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TGF-β1/p53 Signaling in Renal Fibrogenesis

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Abstract

Fibrotic disorders of the renal, pulmonary, cardiac, and hepatic systems are associated with significant morbidity and mortality. Effective therapies to prevent or curtail the advancement to organ failure, however, remain a major clinical challenge. Chronic kidney disease, in particular, constitutes an increasing medical burden affecting >15% of the US population. Regardless of etiology (diabetes, hypertension, ischemia, acute injury, urologic obstruction), persistently elevated TGF-β1 levels are causatively linked to the activation of profibrotic signaling networks and disease progression. TGF-β1 is the principal driver of renal fibrogenesis, a dynamic pathophysiologic process that involves tubular cell injury/apoptosis, infiltration of inflammatory cells, interstitial fibroblast activation and excess extracellular matrix synthesis/deposition leading to impaired kidney function and, eventually, to chronic and endstage disease. TGF-β1 activates the ALK5 type I receptor (which phosphorylates SMAD2/3) as well as non-canonical (e.g., *src* kinase, EGFR, JAK/STAT, p53) pathways that collectively drive the fibrotic genomic program. Such multiplexed signal integration has pathophysiological consequences. Indeed, TGF-β1 stimulates the activation and assembly of p53-SMAD3 complexes required for transcription of the renal fibrotic genes plasminogen activator inhibitor-1, connective tissue growth factor and TGF-β1. Tubular-specific ablation of p53 in mice or pifithrin-α-mediated inactivation of p53 prevents epithelial G_2/M arrest, reduces the secretion of fibrotic effectors and attenuates the transition from acute to chronic renal injury, further supporting the involvement of p53 in disease progression. This review focuses on the pathophysiology of TGF- β1-initiated renal fibrogenesis and the role of p53 as a regulator of profibrotic gene expression.

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Keywords

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Introduction

Sustained inflammation and repeated cycles of kidney injury/repair (or incomplete recovery) leads to tubular atrophy, progressive fibrosis, functional decline and, ultimately, organ failure [1–7]. Episodic acute injury (AKI) to the proximal tubular (largely S3 segment) epithelium is a major factor in the transition to chronic kidney disease (CKD) [e.g., 6–8]; patients who survive AKI have an increased risk of development of CKD [8]. Excessive accumulation of extracellular matrix (ECM; e.g., the fibrillar collagens, fibronectin) in the glomerular, interstitial and vascular compartments is accompanied by a significant decline in glomerular filtration rate and impaired epithelial regeneration [1]. In this regard, interstitial fibrosis is both a pathophysiologic hallmark feature and prognostic biomarker of end-stage renal disease (ESRD) [1,9–12]. The primary sources of ECM synthesis during interstitial fibrogenesis are activated fibroblasts or myofibroblasts [13,14]. Although controversial in origin, recent biomarker analysis and lineage-tracing studies suggest that this cell type-predictor of disease progression likely derives from $FoxD1^{+}$ mesenchymal precursors (i.e., vascular pericytes and tissue-resident fibroblasts) with perhaps minor varying contributions from endothelial cells, completely or partially transdifferentiated tubular epithelia, and bone marrow fibrocytes [15–21]. The persistence of such activated fibroblasts is a critical factor in the initiation and development of renal disease where they likely participate in the silent scarring phase prior to development of significant organ dysfunction [22].

Signaling Transducers of the Renal Fibrotic Phenotype

Transforming growth factor-β1 (TGF-β1) drives the myofibroblastic phenotype, particularly in the context of a stiff microenvironment such as a fibrosing tissue. Signals generated from an increasingly non-compliant stroma, moreover, distort the latency constraints on TGF-β1 releasing the active TGF-β1 dimer facilitating interaction with its receptor complex to promote myofibroblast differentiation and/or retention [23] while activating cellular pathways that impact chromatin architecture and transcription of disease-relevant genes [24,25]. Elevated levels of TGF-β1 in the injured kidney, moreover, orchestrates a program of pathologic renal ECM synthesis and advancing fibrosis in response to diabetes, hypertension, ischemic or repeat tubular injury and urinary tract obstruction [e.g., 12,26–31]. Within hours after ureteral occlusion, for example, the affected kidney exhibits changes in hydrostatic forces and increased oxidative stress [32–34]. Tubular stretch, in turn, further stimulates TGF-β1 expression (>20-fold), increases the epithelial apoptotic index and leads to the development of an inflammatory inflitrate [30,35]. Non-resolving inflammation and continued interstitial ECM deposition accompanies tubular dilation and atrophy, nephron loss and scarring [9,11,13,27,37–41]. Maintenance of renal TGF-β1 expression in response to ischemic or obstructive stimuli, results in escalating tissue injury, impaired regenerative growth, and eventual loss of organ function [28,42,43].

Early findings suggested that renal disease onset and progression could be attenuated by blockade of TGF-β1 expression or function. TGF-β-neutralizing antibodies reduced trauma-initiated inflammation, tubular epithelial apoptosis, and fibrosis [30,44] while retrograde ureteral introduction of TGF-β1 antisense oligodeoxynucleotides or small interfering RNA (siRNA) blunted both collagen I mRNA expression and interstitial involvement [45,46]. Overexpression of latent TGF-β1, to minimize availability of bioactive TGF-β1, resulted in a decrease in both SMAD2/3 activation (the transcriptional effectors of canonical TGF-β1 signaling) and the number α-smooth muscle actin-positive cells (presumably myofibroblasts) in the injured kidney [47] consistent with the finding that SMAD3 knockout mice are protected from renal fibrosis [31,48]. A caveat regarding SMAD involvement in gene control, however, is that positioning of an activated SMAD complex on a target promoter requires repeat SMAD-binding elements (SBEs) and complicated, in part, by recognition of the increasing number of SMAD-interacting transcriptional partners [49]. For SMAD2/3, the most relevant SMADs in fibrotic disorders, these various co-factors contribute to the defined self-enabling, switch enhancer and derepression modes of SMAD-dependent transcription (Hill, 2016) suggesting a model of “contextual” signaling in the varied responses to TGF-β family ligands [50].

The repertoire of TGF-β-dependent non-canonical signaling contributors to normal and dysfunctional tissue repair is expanding and includes the three mitogen-activated protein kinase (MAPK) families (ERK, p38, JNK) as well as the Wnt/β-catenin, Jagged/Notch, Hedgehog, JAK/STAT, Hippo/YAP-TAZ, epidermal growth factor receptor (EGFR), p53, RhoA/ROCK/PTEN, Numb and Toll-like receptor (TLR) networks [e.g., 51–55]. There is, however, considerable pathway cross-talk [56]. Numb increases TGF-β1 expression and promotes a p53-dependent tubular epithelial G_2/M arrest, a prominent profibrotic response in the injured renal epithelium [8,12,40], following ischemia/reperfusion or ureteral obstruction [57] while the Hippo/YAP-TAZ axis integrates mechanochemical and TGF-β/SMAD signaling as a function of YAP-TAZ phosphorylation [24,58]. A progressively non-compliant microenvironment, in fact, induces the YAP-TAZ, SMAD2/3-dependent expression of a subset of profibrotic TGF-β1 target genes including several collagens, plasminogen activator inhibitor-1 (PAI-1) and connective tissue growth factor (CTGF) [e.g., 24,59]. The impact of increasing biomechanical strain, as is likely encountered in a fibrosing tissue, however may well transcend just the YAP-TAZ system since mechanical stress regulates (as least in some cell types) several network “hubs” and their constituent genes by activating the TGF-β1, tumor necrosis factor α (TNFα) and p53 pathways [60,61].

Integration of TGF-β1-Activated p53 in Renal Fibrogenesis

Various species-, tissue- and cell type-specific cis-acting factors regulate the genomic program of fibrosis [62]. Recent findings, however, indicate a further layer of complexity to TGF-β1 signaling and implicate p53 in the transcriptional control of renal disease-causative genes (Figure 1A) [40,55,63]. p53 isoforms are involved in a subset of TGF-β1 responses attributable to, in part, interactions between phosphorylated p53 (p-p53) and SMADs to form transcriptionally-active multi-protein complexes [64–66]. Binding specifically involved the N-terminal MAD homology 1 (MH1) domain of SMAD2/3 and the receptor tyrosine kinase/Ras/MAPK cascade-phosphorylated N-terminus of p53 [63,66]. Increased $p53^{S15}$

phosphorylation, accelerating renal damage and compromised organ function are evident following ureteral obstruction-, ischemia/reperfusion- or nephrotoxin-induced (e.g., cisplatin, aristolochic acid) injury as well as in the dysmorphic tubular epithelium and interstitial cells of renal allograft patients (Figure 1B,C) consistent with a role for p53 in promoting tubular cell apoptosis and proliferative inhibition [67,68]. Recent studies, furthermore, link epithelial growth arrest following both acute (e.g., due to ischemia/reperfusion, nephrotoxins) and more protracted (i.e., ureteral ligation) injury to the development of renal fibrosis via mechanisms involving p53 and JNK with retention of TGF-β signaling [2,40]. p53 inactivation by pifithrin-α or siRNA-directed p53 silencing suppresses p53 phosphorylation, attenuates tubular epithelial apoptosis and G_2/M arrest reducing the severity of cisplatin- or ischemia-induced kidney damage and subsequent renal fibrosis [2,40,69].

p53 is a critical co-factor in the TGF-β1-initiated transcription of a subset of pro-fibrotic genes [54,55,70,71] suggesting widespread involvement in the TGF-β1-directed genomic response to tissue injury. Cluster analysis indicated, moreover, that the p53/TGF-β1 synergy specifically involves genes that regulate growth inhibition, extracellular matrix remodeling and cell substrate attachment [63,72–74]. p53 response element(s) are present in the promoters of the PAI-1, collagen 1α, smooth muscle α-actin and other TGF-β1 target pro-fibrotic genes [75,76]. Oligonucleotide mobility shift and DNase I footprinting/methylation interference analyses confirmed that p53 binds to specific motifs in the PAI-1 promoter, including the two p53 half-sites (AcACATGCCT, cAGCAAGTCC) at −224 bp to −204 bp relative to the transcription start site as well as to the upstream 4G/5G polymorphic sequence [75,77] (Figure 2A). Application of the p53MH algorithm, which identifies genome-wide p53-binding motifs, confirmed that the two PAI-1 half-site motifs meet the >90 cut-off score threshold for potential p53-responsive genes [78]. Induction is due to, in part, the formation of transcriptionally-active p-p53/SMAD multi-protein complexes [54,64–66] with DNA site occupancy reflected in both p53 sequence-driven reporter gene transcription and induced expression of the endogenous PAI-1 gene. Multiple approaches established the involvement of p53 in TGF-β1-stimulated PAI-1 gene expression [54,55,71] and revealed that: (a) TGF-β1 induced binding of p53 to the PAI-1 promoter in human proximal tubular epithelial cells, (b) p53-null fibroblasts do not express PAI-1 upon stimulation with TGF-β1, (c) PAI-1 expression “rescue” was evident in p53-null cells engineered to re-express human p53, (d) pre-treatment of a PAI-1 promoter-luciferase reporter cell line with the p53 inhibitor pifithrin-α suppressed TGF-β1-dependent PAI-1 transcription and protein synthesis, (e) transient siRNA knockdown or pharmacologic blockade of p53 in kidney epithelial cells inhibited PAI-1 induction in response to TGF-β1, and (f) the p53/SMAD2/SMAD3 complex recruits histone acetyltransferase CREB-binding protein to the PAI-1 promoter enhancing H3 acetylation and TGF-β1-stimulated PAI-1 transcriptional activation.

PAI-1 transcripts are short-lived (<2 hours), as is typical of unstable p53-induced mRNAs, and targeted by the microRNAs miR-143-3p and miR-145-5p both of which are also p53-responsive [79–81]. The 3′ untranslated regions (UTRs) of unstable p53 inducible transcripts are typically longer, and have a higher incidence of U-, AU- and GU-rich sequences, than 3′ UTRs from stable mRNAs [79]. Indeed, an AU-rich region is followed by an AUUUA instability pentamer in the 3′ UTR of the short-lived 3.0-kb PAI-1 transcript.

This rather brief PAI-1 mRNA half-life may provide at least a partial basis for the translational utility of designed expression suppression since in vivo delivery of p53 siRNA or pifithrin-α effectively reduced cisplatin- or ischemia/reperfusion-induced renal injury and blunted advancement to CKD [40,69,82].

While the mechanism is uncertain, proximal tubular p53 deficiency down-regulated expression of specific genes involved in apoptosis, signaling and oxidative stress and attenuated ischemia-induced inflammation and interstitial fibrosis [83]. Recent findings, moreover, suggest promoter level competition among certain p53 family members, with p53 target gene control implications. Overexpression of the Δ133p53 isoform (which lacks the N-terminal transactivation domain but retains the C-terminal tetramerization sequence) in human fibroblasts represses specific p53-inducible genes involved in cellular senescence, including PAI-1, p21 and IGFBP7 and enhances reprogramming to an induced pluripotent stem cell phenotype [84]. Δ133p53 physically interacts with p53 and it appears likely that heterodimerization of p53 with Δ133p53 at p53 responsive promoters may constitute a dominant-negative mode of expression regulation. Whether such titration of key transcriptional effectors has clinical efficacy in the context of fibrotic disease remains to be determined.

The PE2 promoter motif in the PAI-1 gene may provide a unique opportunity to probe the intricacies of p53 involvement in gene control. Differential residence of upstream stimulatory factor (USF) family members (involving a USF1→USF2 switch) at the PE2 region E box (CACGTG), which is immediately juxtaposed to three 5′ SBEs, characterized the G_0→G1 transition period and growth state-dependent transcriptional activation of the PAI-1 gene [85] (Figure 2A). USF2, moreover, is up-regulated in the obstructed kidney [70] and a consensus PE2 E box motif at nucleotides -566 to -561 is required for USF/E box interactions and serum-dependent PAI-1 transcription [85]. Site-directed CG→AT substitution at the two central nucleotides inhibited formation of USF/probe complexes and PAI-1 promoter-driven reporter expression, confirming the importance of this site in expression control, while Tet-OFF induction of a dominant-negative USF construct or a double-stranded PE2 “decoy” or “trap” [86] attenuated both serum- and TGF-β1-stimulated PAI-1 synthesis [87]. Phasing analysis, moreover, revealed that certain MYC family bHLH-LZ proteins (including USF) redirect DNA minor grove orientation [88] potentially promoting interactions between p53, bound to its half-site response motif, with SMAD2/3 tethered to the PE2 region SBE (Figure 2B). This conformation would facilitate direct interactions between the MH1 N-terminal domain of SMAD2/3 and the p53 N-terminus transactivation domain [63] consistent with the topographic requirement that p53 transcriptionally activates TGF-β1 target genes with both SBEs and p53 binding motifs in their promoter regions and, perhaps, between the C-terminus of p53 and the MH2 region of SMAD3 [89]. Alternatively, since p53 interacts directly with SMAD2 [65,66,72], the PE2 site (with its 3 SBEs) may also serve as a docking platform for p53/SMAD complexes [54,71] (Figure 2B). p300/CREB-binding protein, a histone acetyltransferase, acetylates SMAD2/3 in response to TGF-β1 [90] facilitating the creation of a SMADs/p53/USF2 transcriptional complex necessary for optimal PAI-1 induction [91–93]. The importance of such interactions is underscored by the finding that RAP250, a protein devoid of intrinsic enzymatic activity yet 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 [94].

While the growing complexity of the PE2-based transcriptional control unit in the PAI-1 promoter, as well as the ability to target the involved *cis*-acting and epigenetic factors [86], promises to provide new opportunities to manage expression of disease-relevant fibrotic genes, p53 may also initiate a profibrotic genomic program indirectly though transcription of specific microRNAs. p53 appears to promote fibrosis following unilateral ureteral obstruction (UUO) by up-regulation of miR199a-3p that, in turn, suppresses expression of SOCS7 stimulating, thereby, STAT3 activation in proximal tubular cells [95]. SOCS1, SOCS3 and SOCS7 are potent inhibitors of STAT1, STAT3 and ERK phosphorylation [96] suggesting a model [95] whereby TGF-β1 activation of p53 stimulates p53-dependent transcription of miR-199a-3p inhibiting SOSC7 expression resulting in STAT3-induced renal fibrosis.

The ancestral p53 family member p73 [97], in particular ΔNp73, also functions in PAI-1 transcriptional control [98]. ΔNp73 transcripts are generated from the alternate P2 promoter site in intron 3 and encode a N-terminal truncated protein lacking the transactivation domain [99,100]. While ΔNp73 generally inhibits gene transcription by p53, and other p73 isoforms, ΔNp73 actually increased expression of the TGF-β1 target genes PAI-1 and collagen 1α [100]. ΔNp73 knockdown attenuated TGF-β1 signaling and reporter anayses confirmed that ΔNp73 induction of the PAI-1 gene, unlike p53, was not dependent on p53 binding motifs but on association with SMADs at the SBEs in the PAI-1 and collagen 1α promoters. DNA pull-down assays indicated, moreover, that ΔNp73 and SMADs form a complex on an SBE oligonucleotide platform and that ΔNp73 enhances SMAD3 binding to the SBE target construct. p53, however, is essential for PAI-1 transcription at least in response to TGF-β1 [54]. It remains to be determined, therefore, if ΔNp73 requires p53 to activate TGF-β1-dependent transcription of the PAI-1 gene. This possibility is supported by the observation that p73 is not sufficient to completely compensate for p53 deficiency in renal development as p53-null mice have defects in nephron differentiation [102] and that p53-p73 cooperation regulates p53 transcriptional activity and genomic impact [100]. p53 family proteins, moreover, form multimeric complexes often described as “dimers on dimers”. It appears that JNK-induced phosphorylation of $p53^{T81}$ drives the formation of transcriptionally-active p53/p73 complexes [103]; it is also conceivable that p73 may, in the context of chromatin, heterodimerize with mutant p53 [104]. The various p53 members, thus, likely foster different transcriptional outcomes by competing for DNA binding sites, acting in a dominant-negative fashion or inhibiting or enhancing function via heterotetramerization or other protein-protein interactions. Addressing these issues will require individual target gene assessments.

Mechanism of p53 Activation by TGF-β1

TGF-β1 regulates p53 activity by serine site phosphorylation, in the N-terminal transactivation domain, and serine/lysine acetylation/methylation (among other post-translational modifications) in the tetramerization and regulatory domains in the C-terminus [105]. Collectively, these promote interactions with activated SMADs and subsequent

binding of p53/SMAD3 to the PAI-1 promoter in human renal proximal tubular epithelial cells [54]. Phosphorylation the p53 amino-terminal serines15/20 and threonine18 residues increases the association of p53 with members of the p300/CREB binding protein (CBP) co-activator family while stimulating p53 transactivation function [106]. p300 and CBP protein/histone acetyltransferases relax chromatin structure facilitating recruitment of accessory transcriptional factors to promoter domains of target genes. The creation of a multi-component p300/p53/SMAD complex preceded optimal TGF-β1-dependent induction of the PAI-1 gene [54]. Similarly, interactions between p53 and SMAD2/3, at their respective binding sites, recruits CBP to the PAI-1 promoter increasing histone H3 acetylation and PAI-1 transcription [89]. Not all p53/SMAD interactions result in gene activation, however. Consistent with the potential opposing actions of SMAD3 (pro-fibrotic) vs. SMAD2 (anti-fibrotic) [107,108], partnering of p53 with SMAD2 in hepatic cells represses expression of the developmentally-dependent alpha fetoprotein gene (AFP). p53 DNA binding is required to anchor TGF-β1-activated SMADs as well as the transcriptional co-repressor mSin3A to the SMAD-binding motifs and the p53 response elements in the AFP promoter [109]. In this context, SnoN (an inhibitor of TGF-β1 signaling) is a critical co-factor in AFP suppression functioning to up-regulate mSin3A levels. Whether other TGF-β1 down-regulated p53-sensitive genes [54] utilize a similar mechanism of repression is unknown.

Recent findings have shed light on the mechanism of p53 activation in response to TGF-β1. One potential regulator of p53 function in the context of tissue injury is the serine/threonine kinase tumor suppressor ataxia telangiectasia mutated (ATM). Activated ATM ($pATM^{S1981}$) increased significantly in the tubulointerstitial region of the UUO-injured kidney correlating with SMAD3 and $p53^{S15}$ phosphorylation, elevation of the $p22^{phox}$ subunit of the NADP(H) oxidases, and expression of the fibrotic markers PAI-1 and fibronectin [71]. This likely reflects elevated levels of TGF-β1 in response to ureteral ligation as ATM is rapidly phosphorylated at the same site (S1981) upon TGF-β1 stimulation of cultured proximal tubular cells. Stable silencing (by lentiviral delivery of short hairpin RNAs) or pharmacological inhibition (with KU-55933) of ATM attenuated TGF-β1-induced p53 activation and markedly reduced expression of the downstream targets PAI-1, fibronectin, CTGF and p21 in human tubular epithelial cells as well as in kidney fibroblasts [71]. The participating elements in TGF-β1-induced ATM mobilization are becoming clarified. Knockdown of the NADPH oxidase (NOX) subunits, $p22^{phox}$ and $p47^{phox}$ in HK-2 cells blocked TGF-β1-stimulated phosphorylation of ATM ($pATM^{S1981}$) and target gene induction via p53- dependent mechanisms. Thus, TGF-β1 promotes NOX-dependent ATM activation leading to TGF-β1-initiated p53 phosphorylation and p53-mediated fibrotic gene reprogramming (Figure 3). Depletion of ATM or p53, moreover, resulted in a bypass of TGF-β1-mediated cytostasis in HK-2 cells [71]. Furthermore, TGF-β1/ATM-stimulated secretion of paracrine factors by the dysfunctional renal epithelium promotes interstitial fibroblast growth, suggesting a role for tubular ATM in mediating epithelial-mesenchymal cross-talk highlighting the translational benefit of targeting the NOX/ATM/p53 axis in renal disease.

Relevant is the recent finding that SMG7 (suppressor with morphological defects in genitalia 7), a regulator of nonsense-mediated mRNA decay, binds to and stabilizes p53 under conditions of genotoxic stress [110]. The mechanism appears to involve promotion of ATM-

dependent phosphorylation and subsequent inhibition of the E3 ubiquitin ligase mouse double minute 2 homolog (MDM2). This role for SMG7 may well have an impact on expression/function of p53 and recovery from AKI, particularly in patients with lupus nephitis who exhibit reduced expression of SMG7 [111]. The participation of MDM2 in fibrosis, however, is complex. MDM2 is a major regulator of p53 function, via inhibition of transcriptional activity, and stability by virtue of its role as a ligase [112]. While TGF-β1 induces p53 activation in vitro and in vivo, this growth factor also increases MDM2 expression in a p53-dependent manner establishing a feedback loop where p53 initiates expression of its negative regulator [54,112,113]. Recent findings suggest that MDM2 also mediates fibroblast activation and renal interstitial fibrosis through a p53-independent pathway perhaps involving Notch I down-regulation [113]. Nevertheless, while underlying events require clarification, it is apparent that the p53-MDM2/murine double minute X (MDMX) axis is required for normal kidney development. Germline p53 depletion results in renal anomalies (with some dependency on genetic background) while MDM2/MDMX deficiencies are associated with acute renal injury, epithelial cell death and fatal dysgenesis [114,115]. The clinical relevance of these findings is highlighted by a recent systems analysis of patients with diabetic nephropathy confirming a marked down-regulation of MDM2 expression in the glomerular and tubular compartments [116].

PAI-1 Involvement in Renal Disease: p53-Dependent/Independent Pathways

Transcriptome profiling highlights the complexity of gene expression patterns in kidney disorders [37,117–124] as well as in TGF-β1-stimulated epithelial cells [54,125,126]. Certain TGF-β1 target genes directly influence the development of the myofibroblastic phenotype and renal fibrogenesis. PAI-1, in particular, is a prominent TGF-β1 response gene in proximal tubular epithelial cells as well as interstitial fibroblasts and is involved in the TGF-β1-induced conversion of fibroblasts to myofibroblasts [71,127–129]. Among its varied functions, PAI-1 negatively regulates the plasmin-dependent pericellular proteolytic cascade effectively limiting ECM degradation and fibrinolytic activity, thereby, contributing to the initiation and/or progression of interstitial fibrosis and vascular thrombosis [62,130]. PAI-1 is a member of both the growth arrest/fibrosis genomic cluster in the diabetic rat kidney [131] and the 11-gene urine mRNA discovery set signature predictive of human renal allograft fibrosis [132]. PAI-1 null mice are, in fact, protected from excessive ECM accumulation as well as lung, liver, kidney and vascular fibrosis and PAI-1 urokinase/tissue-type plasminogen activator domain decoys reduced both UUO-initiated and established interstitial fibrosis [133].

Recent data suggest a rather novel role for PAI-1 in fibrotic disorders apart from its impact on ECM turnover. While it is well established that p53 limits cellular proliferation by inducing a state of replicative senescence, little is known about the mechanisms involved in this growth-limiting response. Suppression of the p53 target gene PAI-1 by RNA interference leads to senescence escape by sustained activation of the PI(3)K-AKT-GSK3β pathway and nuclear retention of cyclin D1 [73]. Genetically-deficient PAI-1 knockout (PAI-$1^{-/-}$) mouse embryonic fibroblasts (MEFs), in fact, proliferate well beyond the senescence checkpoint, albeit at a slower rate than $p53^{-/-}$ MEFs. Moreover, ectopic expression of PAI-1 in p53-null fibroblasts induces a phenotype displaying all the hallmarks

of replicative senescence-induced growth arrest [73]. These data were the first to conclusively indicate that PAI-1 is not merely a marker of senescence, but is both necessary and sufficient for the induction of senescence downstream of p53. Similarly, TGF-β has a significant cytostatic effect on various cell types. p53 knockdown results in escape from TGF-β1-induced growth arrest in various cell types [24,89,134] which exhibit a strong growth inhibitory response to TGF-β1 including those derived from the renal proximal tubular epithelium [71]. Collectively, it appears that p53 plays an important role in TGF-β-induced cytostasis via induction of PAI-1 transcription and that loss of p53 or its target gene PAI-1 confers resistance to TGF-β1-mediated growth inhibition. These findings suggest the utility of p53 pathway disruption in renal disease, perhaps as a strategy to promote compensatory regeneration, and are underscored by very recent findings that loss of phosphatase and tensin homolog (PTEN) expression correlated with increased PAI-1 levels in the obstructed kidney [135]. PTEN knockdown in HK-2 cells, moreover, promoted fibrotic factor expression (PAI-1, fibronectin, CTGF) and G_1 cell cycle arrest [135]. PTEN loss also results in p53, SMAD3, AKT activation and formation of p53/SMAD3 complexes associated with epithelial dysfunction. As is the case for TGF-β1-treated cells, growth restriction was PAI-1-dependent since silencing of PAI-1 expression in PTEN-knockdown HK-2 proximal tubular cells rescued the proliferative response. The increased population density evident in dual-deficient PTEN/PAI-1 cultures was comparable to that of cells with stable silencing of both p53 and PTEN expression. Moreover, the elevated PAI-1 levels evident in PTEN-deficient cultures significantly decreased upon p53 shRNA lentiviral transduction additionally reinforcing a role for p53 in fibrotic gene induction [135]. Furthermore, PCNA expression markedly increased in both dual PTEN+PAI-1 shRNA- and PTEN+p53 shRNA-expressing HK-2 cells compared to similarly seeded PTEN shRNA cultures confirming that depletion of p53 or PAI-1 levels leads to a bypass of cell growth inhibition triggered by PTEN loss in proximal tubular epithelial cells. Since PTEN deficiency is a common event in diabetic-, UUO-, ischemia/reperfusion- and aristocholic acid-induced renal injury and the associated failed regeneration [2,135], approaches designed to limit p53 activation and/or PAI-1 expression may promote tubular epithelial recovery and attenuate nephron loss.

Current data also confirm an unexpected involvement of PAI-1 in innate immunity. Indeed, following kidney injury, PAI-1-null mice develop an attenuated inflammatory/fibrotic response while transgenic PAI-1 over-expressing animals exhibit increased renal interstitial monocyte/macrophage density suggesting that this serine protease inhibitor may promote macrophage and T-cell infiltration and/or immune cell tissue residence time [136,137]. Monocyte adhesion to the aortic intima was significantly reduced in streptozotocin-treated PAI-$1^{-/-}$ mice and accompanied by decreases in the inflammatory mediators TNF-α and monocyte chemotactic protein-1 [138]. Since PAI-1 provides a “don’t eat me” signal, effectively inhibiting neutrophil efferocytosis [139,140], these findings [138–140] suggest that this serine protease inhibitor may impact cellular influx as well as the intensity and/or duration of the injury-initiated inflammatory phase. Indeed, elevated PAI-1 levels closely mirrors systemic and localized inflammation while exogenously-delivered PAI-1 stimulates expression of proinflammatory cytokines (e.g., TNFα and macrophage inflammatory protein-2) in primary bone marrow macrophages [137]. The protease inhibitory-,

vitronectin- or LRP1-binding properties of PAI-1, however, are not necessary for macrophage activation but TLR4 appears to be required, at least in part, since TLR4 neutralizing antibodies or the genetic depletion of TLR4 attenuated PAI-1-induced inflammation. This response was PAI-1 dose-dependent but LPS-independent and reduced in $TLR4^{-/-}$ macrophages [137] suggesting that PAI-1 may function as a matricellular damage-associated molecular pattern (DAMP) TLR ligand [141,142]. There is also evidence for PAI-1 involvement in lipopolysaccharide (LPS) signaling. PAI-1 knockdown attenuated LPS-induced increases in macrophage TLR4, MD-2, MyD88, TNFα, IL-1β and NF-κB levels while vector-driven PAI-1 over-expression enhanced these responses [143,144]. Although the mechanism is unclear, data suggest that PAI-1 is involved in host inflammatory responses via TLR4, at least in macrophages [137]. This is likely to have a significant impact on fibrogenic outcomes following tissue injury as exogenous PAI-1 treatment increased TGF-β1, collagen 1α1, collagen 1α2 and MCP-1 transcripts in renal mesangial and proximal tubular epithelial cells [145–147]. The TLR4/RAGE DAMP-type ligand HMGB1 also activates a subset of genes in the TGF-β1 profibrotic signature that includes PAI-1, CTGF and TGF-β1 [148] suggesting that DAMPs and LPS utilize common and unique signaling pathways that may be exploited in the design of interventional strategies. Collectively, it appears that TLR4 may function as a molecular “switch”, activated by endogenous DAMPs to initiate repair while stimulating TGF-β1 signaling (by down-regulating the TGF-β pseudoreceptor BAMBI) promoting the persistent expression of TGF-β target genes to create and maintain a progressive fibrotic microenvironment [149,150].

Exogenous PAI-1 also activates the JAK/STAT, AKT and focal adhesion kinase (FAK) pathways via LRP1-dependent mechanisms [151–153]. PAI-1 may engage several cellular receptors (TLR4, LRP1), therefore, with differing phenotypic outcomes. Whether PAI-1 occupancy of its binding site on the somatomedin B domain of vitronectin or to urokinase plasminogen activator/urokinase plasminogen activator receptor complexes on the cell surface is required for signaling through TLR4 or LRP1 is not clear. Recombinant PAI-1, however, does mobilize the RhoA/ROCK1/MLC-P pathway stimulating amoeboid cell migration [142] and apparently modulates TGF-β1 signaling, through direct effects on TGF-β1 bioavailability, as PAI-1-null mice subjected to obstructive nephropathy have lower TGF-β1 levels compared to wild-type controls [136,154]. Exogenously-delivered PAI-1 alone, moreover, stimulates TGF-β1 synthesis which could be attenuated by pretreatment with small molecule PAI-1 functional inhibitors, suggesting the existence of a PAI-1/TGF-β1-positive feedback mechanism [145,147]; these same compounds reduced up-regulation of fibronectin, collagen 1 and PAI-1 transcripts in the kidneys of diabetic mice [146]. It appears that PAI-1 may initiate, perhaps maintain, a potential pro-fibrogenic “loop” in the context of renal disease [145,147]. It is tempting to speculate, therefore, that targeted down-modulation of PAI-1 expression or function may provide multi-level therapeutic opportunities to inhibit the onset and progression of tissue fibrosis (Figure 4).

Conclusion

TGF-β1 is the principal driver of tissue scarring leading to interstitial renal fibrosis and eventual organ failure. TGF-β1 stimulates p53 phosphorylation promoting interactions with activated SMADs and subsequent binding of p53/SMAD3 to target promoters. Recent

findings further suggest that PTEN deficiency, perhaps TGF-β1-mediated, is a common event in diabetic-, ischemia/reperfusion-, ureteral ligation- and aristocholic acid-induced renal injury resulting in p53 and SMAD3 activation and formation of p53/SMAD3 complexes. The details in this pathophysiologically-relevant interplay of signaling effectors are only beginning to emerge however p-p53 is required for expression of PAI-1 and CTGF, major TGF-β1 target genes and key causative factors in fibrotic disorders. One regulator of p53 function in the context of renal injury is ATM. $pATM^{S1981}$ levels increase in the injured kidney, as well as in TGF-β1-stimulated tubular epithelial cells, correlating with SMAD3/p53 phosphorylation and expression of the $p22^{phox}$ subunit of the NADPH oxidases. Silencing or pharmacologic inhibition of ATM attenuated TGF-β1-induced p53 activation and subsequent PAI-1, fibronectin, CTGF and p21 up-regulation in human proximal tubular cells and kidney fibroblasts. Engineered reductions in the NOX subunits, $p22^{phox}$ and $p47^{phox}$ blocked TGF-β1-induced ATM^{S1981} phosphorylation and gene induction via p53-dependent pathways. TGF-β1, therefore, appears to promote NADPH oxidase-dependent ATM activation leading to p53-dependent profibrotic genomic reprogramming. Importantly, PAI-1 is a member of the signature gene set predictive of renal allograft fibrosis [132] as well as a prominent p53 target [73]. Increased $p\text{-}p53^{S15}$ immunoreactivity is evident in the epithelial and intertubular compartments in human renal transplants with established tubular dysmorphism and interstitial involvement (Figure 1C). Administration of pifithrin-α to mice with ischemic renal injury reduced both the expression of profibrotic genes and the extent of interstitial fibrosis [40]. Pharmacologic inhibition of p53 function or the p53 activation network, if appropriately managed, may have significant clinical implications. These data collectively highlight the translational potential in targeting the TGF- β1/p53 axis in renal disease but which also may be relevant to the global problem of tissue fibrosis regardless of the involved site.

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Abbreviations

AKT	AKT8 oncogene, alpha serine/threonine-protein kinase or protein kinase B
bHLH-LZ	basic helix-loop-helix/leucine zipper
CREB	cAMP response element-binding protein
ERK	extracellular signal-regulated kinases
JAK	Janus kinases
JNK	c-Jun N-terminal kinases
IGFBP7	insulin-like growth factor-binding protein 7
MLC-P	phospho-myosin light chain
PCNA	proliferating cell nuclear antigen

RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
shRNA	short hairpin RNA
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TAZ	transcriptional coactivator with PDZ-binding motif
YAP	Yes-associated protein

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Highlights for Review

- Fibrotic disorders of the renal, pulmonary, cardiac, and hepatic systems are associated with significant morbidity and mortality..
- TGF-β1 is the principal driver of renal fibrogenesis,.
- TGF-β1 activates the ALK5 type I receptor (which phosphorylates SMAD2/3) as well as non-canonical (e.g., *src* kinase, EGFR, Jak/Stat, p53) pathways that collectively drive the fibrotic genomic program.
- TGF-β1 stimulates the activation and assembly of p53-SMAD3 complexes required for transcription of the renal fibrotic genes plasminogen activator inhibitor-1 (PAI-1, SERPINE1), connective tissue growth factor (CTGF, CCN2) and TGF-β1.
- Focus on TGF-β1-initiated signaling in renal fibrogenesis and the role of p53 as a regulator of profibrotic gene expression.

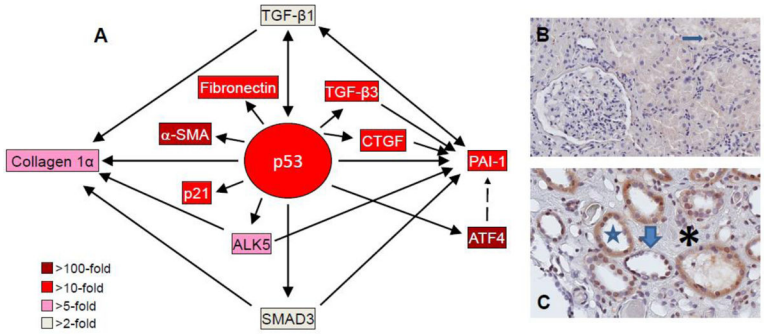

Figure 1. p53 induction and target gene expression in the UUO-injured kidney and in renal allografts

Microarray analyses of TGF-β1-stumulated proximal tubular epithelial cells illustrating the relative expression levels of target genes within the p53 transcriptome node **(A)**. p53-dependent up-regulation of ALK5, SMAD3, TGF-β1, TGF-β3, CTGF, α-smooth muscle actin (α-SMA) and PAI-1 may constitute a complex feed-forward loop that maintains a profibrotic renal microenvironment. Compared to a normal appearing patient allograft **(B)** in which only infrequent p-p53^{S15} tubular epithelial cells were evident **(arrow)**, a renal transplant exhibiting dysmorphic tubules **(star)** with a flattened and occasionally denuded epithelium **(thick arrow)** and a markedly expanded interstitial region **(asterisk)** has abundant nuclear and cytoplasmic p-p53^{S15} immunoreactivity **(C).**

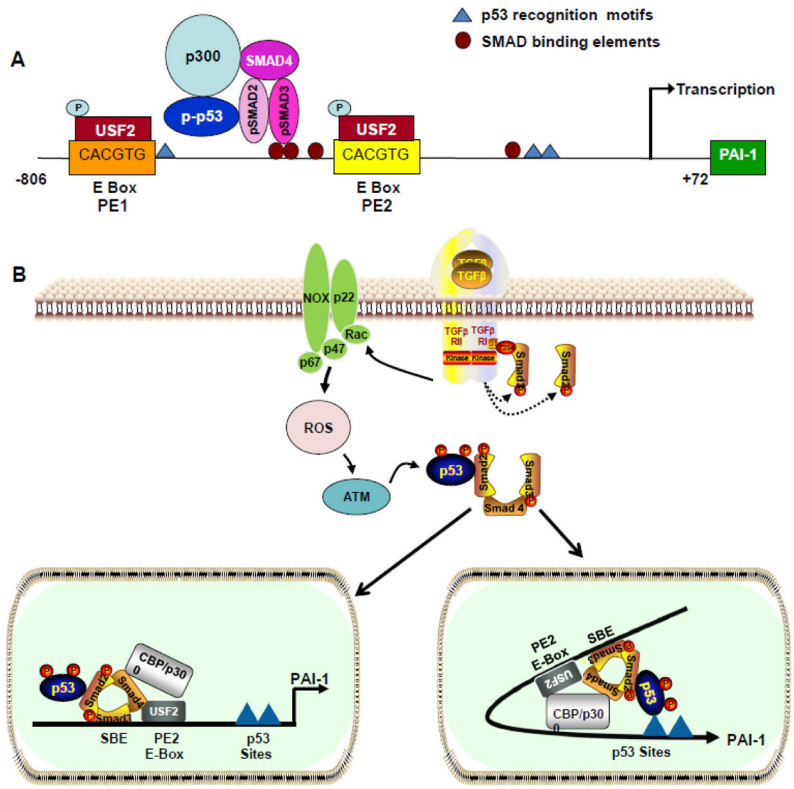

Figure 2. Topography of transcriptional motifs in the PAI-1 promoter and pathways involved in TGF-β1-induced expression

Downstream p53 binding sites (AcACATGCCT, cAGCAAGTCC) map to nucleotides -224 to -204 relative to the transcription start site and the upstream 4G/5G polymorphic sequence (blue triangles) in the PAI-1 promoter. pSMAD2/3/p-p53 interactions, at the PE2 USF2-binding E Box site located immediately 3′ of three clustered SMAD-binding elements (SBEs), are critical for PAI-1 transcription **(A)**. Ligand-dependent TGF-β1 receptor activation initiates SMAD2/3 phosphorylation (by the ALK5/TGF-β1 type I receptor) (**B)**. Rapid TGF-β1-induced generation of ROS stimulates non-SMAD-mediated signaling (e.g., upon p53 phosphorylation/acetylation). The SMAD and non-SMAD pathways collectively regulate target gene expression. In one model **(B, left panel)**, p53 interacts directly with SMAD2; such SMAD2-p53 interactions may occur independently of p53 occupancy of its consensus motif. Alternatively, certain bHLH-LZ factors (including USF) bend DNA toward the minor groove potentially promoting interactions between p53, bound to its two downstream half-site motifs, with SMAD2 tethered to SBE sites immediately upstream of the CACGTG E-Box [72] **(B, right panel)**.

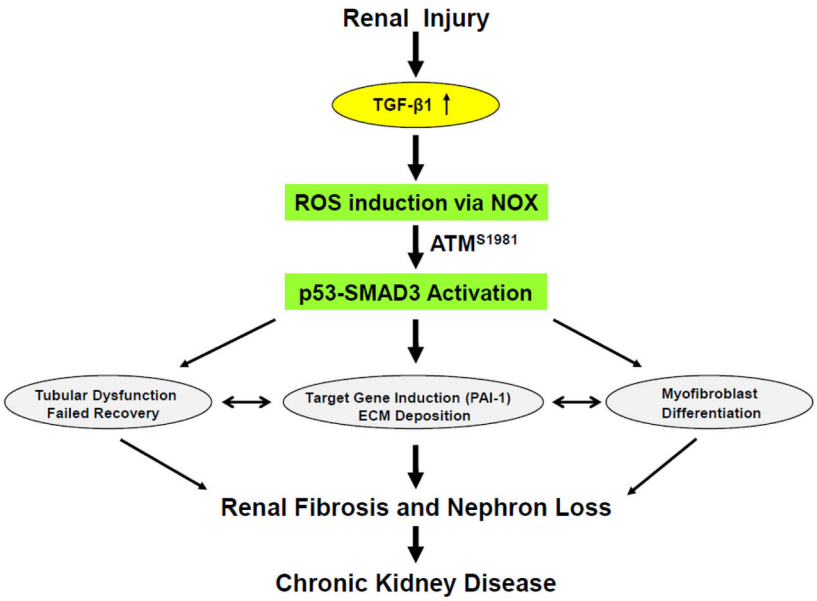

Figure 3. Events downstream of renal injury-induced TGF-β1 expression that contribute to fibrotic disease

Activated ATM (pATM^{S1981}) increased significantly in the tubulointerstitial region of the UUO-injured kidney, likely in response to elevated TGF-β1 levels and expression of the p22^{phox} subunit of the NADP(H) oxidases, correlating with SMAD3 and p53^{S15} phosphorylation and induction of the fibrotic markers PAI-1 and fibronectin [71]. Stable silencing or pharmacological inhibition of ATM attenuated TGF-β1-induced p53 activation and expression of the downstream targets PAI-1, fibronectin, CTGF and p21. Silencing of the NADPH oxidase (NOX) subunits, p22^{phox} and p47^{phox} in HK-2 cells blocked TGF-β1-stimulated phosphorylation of ATM (pATM^{S1981}) and target gene induction via p53-dependent mechanisms. Thus, TGF-β1 promotes NOX-dependent ATM activation leading to p53-mediated fibrotic gene reprogramming and growth arrest in HK-2 cells. Depletion of ATM or p53 in HK-2 cells, moreover, resulted in a bypass of TGF-β1-mediated cytostasis [71]. Furthermore, TGF-β1/ATM-initiated paracrine factor secretion by the dysfunctional renal epithelium promotes interstitial fibroblast growth, suggesting a role for tubular ATM in mediating epithelial-mesenchymal cross-talk highlighting the translational benefit of targeting the NOX/ATM/p53 axis in renal disease.

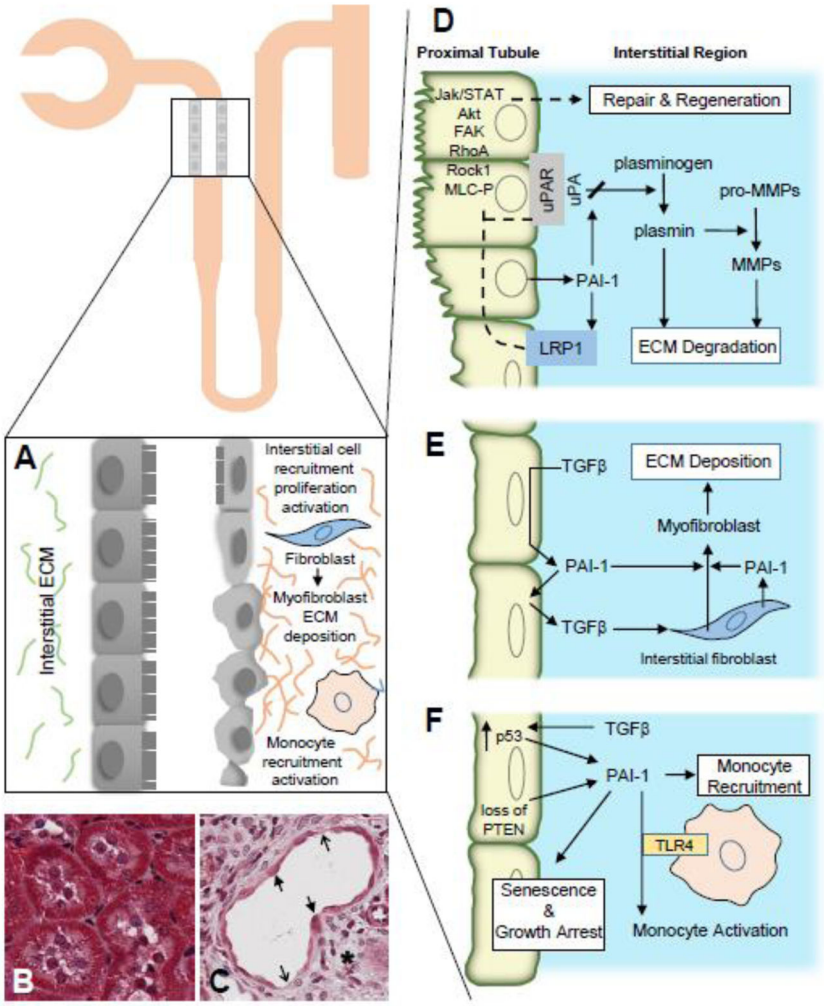

Figure 4. Multifunctional roles of PAI-1 in renal fibrosis

Development of renal disease upon proximal tubule injury is characterized by major functional and morphological changes including epithelial dedifferentiation, ECM accumulation, cell cycle arrest and tubular dysmorphism **(A left, B = normal kidney, A right, C = fibrosis)**. Thick and thin arrows denote flattened epithelium and denuded regions, respectively. Following tissue injury, an inter-dependent plasmin-generating/matrix metalloproteinase (MMP) pericellular proteolytic cascade is finely titered, both temporally and spatially, by local PAI-1 levels **(D)**. Collectively, these highly integrated systems cooperate to regulate ECM degradation and stromal remodeling. Elevated PAI-1 abundance in the wounded kidney compromises ECM degradation and fibrin clearance promoting increased matrix accumulation that contributes to the initiation of the fibrotic process and eventual development of CKD. Increased TGF-β levels or activation in response to trauma facilitate the transition of pericytes and resident interstitial fibroblasts (with perhaps minor contributions from other cell types) toward a myofibroblastic phenotype **(E)**. An increased persistence and/or density of myofibroblasts further accelerates ECM deposition and eventual loss of tissue function. In conjunction with high p53 expression and loss of PTEN, TGF-β expression in the injury microenvironment mediates epithelial growth arrest with loss

of regenerative repair, exacerbating ECM accumulation, likely through elevation of local levels of profibrotic factors including PAI-1 **(F)**. Microenvironmental cues in the injured epithelial and interstitial compartments initiate TGF-β1-dependent monocyte recruitment/activation (perhaps via PAI-1 modulation of TLR4 signaling) **(E,F)**. Additional mechanistic details are provided in the text. Collectively, these events **(D–F)** illustrate the clinical significance of the collaborative effects of TGF-β1 and TGF-β1 target genes (e.g., PAI-1) on the development of renal fibrosis.