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Review

Central role of dysregulation of TGF- β /Smad in CKD progression and potential targets of its treatment

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ABSTRACT

Chronic kidney disease (CKD) has emerged as a major cause of morbidity and mortality worldwide. Interstitial fibrosis, glomerulosclerosis and inflammation play the central role in the pathogenesis and progression of CKD to end stage renal disease (ESRD). Transforming growth factor- β 1 (TGF- β 1) is the central mediator of renal fibrosis and numerous studies have focused on inhibition of TGF- β 1 and its downstream targets for treatment of kidney disease. However, blockade of TGF- β 1 has not been effective in the treatment of CKD patients. This may be, in part due to anti-inflammatory effect of TGF- β 1. The Smad signaling system plays a central role in regulation of TGF- β 1 and TGF- β /Smad pathway plays a key role in progressive renal injury and inflammation. This review provides an overview of the role of TGF- β /Smad signaling pathway in the pathogenesis of renal fibrosis and inflammation and an effective target of anti-fibrotic therapies. Under pathological conditions, Smad2 and Smad3 expression are upregulated, while Smad7 is downregulated. In addition to TGF- β 1, other pathogenic mediators such as angiotensin II and lipopolysaccharide activate Smad signaling through both TGF- β -dependent and independent pathways. Smads also interact with other pathways including nuclear factor kappa B (NF- κ B) to regulate renal inflammation and fibrosis. In the context of renal fibrosis and inflammation, Smad3 exerts pro-fibrotic effect, whereas Smad2 and Smad7 play renal protective roles. Smad4 performs its dual functions by transcriptionally promoting Smad3-dependent renal fibrosis but simultaneously suppressing NF- κ B-mediated renal inflammation via Smad7-dependent mechanism. Furthermore, TGF- β 1 induces Smad3 expression to regulate microRNAs and Smad ubiquitination regulatory factor (Smurf) to exert its pro-fibrotic effect. In conclusion, TGF- β /Smad signaling is an important pathway that mediates renal fibrosis and inflammation. Thus, an effective anti-fibrotic therapy via inhibition of Smad3 and upregulation of Smad7 signaling constitutes an attractive approach for treatment of CKD.

1. Introduction

Renal fibrosis constitutes a common endpoint of various progressive kidney diseases which leads to the loss of nephrons and impairment of

renal function ultimately resulting in end stage renal diseases (ESRD) [1]. Fibrogenesis involves tubulo-interstitial tissues leading to tubulo-interstitial fibrosis and glomeruli leading to glomerulosclerosis [2]. Extensive studies have demonstrated that fibrogenesis can be induced

Abbreviations: ACE2, angiotensin-converting enzyme-2; AGE, advanced glycation end-product; AT1R, angiotensin type 1 receptor; BMP, bone morphogenetic proteins; BMP-7, bone morphogenetic protein 7; CKD, chronic kidney diseases; Co-Smads, common mediator Smads; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; ERK, extracellular regulated protein kinases; ESRD, end stage renal diseases; FSGS, focal and segmental glomerulosclerosis; HIPK2, Homeodomain interacting protein kinase 2; hRPTEC, human renal proximal tubule epithelial cells; ICAM-1, intercellular cell adhesion molecule-1; IgA, immunoglobulin A; interleukin-1 β (IL-1 β) I-Smads, inhibitory Smads; Keap1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinase; monocyte chemoattractant protein-1 (MCP-1) NEDD4-2, neural precursor cell expressed developmentally downregulated gene 4; Nrf2, nuclear factor-erythroid-2-related factor 2; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3 kinase; R-Smads, receptor-regulated Smads; Ski, Sloan-Kettering institute; Smurf1, Smad ubiquitination regulatory factor-1; Smurf2, Smad ubiquitination regulatory factor-2; SnoN, Ski-related novel protein N; TGF- β , transforming growth factor- β ; TGF- β 1, transforming growth factor- β 1; TGF β R, transforming growth factor- β receptor; TGF β RI, transforming growth factor- β receptor type I; TGF β RII, transforming growth factor- β receptor type II; TNF- α , tumor necrosis factor α ; UUO, unilateral ureteral obstruction; WWP1, WW domain containing E3 ubiquitin protein ligase 1; α -SMA, alpha-smooth muscle actin

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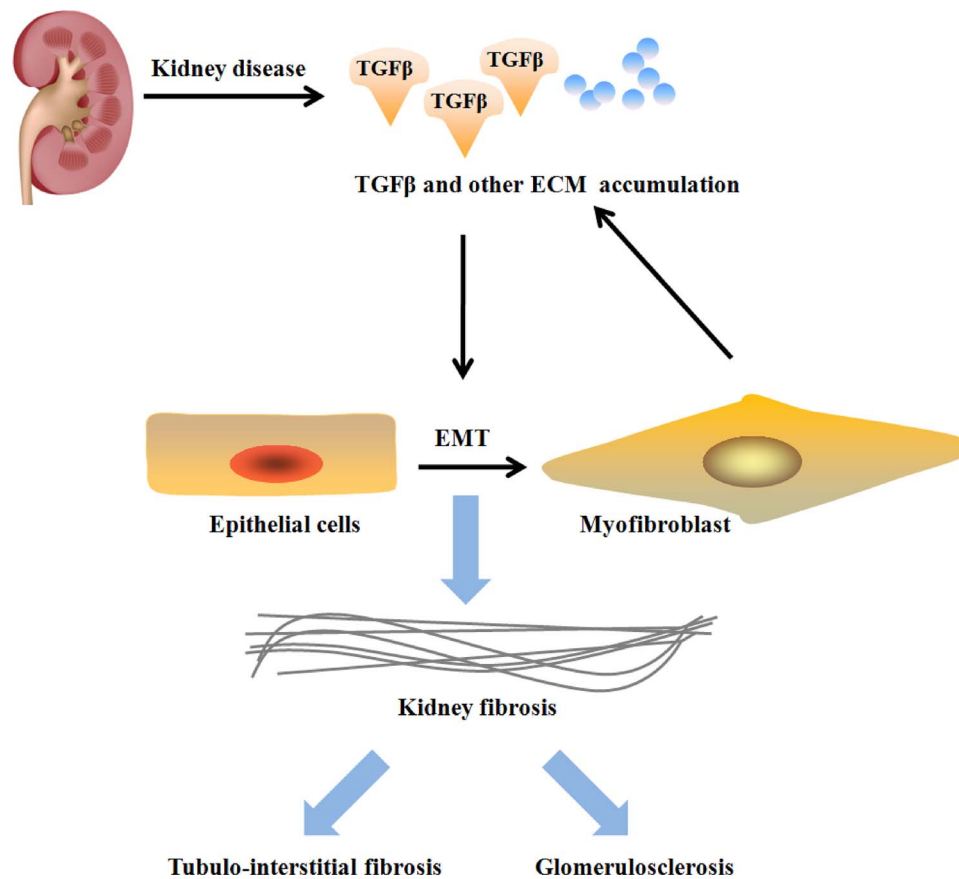


Fig. 1. Hypothesis of progression of fibrosis by EMT in the interstitium or in the glomerulus.

by multiple stimuli or mediators including growth factors, cytokines, toxins and lipid disorders as well as stress molecules *via* multiple mechanisms and signaling pathways [3–13]. Fibrosis is primarily driven by inflammatory cytokines including members of the transforming growth factor- β (TGF- β) superfamily [14,15], various interleukins [16] and oxidative stress [17–20]. Among them, transforming growth factor- β 1 (TGF- β 1) has served as an important and crucial mediator in the pathogenesis of progressive renal fibrosis. TGF- β 1 has been demonstrated to transform tubular epithelial cells into extracellular matrix (ECM) producing fibroblasts or myofibroblasts and to induce epithelial-to-mesenchymal transition (EMT) (Fig. 1). TGF- β is a multi-functional mediator that regulates proliferation, differentiation, apoptosis, adhesion and migration in various cells such as macrophages, activated T and B cells, immature haematopoietic cells, neutrophils and dendritic cells [21].

The TGF- β superfamily is characterized by six conserved cysteine residues. It is encoded by forty-two open reading frames in humans and more than thirty related members in mammals including activins, inhibins, growth factors, differentiation factors and bone morphogenetic proteins (BMP) and anti-müllerian hormone [22,23]. Although the different TGF- β ligands induce very different cellular activities, they share a set of common sequence and structural features [24]. The three mammalian isoforms including TGF- β 1 and its isoforms (TGF- β 2 and TGF- β 3) share 70–82% amino acid homology. Despite their structural similarities, the three TGF- β isoforms induce distinct biological responses. Based on their cell/tissue-specific expression they interact with specific inhibitory molecules and unique combinations of receptors [25]. The active form of TGF- β is a dimer stabilized by hydrophobic interactions. TGF- β evokes intracellular signaling by binding to receptor complexes that contain two distantly related transmembrane serine/threonine kinases called transforming growth factor- β receptor type I (TGF β RI) and transforming growth factor- β receptor type II

(TGF β RII) [21,23]. TGF β RII is a constitutively active kinase, whereas TGF β RI kinase needs to be activated by TGF β RII kinase [21,26]. TGF- β directly binds to TGF β RII in most cell types. The TGF β RII bound TGF- β is then recognized by TGF β RI, which is recruited into the complex and becomes phosphorylated by TGF β RII [14]. The intracellular mediators of TGF- β signaling are known as Smad-dependent and Smad-independent signaling pathways. Three classes of Smads including receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads) and inhibitory Smads (I-Smads) have been identified in biological system. The R-Smads including Smad1–Smad3, Smad5 and Smad8 are directly activated *via* phosphorylation by TGF β RI forming a heterooligomeric complex with the common mediator Smad4. The Smad complex translocate into the nucleus where it is recruited into DNA by specific DNA-binding transcription factors and modulates target gene transcription [23,27,28]. Smad2 and Smad3 are activated by the TGF- β subfamily and Smad1, Smad5 and Smad8 respond to signaling by BMP subfamily [27,29]. I-Smads including Smad6 and Smad7 antagonize the R-Smads' activity by interacting with TGF β RI to prevent the docking and phosphorylation of R-Smads and diverting them for degradation *via* the ubiquitin proteasome degradation mechanisms [23,27] (Fig. 2). In addition to Smad-mediated transcription, TGF- β could activate other signal transduction pathways including mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K) and Rho-like GTPases pathways [14,30]. Since the Smad-dependent signaling pathway plays a critical role in pathogenesis of various forms of chronic kidney disease (CKD) [14], TGF- β 1 has emerged as an attractive target of novel therapeutic interventions.

This review focuses on the molecular mechanisms of TGF- β /Smads-mediated renal fibrosis and inflammation and its role in the progressive kidney injury. The new therapies against renal fibrosis by targeting the downstream Smad3 and Smad7 as well as TGF- β /Smad3-mediated microRNAs are also summarized and discussed.

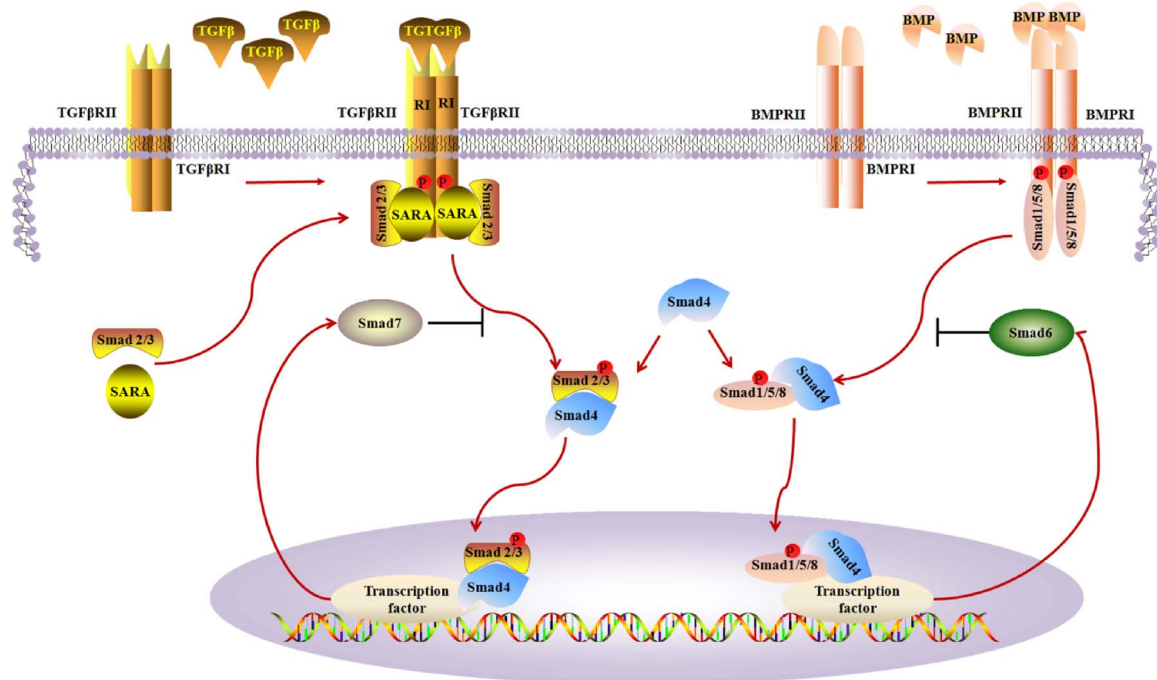


Fig. 2. Crosstalk between TGF-β/Smad and BMP/Smad pathways in kidney fibrosis.

2. Role of TGF-β1/Smad signaling pathway in renal fibrosis and inflammation

2.1. Activation of TGF-β1 and its role in renal fibrosis and inflammation

TGF-β1 is essential for normal development, tissue repair and maintenance for organ functions. TGF-β1 is known as an anti-inflammatory cytokine [14]. It exerts anti-inflammatory effects *via* inhibition of mitogenesis and cytokine responses in glomerular cells and suppression of the infiltrating cells [14]. TGF-β1 knockout mice show multi-organ inflammation and TGF-β1 deficient mice exhibit lethal inflammation and die within three weeks after birth [31]. Similarly, conditional deletion of TGF-β1 or TGFβRII genes from T cells has been shown to cause autoimmune diseases [32,33]. In contrast, mice over-expressing latent TGF-β1 were protected against progressive inflammation and renal fibrosis in obstructive nephropathy and immunologically-induced glomerulonephritis [33–35]. Although TGF-β-induced inhibition of NF-κB-mediated renal inflammation *via* induction of Smad7-dependent I-kappa B alpha (IκBα) expression has been recently demonstrated [33,35], the signaling mechanisms of its anti-inflammatory action remain unclear. Yet, over-expression of TGF-β1 is closely associated with pathological disorders in various kidney diseases [15,23].

There is extensive evidence pointing to upregulation of TGF-β1 and its role in the pathogenesis of renal fibrosis in both animal models and humans with kidney diseases [14,36]. TGF-β1 mediates progressive renal fibrosis by stimulating production and suppressing degradation of ECM. In addition, TGF-β1 induces renal fibrosis by mediating the transformation of tubular epithelial cells to myofibroblasts *via* EMT [35]. The central role of TGF-β1 on EMT and renal fibrosis has been confirmed by experiments which demonstrated the ability of TGF-β1 blockade with decorin, neutralizing TGF-β antibody or anti-sense oligonucleotides to attenuate renal fibrosis [14]. Direct evidence for the causal role of TGF-β1 in renal fibrosis is confirmed in mice over-expressing an active TGF-β1 form [37]. TGF-β has been shown to serve a critical mediator in the pathogenesis of glomerulosclerosis in patients with glomerular diseases, such as immunoglobulin A (IgA) nephropathy, focal and segmental glomerulosclerosis (FSGS), lupus nephritis,

membranous nephropathy, diabetic nephropathy and crescentic glomerulonephritis. Significant upregulation of the three TGF-β isoforms as well as TGFβRI and TGFβRII has been demonstrated in the glomeruli and tubulo-interstitium in kidney diseases [24]. Furthermore, urinary TGF-β1 level is increased and directly correlates with the severity of tubulo-interstitial fibrosis and mesangial matrix abundance in patients with renal diseases. Compared with healthy controls and patients with glomerular disease without proteinuria, urinary TGF-β1 level is increased in patients with proteinuria due to glomerular dysfunction. Urinary TGF-β1 level has been shown to decrease by mitigating proteinuria with immunosuppressive treatment [38]. It is well known that upregulation of TGF-β1 causes excessive ECM productions, decreases ECM-degrading proteinase activity and upregulates proteinase inhibitor, events that lead to excessive ECM deposition. In progressive podocyte-associated glomerular diseases, excessive TGF-β1 expression in the podocytes has been demonstrated indicating the role of TGF-β1 in podocyte injury in patients with IgA nephropathy, FSGS and diabetic nephropathy [39]. Upregulation of TGF-β1 has been demonstrated in experimental animals and patients with diabetic nephropathy. Tubular and glomerular TGF-β expression is increased in early and late stages of diabetic nephropathy and inversely correlates with glycemic control in diabetic patients [40]. TGF-β1 expression is stimulated by glomerular stretch and hyperglycemia in early stage, and by angiotensin II, platelet-derived growth factor (PDGF) and advanced glycation end-product (AGE) in later stages of the disease [40]. Angiotensin II has been demonstrated to raise expression of TGF-β1 and its receptors [41,42]. Unlike CKD, the role of TGF-β1 in acute kidney injury is not completely understood [43]. Earlier studies have shown that TGF-β triggers various pathophysiological processes including epithelial cell apoptosis, cell dedifferentiation and ECM deposition in early stages of acute renal injury, events which contribute to acute deterioration of renal function and progressive renal fibrosis [44,45]. The contribution of TGF-β to severity of acute kidney injury is supported by experiments which demonstrated attenuation of proximal tubule injury in mice with selective deletion of TGFβRII in the proximal tubules [43].

2.2. TGF- β 1 signaling and its role in myofibroblasts transdifferentiation

Extensive studies demonstrated that myofibroblasts are a predominant source for ECM production and myofibroblasts accumulation is a key step in the progressive renal fibrosis [46]. The myofibroblast originate from a wide variety cell types including the resident fibroblasts, fibrocytes, pericytes and epithelial cells [47–49]. A study by Nikolic-Paterson et al revealed that bone marrow-derived macrophages could become myofibroblast phenotype through macrophage-myofibroblast transition in animals with unilateral ureteral obstruction (UUO) and in patients with CKD [50]. It is well recognized that TGF- β mediates transformation of local fibroblasts into myofibroblast [51]. For instance, TGF- β 1 has been shown to promote renal fibrosis by driving the differentiation of quiescent fibroblasts into matrix secreting myofibroblasts and their differentiation into proto-myofibroblast lineage and fully differentiated myofibroblasts [52]. In addition, TGF- β 1 directly stimulates production of collagen by fibrocytes [53]. TGF- β has been shown to result in trans-differentiation of endothelial cells and epithelial cells to myofibroblast-like cells which were inhibited by TGF- β /Smad signaling blockade [54–56]. Moreover, TGF- β 1 promotes renal fibrosis through the cell-cell interaction as TGF- β 1 released from the injured epithelium can activate pericyte-myofibroblast transition [57]. In addition, AGE and angiotensin II can activate Smad3 to mediate the hypertension- and diabetes-induced EMT [41,58,59].

2.3. Role of R-Smads (Smad2 and Smad3) in renal fibrosis and EMT

Extensive studies have identified Smad2 and Smad3 as two major downstream mediators of the biological actions of TGF- β 1. In the context of renal fibrosis, Smad2 and Smad3 are activated in both humans and experimental animals with CKD of diverse etiologies including hypertensive nephropathy [41,60,61], remnant kidney disease [54,62], obstructive kidney disease [63], diabetic nephropathy [59,64,65], chronic renal allograft injury [66] and drug-associated nephropathy [67]. Many fibrogenic genes including plasminogen activator inhibitor-1, proteoglycans, integrins, connective tissue growth factor (CTGF), tissue inhibitor of metalloproteinase-1 as well as collagens such as collagen type 1 α 1 (Col1 α 1), collagen type 1 α 2 (Col1 α 2), collagen type 5 α 2 (Col5 α 2), collagen type 6 α 1 (Col6 α 1) and collagen type 6 α 3 (Col6 α 3) have been shown to be the downstream targets of TGF- β /Smad3 signaling [68]. These observations demonstrate the central role of Smad3 in the TGF- β /Smad signaling-mediated renal fibrosis. An essential role for Smad3 in renal fibrosis is confirmed by the findings that fibrogenesis is markedly reduced with Smad3 deletion in mice with diabetic nephropathy [69] and drug toxicity-related nephropathy [67]. In addition, inhibition of TGF- β 1-mediated phosphorylation and nuclear translocation of Smad3 by heat shock protein-72, has been shown to ameliorate tubulo-interstitial fibrosis in UUO rats [70]. A recent study demonstrated that N-Myc downstream-regulated gene-2 knockdown promoted renal fibrosis through TGF- β 1/Smad3 pathway in TGF- β 1-stimulated HK-2 cells [71]. Furthermore, the demonstration of efficacy of the Smad3 inhibitor to attenuate EMT and renal fibrosis has identified the Smad3 signaling as a novel therapeutic target for treatment of diabetic nephropathy [72]. Although the effect of TGF- β 1/Smad3 signaling as promoters of renal fibrosis is well defined, the effect of Smad2 on kidney disease remains uncertain. This is partly due to the lack of availability of Smad2 knockout mice owing to its embryonic lethality [73]. However, transgenic mice with conditional Smad2 deletion in the renal tubular epithelial cells have been developed by crossing the Smad2 floxed mouse with kidney specific promoter (Cadherin 16)-driven Cre transgenic mouse [74]. The *in vivo* and *in vitro* studies have shown accelerated Smad3-dependent renal fibrosis in mice with Smad2-null tubular epithelial cells. This was mediated by upregulations of phosphorylation and nuclear translocation of Smad3 and its binding to the Col1 α 2 promoter leading to excessive ECM production [75]. Although it is well

accepted that Smad2 and Smad3 bind together and act in embryonic development, Smad2 may suppress Smad3 phosphorylation in response to TGF- β 1. Another possibility is that physical interaction of Smad2 and Smad3 with Smad4 may affect phosphorylation, nuclear translocation and subsequent binding of Smad3 to its target genes. Therefore, the combination of Smad2 deficiency, upregulation of Smad3 signaling, and other fibrotic mediators including angiotensin II and AGE work in concert to promote ECM accumulation and fibrosis of the renal tissue [41,54,59,67].

EMT has been well recognized as an important and key process in renal fibrosis. Extensive investigations have illuminated that Smad3 plays a crucial role in EMT process in kidney [41,76–78]. To identify the role of Smad2 and Smad3 on EMT, Smad2 or Smad3 were conditionally knocked down in the renal tubular epithelial cells. Interestingly, disruption of Smad3, but not Smad2, upregulated E-cadherin expression and downregulated α -smooth muscle actin (α -SMA) in the angiotensin II-induced EMT process [54]. Similarly, knockdown of Smad3, but not Smad2, mitigated AGE- and angiotensin II-induced CTGF protein expression and renal fibrosis [58,59]. Recent study demonstrated that angiotensin II promoted TGF- β 1/Smad3-mediated renal fibrosis and NF- κ B-driven renal inflammation in the hypertensive nephropathy [41]. As we all know, many fibrogenic genes (collagens) and EMT markers (α -SMA and E-cadherin) are Smad3-dependent and Smad3, but not Smad2, directly binds to their DNA sequences to modulate expression of these target gene. Therefore, knockdown of Smad3, but not upregulation of Smad2, suppressed CTGF expression and EMT and provided a novel evidence for the essential role of Smad3 in the EMT process.

Podocyte loss is an important feature of FSGS, membranous nephropathy, minimal change disease, collapsing glomerulopathy and diabetic nephropathy [79–82]. Podocytes are paracrine-regulated commander cells that maintain the structure of the glomerular tuft [79–82]. It has been demonstrated that inhibition of TGF- β 1 expression, Smad2/3 phosphorylation and Smad7 over-expression lower immune complex deposition, restore podocyte injury, and prevent tubulo-interstitial fibrosis in kidney [83]. Additionally, bone morphogenetic protein 7 (BMP-7) has a protective effect in podocyte differentiation via Smad signaling [84].

2.4. Role of co-Smad4 in regulation of renal fibrosis and inflammation

Smad4 simultaneously promotes Smad3-mediated renal fibrosis and attenuates inflammation via Smad7-dependent inhibition of NF- κ B. Smad4 is a critical regulator for shuttling R-Smads and BMP-Smads into the nucleus and modulating TGF- β -induced Col1 α 1 expression [36]. However, Smad4 may be more important in regulating the Smad3 activity to initiate transcription of its target genes' expression instead of the nuclear shuttling [36]. During TGF- β signaling, activation of Smad4 is driven by ligand-mediated R-Smads phosphorylation, mainly at MH2 domains of Smad2 and Smad3 in their SSXS motif by TGF β RI [85,86]. TGF- β 1 induces renal fibrosis by phosphorylation of Smad2 and Smad3, which form a complex with Smad4 that translocates to nuclei to bind and regulate the target gene expression [87]. It has been reported that AMP-activated protein kinase activator (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside) could inhibit upregulation of Co-Smad4 and reduce the excessive ECM accumulation in diabetic nephropathy [88]. Although Smad4 is the common Smad in the TGF- β family's signal transduction pathway, its functional role in TGF- β 1-mediated renal fibrosis and inflammation remains unclear. This is partly attributed to the lethality of Smad4 knockout mice [89]. Conditional Smad4 knockout mouse was established by crossing the Smad4 floxed mouse to the renal specific promoter-driven Cre transgenic mouse. Smad4 was deleted from tubular epithelial cells upon Cre recombination [90]. The findings showed that Smad4 disruption in kidney enhanced kidney inflammatory response as evidenced by a greater CD45⁺ leukocyte and F4/80⁺ macrophage infiltration and up-regulation of tumor necrosis

factor α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), interleukin-1 β (IL-1 β) and intercellular cell adhesion molecule-1 (ICAM-1) in IL-1 β -induced macrophages and in ureteral obstructed kidney [90]. Interestingly, the mechanism by which Smad4 deletion mitigated renal fibrosis was not related to the inhibition Smad2/3 activation because Smad4 disruption did not alter Smad2 and Smad3 phosphorylation levels or nuclear translocation of p-Smad2 and p-Smad3. However, Smad4 deletion affected Smad3-induced promoter activity and the binding of Smad3 to the COL1 α 2 promoter [90]. Several studies have demonstrated that Smad3 binding sites are located in the promoter regions of COL1 α 2, Col2 α 1, collagen type 3 α 1 (Col3 α 1), collagen type 5 α 1 (COL5 α 1), COL6 α 1, COL6 α 3 and tissue inhibitor of metalloproteinase-1 [91–93]. Thus, disruption of Smad4 can limit Smad3 binding to the collagen promoter, thereby suppressing the fibrotic process.

Further studies have indicated that Smad4 deletion restrains Smad7-mediated inhibition of NF- κ B signaling [90]. Smad7 transcription is regulated by TGF- β 1 via direct binding of Smad3 and Smad4 to Smad7 promoter. Hence, Smad4 disruption leads to the loss of transcriptional Smad7 expression and inhibits Smad7 promoter activity [90]. Because Smad7 could induce expression of I κ B α which inhibits NF- κ B [94], Smad4 disruption reduces renal Smad7, thereby accelerates NF- κ B-mediated renal inflammation and TGF- β 1 inhibition in IL-1 β -activated macrophages [90]. Taken together, Smad4 is a key modulator of the TGF- β 1-mediated fibrosis and inflammation by interplaying with Smad3 and Smad7 to affect their transcriptional activity in renal inflammation and fibrosis. Taken together, the various biological roles of Smad4 in renal inflammation and fibrosis indicated that Smad4 could not be a therapeutic target for kidney diseases.

2.5. Regulation of I-Smad7 in renal fibrosis and inflammation

Smad7 serves as negative feedback regulator of TGF- β 1/Smad pathway thereby protects against TGF- β 1-mediated fibrosis via receptor degradation that halts recruitment and phosphorylation of Smad2 and Smad3 [95]. In anti-Thy1 rodent models of acute kidney injury, chronic allograft kidney rejection, remnant kidney and obstructive kidney disease, Smad7 expression is downregulated which by enhancing TGF- β 1 signaling lead to progressive renal fibrosis [41,96–98]. In CKD, both angiotensin II and TGF- β 1 activate Smurfs and arkadia-dependent ubiquitin-proteasome signaling pathways that, in turn, degrade Smad7 protein through a post-transcriptional mechanism [41,54,99]. Smad ubiquitination regulatory factor-1 (Smurf1), Smad ubiquitination regulatory factor-2 (Smurf2) and arkadia are E3 ubiquitin ligases for Smad7 [99] and have been demonstrated to physically interact with Smad7 [100]. Smad7 acts as an adaptor protein to recruit E3 ubiquitin ligases including Smurf2 and arkadia to the TGF- β receptor complex to accelerate its degradation via proteasomal-ubiquitin degradation pathway [99,100]. The recent study showed that renal fibrosis in angiotensin-converting enzyme-2 (ACE2) knockout mice was related to increased Smurf2 and marked activation of TGF- β /Smad3 and NF- κ B signaling, indicating that ACE2 loss promotes angiotensin II-induced TGF- β /Smad3 and NF- κ B-mediated hypertensive nephropathy [41]. Once Smad7 was decomposed, Smad2/3 activation and renal fibrosis was enhanced. Upregulated Smurf2 resulted in a ubiquitin-dependent Smad7 degradation in kidney, caused upregulation of TGF- β /Smad signaling and enhanced renal fibrosis [101]. Furthermore, Smurf2 interacts with Smad2 and specifically targets Smad7 and Smad transcription co-repressors such as Sloan-kettering institute (Ski), Ski-related novel protein N (SnoN) and TG-interacting factor for degradation in tubular epithelial cells, causing renal fibrosis and EMT in the mouse models of diabetic nephropathy and obstructive kidney disease [102–104]. Thus, ubiquitin-mediated degradations of Smad7 and Smad transcription co-repressors such as SnoN, Ski and TG-interacting factor promote further activation of TGF- β 1 signaling and renal fibrosis as shown in many animal models of CKD [54,103,105]. This conclusion is further supported by the findings that Smad7 knockout mice exhibit

more severe renal fibrosis in both diabetic and obstructive nephropathies [64,105].

Renal Smad7 loss not only promoted TGF- β 1/Smad3-induced progressive renal fibrosis, but also enhanced renal inflammation by activating NF- κ B-mediated inflammatory pathway [64,105,106]. NF- κ B activation (nuclear translocation of NF- κ B, p65) which mediates renal inflammation is observed in obstructive nephropathy, diabetic nephropathy and glomerulonephritis [64,94,105,106]. Smad7 over-expression inhibited nuclear translocation, DNA binding and transcriptional activities of NF- κ B p65 and NF- κ B-mediated inflammation induced by TNF- α and IL-1 β [94], indicating a functional link between the Smad7 and NF- κ B. Smad7 has been shown to increase expression of the NF- κ B inhibitor, I κ B α , indicating that TGF- β 1 may inhibit NF- κ B activity via Smad7-mediated upregulation of I κ B α expression [94].

The potential role of Smad7-NF- κ B crosstalk in renal inflammation was confirmed by the ability of Smad7 over-expression to inhibit NF- κ B activation and inflammation in hypertensive nephropathy [41,61], obstructive nephropathy [98], remnant kidney disease [107] and diabetic nephropathy [64,69]. Compared with the wild-type mice Smad7 knockout mice with UUO and diabetic nephropathy exhibit severe inflammation, nuclear NF- κ B p65 translocation, upregulation of TNF- α , IL-1 β , ICAM-1, MCP-1 and macrophage infiltration in the renal tissue pointing to the anti-inflammatory effect of Smad7 within the kidney [64,90]. Furthermore, Smad7 over-expression in mesangial cells, tubular epithelial cells and vascular smooth muscle cells has been shown to inhibit TGF- β /Smad signaling and reduce ECM deposition in response to TGF- β 1, AGE, angiotensin II and high glucose levels [54,58,108]. Therefore, Smad7 mitigates renal inflammation via I κ B α -induced inhibition of NF- κ B signaling.

The therapeutic effect of Smad7 on renal inflammation and fibrosis has been investigated in the rat models of hypertensive nephropathy [41,61], obstructive nephropathy [98], remnant kidney disease [107] and diabetic nephropathy [64,69]. Smad7 was transferred into the kidney using the ultrasound-microbubble-induced gene therapy approach. Smad7 over-expression not only blocked Smad3-mediated renal fibrosis, but also inhibited NF- κ B-mediated inflammation as evidenced by significant reduction of tubulo-interstitial and glomerular macrophage and T cell infiltration and IL-1, ICAM-1, TNF- α and inducible nitric oxide synthase (iNOS) levels. Therefore, restoration of the renal Smad7 retarded progression of renal disease by attenuating dysregulation of the TGF- β /Smad and NF- κ B pathways (Fig. 3). Given the documented efficacy of Smad7 in suppressing renal fibrosis and inflammation, it has emerged as a potential therapeutic target for treatment of kidney disease [109].

2.6. TGF- β 1-dependent and independent Smad signaling in renal fibrosis and inflammation

Considerable evidence has emerged indicating that TGF- β 1 is not the sole mediator of the Smad signaling activation in CKD [14,30]. In fact, many other mediators can activate Smad2 and Smad3 in the TGF- β 1-independent manner. This is because Smads interact with other signaling pathways such as MAPK which participate in the pathogenesis and progression of CKD [14,30]. For instance, in presence of hypertension, angiotensin II can increase ECM production by activating Smad signaling through the angiotensin type 1 receptor (AT1R) and extracellular regulated protein kinase (ERK)/p38MAPK-Smad pathway [41,110]. In fact, downregulation of intrarenal angiotensin II and AT1R can attenuate fibrosis and inflammation by inhibiting activation of TGF- β -Smad and NF- κ B [110]. The impact of MAPK-Smad pathway on renal fibrosis has been explored by stimulation of angiotensin II and AGE to activate Smad2/3 mediated CTGF expression in renal cells in the absence of TGF- β 1 gene or TGF β RII [41]. Similarly, under the diabetic conditions, AGE could activate Smad2 and Smad3 by a TGF- β 1-independent pathway through ERK/p38MAPK-dependent mechanism [59,111]. This finding was further supported by the results of the study

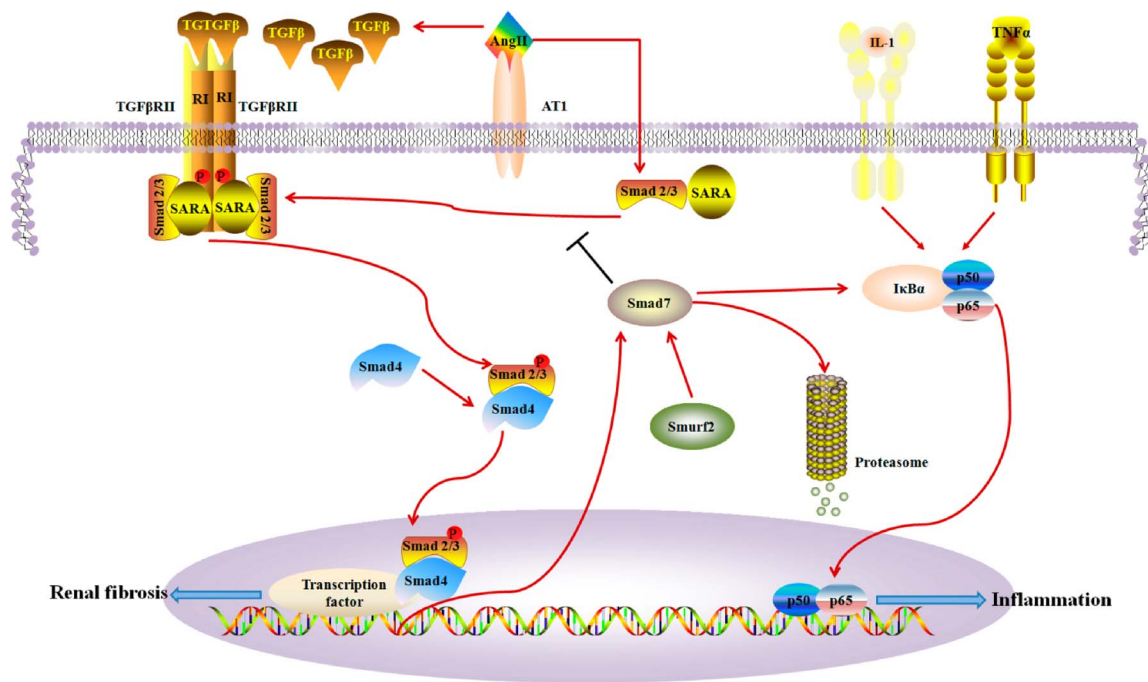


Fig. 3. TGF- β /Smad and crosstalk pathways in renal fibrosis and inflammation.

which showed that TGF- β 1 or TGF β RII deletion could not prevent AGE-induced Smad2 and Smad3 activation and fibrosis development [59]. On the contrary, blockade of the AGE receptor could prevent AGE-induced p-Smad2/3 expression and nuclear translocation [59]. Taken together, these findings demonstrate that Smad activation is complex and targeting the TGF- β 1 signaling per se is not an effective therapy due to the existing intracellular signaling pathways.

TGF- β /Smad also interacts with the BMP/Smad to counteract each other in order to maintain the balance between two pathways during pathological process. As we know, Smad2 and Smad3 induce TGF- β 1 activity, whereas Smad1, Smad5 and Smad8 cause BMP activation and the interaction of two pathways can occur at multiple levels including individual Smads and receptors [112]. Upregulation of BMP by Smad1/5/8 activation can inhibit TGF- β 1 mediated fibrotic gene expression [112]. TGF- β -mediated activation of Smad2/3 promotes fibrosis, whereas increased BMP/Smad1/5/8 activity inhibits fibrosis. The study showed that TGF- β -activates Smad2/3 to cause EMT which was reversed by addition of human recombinant BMP-7 via the Smad1-dependent pathway [113].

2.7. Role of TGF- β /Smad-dependent microRNAs in renal fibrosis

A number of studies have demonstrated that TGF- β 1 modulates several microRNAs (miR) to promote renal fibrosis. TGF- β 1 has been shown to upregulate miR-21 and miR-192, -377, -382 and -491-5, but downregulates miR-29, -200 and -378 causing renal fibrogenesis [114–116]. The miR-21 level is markedly elevated in fibrotic kidneys [117,118], and its inhibition has been shown to attenuate ECM and retard the progressive renal fibrosis [119,120]. In contrast, the miR-29 and miR-200 which are anti-fibrotic are suppressed in fibrotic renal tissues [121]. It is noteworthy that more than 20 ECM genes some of which are modulated by the TGF- β 1 signaling are potential targets of miR-29 [122]. The miR-29 over-expression has been shown to mitigate renal fibrogenesis in obstructive nephropathy and diabetic nephropathy and suppress the fibrotic genes driven by high glucose, TGF- β 1 or salt-induced hypertension [121,123–125].

Gene therapy using microRNA targeting Smad signaling has shown therapeutic potential in a variety of kidney diseases. TGF- β 1 can up-regulate expression of miR-21, miR-93, miR-192, miR-216a, miR-377,

miR-382 and miR491-5p, and down-regulate expression of miR-29 and miR-200 [36]. Among these, miR-21, miR-29 and miR-192 expression are Smad3-dependent and could effectively downregulate fibrotic protein expression suggesting that TGF- β /Smad3 might exert pro-fibrotic effect via miR-21, miR-29 and miR-192 *in vivo* [115,126–129]. The miR-21 and miR-192 can downregulate TGF- β -induced collagen expression *in vitro* [115,126–129], whereas miR-377 upregulate fibronectin expression [130]. In addition, miR491-5p promotes Par-3 degradation in tubular epithelial cells [131] and miR-382 downregulates E-cadherin expression through TGF- β 1 [132]. In the kidney tissue of patients with CKD, miR-214 was found in the tubular and glomerular and infiltrating immune cells mediating a Smad2/3-independent profibrotic effect. Treatment of mice with an anti-miRNA directed against miR-214 (anti-miR-214) prior to UUO has been shown to exert and antifibrotic effects [133]. Moreover, miRNA let-7 family members (let-7b/c/d/g/i) were identified to inhibit TGF- β 1-induced Smads, COL1 α 2 and COL4 α 1 expressions in diabetic nephropathy [134].

As described in the review article by Kantharidis et al [135], TGF- β regulates specific microRNAs to influence renal fibrosis in kidney diseases. Several studies have demonstrated that in renal fibrosis, expression of miR-21, miR-192 and miR-29 is regulated by TGF- β 1 through the Smad3, but not Smad2. UUO causes severe renal fibrosis which is associated with miR-29 depletion in Smad3 wild-type mice [136]. In contrast silencing of miR-21 either by gene knockout or by anti-miR administration significantly ameliorates fibrosis in UUO mice [136]. Renal fibrosis prevention in Smad3 knockout mice is mainly attributed to the increased expression of renal miR-29 [121]. *In vitro* studies showed that deletion of Smad3, not Smad2, halted miR-21 and miR-192 expressions, but promoted miR-29 family expression by TGF- β 1 stimulation in renal tubular epithelial cells and mouse embryonic fibroblasts [108,136,137]. Another study reported that miR-200a overexpression could inhibit Smad3 activity and ameliorate TGF- β 1-mediated renal fibrosis [138]. These results revealed the complex association of TGF- β /Smads and microRNA in the pathogenesis of renal fibrosis.

It should be noted that Smad7 regulates TGF- β 1/Smad-dependent microRNA expression. In fact, by promoting Smad3 signaling, Smad7 deletion enhances miR-192 expression, thereby, facilitates renal fibrosis in UUO. This assumption is supported by the findings that Smad7 over-

expression ablated TGF- β /Smad signaling and, thereby, suppressed miR-192 expression and alleviated renal fibrosis in 5/6 nephrectomized rats [139]. *In vitro* study revealed that Smad7 over-expression in tubular epithelial cells abrogated TGF- β 1-induced miR-192 expression [108]. *In vitro* findings further demonstrated that miR-21 and miR-192 over-expression enhanced ECM deposition, but miR-21 or miR-192 knockdown alleviated ECM deposition in TGF- β /Smad3-dependent pathway [108,137]. In contrast, miR-29 knockdown promoted renal fibrosis, while miR-29 over-expression inhibited collagen I expression in response to TGF- β 1 [121]. Taken together, these data revealed that specific targeting of the Smad3-dependent microRNA represents a novel and specific anti-fibrosis therapy for renal fibrosis.

2.8. Post-translational regulation of TGF- β /Smad signaling by ubiquitination

TGF- β /Smad signaling is tightly controlled by the components of ubiquitin-proteasome system and aberrations in specific ubiquitin modifying enzymes can cause renal fibrosis [140]. Ubiquitylation leads to the sequential actions of E1–E3 ubiquitin ligases that provide substrate specificity. The stability and levels of TGF β R complexes are determined by ubiquitylation [141]. Specifically, Smad7 recruits E3 ubiquitin ligases to TGF β RI, leading to TGF β RI ubiquitylation and degradation. Multiple E3 ligases were demonstrated to be closely associated with TGF β RI ubiquitylation, such as Smurf1, Smurf2, WW domain containing E3 ubiquitin protein ligase 1 (WWP1) and NEDD4-2 (Neural precursor cells express developmentally downregulated gene 4-2), which relate to the E2 ligase UbcH7 and an N-terminal sequence in Smad7 [140,141].

Smurf2 is an E3 ubiquitin ligase that plays a key role in regulation of TGF- β /Smad pathway by selectively targeting the key components of the Smad signaling for degradation. Smurf2 downregulates expression of Smad7 and the Smad transcriptional co-repressors including SnoN and Ski [142]. Upon TGF- β /Smad stimulation, the p-Smad2 interacts with smurf2. SnoN inhibits the TGF- β 1 target gene pre-transcriptionally and decreases the potency and duration of TGF- β 1/Smad signaling. SnoN deficiency plays an important role in the pathogenesis of diabetic nephropathy. Degradation of SnoN by ubiquitination is activated by its binding to Smurf. Smurf2 also promotes the TGF- β /Smad signaling functions by enhancing degradation of TGF β R and Smad2, leading to reduction of biological function of TGF- β 1 [141]. The TGF- β /Smad downregulate expression and heightens degradation of SnoN by smurf2 in diabetic nephropathy [143]. Similarly, SnoN protein level is reduced while its mRNA level is increased and protein and mRNA levels of Smurf2 are increased in the human renal proximal tubule epithelial cells (hRPTEC) exposed to high glucose concentrations. The knockdown of Smurf2 upregulates SnoN expression in hRPTEC. Treatment with MG132 has been shown to partially ameliorate high glucose-induced downregulation of SnoN and treatment with SB-431542 downregulates expression of p-Smad2 and Smurf2 in the hRPTEC [144]. The proteasome inhibitor MG132 has been shown to ameliorated renal fibrosis by inhibiting SnoN degradation and TGF- β activation in rats with diabetic nephropathy [145]. Another study demonstrated that treatment with MG132 attenuated downregulation of Smad7 protein expression and the upregulations of Smurf2 mRNA and TGF- β protein expressions in rats with streptozotocin-induced diabetic nephropathy [146]. Arkadia, a member of the RING finger ubiquitin ligase superfamily, promotes activation of the TGF- β 1 signaling. Once TGF- β 1 signaling is activated, Arkadia binds to p-Smad2/3 and induces degradation of the Smad7 and SnoN/Ski, enabling transcription of TGF- β 1 target genes [140]. Previous studies have demonstrated that TGF- β 1 expression is significantly increased, while SnoN expression is significantly decreased in rats with diabetic nephropathy and renal tubular epithelial cells exposed to high glucose concentration, events which enhance activation of TGF- β 1/Smad pathway and cause renal fibrosis [147,148]. Another study indicated that SnoN expression was significantly decreased, but Arkadia

and p-Smad2/3 expressions were significantly increased in NRK-52E cells exposed to high glucose concentrations [149]. Exposure to the high glucose levels induced histone H2A ubiquitination and reduced histone H2B ubiquitination in rat glomerular mesangial cells [150]. The changes of histone ubiquitination may be due, in part, to activation TGF- β signaling by diabetic nephropathy. In addition, PI3K-dependent ubiquitin C-terminal hydrolase-L5 is required for high glucose-mediated upregulation of TGF β RI protein in mouse mesangial cells. PI3K-dependent ubiquitin C-terminal hydrolase-L5 is also required for high glucose-induced TGF β RI protein deubiquitination [151].

Ubiquitin-dependent degradation results in the downregulation of Ski and SnoN proteins expression in the tubulointerstitial fibrosis [152]. Downregulation of SnoN expression in UO mice is mediated by enhanced ubiquitin-dependent degradation [153]. The Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor- erythroid-2-related factor 2 (Nrf2) antioxidant system inhibited TGF β 1-stimulated renal epithelial cell to fibroblast transition *via* the Smurf1-Smad7 signaling in the HK-2 cells, indicating the protective role of Nrf2 against renal fibrosis [154]. In autosomal recessive polycystic kidney disease, deletion of PKHD1 led to altered localization and function of the C2-WWW-HECT domain of E3 family of ligases. Vesicles contained the PKHD1/Pkhd1 gene product, FPC and NDFIP2, which interacted with multiple members of the C2-WWW-HECT domain E3 family of ligases [155]. Usp2-69 overexpression mitigated progression of the anti-Thy1.1 nephritis in rats [156]. Inhibition of ubiquitin carboxyl-terminal hydrolase 4 reduced TGF- β 1-induced TGF β RI expression and ameliorated the altered Smad7 and Smurf2 expression in mice undergoing peritoneal dialysis [157]. Taken together, these studies demonstrated that post-translational regulation of TGF- β 1/Smad signaling by ubiquitination is involved in the progression of renal fibrosis and provides a novel target for treatment of CKD and renal fibrosis.

3. Targeting TGF- β 1/Smad signaling as a therapeutic potential for renal fibrosis

3.1. Blockade of TGF- β 1 signaling

Given the central role of TGF- β 1 in fibrogenesis the effect TGF- β 1 blockade on control of renal fibrosis has been widely explored. Anti-sense TGF- β oligodeoxynucleotides, TGF- β neutralizing antibodies, soluble human TGF β RII and specific inhibitors of TGF β R kinases including IN-1130 and GW788388 have been shown to effectively inhibit the progressive renal fibrosis in different CKD models. Several TGF- β 1 inhibitors have been studied in pre-clinical and clinical trials [158]. The blockade of TGF- β 1 receptor post-translational core fucosylation has been shown to retard renal tubulo-interstitial fibrosis in rats with unilateral ureteral obstruction [159]. Treatment with Pirfenidone, a small molecule that blocks TGF- β 1 promoter, has been shown to attenuate the decline in estimated glomerular filtration rate in patients with diabetic nephropathy and FSGS [160,161]. Fresolimumab, a high-affinity neutralizing antibody that targets TGF- β isoforms has been shown to reverses markers of skin fibrosis in patients with systemic sclerosis [162]. In addition, the efficacy of TGF- β 1 neutralizers, Fresolimumab and LY2382770, for treatment of patients with FSGS and diabetic nephropathy has been explored [163]. Klotho is a transmembrane protein predominantly expressed on the basal membrane of the renal tubular epithelial cells. By directly binding TGF β RII it blocks the TGF- β -induced signaling, thereby inhibits renal fibrosis [164]. However, the major risk and barrier for its use as a potential therapeutic tool is that by blocking TGF- β signaling it may result in the loss of the body's anti-inflammatory and anti-tumorigenic capacities.

3.2. Selective inhibition of Smads signaling

Since blockade of TGF- β 1 signaling inhibits its anti-inflammatory effect, several studies have focused on inhibition of the downstream

targets of the TGF- β 1 signaling such as Smad2–Smad4, Smad7, and Smad-dependent microRNAs as potential strategies for prevention/treatment of renal fibrosis [165]. Selective inhibitor of Smad3 phosphorylation (SIS3) has been shown to ameliorate renal fibrosis in diabetic nephropathy. SIS3 also attenuated excessive ECM production by TGF- β 1-treated normal fibroblasts and scleroderma fibroblasts *in vitro* [166]. Increasing evidence uncovered that targeting Smad3 by over-expressing Smad7 in the kidney inhibits renal inflammation and fibrosis in different models of kidney disease [61,64,106,167]. TGF- β which is upregulated in CKD is normally inhibited by its natural endogenous antagonist BMP-7 [29]. The latest study has revealed that BMP-7 strengthens SnoN mRNA expression in HK-2 cells exposed to high-glucose concentrations [168]. Kindlin-2, an adaptor protein, recruits Smad3 to TGF β RI, thereby contributes to TGF- β /Smad3-induced tubulo-interstitial fibrosis [169]. Homeodomain interacting protein kinase 2 (HIPK2) is a critical regulator of multiple pro-fibrotic pathways, including TGF- β 1/Smad3. Genetic ablation of HIPK2 was shown to reduce renal fibrosis in UUO and Tg26 mice [170]. BT173 was synthesized as a small molecule inhibitor with a structure similar to HIPK2. Treatment with BT173 inhibited TGF- β 1-induced Smad3 phosphorylation and Smad3 target gene expression in HK-2 cells and downregulated Smad3 phosphorylation, renal fibrosis and ECM deposition in UUO and Tg26 mice [171]. BT173 is a novel HIPK2 inhibitor that mitigates renal fibrosis by inhibiting the TGF- β 1/Smad3 pathway. Another study revealed that phosphate niclosamide attenuates activation of Smads and NF- κ B pathways in rats with UUO and adriamycin-induced nephropathy by preventing the binding of Smad3 to HIPK2 gene promoter and consequent inhibition of HIPK2 expression [172]. Hence, phosphate niclosamide may be a potential therapeutic agent for renal fibrosis. Two Smad transcriptional co-repressors Ski and SnoN, exert their anti-fibrotic effects on TGF- β 1 by antagonizing Smad-induced gene transcription [104]. Moreover, some microRNAs including let-7b and miR-29 could suppress TGF- β 1 signaling and retard the progressive renal fibrosis [122,173,174]. Additionally, by attenuating expression of intrarenal angiotensin II and AT1R, a combination of telmisartan and pitavastatin could inhibit renal fibrosis and inflammation by suppressing activations of TGF- β 1-Smad and NF- κ B [110]. Fingolimod (FTY720), an analogue of sphingosine 1-phosphate, has been shown to attenuate collagen deposition, inflammation and tubulo-interstitial fibrosis in UUO mice [175]. Furthermore, FTY720 mitigated TGF- β -mediated α -SMA expression and collagen synthesis by inhibiting both Smad2/3 and PI3K/AKT/glycogen synthase kinase 3 beta pathways in NRK-49F cells [175]. Ki16425, a LPA receptor 1/3 antagonist, has been shown to block upregulation of TGF- β 1 expression and Smad2/3 phosphorylation in SV40 MES13 cells by lysophosphatidic acid stimulation and in db/db mice [176]. Likewise, JQ1, a selective bromodomain and extra-terminal protein inhibitor, has been shown to inhibit TGF- β /Smad-induced tubulo-interstitial fibrosis in UUO rats [63]. Finally, Nicousamide can protect podocyte by inhibiting the TGF β RII and AGE-RAGE signaling [177].

3.3. Specific inhibition of Smads signaling by natural products

A number of natural products have been widely used as anti-fibrotic agents. Poricoic acid ZG and poricoic acid ZH, isolated from the surface layer of *Poria cocos*, exhibited a strong inhibitory effect on renal fibrosis and podocyte injury. Poricoic acid G and poricoic acid H suppressed TGF- β /Smad pathway by selectively inhibiting the phosphorylation of Smad3 via blocking the interactions of SARA with TGF β RI and Smad3 [178]. Similarly, renal fibrosis in a variety of animal models were attenuated or suppressed through TGF- β /Smads and NF- κ B-mediated pathways by administration of compounds isolated from various natural products, such as GQ5 [179], arctigenin [98], curcumin [180,181], resveratrol [182], berberine [183,184], sinomenine [185], rutin [186], oxymatrine [149,187], bergenin [188], oleanolic acid [189], tan-shinone IIA [190], leonurine [191]; (+/-)-sinensilactam A [192],

epigallocatechin-3-gallate [193] and astragaloside IV [194,195].

4. Concluding remarks

Renal fibrosis represents the common pathway of progression of CKD to end-stage renal disease. Current treatment of CKD is primarily focused on inhibition of the renin-angiotensin system by angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers. However, the efficacy of renin-angiotensin system blockers in preventing progression of CKD is limited and is estimated to be about a 20%. TGF- β 1 remains an attractive target for treatment of renal fibrosis which is the driving mediator of CKD progression. However, complete blockade of TGF- β is not sufficient to mitigate renal disease and may deteriorate the disease by intensifying inflammation. The TGF- β 1 function depends on multiple components, including several distinct Smad and non-Smad signaling pathways which constitute potentially safer and more effective therapeutic targets for treatment of CKD.

Within the TGF- β /Smad signaling pathway, Smad3 plays a central role in renal inflammation and fibrosis via its downstream specific microRNAs. On the contrary, Smad2 mitigates renal fibrosis by competitively suppressing Smad3 phosphorylation and nuclear translocation. Smad4 can promote Smad3-mediated renal fibrosis but suppress NF- κ B-mediated inflammation by stimulating transcriptional expression of Smad7. Smad7 is a negative regulator of both renal inflammation and fibrosis. Smad7 inhibit TGF- β /Smad pathway by recruiting the Smurf2 or arkadia to the TGF- β receptor complex or Smads to accelerate their degradation via the proteasomal-ubiquitin degradation pathway. Additionally, Smad7 can induce I κ B α expression and prevent phosphorylation of NF- κ B subunit. However, Smad7 is downregulated and Smad2 and Smad3 are activated in kidney diseases, which intensify renal fibrosis and inflammation via TGF- β /Smad and NF- κ B activation. Therefore, strategies to improve the TGF- β /Smad signaling by inhibition of Smad3 and up-regulation Smad7 using specific Smad3-regulating miRNAs may provide an effective therapy to prevent renal fibrosis and CKD progression.

Conflict of interest

The authors declare that there is no conflict of interest.

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