., Ph.D., Department of Nephrology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China. E-mail: hylan@cuhk.edu.hk or yuxq@mail.sysu.edu.cn.

Peritoneal fibrosis is a major cause of ultrafiltration failure in patients receiving continuous ambulatory peritoneal dialysis. Transforming growth factor (TGF)-β1 is an important mediator in this process; however, its signaling mechanisms had not been explored. Thus, we examined TGF-β1/Smad signaling in human peritoneal biopsy specimens associated with continuous ambulatory peritoneal dialysis. We found that TGF-β/Smad2/3 signaling was highly activated in patients with increased collagen deposition and thickening of the peritoneal membrane who were receiving continuous ambulatory peritoneal dialysis. Long-term exposure of wild-type mice to 4.25% peritoneal dialysis solution for 30 days induced significant peritoneal fibrosis with impaired peritoneal equilibrium, which was prevented in Smad3 knockout mice. In contrast, conditional Smad2 gene deletion in the peritoneum exacerbated peritoneal fibrosis and dysfunction. The contrasting roles of Smad2 and Smad3 in peritoneal fibrosis were also examined in vitro. Cultured mesothelial cells from Smad3 knockout mice were resistant to TGF-β1-induced collagen I production and the transition toward a myofibroblast phenotype as seen in wild-type cells, whereas Smad2 deficiency in mesothelial cells failed to modulate the profibrotic response to TGF-β1. In conclusion, this study found activation of TGF-β1/Smad signaling in peritoneal fibrosis in patients receiving continuous ambulatory peritoneal dialysis and identifies opposing roles for Smad2 and Smad3 in peritoneal dialysis—associated peritoneal fibrosis. These findings provide a mechanistic basis for future therapies targeting TGF-β1/Smad signaling in peritoneal fibrosis. (Am J Pathol 2014, 184: 2275–2284; http://dx.doi.org/10.1016/j.ajpath.2014.04.014)

Continuous ambulatory peritoneal dialysis (CAPD) is a widely used method of renal replacement therapy. However, long-term continuous exposure to dialysis fluid results in a reduction in ultrafiltration across the peritoneal membrane, ultimately leading to technique failure in many CAPD patients.1 Pathologic changes in the peritoneal membrane in response to long-term dialysis include a loss of the mesothelial cell layer and a thickening of the submesothelial layer that features increased myofibroblasts, collagen deposition, and new blood vessels.2–4 These morphologic changes are associated with loss of ultrafiltration function, but the mechanisms responsible for this loss are challenging to study in patients because of the practical and ethical difficulties in obtaining additional samples of peritoneal tissue. Therefore, progress in this area often relies on the use of animal models in which genetic and pharmacologic manipulation can establish causative roles for individual mechanisms.

Transforming growth factor (TGF)-β1 is a major profibrotic factor in many different forms of tissue fibrosis, including peritoneal fibrosis.5 A role for TGF-β1 in promoting peritoneal fibrosis has been proposed based on a range of evidence.6 Significant levels of TGF-β1 can be detected in peritoneal dialysate, although it is difficult to establish a cause and effect relationship with peritoneal fibrosis.7,8 However, long-term administration of PD fluid in animal studies can induce TGF-β1 production in the peritoneum and progressive peritoneal fibrosis.9 In addition,

Supported by the Major State Basic Research Development Program of China 973 program, grant 2012CB517700 (H.Y.L. and X.Y.), National Natural Scientific Foundation of China grant 81130012 (X.Y.), Research Grant Council of Hong Kong grants CUHK5/CRF/09 and CUHK3/CRF/12R (H.Y.L.), and Chinese University of Hong Kong Focused Investment Scheme A and B Programs (H.Y.L.).

Disclosures: None declared.
adenovirus-mediated overexpression of active TGF-β1 in mesothelial cells is a potent inducer of peritoneal fibrosis and loss of ultrafiltration function. Finally, the addition of TGF-β1 to cultured human mesothelial cells induces collagen production and epithelial to mesenchymal transition (EMT), providing an in vitro model to dissect mechanisms relevant to peritoneal fibrosis.

TGF-β1 can signal through Smad-dependent and Smad-independent pathways. However, most profibrotic actions of TGF-β1 operate via Smad signaling. This involves phosphorylation of Smad2 and Smad3, which form a homodimer and then bind with Smad4 and translocate to the nucleus to regulate many different genes involved in the fibrotic response, which is counterregulated by an inhibitory Smad7. Many studies have established a critical role of TGF-β/Smad signaling in peritoneal fibrosis. It is reported that Smad3-deficient mice are protected from peritoneal fibrosis caused by adenovirus-mediated gene transfer of active TGF-β1 in the absence of PD conditions. In contrast, overexpression of Smad7 is capable of blocking PD-induced TGF-β/Smad activation and peritoneal fibrosis. Interestingly, our recent study identified that Smad2 and Smad3 play a diverse role in renal fibrosis under a variety of disease conditions in vivo and in vitro. For example, an antifibrotic role for Smad2 in the development of interstitial fibrosis directly opposes the profibrotic role of Smad3 in the obstructive nephropathy. However, whether this unique observation is more generally applicable and whether Smad2 and Smad3 play opposing roles in peritoneal fibrosis are unknown.

Thus, the current study aimed to establish the importance of TGF-β1/Smad signaling in human peritoneal fibrosis and to dissect the relative contribution of Smad2 versus Smad3 in a mouse model of peritoneal dialysis (PD) fluid–induced peritoneal fibrosis.

Materials and Methods

Peritoneal Biopsy

Posterior peritoneal tissues were obtained from 15 CAPD patients who were subjected to PD withdrawal because of the technical failure of PD, such as catheter dysfunction after long-term PD as previously described. Of them, five patients had IgA nephropathy, five had diabetic nephropathy, two had hypertensive nephropathy, and three had crescentic glomerulonephritis. All patients (8 male and 7 female; mean ± SD, 52 ± 6.7 years) had undergone PD for 25 to 66 months using a conventional glucose-based PD solution (Dianeal; Baxter Healthcare, Deerfield, IL) with a regimen of three to five exchanges daily. In addition, five normal omental tissues were obtained by surgical removal of paragaric cancer tissues as healthy controls. All the participants had signed the informed consent forms. The study was approved by the Institutional Review Board and Ethics Committee of The First Affiliated Hospital (Sun Yat-sen University).

Mouse Model of High Glucose–Induced Peritoneal Fibrosis and PET

Mice with a floxed Smad2 gene (Smad2 FF) and Smad3 gene knockout (KO) or wild-type (WT) on the C57BL/6J background mice were maintained in the animal facility of the Chinese University of Hong Kong. The characterization of Smad2 FF and Smad3 KO mice was described previously. Groups of eight mice were given once daily i.p. injections of 3 mL of 4.25% PD solution (Baxter Healthcare) for 30 days. A peritoneal equilibrium test (PET) was performed in each mouse before being euthanized. Briefly, after i.p. injection of 3 mL of the PD solution, dialysate samples were collected at 60 and 120 minutes of dwell time, respectively. Peritoneal tissues, including the anterior abdominal wall and the mesentery, were examined by histologic analysis, immunohistochemistry (IHC), real-time PCR, and Western blot analysis. The study was approved by the Animal Experimental Ethic Committee at the Chinese University of Hong Kong.

Conditional Smad2 Gene Deletion

To delete Smad2 from the peritoneal tissue, a plasmid expressing a green fluorescence protein (GFP)-Cre fusion protein was transfected into the peritoneal tissue of Smad2 FF mice using ultrasonography-based gene therapy as previously described. Briefly, the mixture of plasmid and lipid microbubbles (Sonovue, Diagnostics, Princeton, NJ) in 1:1 ratio (v/v) was prepared, and 1 mL of mixture containing 200 μg of plasmid with or without Cre (as control) was injected into the abdominal cavity and followed immediately by the continuous-wave ultrasonographic treatment (Therasonic; Electro-Medical Supplies, Wantage, England) at 1 MHz, 2 W/cm², for 4 minutes in a 1-minute interval. The ultrasonographic transducer was placed onto the anterior abdominal wall and moved over the entire anterior abdominal surface at the face-up and -down positions. GFP-Cre expression and degree of Smad2 deletion from the peritoneal tissues were confirmed in a pilot study of three mice sacrificed at 5 days after ultrasonographic gene therapy. The study was approved by the Animal Experimental Ethic Committee at the Chinese University of Hong Kong.

Primary Culture of Mouse Mesothelial Cells

Primary cultures of mouse peritoneal mesothelial cells (PMCs) were obtained from Smad3 WT or KO mice and Smad2 FF mice by trypsin digestion from peritoneal tissue using an established protocol. Briefly, anesthetized mice were given an i.p. injection of 10 mL of 0.25% trypsin and 0.02% EDTA solution and sacrificed 60 minutes later for collecting the peritoneal cells for in vitro studies. The PMCs were polygonal and exhibited a characteristic cobblestone pattern when confluent and stained positive for cytokeratin. To delete Smad2 from PMCs isolated from Smad2 FF mice,
cells were infected with a Cre-expressing adenovirus or a control adenovirus (multiplicity of infection = 50), as previously described.24 The PMCs at passages 2 or 3 were used for studies. After serum starvation for 12 hours, cells were treated with 2.5 ng/mL of TGF-β1 (R&D System Inc., Minneapolis, MN) for varying periods as indicated. Cells were then harvested for real-time and Western blot analysis.

Histopathologic Analysis and IHC

Histologically, 3-μm paraffin sections from the anterior abdominal wall were stained with periodic acid-Schiff (PAS) and Masson’s trichrome. The thickness of the peritoneum was measured in each group using a micrometer fitted into the eyepiece of the microscope and expressed as means ± SD. Each section was measured at five random sites.

Immunohistologically, 4-μm paraffin sections from the anterior or posterior peritoneal tissues were stained for collagen I (Southern Biotech, Birmingham, AL), α-smooth muscle actin (α-SMA; Dako, Glostrup, Denmark), cytokeratin-18 (Sigma, St. Louis, MO), and TGF-β1, Smad2, and phospho-Smad2/3 (p-Smad2/3; Santa Cruz Biotechnology Inc., Santa Cruz, CA) with a microwave-based antigen retrieval method. Briefly, after microwaving, sections were incubated with the primary antibody overnight, followed by the secondary antibody, and developed with diaminobenzidine. Sections were then counterstained with hematoxylin.

Real-Time PCR Analysis

Total RNA from tissue samples or cultured PMCs was extracted using the RNeasy Kit (Qiagen, Valencia, CA). Real-time PCR for collagen I, α-SMA, and Smad2 mRNA expression was performed with SYBR Green PCR reagents (Bio-Rad, Hercules, CA) on an Opticon real-time PCR machine (MJ Research Inc., Waltham, MA) using the primers as previously described.19,23,24

Figure 1 Evidence of activation of TGF-β/Smad signaling in peritoneal fibrosis in patients undergoing long-term CAPD. A, C, E, and G: Healthy posterior peritoneal tissues (omentum). B, D, F, and H: Diseased posterior peritoneal tissues from patients undergoing long-term CAPD. Note that compared with healthy tissues, severe peritoneal fibrosis with many α-SMA+ myofibroblasts (B) and collagen I accumulation (D) in the thickened peritoneal tissues are associated with a marked up-regulation of TGF-β1 (F) and activation of Smad signaling as evidenced by p-Smad2/3 nuclear location (H). Cells exhibited mesothelial to mesenchymal transition are clearly identified using two-color immunohistochemistry with CK18+ α-SMA+ in fibrotic (E) but not in healthy (A) peritoneal tissues. Scale bars: 100 μm. Original magnification: ×800 (insets, A and B). α-SMA, α-smooth muscle actin; CAPD, continuous ambulatory peritoneal dialysis; CK, cytokeratin; PD, peritoneal dialysis; p-Smad2/3, phosphorylated Smad2/3; TGF, transforming growth factor.
Western Blot Analysis

Proteins from peritoneal tissues and PMCs were extracted with radioimmunoprecipitation assay lysis buffer for Western blot analysis as previously described.\textsuperscript{16,17} Briefly, after blocking with 5% dried nonfat milk in Tris-buffered saline that contained 0.1% Tween 20, membranes were incubated overnight at 4°C with primary antibodies against p-Smad2/3, Smad2, total Smad2/3, collagen type I, α-SMA, E-cadherin, or glyceraldehyde-3-phosphate dehydrogenase.
After washing, membranes were incubated with LI-COR IRDye 800 e-labeled secondary antibodies (Rockland Immunocatalysts, Gilbertsville, PA). The signal was detected with Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE) and quantified against the internal loading control glyceraldehyde-3-phosphate dehydrogenase with ImageJ version 1.48 (NIH, Bethesda, MD).

Statistical Analysis

Data obtained from the study are expressed as means ± SEM. Statistical analyses were performed using one-way analysis of variance followed by the Newman-Keuls Post Test using the Prism Program version 5.0 (GraphPad Software, San Diego, CA).

Results

TGF-β/Smad Signaling Is Highly Activated in CAPD Patients with Peritoneal Fibrosis

IHC identified a single layer of cytokeratin-positive mesothelial cells with low expression of TGF-β1 and minimal extracellular matrix accumulation (collagen I) without detectable p-Smad2/3 in normal posterior peritoneal tissues (omentum) collected from surgically removed gastric cancer tissues (Figure 1, A, C, E, and G). In contrast, patients undergoing PD for >2 years had a marked peritoneal thickening with excessive collagen I deposition, up-regulation of TGF-β1, and activation of Smad2/3 identified by many nucleated p-Smad2/3-positive cells (Figure 1, B, D, F, and H). In addition, severe peritoneal fibrosis in CAPD patients was associated with mesothelial-myofibroblast conversion as demonstrated by cytokeratin-positive, α-SMA-positive cells (Figure 1B).

Smad3 KO Mice Are Protected from PD-Induced Peritoneal Fibrosis

Compared with healthy Smad3 WT mice, daily i.p. injection of PD solution for 30 days in WT mice induced significant peritoneal damage with thickening of the mesothelial layer and increased cellularity with a marked collagen matrix deposition as shown by PAS and Masson’s trichrome staining and collagen I IHC (Figure 2). This was associated with up-regulation of peritoneal TGF-β1 and activation of TGF-β/Smad signaling identified by many p-Smad2/3+ cells in the thickened mesothelial layer (Figure 2). In contrast, Smad3 KO mice were protected from PD-induced peritoneal fibrosis and...
activation of TGF-β/Smad signaling (Figure 2). These results were further confirmed by real-time PCR and Western blot analysis. PD-induced up-regulation of collagen I and α-SMA was prevented in Smad3 KO mice at the mRNA and protein levels (Figure 3). Interestingly, deletion of Smad3 also prevented a loss of E-cadherin in response to PD (Figure 3B). Furthermore, as determined by PET, PD-induced loss of peritoneal function in WT mice was completely prevented in Smad3 KO mice (Figure 4). Of note, Smad3 KO mice had a lower baseline peritoneal function because of the small size (40% reduced) when compared with WT mice; however, Smad3 KO mice did not have a reduction in the PET after administration of high glucose dialysis solution (Figure 4).

Conditional Deletion of the Smad2 Gene from Peritoneal Tissues Exacerbates Peritoneal Fibrosis in Response to PD in Vivo

Global Smad2 gene deletion in mice is lethal; therefore, to examine the role of Smad2 in experimental PD, we used conditional Smad2 deletion mice. Five days before commencing the PD model, Smad2 FF mice were given an i.p. injection of microbubbles complexed with a GFP-Cre fusion protein expression plasmid (or a control plasmid) followed by ultrasonographic treatment to conditionally delete the Smad2 gene from the peritoneal membrane. To confirm the transfection efficiency, two mice were sacrificed 3 days after ultrasonography-based gene transfer of GFP-Cre plasmid. Under a fluorescent microscope, we found that strong GFP-positive cells were detected in visceral and parietal mesothelial cells (Supplemental Figure S1). Interestingly, many cells in submesothelial layers also expressed GFP, indicating GFP-Cre transfected cells (Supplemental Figure S1A). Consistent with GFP-positive cell data, IHC detected that ultrasonography-mediated Cre expression resulted in Smad2 deletion from the mesothelial layers in healthy and PD-treated Smad2 FF mice (Supplemental Figure S1B). Further studies using real-time PCR and Western blot analysis also revealed that there was a threefold increase in peritoneal Smad2 mRNA and protein expression in mice receiving PD for 30 days, which was

Figure 5  Conditional deletion of Smad2 from the peritoneum enhances TGF-β/Smad-mediated peritoneal fibrosis in a mouse model of PD. A: PAS staining. B: Masson’s trichrome staining. C: Collagen I immunostaining. D: TGF-β1 immunostaining. E: p-Smad2/3 immunostaining. F: Quantitative analysis of peritoneal thickness from PAS-stained sections. Note that deletion of Smad2 largely enhances PD-induced activation of TGF-β/Smad signaling, thereby promoting peritoneal fibrosis. Data are expressed as means ± SEM from groups of six mice. *P < 0.001 versus healthy controls; †P < 0.001 versus Smad2 FF PD mice treated with CV (PD + CV). Scale bars: 100 μm. Original magnification: ×800 (E, inset). ΔS2K0, conditional Smad2 gene deletion; CV, control vector; p-Smad2/3, phosphorylated Smad 2/3; PAS, periodic acid-Schiff; PD, peritoneal dialysis; Smad2 FF, Smad2 flox/flox; TGF, transforming growth factor.
virtually blocked by ultrasonography-mediated Cre expression (Supplemental Figure S1, C and D), establishing effective Smad2 gene deletion from the peritoneal tissues.

We then investigated the functional role of Smad2 in PD. By comparison with WT mice, PD solution administration in mice with conditional Smad2 gene deletion exacerbated peritoneal fibrosis as indicated by a significant increase in the thickening of peritoneum, with abundant collagen I deposition and a marked up-regulation of TGF-β1, and, in particular, an increase in the number of cells exhibiting nuclear p-Smad2/3 staining (Figure 5). Analysis by real-time PCR and Western blotting confirmed the significant increase in collagen I mRNA expression and protein deposition in mice with conditional Smad2 deletion (Figure 6). In addition, Western blotting confirmed that the marked increase in Smad2/3 phosphorylation in mice with conditional Smad2 deletion was associated with a loss of E-cadherin and a marked increase in α-SMA expression, indicating an increase in the EMT process (Figure 6). Furthermore, conditional Smad2 deletion resulted in a worse outcome on the PET (Figure 7).

Differential Role of Smad3 and Smad2 in the Primary Culture of Mesothelial Cells in Response to TGF-β1 in Vitro

The functional role of Smad2 and Smad3 was further examined in primary cultures of mesothelial cells. TGF-β1 was found to induce Smad2/3 phosphorylation in mesothelial cells and to induce an increase in collagen I expression and a transition toward a myofibroblast phenotype based on the loss of E-cadherin expression and de novo expression of α-SMA (Supplemental Figures S2 and S3). Consistent with the protection of Smad3 KO mice from peritoneal fibrosis in vivo, mesothelial cells isolated from Smad3 KO mice were resistant to TGF-β1-induced collagen I expression and did not transition toward a myofibroblast phenotype (Figure 8).

We next examined the function of Smad2. By using an adenovirus expressing the Cre enzyme, Smad2 was efficiently deleted from mesothelial cells isolated from Smad2 FF mice compared with a control adenovirus (Supplemental Figure S4). Deletion of Smad2 did not prevent TGF-β1-induced Smad3 phosphorylation (Supplemental Figure S4), TGF-β1—induced collagen I and α-SMA expression, and loss of E-cadherin (Figure 9).

Discussion

This study has identified TGF-β1/Smad signaling in peritoneal fibrosis in biopsy tissues from patients undergoing PD. In addition, we found contrasting roles for Smad2 and Smad3 in peritoneal fibrosis in a mouse model of PD. The significance of these findings and potential mechanisms underlying the opposing functional roles of Smad2 and Smad3 are considered below.

Investigation of patients undergoing PD has identified increased TGF-β1 levels in peritoneal fluid, but it has proven difficult to establish the causal link between levels of
TGF-β1 and peritoneal fibrosis.6–8 The clinical data supporting TGF-β1 as a major driver of peritoneal fibrosis comes from studies of isolated human mesothelial cells in which TGF-β1 induces increased collagen production and EMT and from peritoneal biopsy specimens in which mesothelial to myofibroblast transition is evident.11 This study provides in vivo evidence that increased TGF-β1/Smad signaling occurs in patients with peritoneal fibrosis. In addition, it signifies the clinical relevance for studies in cultured mesothelial cells in which high glucose exposure induces TGF-β1 and extracellular matrix production8,9,26 and in animal models in which long-term exposure to high glucose dialysate induces TGF-β1/Smad signaling in association with peritoneal fibrosis.9,16–18 Furthermore, the present finding is also consistent with previous studies with adenovirus-mediated TGF-β1 overexpression in the peritoneal cells or chlorhexidine gluconate induced peritoneal fibrosis.10,16,27

Our most significant finding is that peritoneal fibrosis is mediated by Smad3 but not Smad2 in vivo and in vitro. Many studies have found that peritoneal fibrosis in response to high glucose dialysate can be inhibited by TGF-β1—blocking peptides or through overexpression of the inhibitory Smad7,17,18,28 establishing a functional role for TGF-β1/Smad signaling in peritoneal fibrosis. However, whether high glucose dialysate induces peritoneal fibrosis via Smad2 and/or Smad3 has not been addressed. We report that Smad3 is critical for PD-induced peritoneal fibrosis because Smad3 KO mice were protected from peritoneal damage. Of interest was the finding that untreated Smad3 KO mice have a reduced capacity apparent on PET compared with WT mice. Whether this relates to smaller body size and/or subtle differences in peritoneal membrane structure was not explored. In addition, Smad3 KO mesothelial cells were resistant to TGF-β1—induced up-regulation of collagen production and EMT, which is consistent with previous studies in cultured mesothelial cells and in vivo by adenoviral-based overexpression of active TGF-β1 in Smad3-deficient mice.16,29 Indeed, Smad3-deficient mice are protected from peritoneal fibrosis and angiogenesis caused by adenovirus-mediated gene transfer of active TGF-β1. However, there is still evidence of mesothelial transition to α-SMA—positive myofibroblasts, suggesting a Smad3-independent mechanism of EMT that operates via the TGF-β/Akt pathway.16 We did not find...
data are expressed as means ± SEM from four independent studies. *P < 0.05, **P < 0.01 versus healthy medium controls. Smad2 gene deletion; α-SMA, α-smooth muscle actin; Adv, adenovirus; CV, control vector; E-cad, E-cadherin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Smad2 FF, Smad2 flox/flox; TGF, transforming growth factor.

**Figure 9** Effect of TGF-β1 on peritoneal fibrosis in mesothelial cells conditionally deleted for Smad2 in vitro. Western blots reveal that the addition of 2.5 ng/mL of TGF-β1 induces a loss of E-cad but up-regulates α-SMA and collagen I in mesothelial cells isolated from Smad2 FF mice in a time-dependent manner, which is not altered in conditional Smad2 KO mesothelial cells. White bars, 0 days; gray bars, 3 days; black bars, 7 days. Data are expressed as means ± SEM from four independent studies.

Evidence of a Smad3-independent mechanism of peritoneal fibrosis because mice lacking Smad3 abolished peritoneal fibrosis induced by high glucose dialysate. The apparent difference between these studies likely relates to the different insults used to induce peritoneal fibrosis. In this study, mice were exposed in the long term to high glucose dialysate, and Smad3 KO mice failed to up-regulate TGF-β1 expression in the peritoneum, thereby preventing autoinduction of TGF-β1-driven fibrosis and EMT. In contrast, the use of adenoviral-based overexpression of active TGF-β1 in peritoneal tissues of Smad3 KO mice by Patel et al.13 overcomes the failure to up-regulate TGF-β1 as seen, thereby driving the EMT process via a Smad3-independent mechanism in response to higher levels of TGF-β1 produced in the peritoneal tissue.

Unlike Smad3, defining the role of Smad2 in TGF-β1-induced fibrosis has been difficult because of the lethality associated with Smad2 deficiency. However, there are clear differences in Smad2 and Smad3 function despite their close association in TGF-β signaling. Smad3 can bind directly to Smad-binding elements in many gene promoters, including collagens I, III, and IV, but Smad2 is unable to directly bind to DNA.30 In addition, the Smad3/4 complex is sufficient to bind gene promoters for transcription,15 whereas the Smad2/Smad4 complex requires binding to additional co-activators or co-repressors to regulate gene expression.31 Furthermore, it is also likely that Smad2 and Smad3 may exert their differential roles in a cell type–dependent manner. For example, silencing Smad3 in human keratinocytes inhibits TGF-β1–induced cell proliferation, but silencing Smad2 has no effect on the proliferative response.30 The exacerbation of peritoneal fibrosis in mice with conditional Smad2 gene deletion may be associated with the enhancement of the TGF-β1 autoinduction loop as evidenced by increased Smad3 phosphorylation and TGF-β1 production. This is consistent with our previous finding that deletion of Smad2 enhances Smad3 signaling and renal fibrosis in the obstructive kidney.19 Thus, Smad2 plays an antifibrotic role in two different tissues in response to two different insults. However, it remains to be determined whether Smad2 plays a general antifibrotic role in other forms of tissue fibrosis.

Interestingly, although conditional deletion of Smad2 enhanced peritoneal fibrosis in response to high glucose dialysate in vivo, this was not evident in Smad2-deficient mesothelial cells in vitro. This discrepancy may imply that unlike in vitro studies that use only TGF-β1, additional factors, such as advanced glycation end products, angiotensin II, and osmotic stress, may be involved in the in vivo study, thereby enhancing TGF-β1/Smad3 signaling in the absence of Smad2 in the peritoneum under the more complex in vivo situation occurring in PD.

In contrast to the present study, a recent report found that siRNA-based knockdown of the Smad2 partially inhibited TGF-β1–stimulated expression of collagen III and fibronectin in cultured human mesothelial cells.32 This finding appears at odds with the current study in which Smad2-deficient mesothelial cells had no reduction in TGF-β1-induced collagen production or EMT. The reasons for these apparently discrepant results are not immediately apparent, although the study by Lv et al.32 did not quantify the degree of inhibition of the TGF-β1 response in Smad2 knock-down cells or examine whether expression of TGF-β receptors or Smad3 was affected by the Smad2 knock-down technique.

**Conclusions**

We identified a marked increase in TGF-β/Smad signaling in human peritoneal fibrosis. We also identified opposing roles for Smad2 and Smad3 in the development of peritoneal fibrosis in mice in response to high glucose dialysate during PD. These findings provide a mechanistic basis for future therapies that target TGF-β/Smad signaling in peritoneal fibrosis.
Acknowledgment

We thank David J. Nikolic-Paterson (Monash Medical Center) for expert editing of the manuscript.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.04.014.

References

7. Mlambo NC, Hylander B, Brauner A: Increased levels of transforming growth factor beta 1 and basic fibroblast growth factor in patients on CAPD: a study during non-infected steady state and peritonitis. Inflammation 1999, 23:131–139