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TGF-β1 → SMAD/p53/USF2 → PAI-1 transcriptional axis in ureteral obstruction-induced renal fibrosis

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Abstract

Chronic kidney disease constitutes an increasing medical burden affecting 26 million people in the United States alone. Diabetes, hypertension, ischemia, acute injury, and urological obstruction contribute to renal fibrosis, a common pathological hallmark of chronic kidney disease. Regardless of etiology, elevated TGF-β1 levels are causatively linked to the activation of profibrotic signaling pathways initiated by angiotensin, glucose, and oxidative stress. Unilateral ureteral obstruction (UUO) is a useful and accessible model to identify mechanisms underlying the progression of renal fibrosis. Plasminogen activator inhibitor-1 (PAI-1), a major effector and downstream target of TGF-β1 in the progression of several clinically important fibrotic disorders, is highly up-regulated in UUO and causatively linked to disease severity. SMAD and non-SMAD pathways ($pp60^{c\text{-}src}$, epidermal growth factor receptor [EGFR], mitogen-activated protein kinase, p53) are required for PAI-1 induction by TGF-β1. SMAD2/3, $pp60^{c\text{-}src}$, EGFR, and p53 activation are each increased in the obstructed kidney. This review summarizes the molecular basis and translational significance of TGF-β1-stimulated PAI-1 expression in the progression of kidney disease induced by ureteral obstruction. Mechanisms discussed here appear to be operative in other renal fibrotic disorders and are relevant to the global issue of tissue fibrosis, regardless of organ site.

Keywords

Fibrosis; PAI-1; TGF-β1; p53; Transcription

Cellular events in obstructive renal disease

Interstitial fibrosis is a common hallmark and major prognostic indicator of end-stage renal disease (Eddy 2000; Strutz and Neilson 2003; Eddy 2005; Bonventre 2010). The extent of associated tubulointerstitial pathology (i.e., inflammation, tubular atrophy, progressive fibrosis) has prognostic significance regardless of etiology (Grande et al. 2010; Truong et al. 2011). Congenital obstructive nephropathy, frequently involving the ureteropelvic junction, is the most significant contributor to chronic renal disease and eventual organ failure in children (Chevalier et al. 2010; Klein et al. 2010). Obstructive uropathy in adults generally reflects several age-associated disease processes, both benign and malignant. Whereas the pathophysiology of nephrofibrosis is complex regardless of origin, unilateral ureteral obstruction (UUO) in both neonatal and adult rodents is a well-established in vivo model of disease progression (Klahr and Morrissey 2002; Bascands and Schanstra 2005; Chevalier et al. 2009; Puri et al. 2010). Tubulointerstitial inflammatory and fibrotic responses following

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experimental UUO, orchestrated predominantly by dramatically elevated transforming growth factor-β1 (TGF-β1) levels in the injured kidney (Miyajima et al. 2000; Inazaki et al. 2004), closely mirror human obstructive uropathy (Hruska 2002; Moller et al. 1984) providing an accessible, translationally relevant, in vivo platform for the dissection of critical events and the identification of potential therapeutic targets. The extent of renal impairment or progression to eventual organ failure depends, to some degree, on the duration of obstruction but may be pronounced even upon short periods of blockage, and in many cases, injury progresses even upon release of the restriction (Truong et al. 2011). Persistence of elevated renal TGF-β1 expression, a potent profibrotic cytokine, even after relief of UUO (depending on the timing of release) leads to progressive tissue injury, impaired growth, and eventual loss of kidney function (Chevalier et al. 1999, 2009, 2010). Inflammation, interstitital fibrosis, tubular atrophy, and glomerular sclerosis all increase in the post-obstructed organ (Chevalier et al. 2009). Progressive fibrosis, moreover, is associated with capillary rarefaction and tissue hypoxia, exacerbating the fibrotic response (Boor 2010).

Within hours after experimental ureteral occlusion, the affected kidney exhibits changes in hydrostatic forces and increased oxidative stress (Schreiner et al. 1988; Klahr and Morrissey 2002; Dendoovan et al. 2010). Tubular stretch stimulates TGF-β1 expression (>20-fold), apoptosis, and inflammatory responses dependent upon nuclear factor kappa-B (Miyajima et al. 2000; Rohatgi and Flores 2010). Marked inflammation and excessive extracellular matrix (ECM) accumulation, the latter being attributable to the increased synthesis of collagen and fibronectin (predominantly by activated fibroblasts or myofibroblasts) coupled with reduced matrix degradation, accompanies tubular dilation, atrophy, progressive fibrosis, nephron loss, and scarring with eventual impairment of tissue function (Strutz and Neilson 2003; Eddy and Fogo 2006; Eddy 2000; Grande and Lopez-Novoa 2009; Higgins et al. 2003; Yabuki et al. 2005; Meng et al 2010; Manucha 2007; Yang et al. 2010; Truong et al. 2011). Myofibroblast density and/or persistence correlates with the severity of tubulointerstitial fibrosis and progression to renal failure (Qi et al. 2006b). Although their origin is uncertain, myofibroblasts are probably derived (1) from resident interstitial fibroblasts (e.g., Picard et al. 2008), (2) by Snai1- or Id1-dependent transcriptional reprogramming of renal pericytes or perivascular fibroblasts (Humphreys et al. 2010; Cook 2010; Roufosse et al. 2006; Lin et al. 2008), (3) as a consequence of tubular epithelial-to-mesenchymal transdifferentiation (EMT; Yamashita et al. 2005; Kalluri and Neilson 2003; Iwano et al. 2002; Eddy 2009; Iwano and Neilson 2004; Liu 2006, 2010; Grande and Lopez-Novoa 2009), or (4) by extra-renal recruitment from the circulation or bone marrow (Ricardo et al. 2008). Indeed, the mobilization of stem-like cells accompanies post-obstructive kidney regeneration suggesting that renal capsule-derived, hematopoietic, or mesenchymal progenitor cells participate in repair (Park et al. 2010aPark et al. 2010b; Yamashita et al. 2005). Whether progenitor involvement has a positive or negative effect on outcome, however, is controversial, although renal-artery-delivered human mesenchymal stem cells appear to attenuate UUO-induced fibrosis (Asanuma et al. 2010).

TGF-β/SMAD signaling as a transducer of the fibrotic phenotype in obstructive nephropathy

TGF-β and downstream receptor-regulated SMAD transcriptional effectors are among the most significant inducers of fibrosis in multiple organ systems integrating, in some cases, signaling pathways initiated by other stimuli (Kalluri and Neilson 2003; Bottinger and Bitzer 2002; Stahl and Felsen 2001; Iwano and Neilson 2004; Ricardo et al. 2008; Bottinger 2007). Indeed, several factors that drive EMT in kidney cells and that promote myofibroblast differentiation and fibrosis in animal models of chronic renal disease utilize, at least in part, TGF-β/SMAD signaling pathways (Oldfield et al. 2001; Mezzano et al 2003;

Fan et al. 2001; Liu 2010). Intraperitoneal injection of TGF-β alone, moreover, is sufficient to initiate a prominent renal fibrotic response (Ledbetter et al. 2000), and TGF-β-activated SMAD pathways are pivotal for the induction of EMT, myofibroblast differentiation, and disease progression (Derynck and Zhang 2003; Kalluri and Neilson 2003; Iwano and Neilson 2004). In the setting of UUO, tubulointerstitial fibrosis appears to be dependent on the interstitial upregulation of TGF-β1 expression (Miyajima et al. 2000). Prominent TGF-β1 reactivity in the tubular epithelium of senescence-prone SAMP1/Sku male mice following UUO is accompanied by an accelerated and severe onset of all the pathophysiologic hallmarks of mononuclear cell infiltration/fibrosis with rapid progression of EMT (Yabuki et al. 2005). Given the causative association between TGF-β1 and fibrotic disease, it is not surprising that SMAD3-deficient mice are protected from renal fibrosis by, at least in part, reduced EMT, inflammation, collagen deposition, tubular apoptosis, and the impaired expression of profibrotic TGF-β1 target genes (Sato et al. 2003; Arany et al. 2007; Zeisberg et al. 2003; Inazaki et al. 2004).

These findings provide a rationale for targeted intervention therapies directed to the TGF-β signaling network. Disease onset and progression is certainly attenuated by the blockade of TGF-β1 expression or function (Klahr 1991; Kaneto et al. 1993). The introduction of TGF-β1 antisense phosphorothioate oligodeoxynucleotides, by retrograde ureteral injection, or TGF-β1 small interfering RNA (siRNA) effectively inhibits collagen I mRNA expression and interstitial fibrosis in the obstructed kidney (Isaka et al. 2000; Hwang et al. 2006). The administration of TGF-β neutralizing antibodies significantly reduces UUO-initiated inflammation, tubular epithelial apoptosis, and fibrosis (Miyajima et al. 2000; Gagliardini and Benigni 2006). The overexpression of the latent form of TGF-β1, as one strategy to minimize active TGF-β1 in the tissue microenvironment, decreases the incidence of cells positive for α-smooth muscle actin (presumably myofibroblasts) in UUO and blocks SMAD2/3 activation (Huang et al. 2008). The peroxisome proliferator-activated receptor gamma agonist troglitazone, moreover, attenuates UUO-induced renal interstitial fibrosis and inflammation through suppression of TGF-β1 expression (Kawai et al. 2009). Collectively, these data are consistent with the current concept that TGF-β1 is, indeed, the key initiator of fibrosis in UUO (Inazaki et al. 2004), either directly or as a consequence of elevated angiotensin II expression (Shin et al. 2005; Pimentel et al. 1995; Gu et al. 2001; Fern et al. 1999; Satoh et al. 2001; Ishidoya et al. 1995). Moreover, angiotensin stimulates the expression of genes encoding both ECM structural elements, such as collagen, fibronectin, and laminin, and specific inhibitors of matrix degradation, including plasminogen activator inhibitor-1 (PAI-1; a clade E member of the serine protease inhibitor gene family, SERPINE1), via TGF-β1-dependent mechanisms, thus leading to ECM accumulation and tissue fibrogenesis (Kagami et al. 1994; Wolf et al. 1993; Wolf 2006). Angiotensin also increases TGF-β1 transcription in renal cells and its bioactivation via thrombospondin-1 (Naito et al. 2004). Obstructive nephropathies are additionally accompanied by an increased expression of TGF-β receptors (Sutaria et al. 1998) presenting, perhaps, another therapeutic opportunity. Oral administration of the TGF-β type I receptor (ALK5) kinase inhibitor IN-1130, indeed, suppresses UUO-induced SMAD activation, matrix accumulation, and interstitial fibrosis (Moon et al. 2006). Caution as to the overall usefulness of TGF-β1 network-neutralizing or signal-inhibitory approaches in the setting of obstructive renal disease, however, is highlighted by the finding that high-dose antibody administration reduces efficacy because of an exacerbated inflammatory reaction (Ma et al. 2004).

TGF-β1 signaling regulation in renal fibrosis

Once engaged, TGF-β1-mediated signaling mechanisms are highly-regulated, positively and negatively, at multiple levels, e.g., receptor activity, internalization, SMAD recruitment,

activation, translocalization and promoter binding, nuclear retention/exit of SMADs, and SMAD partnering with co-factors and non-SMAD transcriptional factors (Moustakis and Heldin 2009; Massagué 1998). Mechanisms of SMAD activation and the canonical pathway of TGF-β signaling have been comprehensively reviewed previously (e.g., Bottinger and Bitzer 2002; Miyazono 2009; Moustakis and Heldin 2009; Itoh and ten Dijke 2007; Ikushima and Miyazono 2010a, 2010b). TGF-β1-stimulated receptor SMAD activation is negatively regulated by SMAD7 and by the SMAD co-repressors Ski and SnoN effectively suppressing transcriptional responses (Itoh and ten Dijke 2007). In obstructive nephropathies and chronic renal disease, endogenous levels of SMAD7, Ski, and SnoN are reduced (by ubiquitin-dependent degradation), probably leading to persistent TGF-β1 signaling (Fukasawa et al. 2004, 2006; Yang et al. 2003). Gene transfer of SMAD7 to the kidney dramatically reduces interstitial fibrosis and SMAD activation induced by UUO (Lan et al. 2003; Li et al. 2002). SnoN targeting might also have translational implications. Indeed, the inhibition of SnoN degradation effectively attenuates TGF-β-induced renal fibronectin and α-smooth muscle actin in response to UUO (Tan et al. 2006; Fukasawa et al. 2006). An additional level of control involves bone morphogenetic protein-7 (BMP-7), a member of the TGF-β superfamily of ligands (e.g., Wang and Hirschberg 2003; Tanaka et al. 2008). Expression of BMP-7 and its receptors are reduced during the progression of renal fibrosis (Wang and Hirschberg 2003). BMP-7 largely signals through SMAD1/5/8 to antagonize SMAD2/3-dependent transcription (Mitu and Hirschberg 2008; Patel and Dressler 2005). BMP-7 administration in animal models of progressive renal failure (e.g., UUO, diabetic nephropathies) significantly reverses interstitial fibrosis, EMT, and inflammation (Zeisberg et al. 2003) highlighting the potential therapeutic value of BMP-7 as an inhibitor of disease progression. Moreover, BMP-7 attenuates the TGF-β1 induction of matrix elements (e.g., collagen and fribonectin), certain matrix metalloproteinases, and the potent profibrotic factors CCN2 (previously known as connective tissue growth factor) and PAI-1 in both interstitial fibroblasts and mesengial cells. The mechanism appears to involve the suppression of TGF-β1-activated SMAD3 pathways via the upregulation of the inhibitory SMAD6 through SMAD5 signaling in response to BMP (Wang and Hirschberg 2003; Wang et al. 2005). BMP-7, moreover, promotes mesenchymal-to-epithelial conversion (MET) in renal fibroblasts (reminiscent of the early stages of nephron development) and reduces TGF-β1-mediated EMT in tubular epithelial cells, collectively contributing to kidney regeneration and inhibiting disease progression (Patel and Dressler 2005; Zeisberg et al. 2003; Zeisberg et al. 2005). BMP-7 action in the context of renal disease, however, is itself subject to complex controls. KCP (Kielin/chordin like protein), a secreted protein with cysteine-rich domains, positively regulates BMP-7 signaling by enhancing the binding of BMP-7 to its receptor (Lin et al. 2005). KCP stimulation of BMP-7 signaling to Smad1 transcriptional targets reduces renal fibrosis and EMT in response to UUO or acute ischemic tubular injury, and consistent with this putative protective role, $KCP^{-/-}$ mice are susceptible to the development of interstitial fibrosis (Lin et al. 2005). Conversely, ectodin (uterine sensitization-associated gene-1 [USAG-1] or sclerostin) is a natural suppressor of BMP-7 signaling expressed in the kidney. As predicted from this role, $ectodin^{-/-}$ mice have significantly less renal fibrosis and EMT attributable to reduced TGF-β1 activity and enhanced BMP-7 signaling compared with wild-type mice subjected to UUO or cisplatin-initiated renal damage (Yanagita et al. 2006). Thus, the magnitude of BMP-7 signaling, regulated by agonist (e.g., KCP, BMP-7) and antagonist (ectodin, TGF-β1) interplay, profoundly influences the progression of renal disease but also provides additional therapeutic candidates to address the pathophysiologic consequences of persistent TGF-β1 signaling in the kidney.

TGF-β target genes: causative roles in pathogenesis

TGF-β1 regulates the expression of subsets of genes involved in epithelial "plasticity", morphologic transdifferentiation, and stromal remodeling (e.g., Zavadil et al. 2001; Higgins 2006; Freytag et al. 2009, 2010). Many of these same morphologic transitions reflect, in large part, changes in transcriptional outputs in response to increasing levels of TGF-β1 in the renal parenchyma and, therefore, are superimposed on the time course of tubulointersitial fibrosis. Several comprehensive databases, derived from microarray profiling, provide critical insights into the spectrum of differential gene expression patterns in renal disease (Murphy et al. 2008; Ju et al. 2009; Matsuo et al. 2005; Silverstein et al. 2003; Higgins et al. 2003; Eikmans et al 2003; Sadlier et al. 2004; Seseke et al. 2004) and in response to TGF-β1 (for reviews, see Freytag et al. 2009, 2010). PAI-1, in particular, is a prominent member of, if not the most highly upregulated gene in, the TGF-β1-induced gene set (Freytag et al. 2009; Zavadil et al. 2001; Akiyoshi et al. 2001) and is causatively involved in the development of the EMT phenotype (Freytag et al. 2010). Importantly, and consistent with in vitro data, TGF-β1 siRNA suppresses UUO-initiated interstitial pathology through the reduced expression of the profibrogenic TGF-β1 target genes PAI-1 and collagen I (Hwang et al. 2006). PAI-1 is a prominent downstream target of the TGF-β1/SMAD3 pathway (for reviews, see Samarakoon and Higgins 2008; Ha et al. 2009) and an established contributor to fibrogenesis in several organ systems (e.g., Eddy and Fogo 2006; Chuang-Tsai et al. 2003). A major inhibitor of plasmin generation, PAI-1 decreases ECM degradation and promotes its accumulation, contributing thereby to renal fibrotic disease (Oda et al. 2001; Eddy 2009). Indeed, the delivery of small PAI-1 decoy peptides that mimic uPA/tPA domains effectively reduces both UUO-initiated and established interstitial fibrosis (Gonzalez et al. 2009). Consistent with the activation of TGF-β1 signaling in UUO, a dramatic increase of SMAD2/3 phosphorylation and PAI-1 protein expression occurs in the obstructed kidney compared with contralateral controls (Fig. 1).

Increased PAI-1 expression occurs not only in UUO (Higgins et al. 2003), but also in other animal models of renal diseases including streptozotocin-initiated diabetic nephropathy (Nicholas et al. 2005) in which its induction is initiated by a number of profibrogenic and pro-inflammatory mediators including angiotensin, TGF-β, CCN2, interleukins, and tumor necrosis factor-α (Eddy and Fogo 2006). Although UUO is an important approach for identifying and assessing pathophysiologic events underlying induced renal fibrosis, some acknowledgement should be made that the underlying mechanisms might differ from those operative in diabetic animal models or human diabetic disease. In tubulointerstitial fibrosis induced by UUO, PAI-1 deficiency is renal-protective (Oda et al. 2001), whereas PAI-1 overexpression (in transgenic mice) promotes a fibrotic response with associated recruitment of macrophages and myofibroblasts (Matsuo et al. 2005). PAI-$1^{-/-}$ mice subjected to UUO, moreover, exhibit a significantly reduced inflammatory response compared with their wild-type counterparts suggesting that PAI-1 promotes the infiltration of macrophages and T-cells (Oda et al. 2001). PAI-1 also modulates TGF-β1 signaling, as PAI-$1^{-/-}$ animals have reduced TGF-β1 levels compared with wild-type mice similarly subjected to UUO (Krag et al. 2005). Indeed, recombinant PAI-1 stimulates TGF-β1 synthesis, perhaps through a urokinase plasminogen activator receptor (uPAR)-dependent mechanism involving downsteam mitogen-activated protein kinase (MAPK) signaling, to activate the TGF-β1 promoter suggesting the existence of a PAI-1/TGF-β1-positive feedback loop (Seo et al. 2009; Nicholas et al. 2005). However, significant examples exist in which PAI-1 can activate MAPK or Janus kinase(JAK)/signal transducer and activator of transcription (STAT) pathways via other co-receptors (e.g., low-density-lipoprotein receptor-related protein 1 [LRP1]; Degryse et al. 2004; Providence et al. 2008; Czekay et al. 2011). Modulation of PAI-1 expression levels may, therefore, have relevant effects on matrix

accumulation through several proteolytic and non-proteolytic pathways, each of which may prove to have significant therapeutic value in retarding fibrosis (Huang et al. 2003).

Cooperative SMAD and non-SMAD factors mediate PAI-1 induction by TGF-β1

Although SMAD2/3 activation might be necessary, it is not sufficient for TGF-β1-stimulated PAI-1 expression in the absence of epidermal growth factor receptor (EGFR) signaling (Samarakoon et al. 2008; Samarakoon and Higgins 2008). These data are consistent with the realization that the progression of chronic renal disease involves the participation of several receptor and non-receptor tyrosine kinases (e.g., EGFR, platelet-derived growth factor, Abl, c-Src; see Wang et al. 2005; Floege et al. 2008; Eitner et al. 2008; Francois et al. 2004). Angiotensin-activated EGFR-mediated signaling (via the sheddase Adam 17), for example, is critically important in renal fibrosis induced by angiotensin infusion in mice (Lautrette et al. 2005). Transgenic mice expressing a dominant-negative EGFR (DN-EGFR) construct in the kidney, moreover, are also resistant to the development of renal fibrotic lesions following ischemic injury or subtotal nephrectomy (Terzi et al. 2000). Inhibition of EGFR by Gefitinib retards gromerular damage and collagen deposition in response to L-NAME-induced renal injury in rats (Francois et al. 2004). TGF-β1 also activates several tyrosine kinases including $pp60^{c\text{-}src}$, c-Abl, and the EGFR (Wang et al. 2005; Samarakoon and Higgins 2008; Moustakis and Heldin 2009), and similar events occur in the setting of UUO (Fig. 1).

The available data suggest that SMADs and specific MAPK-targeted transcription factors occupy separate binding motifs at the TGF-β1-responsive PE2 region E-box in the human PAI-1 promoter (Dennier et al. 1998; Allen and Higgins 2004; Allen et al. 2005; Kutz et al. 2006; Samarakoon et al. 2005, 2008; Qi and Higgins 2003; Qi et al. 2006a). Complex formation at the PE2 site, moreover, requires cooperative signaling by the EGFR → extracellular signal-regulated kinase (ERK) (upstream stimulatory factor [USF]) and Rho/Rho-associated protein kinase (ROCK) (SMAD) pathways (Samarakoon and Higgins 2008). Extract immunodepletion and super-shift/complex-blocking experiments identified one PAI-1 E-box-binding protein to be USF2, a member of the basic helix-loop-helix-leucine zipper (bHLH-LZ) family of MYC-like proteins (Allen et al. 2005; Qi et al. 2006a). Replacement of USF1 homodimers with USF1/2 heterodimers or USF2 homodimers is associated with transcriptional activation of the PAI-1 gene (Qi et al. 2006a). Dominant-negative interference with USF DNA-binding ability significantly attenuates TGF-β1-mediated PAI-1 transcription (Qi and Higgins 2003; Qi et al. 2006a). Since MAPKs regulate the DNA-binding and transcriptional activities of USF (for a review, see Kutz et al. 2006), TGF-βR signaling through SMAD2/3 appears to require EGFR/mitogen-activated protein kinase kinase (MEK)-ERK-activated USF to attain high level PAI-1 expression (Samarakoon et al. 2005; Higgins 2006). SMADs might also interact with other E-box-binding HLH-LZ factors including TFE3 at the PE2 site, at least in one cell type (Hua et al. 1998). Such interacting complexes are likely to be important in PAI-1 gene control, since USF occupancy of the PAI-1 PE2 region E-box site modulates transcription in response to TGF-β1 or serum (Dennier et al. 1998; Qi and Higgins 2003), and USF2, but not USF1, levels are significantly increased in response to renal obstructive injury (Fig. 1).

The tumor suppressor p53 has also been implicated in the transcriptional regulation of profibrotic effectors (Vousden and Prives 2009). p53 family members are involved in a subset of TGF-β1 responses attributable to, in part, interactions between phosphorylated p53 and SMAD2, forming transcriptionally active multi-protein complexes (Piccolo 2008; Cordenonsi et al. 2003, 2007). DNase I footprinting/methylation interference and oligonucleotide mobility shift analyses have confirmed that p53 binds to motifs in the PAI-1

promoter, possibly the two p53 half-sites (AcACATGCCT, cAGCAAGTCC) at −224 bp to −204 bp relative to the transcription start site (Kunz et al. 1995; Riley et al. 2008). The DNA binding reflects both p53 sequence-driven reporter gene transcription and induced expression of the endogenous PAI-1 gene. TGF-β1-initiated PAI-1 expression is significantly attenuated in p53 knockdown cells (Cordenonsi et al. 2003). Consistent with these findings, $p53^{-/-}$ fibroblasts are not inducible for increased PAI-1 expression in response to TGF-β1 and pretreatment of Mv-1Lu mink lung cells (stably expressing a PAI-1 promoter-luciferase reporter construct) with the p53 inhibitor pifithrin-α effectively suppressed TGF-β1-dependent PAI-1 transcription (Fig. 2). Stimulation of PAI-1 gene activation in mouse fibroblasts by the nephrotoxin cisplatin, moreover, is p53-dependent (Kortlever et al. 2008). One hypothesis suggests that p53 interacts directly with SMAD2 (Dupont et al. 2004; Cordenonsi et al. 2003, 2007). In TGF-β1-stimulated cells, the binding of USF to the PE2 site might facilitate DNA bending. Phasing analysis has revealed that certain bHLH-LZ of the MYC family (including USF) orient the DNA bend toward the minor groove (Fisher et al. 1992); this could potentially promote interactions between p53, bound to its downstream half-site motif, with SMAD2 tethered to the upstream PE2 region SMAD site. Radiation-associated PAI-1 expression also requires TGF-β1-mediated p53/SMAD cooperative transcriptional interactions, further highlighting SMAD and non-SMAD interplay in TGF-β1-mediated signaling events (Milliat et al. 2008). Regardless of the precise mechanism, the available data suggest a central role for p53 in TGF-β1-initiated PAI-1 gene control (Figs. 1, 2).

Increased expression and serine 15 phosphorylation (activation) of p53 is evident in the kidney following UUO (Fig. 1), ischemic-reperfusion- or nephrotoxin-induced (e.g., cisplatin, aristolochic acid) renal injury (Zhou et al. 2010; Wei et al. 2007). $p53^{-/-}$ mice largely retain their renal architecture and function following treatment with cisplatin or aristolochic acid, whereas their wild-type counterparts develop severe renal damage and compromised organ function consistent with a role for p53 in promoting tubular cell apoptosis and growth arrest (Wei et al. 2007; Zhou et al. 2010). siRNA-directed silencing of p53 in mice, moreover, abates the severity of cisplatin- and ischemic-induced kidney damage (Molitoris et al. 2009). Similarly, the p53 inhibitor pifithrin-α suppresses both the accumulation and phosphorylation of p53 and attenuates tubular apoptosis and renal damage (Wei et al. 2007). Recent studies, furthermore, link tubular epithelial growth arrest in response to both acute (e.g., ischemia-reperfusion, nephrotoxins) and more protracted (UUO) injury to the progression of renal fibrosis via p53 and JNK signaling with the retention of TGF-β signaling (Yang et al. 2010). Blockade of p53 activation by pifithrin-α effectively attenuates tubular cell G2/M arrest, decreases TGF-β1 and CCN2 levels, and reduces overall fibrosis (Yang et al. 2010).

Concluding remarks

MAPKs (e.g., p38, ERK1/2) phosphorylate and transcriptionally activate USF proteins (Higgins 2006), and molecular interference with the DNA-binding ability of USF (by dominant-negative approaches) significantly attenuates PAI-1 transcription in response to TGF-β1 in non-kidney cells (Qi et al. 2006a). Similarly, PAI-1 induction in renal and non-renal cells by TGF-β1 requires EGFR-ERK1/2 signaling (Cho et al. 2009; Samarakoon et al. 2005, 2008) and a USF1 → USF2 isoform switch at the PE2 E-box site in the PAI-1 promoter (Qi et al. 2006a). In this regard, the TGF-β1-initiated engagement of the MAPK (ERK, p38) pathways in UUO (Masaki et al. 2003) could result in USF2 mobilization and the induction of USF target genes, such as PAI-1, in cooperation with (TGF-β1-activated) SMADs and (MAPK-phosphorylated) p53 (summarized in Fig. 3). In human renal cells, moreover, autostimulation of TGF-β1 gene transcription by TGF-β1 also requires both SMAD3 and MAPK/ERK activity (Zhang et al. 2006).

Activated MEK/ERK, ataxia telangiectasia-mutated (ATM), and casein kinase sites 1 and 2 (CK1 and CK2) can phosphorylate p53 (at serine 15) promoting the acetylation of p53 (by CREB-binding protein/p300) necessary for subsequent transcriptional activation (Meek and Anderson 2009). p300/CREB-binding protein, a histone acetyltransferase, also interacts with and acetylates SMAD2/3 in response to TGF-β1 (Feng et al. 1998) facilitating the creation of a transcriptional complex (SMADs/p53/USF2) necessary for optimal PAI-1 induction (Tu and Luo 2007; Simonsson et al. 2006; Das et al. 2008). The importance of such interactions is underscored by the finding that RAP250, a protein that has no intrinsic enzymatic activity but that effectively recruits histone acetyltransferases and methylases to chromatin complexes, also interacts with SMAD2/3 and is essential for maximal TGF-β1-stimulated PAI-1 expression (Antonson et al. 2008). Selective targeting of p53/SMAD/USF2 interactions, therefore, might provide an attractive strategy to intervene in the progression of obstructive renal disease. Phosphorylation of p53, moreover, is critical for SMAD2/3 interactions, further demonstrating cross-talk between SMAD and non-SMAD elements in TGF-β signaling (Cordenonsi et al. 2007). USF2, which is upregulated in the obstructed kidney (Fig. 1), and the PE2 E-box motif with its adjoining SMAD-binding sequences probably constitute a platform for transcriptional complex formation with SMAD2/3 and p53. This hypothesis is supported by the finding that transgenic USF-2 mice overexpress TGF-β1 and components of the renin-angiotensin system, thereby modulating the progression of kidney disease in streptozotocin-induced diabetic nephropathies (Shi et al. 2009; Liu et al. 2007). Whether stimulation of the MAPK (ERK, p38) pathways in UUO (Masaki et al. 2003) by TGF-β1 with downstream USF activation subsequently induces the expression of USF-dependent genes, such as PAI-1, in cooperation with SMAD and p53 remains to be established. Clarification of this axis would have generalized implications with regard to current therapeutic options for the clinical management of renal fibrotic disease.

Acknowledgments

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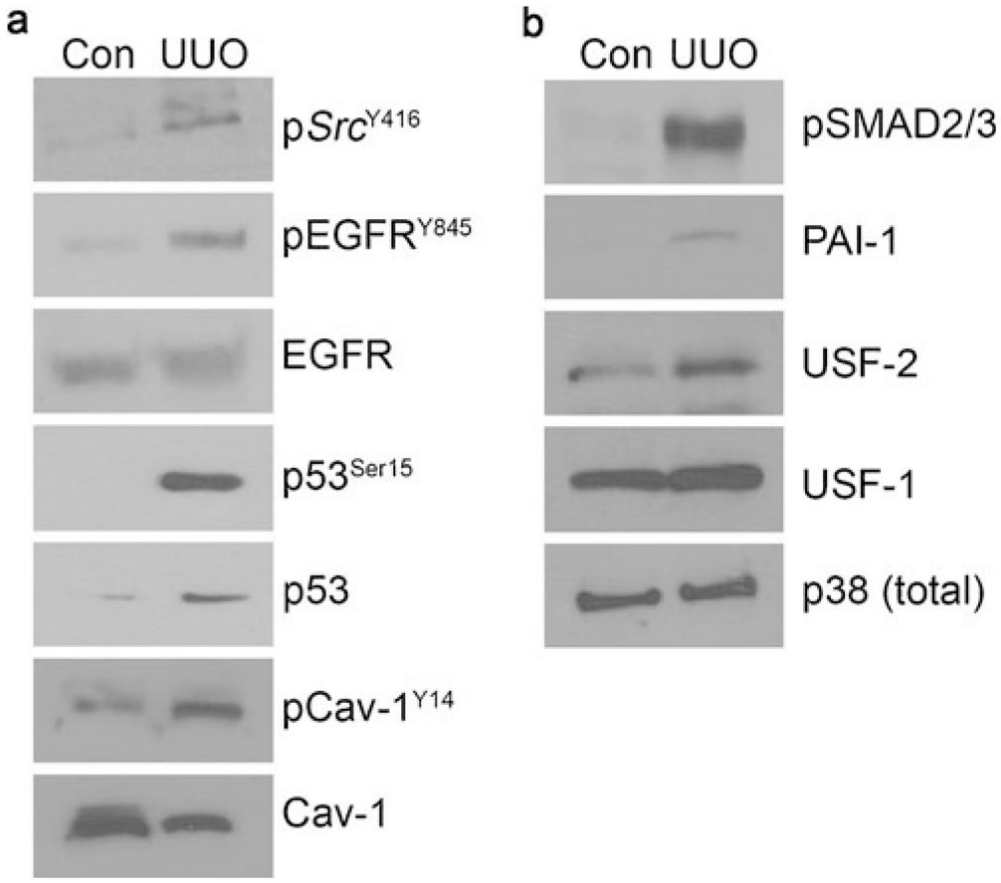

Fig. 1.

Activation of various profibrotic effector pathways in unilateral ureteral obstruction (UUO) in mice. UUO involved the ligation of the left ureter for 3 or 7 days; the contra-lateral (right) kidney that remained without surgical manipulation for the same period served as a control (*Con*). Obstructed and control kidneys were isolated, homogenized directly in SDS-containing buffer for Western analysis of tissue extracts. At 3 days after ureteral ligation, phosphorylation of c-Src^{Y416}, epidermal growth factor receptor^{Y845} (EGFR^{Y845}), and caveolin-1^{Y14} (Cav-1^{Y14}; the two last-mentioned molecules being established targets of activated src family kinases) were increased, whereas total levels of EGFR and caveolin-1 largely remained unchanged providing relevant loading standards. p53^{Ser15} phosphorylation and total p53 levels were both markedly elevated in the obstructed kidney compared with the contra-lateral controls at day 3 (**a**). Consistent with increased TGF-β1 signaling in renal fibrotic disease, a robust induction of SMAD2/3 phosphorylation and an increase in plasminogen activator inhibitor-1 (PAI-1) expression were evident in the obstructed kidney at 7 days following UUO (**b**). A fivefold increase was noted in upstream stimulatory factor 2 (USF2) but not USF1 in the obstructed kidney. Total p38 levels remained constant providing a loading control

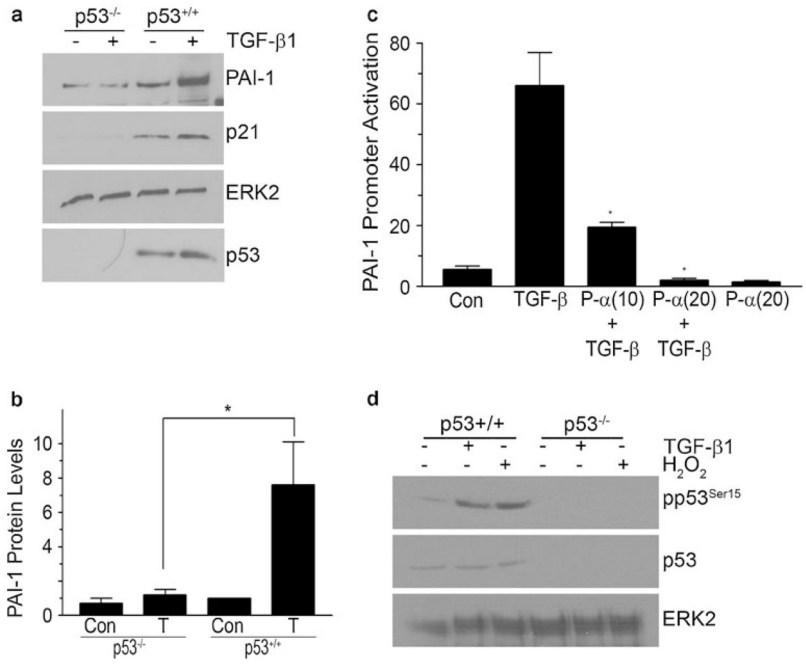

Fig. 2.

Role of p53 in TGF-β1-induced PAI-1 expression. Confluent and serum-deprived $p53^{-/-}$ or wild-type ($p53^{+/+}$) mouse embryonic fibroblasts (MEFs) were stimulated with TGF-β1 (0.1 ng/ml) for 24 h and disrupted in SDS-containing buffer; lysates were immunoblotted for the indicated proteins. Increases in PAI-1 (**a**, **b**) and p21 (**a**) protein 24 h after addition of TGF-β1 (*T*) were evident in wild-type but not in $p53^{-/-}$ MEFs (Con=control). ERK2 provided a loading control; p53 genetic status was confirmed by Western analysis (**a**). The histogram in **c** represents the mean±SD of triplicate studies highlighting the p53 requirement for transcriptional activation of the PAI-1 promoter in response to TGF-β1. Mink lung epithelial cells (Mv-1Lu) stably expressing a PAI-1 promoter-driven luciferase reporter (p800-Luc) were passaged and stimulated 8 h later with various doses of the p53 inhibitor pifithrin-α (10–20 μM; *P-α(10)*, *P-α(20)*) for 24 hours prior to addition of TGF-β1 for an additional 18 h. Cellular lysates were used for luciferase measurements. TGF-β1-induced PAI-1 promoter activation was dose-dependently suppressed by pifithrin-α suggesting a requirement for p53 in PAI-1 transcription (**c**). TGF-β1 (0.1 ng/ml) and 200 μM H_2O_2 (serving as a positive control for oxidative stress) induced $p53^{Ser15}$ phosphorylation, consistent with activation of this transcription factor by both stimuli in wild-type ($p53^{+/+}$) but not p53 deficient MEFs (**d**). ERK2 provided a loading control

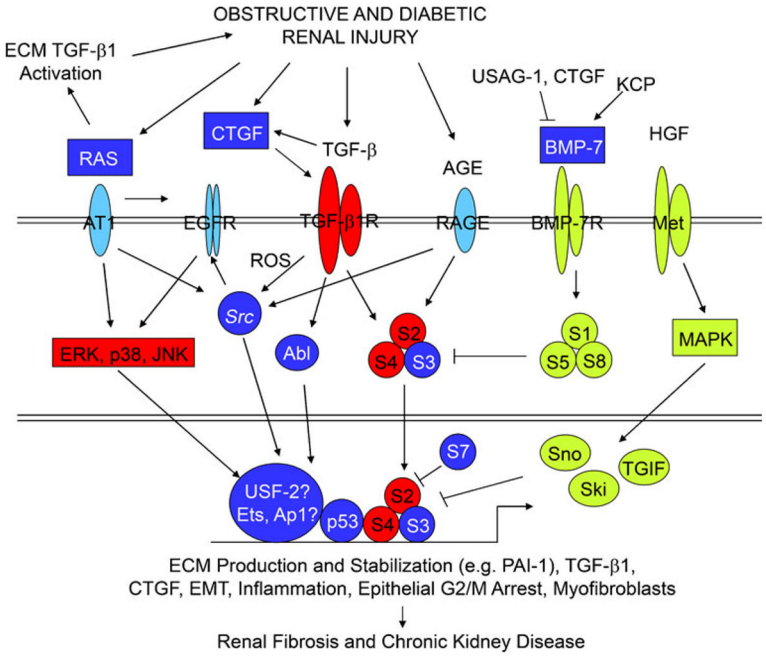

Fig. 3.

TGFβ1/SMAD3 signaling as an integrator of fibrotic mechanisms in the kidney. The pathophysiology of obstructive and diabetic renal fibrosis involves the activation of multi-component receptor-initiated signaling events. Representation of the individual interacting systems discussed in this review. Whereas unique elements are found in each, TGF-β1/SMAD2/3 signaling appears to integrate downstream effectors of these collateral pathways, in certain circumstances, to promote a profibrotic microenvironment. Persistent TGF-β1 expression and SMAD2/3 phosphorylation consistently accompany progression of chronic renal disease in animal models and humans. Expression of negative regulatory TGF-β1/SMAD pathway elements (inhibitory SMAD-7, Ski, Sno) are reduced in the context of tissue fibrosis, thus, further amplifying TGF-β1-initiated fibrogenic processes (e.g., ECM synthesis, EMT, inflammation, CNN2 [previously designated connective tissue growth factor] and PAI-1 induction, and myofibroblast differentiation/persistence). Moreover, both the expression and signaling ability of negative effectors of TGF-β1 signaling (e.g., BMP-7, hepatocyte growth factor [HGF]) are also suppressed by a variety of mechanisms (detailed in the text) facilitating maintenance of TGF-β1 signaling in the injured tissue leading to excessive deposition of ECM components, disruption of renal parenchyma, and progressive loss of kidney function