TGF-β Signaling in the Kidney: Pro-fibrotic and Protective Effects

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Running Title: Pro-fibrotic and Protective Effects of TGF-β

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Abstract

Transforming Growth Factor-beta (TGF-β) is generally considered as a central mediator of fibrotic diseases. Indeed, much focus has been placed on inhibiting TGF-β and its downstream targets as ideal therapeutic strategies. However, pharmacological blockade of TGF-β has not yet translated into successful therapy for humans, which may be due to pleiotropic effects of TGF-β signaling. Equally, TGF-β signaling as a protective response in kidney injury has been relatively underexplored. Emerging body of evidence from experimental kidney disease models indicates multifunctionality of TGF-β capable of inducing pro-fibrotic and protective effects. This review article discusses recent advances highlighting the diverse roles of TGF-β in not only promoting renal fibrosis, but also protective responses of TGF-β signaling. We review, in particular, growing evidence that supports protective effects of TGF-β by mechanisms which include inhibiting inflammation and induction of autophagy. Additional detailed studies are required to fully understand the diverse mechanisms of TGF-β actions in renal fibrosis and inflammation that will likely direct towards effective anti-fibrotic therapies.

Keywords: TGF-β, Smad, BMP, Kidney, Fibrosis, Autophagy, Apoptosis, Inflammation
Introduction

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that is well recognized to regulate a broad spectrum of cellular processes such as growth, differentiation, apoptosis, wound repair, and the pathogenesis of fibrosis. It is a member of a superfamily of 38 cytokines that include TGF-β, bone morphogenetic proteins (BMP), growth differentiation factors, inhibins, and activins. TGF-β superfamily members, in particular, TGF-β and BMP have been implicated to play important and diverse roles in chronic kidney disease (CKD) (71, 100).

The hallmark of progressive CKD is the excessive extracellular matrix (ECM) production leading to renal fibrosis and TGF-β has been generally considered a potent pro-fibrotic mediator in this process (17, 48).

Mammalian TGF-β includes three major isoforms that are encoded by distinct genes (TGF-β1, TGF-β2, and TGF-β3). The isoforms are synthesized as large precursor proteins (pro-TGF-β) forming dimeric complexes in the endoplasmic reticulum, and are subsequently cleaved near the carboxy-terminus to yield mature 112 amino acid polypeptides, which share 60-80% conservation across the three TGF-β isoforms (99). The mature TGF-β dimer remains associated with cleaved latency peptide portion of the precursor as an inactive latent complex, and dissociation from the complex results in biologically active TGF-β (76). Thus, newly synthesized TGF-β bound to the latency associated peptide (LAP) forming a small latent complex (SLC) is biologically inactive and cannot bind to its receptors. Through the formation of disulfide bonds, this complex loosely binds to a latent TGF-β binding protein (LTBP) to form a large latent complex (LLC). TGF-β is then secreted in a latent state and is stored in the ECM (2, 76).

Activation of TGF-β involves release from the latent complex following exposure to a number of different factors including integrins, proteases, metalloproteinases, reactive oxygen species
(ROS), plasmin, and acid, that allows binding to its cell surface receptors for initiation of TGF-β signaling (25, 76, 81, 87).

It is well established that activation of TGF-β leads to pleiotropic cellular responses that are mediated by TGF-β signaling via interactions with TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII) and subsequent activation of intracellular Smad and non-Smad dependent signaling pathways (13, 16, 57). In this review, we describe emerging body of evidence from experimental kidney disease models demonstrating multifunctional nature of TGF-β capable of inducing pro-fibrotic and protective effects. We provide an overview on recent advances highlighting the diverse roles of TGF-β in not only promoting renal fibrosis but also protective responses, focusing on growing evidence for protective effects of TGF-β by mechanisms which include inhibiting inflammation and induction of autophagy.

**TGF-β isoforms in the kidney**

Studies of human kidney specimens have confirmed that the three major isoforms TGF-β1, TGF-β2, and TGF-β3 are expressed in the kidney (33). While functional redundancy between the TGF-β isoforms has been long recognized, there is a growing body of evidence for the existence of non-redundant functions in inflammation and organ development (76). TGF-β1, the major focus of this review, is the predominant and best-characterized isoform, while TGF-β2 and TGF-β3 are less known. Some clear differences among the isoforms have been noted. In the normal human adult kidney, glomerular expression of TGF-β2 and TGF-β3 is seen mainly in podocytes, whereas TGF-β1 is primarily detected in the tubules but not in the glomeruli (33). Interestingly, glomerular expression of TGF-β1, generally with TGF-β2 and TGF-β3, was detected in podocytes in kidney biopsy specimens from patients with proliferative
glomerulonephritis and in mesangial cells in diabetic nephropathy and IgA nephropathy (33). Moreover, increased expression of TGF-β1 was associated with development of severe glomerulonephritis and glomerulosclerosis (33).

Biological actions of TGF-β isoforms are mediated by ligand binding to its receptors for the initiation of signaling. Both TGF-β1 and TGF-β3 bind directly with TβRII, whereas TGF-β2 requires the presence of a type III TGF-β receptor (TβRIII) for ligand binding to TβRII (99). Given the differences in the expression patterns and the mechanism of ligand binding, together with apparent non-overlapping phenotypes of the three TGF-β isoform knockout mice, it is not unreasonable that some cellular responses may differ among the TGF-β isoforms.

All three TGF-β isoforms have been shown, in vitro, to induce ECM protein production in various renal cells, including glomerular mesangial cells, renal fibroblasts, and renal tubular epithelial cells (91, 99). While most studies have demonstrated similar pro-fibrotic effects of the TGF-β isoforms, a number of recent studies have suggested that TGF-β2 and TGF-β3 can exert anti-fibrotic effects (72, 75, 96, 99). Studies in human podocytes demonstrated anti-fibrotic effects of TGF-β2 through upregulation of sphingosine kinase-1 (SK-1) activity, thereby suppressing pro-fibrotic connective tissue growth factor (CTGF) expression (75). It is important to note that similar anti-fibrotic effects of TGF-β2 were not observed in other kidney cell types, as all three isoforms equally induced upregulation of CTGF mRNA in cultured mesangial cells and glomerular visceral epithelial cells (33). Moreover, TGF-β2 stimulated the expression of ECM proteins and induced EMT in tubular epithelial cells, whereas neutralizing antibody to TGF-β2 or repression of TGF-β2 expression inhibited renal fibrogenesis (91). Further investigations are warranted to clarify the seemingly opposite findings regarding the anti-fibrotic
roles of TGF-β2 and TGF-β3, which carry important implications for therapeutic targeting strategy.

Activation of TGF-β1 Signaling

TGF-β1 signaling involves activation of complex intracellular networks to regulate pleiotropic biological functions. As depicted in Figure 1, the active form of TGF-β1 transmits signals through type I and type II receptors, TβRI and TβRII. These cell surface receptors contain serine/threonine kinases that phosphorylate transcription factor proteins called Smads to initiate the canonical TGF-β signaling pathway. TGF-β1 ligand assembles a heteromeric complex through binding to TβRII, which then phosphorylates the kinase domain of TβRI, and leads to the downstream activation of the receptor-activated or regulatory Smads (R-Smads), namely Smad2 and Smad3. The activated R-Smads form an oligomeric complex with the common mediator Smad4 and undergo nuclear translocation to regulate transcription of target genes (57). On the other hand, inhibitory Smads (Smad6 and Smad7) bind to activated TβRI and interfere with R-Smad activation. Smad7 has also been shown to negatively regulate TGF-β signaling through recruitment of E3 ubiquitin ligases, Smurf1 and Smurf2, which target TGF-β receptors and Smad7 to undergo degradation via proteasomal and lysosomal pathways (22, 36).

Additionally, TGF-β1 signaling is mediated via non-canonical, non-Smad pathways (Figure 1) including the mitogen-activated protein kinases (MAPKs), mediated by Ras-Raf activating MEK (also called MAPKK) and extracellular signal-regulated kinase (ERK), (16, 68, 90) or TAK1 (TGF-β activated kinase 1) pathway, the upstream MAPK kinase kinase (MKKK) activating MKK3-p38 and MKK4-JNK (c-Jun N-terminal kinase) signaling cascades (13, 37, 38, 69). TGF-β1 signaling also activates Rho family small GTPases, Rho, Rac, Cdc42 as well as
Phosphatidylinositol 3 Kinase (PI3K)/AKT and Integrin Linked Kinase (ILK) (3, 23, 50, 66, 95, 98).

Although the precise mechanisms underlying progressive renal fibrosis are not completely understood, studies have shown that TGF-β1, mediated via its canonical Smad signaling pathway, drives development of renal fibrosis (77). Recent investigations have also demonstrated that the non-canonical, non-Smad pathways can also mediate TGF-β1 fibrogenic responses (13, 38). However, new findings show that blocking TGF-β1 signaling in renal cells \textit{in vitro} or in experimental animal models \textit{in vivo} does not necessarily reduce renal fibrosis.

\textbf{Anti-TGF-β therapy in Renal Fibrosis}

Based on significant pre-clinical evidence showing TGF-β1 as a key mediator of fibrotic diseases, much effort had been placed on inhibiting TGF-β1 as a therapeutic strategy. Several recent clinical trials using pharmacological blockade of TGF-β in fibrotic kidney diseases are summarized in Table 2. Therapeutic approach using neutralizing anti-TGF-β antibody has been explored in models of experimental diabetes induced by STZ and \textit{Lepr}^{db/db} diabetic mice, demonstrating significant reduction in TGF-β1 levels and inhibition of glomerular mesangial matrix expansion with suppression of collagen and fibronectin expression (10, 80, 102). However, the recent multicenter phase II trial sponsored by Eli Lilly using LY2382770, a humanized neutralizing monoclonal antibody against TGF-β1 for the treatment of diabetic nephropathy had to be prematurely terminated due to futility in efficacy (clinicaltrials.gov: NCT01113801). Fresolimumab (GC-1008, Genzyme) is a human monoclonal antibody that neutralizes all three isoforms (TGF-β1, 2, and 3). A single-dose infusion was shown to be relatively safe and well tolerated in a phase I multicenter, open-label study conducted in patients
with treatment-resistant primary FSGS. A subsequent phase II multicenter, double-blind, randomized study of fresolimumab in patients with steroid-resistant primary FSGS was recently completed but no study results have been reported to date (clinicaltrials.gov: NCT01665391).

Pirfenidone is a small-molecular weight synthetic compound that has attracted much attention as an orally active anti-fibrotic agent recently approved by the U.S. Food and Drug Administration for the treatment of idiopathic pulmonary fibrosis (35). Pirfenidone effects are, in part, mediated via inhibition of TGF-β1 synthesis (78). In the kidney, the effects of pirfenidone in suppressing renal fibrosis have been shown in several experimental models including diabetic nephropathy, UUO, and subtotal nephrectomy (9, 74, 82). An open-label, single-center pilot study demonstrated that pirfenidone significantly slowed renal function decline rate in patients with FSGS, with a median improvement of 25% in patients who have moderate to severe CKD and are already being treated with angiotensin antagonists (11). However, the phase II trial failed to show an improvement in proteinuria (clinicaltrials.gov: NCT00001959). Limitations of the trial included the small-scale, exploratory nature of the study and the lack of control group. A phase I/II randomized, placebo-controlled trial conducted in 77 patients with overt diabetic nephropathy who had albuminuria and reduced estimated glomerular filtration rate (eGFR) (20 to 75 ml/min per 1.73 m²) demonstrated encouraging results of pirfenidone in improving kidney function, but without improvement in proteinuria (clinicaltrials.gov: NCT00063583). The mean eGFR increased in the pirfenidone (1200 mg/d) group (+3.3 ± 8.5 ml/min per 1.73 m²), whereas, the mean eGFR decreased in the placebo control group (-2.2 ± 4.8 ml/min per 1.73 m²) (79).

The results from these clinical trials portend some promise of pirfenidone as an anti-fibrotic therapy to improve or slow the decline in kidney function associated with CKD. It should be noted, however, that in the above studies, the assessment of kidney function was by eGFR
using the Modification of Diet in Renal Disease (MDRD) equation, and not by direct measurement of GFR. In addition, no serial kidney biopsies were performed to confirm histologic evidence of regression of renal fibrosis. Optimal dosage also may need to be further identified as well.

Hence, anti-TGF-β therapy has not yet translated into successful treatment in CKD patients, and unfortunately, many of the clinical trials that have been completed to date have been small and/or uncontrolled, and conclusive evidence regarding efficacy is limited and disappointing. Below, we describe studies that provide evidence for pro-fibrotic effects as well as evidence that supports protective effects of TGF-β1.

**Pro-fibrotic Effects of TGF-β1**

Renal fibrosis is characterized by excessive production and accumulation of ECM proteins in the kidney that leads to parenchymal scarring, renal dysfunction, and ultimately end-stage kidney disease. TGF-β1 is the most extensively studied prototype member of the TGF-β superfamily in the context of fibrosis. TGF-β1 is well known as a central mediator of renal fibrosis and has long been considered to play potent roles in the progression of CKD (7, 17, 48). Increased expression of TGF-β1 mRNA and protein is seen in patients with fibrotic kidney diseases, including IgA nephropathy, focal and segmental glomerulonephritis, lupus nephritis, and diabetic and human immunodeficiency virus–associated nephropathy (5). Moreover, urinary TGF-β1 excretion is significantly elevated in patients with glomerular disease and heavy proteinuria (24).

The overexpression of TGF-β1 in transgenic mice with increased levels of circulating active TGF-β1 induces progressive renal disease characterized by mesangial expansion,
accumulation of ECM proteins, and development of glomerulosclerosis and tubulointerstitial fibrosis (44). The accumulation of ECM proteins is stimulated by TGF-β1 acting as both an inducer of ECM synthesis and an inhibitor of ECM degradation by reduced synthesis of matrix metalloproteinases, and increased synthesis of proteinase inhibitors, such as plasminogen activator inhibitor-1 (PAI-1).

It has been shown that TGF-β1 activation is required for its biological actions and important in the development of renal fibrosis. Active TGF-β1 is released when the latent complex anchored to ECM is cleaved through proteolysis, following exposure to factors such as integrins (2). Integrin αvβ6 is a heterodimeric matrix receptor expressed in renal epithelia which binds and activates latent TGF-β1. Studies using an in vivo model of progressive renal fibrosis induced by unilateral ureteral obstruction (UOO) in β6 integrin-null mice have demonstrated that blockade of αvβ6-mediated TGF-β1 activation was associated with lower expression of type I and type III collagen, PAI-1 and collagen content, and protected against tubulointerstitial fibrosis (56). Thus, these studies provide evidence that local activation of TGF-β1 is important in the development of renal fibrosis.

A significant body of evidence exists in the literature that various approaches that disrupt TGF-β1 signaling protect against renal fibrosis. Inhibition of TGF-β1 by using the proteoglycan decorin which is a known inhibitor of TGF-β1 or anti-TGF-β antibodies abrogated development of renal fibrosis (32, 62). BMP-7 is a TGF-β superfamily member that has been shown to play a protective role in renal fibrosis through anti-inflammatory, anti-oxidative, and anti-fibrotic effects by counteracting the effects of TGF-β1 (100). BMP-7 signaling pathway and its role in fibrosis have been the subject of several recent reviews (6, 48, 71). In murine mesangial cells, BMP-7 decreases Smad3 accumulation and inhibits the transcriptional upregulation of Smad3
targets such as plasminogen activator inhibitor-1 (PAI-1), thereby, suppresses the pro-fibrotic
effects of TGF-β1 (93). Administration of exogenous recombinant human BMP-7 reduced renal
fibrosis in experimental models of renal injury in mice, including UUO and streptozotocin
(STZ)-induced diabetic kidney disease (64, 84, 92). Thus, BMP-7 opposes TGF-β1/Smad3-
mediated renal fibrosis. Studies in Smad3-null mice revealed that targeted deletion of Smad3
signaling resulted in reduced ECM protein accumulation and attenuation of tubulointerstitial
fibrosis following UUO injury (31, 73, 77). They also showed that the numbers of
myofibroblasts, macrophages, and CD4/CD8 T cells, and monocyte influx into the kidney after
UUO were significantly decreased in the obstructed kidneys of Smad3-null mice (31, 77). These
findings suggest that TGF-β1/Smad3 signaling mediates development of renal fibrosis and
inflammation.

TGF-β1 signaling via the non-Smad pathways also participates in mediating pro-fibrotic
responses. Data from in vitro studies indicate that TGF-β1-stimulated collagen expression in
mesangial cells is mediated via TAK1/MKK3/p38 signaling cascade, and fibronectin expression
in fibroblasts (13, 38, 69). Moreover, investigations using pharmacologic and genetic blockade in
in vivo models of glomerular and tubulointerstitial injury in mice have shown that the
TAK1/MKK3/p38 and JNK pathways promote renal fibrosis (13, 39). Both the p38 and JNK are
downstream targets of TAK1 activation. Conditional Tak1 gene deletion in mice suppressed
interstitial myofibroblast accumulation, collagen deposition, and expression of pro-fibrotic
molecules following UUO injury (55). These studies strongly suggest that the non-Smad
signaling pathway via TAK1 plays a critical role in ECM production and the pathogenesis of
kidney fibrosis.
Protective Effects of TGF-β1

Given the abundance of evidence from the preclinical studies, targeting TGF-β signaling pathways seems logical in order to attenuate the development of renal fibrosis and progression of CKD. However, contrary to highly anticipated results, data from clinical trials to date have not been as strong as was hoped, and anti-TGF-β1 treatment directed at pharmacological blockade of TGF-β1 has not yet translated into successful therapy for humans. Recently emerging evidence suggests that it may be due, at least in part, to the fact that TGF-β1 is capable of inducing not only pro-fibrotic effects, but also protective effects (Table 1).

Numerous in vitro studies have shown that TGF-β1 acts as a potent stimulator of ECM production, including the synthesis of type I collagen and fibronectin. However, a recent report by Neelisetty and colleagues demonstrated surprising findings that blocking TGF-β signaling through deletion of TβRII in matrix-producing renal interstitial cells failed to suppress renal fibrosis in mouse models kidney injury induced by UUO or aristolochic acid (67). The studies showed that TβRII deleted renal interstitial cells had significantly reduced type I collagen production, but the overall renal fibrosis following kidney injury was not decreased in the conditional knockout mice (67). These findings indicate that inhibiting TGF-β signaling in matrix-producing renal interstitial cells is not sufficient to protect against development of fibrosis after kidney injury.

Interestingly, mice overexpressing latent (inactive) form of TGF-β1 in keratinocytes demonstrated protection against renal fibrosis in experimental obstructive kidney disease and crescentic glomerulonephritis (28, 29). These mice had increased levels of latent, but not active, TGF-β1 in plasma and kidney tissue, and upregulation of renal Smad7. The observed protective effects in the latent TGF-β1 transgenic mice may be, at least in part, due to Smad7-mediated
inhibition of nuclear factor-κB (NF-κB) as well as inhibiting TGF-β1 activation, consequently
protecting the kidney against inflammation and fibrosis.

Taken together, these studies suggest that TGF-β1 is capable of exerting protective
effects by opposing the pro-fibrotic pathway via negative feedback effects of Smad2 and Smad7.
The maintenance of balance through this negative self-regulation of pro-fibrotic effects may be
important for tissue homeostasis. Under pathological conditions, it is possible that the balance
tips towards the pro-fibrotic pathway through dysregulation of the inhibitory mechanisms to
cause renal fibrosis, rather than solely through excess TGF-β1, and that this may perhaps account
for the disappointing results of targeting TGF-β in humans. Furthermore, there is emerging
evidence for the protective effects of TGF-β by mechanisms that include inhibiting inflammation
and induction of autophagy, which will be discussed in the following sections.

TGF-β Signaling and Renal Inflammation

Renal inflammation is implicated as a critical event in the initiation and progression of
renal fibrosis in CKD. Inflammatory response to renal injury involves infiltrating immune cells
as well as activated resident renal cells, which stimulate the production and release of pro-
fibrotic cytokines and growth factors and drive the pro-fibrotic process. Interestingly, despite the
established pro-inflammatory role, TGF-β1 also possesses anti-inflammatory effects (49). Its
anti-inflammatory properties are evidenced by findings that targeted deletion of TGF-β1 gene
results in profound multifocal inflammatory disease in mice (48, 83). Tgf-β1-null mice develop a
rapid wasting syndrome and early death by 3–4 weeks of age, and display excessive
inflammatory responses with massive infiltration of lymphocytes and macrophages in many
organs (45). These data indicate that TGF-β1 is a potent anti-inflammatory molecule.
It has been proposed that the reason why therapies directed against TGF-β1 in general have yielded disappointing results is because blockade of TGF-β1 signaling not only abrogates its pro-fibrotic effects, but also inhibits its anti-inflammatory effects, and thereby can promote renal fibrosis. Indeed, in vivo studies in mice have confirmed that conditional deletion of TβRII from the kidney tubular epithelial cells enhanced NF-κB signaling and renal inflammation, with upregulation of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the kidney following UUO injury (59). Similarly, disruption of TβRII in cultured renal tubular epithelial cells and fibroblasts resulted in impairment of the anti-inflammatory effect of TGF-β1 (59). Furthermore, conditional Smad4 knockout mice in which Smad4 was specifically deleted from the kidney tubular epithelial cells displayed significantly enhanced renal inflammation as evidenced by increased infiltration of CD45+ leukocytes and F4/80+ macrophages, and upregulation of pro-inflammatory cytokines IL-1β, TNF-α, monocyte chemoattractant protein-1 (MCP-1), as well as intercellular adhesion molecule-1 (ICAM-1) in the UUO kidney (60). Thus, inhibition of TGF-β signaling through either disruption of TβRII or Smad4 resulted in enhanced renal inflammation, suggesting that TGF-β possesses anti-inflammatory effects in the kidney.

Further evidence indicates that loss of Smad4 represses Smad7 transcription that leads to decreased IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) expression but enhanced NF-κB activation in the tubulointerstitium after UUO, thereby promoting renal inflammation (60). Moreover, Smad7 knockout mice display enhanced renal inflammation with increased NF-κB/p65 phosphorylation and increased macrophage infiltration in models of UUO and STZ-induced diabetes (8, 14). In contrast, Smad7 gene transfer into the kidney of STZ-induced diabetic rats using an ultrasound microbubble-mediated technique
significantly attenuated NF-kB/p65-driven renal inflammation and macrophage infiltration in diabetic rats (8). Latent TGF-β1 overexpression in mice also protected against renal inflammation, via up-regulation of renal Smad7 and IκBα and suppression of NF-κB activation in models of UUO and crescentic glomerulonephritis (29, 94). Thus, Smad7-mediated inhibition of NF-κB activation via the induction of IκBα may be a central mechanism to abrogate renal inflammation (Figure 2).

Recent investigations have also explored the role of TGF-β signaling and macrophage differentiation in inflammation. Kidney injury stimulates the recruitment and induction of macrophages to undergo classical activation and differentiate into pro-inflammatory M1 and anti-inflammatory M2 macrophages (1, 47). More recently, M2 macrophages have been further subdivided into three subsets namely M2a, M2b and M2c macrophages. Regulatory M2c macrophages are induced by TGF-β or IL-10 and recent evidence reported that this particular subset displayed protection against renal fibrosis (54, 65). While M2c phenotype is induced by TGF-β and generally considered to be anti-inflammatory, M2c macrophages in turn produce TGF-β, and it has been proposed that this might be a mechanism by which macrophages drive the development and progression of fibrosis (54). However, a recent study using conditional deletion of TGF-β1 in macrophages suggest that macrophage-derived TGF-β may not serve as a functionally important source of TGF-β for renal fibrosis (30).

**TGF-β Signaling and Autophagy**

Recent evidence proposes a new mechanism by which TGF-β1 can provide cytoprotective effects via induction of a highly conserved cellular process known as macroautophagy, hereafter referred to as autophagy. It has long been known that autophagy is an
intracellular process that occurs in all eukaryotes and results in the degradation of sequestered cytoplasmic elements in lysosomes (12). The execution of autophagy begins with the establishment of protein complexes that function in the formation of the phagophore, also known as the isolation membrane. The next major step, elongation of the autophagosome includes two ubiquitin-like protein complexes, namely autophagy-related gene (ATG)5 – ATG12 and microtubule-associated protein 1 light chain 3 (LC3) conjugation systems. These two major steps are sequentially followed by autophagosome-lysosomal fusion, lysosomal degradation and release of free amino acids and fatty acids (12, 85, 86).

Accumulating data implicate critical roles of autophagy in health and kidney disease (17). TGF-β1 stimulation induced the accumulation of autophagosomes and conversion of LC3 to the lipidated form, LC3-II and upregulated autophagy-related genes, ATG5, ATG7, LC3 and Beclin 1 in renal tubular epithelial cells (19, 42, 97). We also recently reported that TGF-β1 induced autophagy in glomerular mesangial cells (18, 40). Hence, these studies provide strong evidence that TGF-β1 acts as an inducer of autophagy.

Important advances have also been made in our understanding of the mechanisms of autophagy activation by TGF-β1 signaling pathways (18, 40, 88). In primary mesangial cells, we further showed evidence that TGF-β1 induces autophagy through the TAK1-MKK3-p38 signaling pathway, and autophagy promotes intracellular degradation of collagen and aggregated, insoluble procollagen (40). Moreover, TGF-β1 protects against serum deprivation-induced mesangial cell apoptosis through the induction of autophagy via TAK1 and AKT activation (18). The role of AKT-mTOR signaling pathway has also been demonstrated to mediate the activation of autophagy in vivo following UUO injury in rats (41).
Autophagy is increasingly recognized as an important cellular defense against various stress stimuli, and dysregulated autophagy is implicated in diseases characterized by progressive kidney fibrosis (17). Upregulation of autophagy expression was noted in human kidney biopsies of patients with acquired proteinuric diseases such as focal segmental glomerulosclerosis (FSGS) (27). We and others have demonstrated induction of autophagy following UUO-induced kidney injury (19, 40, 41). Using GFP-LC3 transgenic mice, we confirmed that autophagy is induced in renal tubular epithelial cells of obstructed kidneys after UUO (19). Autophagy deficiency led to enhanced kidney fibrosis following UUO injury. For instance, mice deficient in autophagic protein Beclin 1 by heterozygous deletion of Beclin 1 displayed increased collagen deposition in the kidney (40). Both Lc3b-null mice and Beclin 1-heterozygous mice displayed enhanced collagen deposition in the obstructed kidneys after UUO (19). Treatment with autophagy inhibitor 3-Methyladenine (3-MA) also enhanced renal tubular cell apoptosis and tubulointerstitial fibrosis in the obstructed kidneys after UUO in rats (41).

Podocytes exhibit a high basal level of autophagy, important in cell survival, given that they are terminally differentiated and have a very limited capacity for cell division and replacement (27). Podocyte-specific deletion of the Atg5 gene resulted in increased susceptibility to injury with more severe albuminuria, foot process effacement, loss of podocytes, and glomerulosclerosis, following exposure to inducers of proteinuric glomerular injury, such as puromycin aminonucleoside, adriamycin, or LPS (27). Induction of autophagy has also been shown to be protective against cyclosporine-induced renal tubular cell death (70). Collectively, these findings provide evidence that TGF-β1-induced autophagy provides cytoprotective effects that promote renal cell survival and negatively regulate and limit ECM accumulation in the kidney, and thereby protect against kidney fibrosis (Figure 3).
**Negative Feedback Regulation of TGF-β Signaling**

The diverse roles of TGF-β1 necessitate that its signaling is tightly regulated at various levels. TGF-β1 induces Smad7, which is an inhibitory Smad that acts in a negative feedback loop to negatively regulate TGF-β1 (22). Overexpression of Smad7 has been shown to oppose TGF-β1-mediated renal fibrosis following UUO or STZ-induced diabetes, whereas deficiency of Smad7 aggravated the severity of the renal fibrosis (8, 14, 46). Smad7-null mice display enhanced TGF-β/Smad3 signaling and worsened progressive renal fibrosis in a model of angiotensin II-mediated hypertensive nephropathy (51), whereas treatment with Smad7 prevented angiotensin II-induced hypertensive nephropathy (52). Disruption of Smad7 also exacerbated chronic aristolochic acid nephropathy (AAN) in mice, a progressive CKD related to herb medicine (15). Furthermore, overexpression of Smad7 in the kidneys with established chronic AAN attenuated progression of chronic AAN through inactivation of TGF-β/Smad3 signaling (15).

Similarly, studies utilizing other approaches to inhibit TGF-β signaling, downstream of the cell-surface expressed TGF-β receptors, have also shown failure to protect against kidney fibrosis. Findings in mice with targeted conditional deletion of Smad2 in the kidney show that blockade of Smad2 signaling pathway not only failed to protect against the development of fibrosis after UUO injury but actually enhanced renal fibrosis (58). Moreover, knockdown of Smad2 in cultured renal tubular epithelial cells and fibroblasts resulted in enhanced ECM production in response to TGF-β1 stimulation, with increased expression of type I and type III collagen, and TIMP-1, and decreased expression of MMP-2, a major matrix-degrading enzyme (58). Smad2 deletion was associated with increases in Smad3 phosphorylation, nuclear
translocation, and Smad3 binding to collagen (COL1A2) promoter, and autoinduction of TGF-β1 (58). These studies indicate that inhibition of Smad2 promotes renal fibrosis via enhanced Smad3 signaling. On the other hand, overexpression of Smad2 resulted in attenuation of TGF-β1-induced Smad3 phosphorylation and type I collagen production in renal tubular epithelial cells (58). Taken together, these findings suggest that Smad2 functions to protect against development of renal fibrosis through countering the pro-fibrotic function of Smad3 signaling.

Our recent investigations have uncovered a novel mechanism that autophagy regulates TGF-β1 expression and suppresses kidney fibrosis induced by UUO. Increased levels of mature TGF-β1 were seen in the obstructed kidneys of LC3 deficient mice upon UUO injury (19). Similarly, mature TGF-β1 levels were increased in LC3 deficient renal tubular epithelial cells, and in cells treated with autophagy inhibitor bafilomycin A1, without alterations in TGF-β1 mRNA (19). These findings suggest a novel feedback mechanism to limit TGF-β signaling through autophagic degradation and suppress kidney fibrosis.

A number of endogenous modulators of TGF-β signaling has been described, such as secreted Klotho, which may serve as a promising therapeutic target against renal fibrosis. Klotho is a transmembrane protein expressed in renal tubular epithelial cells, and its extracellular domain is secreted by ectodomain shedding (4). Secreted Klotho inhibited TGF-β1-induced EMT in cultured cells, and administration of secreted Klotho protein to mice suppressed renal fibrosis induced by UUO (20). Interestingly, it has been suggested that the Klotho-mediated renoprotective effects may be, at least in part, due to upregulated autophagy flux (4). Secreted Klotho protein directly binds to TβRII, thereby inhibiting TGF-β1 binding to cell surface receptors, and inhibit TGF-β1 signaling (20). In contrast, high plasma levels of Lipoprotein(a) [Lp(a)], a low-density lipoprotein (LDL)-like particle, can inhibit the activation of latent TGF-β1
by competing with the binding of plasminogen to cell or matrix surfaces (26, 43). The proteoglycan decorin is another natural antagonist of TGF-β1 that binds active TGF-β1 and can neutralize the biological effects of TGF-β1 (32). Retinoic acid, particularly all-trans retinoic acid (ATRA), has also been shown to suppress renal expression of TGF-β1 and TβRII, leading to decreased ECM accumulation and inhibit progression of renal fibrosis (53, 63, 101). Other studies have indicated that ATRA exacerbes kidney injury with increased glomerular ECM, mesangial cell activation, glomerular macrophage influx, and immune complex deposition in a model of membranoproliferative glomerulonephritis in mice (34, 101). Thus, the therapeutic effects of retinoic acid in fibrotic kidney disease remain somewhat controversial, suggesting that caution is needed in applying retinoid therapy to human disease. No published clinical studies using soluble Klotho protein or ATRA administration in patients with CKD have been reported to date, despite the preclinical data supporting their therapeutic potential. Recently, a phase I/II trial has been initiated to evaluate the safety and efficacy of isotretinoin (13-cis retinoic acid) treatment in patients with biopsy-proven FSGS, minimal change disease, or collapsing glomerulopathy (clinicaltrials.gov: NCT00098020). The results of this clinical study will be of great interest.

Concluding Remarks

Renal fibrosis represents the common pathway in kidney injury of most progressive CKD leading to renal failure and end-stage kidney disease. Unfortunately, there are no effective therapies that prevent progressive renal fibrosis. Current treatment for patients with CKD primarily relies on inhibition of the renin-angiotensin-aldosterone system (RAAS) by angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs)
(61). However, the magnitude of the renoprotective effects of RAAS blockade is estimated to be only about a 20% risk reduction of progression (21).

TGF-β1 remains an attractive target for treating fibrotic kidney diseases, to counter its potent pro-fibrotic effects. However, as this review highlights its opposing protective effects, indiscriminate complete blockade of TGF-β functions is not sufficient to reduce fibrosis, and may actually aggravate the disease in some pathological settings. Perhaps, there are also interspecies differences suggesting that results derived from preclinical studies in mice and in vitro may not necessarily translate to humans. TGF-β1 actions depend on multiple factors including the involved cell type and context, the dose, isoform, and the distinct Smad and non-Smad signaling pathways. Therefore, therapeutic targeting of TGF-β1 requires more optimal strategies such as those selectively directed at specific signaling pathway(s), and future investigations to better understand the precise molecular mechanisms of TGF-β1 signaling are warranted.

Acknowledgements

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Figure 1. Smad and non-Smad TGF-β1 signaling pathways. (A) TGF-β1 activation occurs with the release from latent TGF-β binding protein (LTBP) complex by proteases. TGF-β1 signaling is initiated upon binding of active TGF-β1 with TβRII and forming TβRI-TβRII heteromeric complex, leading to phosphorylation of Smad2/3, oligomerization with Smad4, and subsequent nuclear translocation to regulate the transcription of ECM genes. Smad7 serves as a negative regulator of TGF-β1 signaling. TGF-β1 also activates Smad-independent signaling such as the MAPK, mediated by Ras-Raf-MEK-ERK, pathway, and TGF-β-activated kinase 1 (TAK1) mediated by TAK1-TAK1-binding protein 1 (TAB1) pathway. TGF-β1-induced TAK1 activation leads to signaling via M KK4-JNK and M KK3-p38 pathways and activation of transcription factors activator protein-1 (AP-1) and activating transcription factor 2 (ATF-2), respectively, and activation of NF-κB to mediate pro-fibrotic responses.

Figure 2. An overview of Smad signaling and inflammatory pathways of TGF-β1 signaling. Binding of Smad4 to phosphorylated Smad2/3 leads to the nuclear translocation of Smad complex to regulate gene transcription including Smad7. Smad7 induces IκBα expression which inhibits phosphorylation of p65/p50 proteins of NFκB signaling complex to prevent NFκB-driven renal inflammation.

Figure 3. An overview of pro-fibrotic and protective pathways of TGF-β1 signaling. Activation of TGF-β1 in response to kidney injury can signal both pro-fibrotic and protective effects via Smad and non-Smad signaling pathways. Overexpression of Smad2 attenuates TGF-
β1-induced Smad3 phosphorylation and type I collagen (Col-1α1) expression, whereas inhibition of Smad2 promotes renal fibrosis via enhanced TGF-β1/Smad3 signaling. TGF-β1-induced autophagy negatively regulates TGF-β1-stimulated collagen accumulation in the kidney by promoting collagen degradation. Autophagy also negatively regulates mature TGF-β1 expression, thereby protects against kidney fibrosis.
**Table 1.** Protective roles of TGF-β signaling in chronic kidney diseases.

<table>
<thead>
<tr>
<th>Strategy and Results</th>
<th>Disease Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditional deletion of TβRII in renal interstitial cells significantly reduces collagen production, but does not ameliorate overall renal fibrosis</td>
<td>UUO, Aristolochic acid-induced nephropathy</td>
<td>(67)</td>
</tr>
<tr>
<td>Conditional deletion of Smad2 in renal tubular epithelial cells enhances renal fibrosis</td>
<td>UUO</td>
<td>(58)</td>
</tr>
<tr>
<td>Overexpression of latent TGF-β1 in keratinocytes reduces renal fibrosis</td>
<td>UUO</td>
<td>(28)</td>
</tr>
<tr>
<td>Overexpression of latent TGF-β1 in keratinocytes protects against glomerular crescentic formation and severe tubulointerstitial damage</td>
<td>Crescentic glomerulonephritis</td>
<td>(29)</td>
</tr>
<tr>
<td>Overexpression of Smad7 suppresses renal fibrosis, whereas deficiency of Smad7 aggravates the severity of renal fibrosis</td>
<td>Diabetic nephropathy</td>
<td>(8)</td>
</tr>
<tr>
<td>Smad7 overexpression in kidneys suppresses renal fibrosis and disruption of Smad7 enhances renal fibrosis</td>
<td>UUO</td>
<td>(14, 46)</td>
</tr>
<tr>
<td>Smad 7 deficiency aggravates angiotensin II-mediated renal fibrosis, whereas Smad7 treatment prevents progressive renal injury</td>
<td>Hypertensive nephropathy</td>
<td>(51, 52)</td>
</tr>
<tr>
<td>Smad7 disruption exacerbates nephropathy, whereas Smad7 overexpression in kidneys attenuates nephropathy</td>
<td>Aristolochic acid-induced nephropathy</td>
<td>(15)</td>
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<td>Conditional deletion of TβRII in renal tubular epithelial cells and renal fibroblasts enhances NF-κB signaling and renal inflammation</td>
<td>UUO</td>
<td>(59)</td>
</tr>
<tr>
<td>Conditional deletion of Smad4 in renal tubular epithelial cells enhances renal inflammation</td>
<td>UUO</td>
<td>(60)</td>
</tr>
<tr>
<td>Treatment with autophagy inhibitor 3-Methyladenine enhances renal tubular cell apoptosis and tubulointerstitial fibrosis</td>
<td>UUO</td>
<td>(41)</td>
</tr>
<tr>
<td>Heterozygous deletion of Beclin 1 and LC3b-null mice enhances collagen deposition</td>
<td>UUO</td>
<td>(19, 40)</td>
</tr>
<tr>
<td>Conditional deletion of Atg5 in podocytes increases susceptibility to renal injury</td>
<td>Puromycin, Adriamycin, Lipopolysaccharide</td>
<td>(27)</td>
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Table 2. Recent clinical trials using anti-TGF-β therapy in fibrotic kidney diseases.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Company</th>
<th>Phase</th>
<th>Disease</th>
<th>References or NCT Identifier</th>
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<tbody>
<tr>
<td>LY2382770</td>
<td>TGF-β1</td>
<td>Eli Lilly</td>
<td>Phase II</td>
<td>Diabetic nephropathy</td>
<td>NCT01113801</td>
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<tr>
<td>Fresolimumab (GC1008)</td>
<td>TGF-β1,2,3</td>
<td>Genzyme</td>
<td>Phase I</td>
<td>Focal Segmental Glomerulosclerosis</td>
<td>(89)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Phase II</td>
<td></td>
<td>NCT01665391</td>
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<tr>
<td>Pirfenidone</td>
<td>TGF-β1,2,3</td>
<td>InterMune</td>
<td>Phase II</td>
<td>Focal Segmental Glomerulosclerosis,</td>
<td>NCT00001959 (11)</td>
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<td></td>
<td></td>
<td></td>
<td>Phase I/II</td>
<td>Diabetic nephropathy</td>
<td>NCT00063583 (79)</td>
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</table>
Figure 2
Figure 3