# A Review on Novel Target of TGF-B/Smads and EPO in Renal Disease.

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**Abstract**
The purpose of this article to review the role transforming growth factor (TGF-β) signaling that can manifest chronic kidney disease (CKD). It is a vital public health complication in all countries, because of both the number of people affected and the high cost of care when anticipation strategies are not effectively implemented. Public health strategies to prevent diabetes, hypertension, and obesity as risk factors for CKD are important. In recent years, accumulating evidence has indicated that genetic factors, particularly transforming growth factor (TGF-β) signaling, play a key role in the development of CKD, several mutated genes have been identified from patients with CKD, many of these mutated genes belong to members of the TGF-β signaling family. However, the role of TGF-β signaling in CKD is controversial. Transforming growth factor-β (TGF-β) regulates a variety of cellular functions in different cell types. This concise review will begin with a synopsis of the colorful history of TGF-β signaling in CKD, then covers in more detail the contemporary understanding of Epo's physiology as well as its structure and interaction with its receptor. A major part of this article focuses on the regulation of the TGF-β signaling, SMAD protein & Epo gene, a transcription factor that plays a cardinal role in molecular adaptation to renal disorder. In the concluding section, a synopsis of Epo's role in disorders of kidney will be followed assessment of the remarkable impact of Epo therapy in the treatment of CKD, as well as concerns that provide a strong impetus for the development of even safer and more effective treatment. TGF-β has been shown to modulate cell growth, extracellular matrix production and cell death via apoptosis, and inflammation. Advance research during recent years have been identified as the SMAD family of proteins is the key components in intracellular signaling of the TGF-β family. in this review we find out the complication associated with the novel targets regulating the signaling pathway of the transforming growth factor (TGF-β) along with the involvement of the SMAD proteins.

**Keywords:** Chronic kidney disease, transforming growth factor (TGF-β), SMAD, Epo

## 1 Introduction

Complication in the renal function is a worldwide has been seen from the last 10-year decay, disease is defined as the health of an individual, which can occur abruptly, and either resolve or become chronic. CKD is a general term for heterogeneous disorders affecting kidney structure and function with variable clinical presentation, in part related to cause, severity and the rate of progression The concept of CKD evolved after the recognition of the contribution of disordered kidney structure and function on the health of individuals across a wide range of severity. Earlier stages of kidney disease are often asymptomatic, are detected during the evaluation of comorbid conditions, and may be reversible (levey et al 2003). Rapidly progressive diseases may lead to kidney failure within months but most diseases evolve over decades, and some patients do not progress during many years of follow-up. Transforming growth factor-β1 (TGF-β1) is considered as a crucial mediator in tissue fibrosis and causes tissue scarring largely by activating its downstream small mother against decapentaplegic (Smad) signaling. TGF-β1 directly activates Smad signaling which triggers pro-fibrotic gene overexpression. In previous studies it has been demonstrated that dysregulation of TGF-β1/Smad pathway was an important pathogenic mechanism in tissue fibrosis. Smad2 and Smad3 are the two major downstream regulator that promote TGF-β1-mediated tissue fibrosis, while Smad7 serves as a negative feedback regulator of TGF-β1/Smad pathway thereby protects against TGF-β1-mediated fibrosis. (Lan et al ., 2011). Erythropoietin (EPO) is a 30.4-kDa glycoprotein hormone produced primarily by the kidneys that regulates red blood cell production in response to anemia. In previous studies it has been reported that EPO ameliorate cisplatin-induced and ischemia-reperfusion renal injuries without affecting hemoglobin and inhibit the apoptosis of TGF-β. EPO treatment has also been found to reduce renal fibrosis in a murine model of unilateral ureteral obstruction (UUO) by inhibiting TGF-β1-induced EMT [15], suggesting that inhibition of EMT by EPO administration could be a potential therapeutic strategy to inhibit or slow CKD progressionz. The SMAD proteins can be divided into three major groups, i.e. (i) receptor-regulated Smads (Smad2, Smad3 in TGF-β/activin-pathway; Smadl, Smad5 and Smad8 in the BMP-pathway), (ii) inhibitory Smads (Smad6 and Smad7), and (iii) a common mediator Smad

**1.1 Causes of Acute kidney Diseases**

**1.1.1.** Nonsteroidal anti-inflammatory drugs (NSAIDs) have long been regarded as dangerous for use in patients with CKD because of their risk for nephrotoxicity and thus alternative classes of analgesics, including opioids, have become more commonly used for pain control in this population. NSAID use has been associated with acute kidney injury, progressive loss of glomerular filtration rate in CKD, electrolyte derangements, and hypervolemia with worsening of heart failure and hypertension. The risk for these nephrotoxicity syndromes is modified by many co morbid conditions, risk factors, and characteristics of use, and in patients with CKD, the risk differs between levels of glomerular filtration rate (baker & prazella et l., 2020).

**1.1.2. Vascular calcification**

Activation of the TGFBR1/TAK1 pathway, play a key role in the development of CKD, several mutated genes have been identified from patients with CKD, many of these mutated genes belong to members of the TGF-β signaling family. However, the role of TGF-β signaling in CKD is controversial. Chronic inflammation or progressive inflammation under high-phosphate condition leads to vascular calcification in chronic kidney disease (CKD) patient's resulting,. The evidence of calcification in CKD rats or osteogenic medium-cultured human aortic smooth muscle cells (HASMCs) has been reported previously (li et al., 2020).

**1.1.3. Renal Inflammation due to Cytokines**

Cytokines are the major neurotransmitter that cause the inflamtion but the role of TGF-β1 can be neglected in the inflamtion of the kidney, little attention has been paid to the role of TGF-β1 in renal inflammation studies shown that, conditional deletion of TβRII or TGF-β1 gene from T cells develops autoimmune diseases whereas mice overexpressing human latent TGF-β1 are protected against progressive renal inflammation and fibrosis in glomerulonephritis caused by the obstructive and immunologically. The involvement Smad7-dependent IκBα expression isthe other aspect of the renal inflamtion by inhibition of NF-κB-. However, signaling mechanisms by which TGF-β1 exerts its anti-inflammatory properties remain unclear.

**1.1.4. Mitogen-Activated Protein Kinase (MAPK) signaling pathway.**

Mitogen-Activated Protein Kinase (MAPK) signaling pathway and play a role in the pathophysiological processes of kidney diseases. Under the diabetic conditions, advanced glycation end-products (AGEs), an critical mediator in diabetic complications, are able to activate Smad2 and Smad3 independently from TGF-β1 via the ERK/p38 MAP kinase-dependent mechanism. This is supported by the findings that deletion of TGF-β1 or TGF-β receptor II is unable to prevent AGE-induced Smad2 and Smad3 from activation and fibrosis response. In contrast, blockade of the engagement of AGE to its receptor (RAGE) with the soluble RAGE or ERK/p38 MAP kinases with the specific inhibitors or dominant negative ERK1/2 or p38 is able to prevent AGE-induced Smad2/3 phosphorylation and nuclear translocation. Similarly, under the hypertensive conditions, Ang II can activate the Smad signaling pathway to stimulate ECM production via the AT1 receptor-mediated, ERK/p38 MAP kinase-Smad cross talk pathway, in addition to the TGF-β-dependent mechanism. The important role for the MAPK-Smad crosstalk pathway in renal fibrosis is further demonstrated by the ability of Ang II and AGE to activate Smad2/3 to stimulate connective tissue growth factor (CTGF) expression in kidney cells lacking TGF-β1 gene or TβRII, but not in those with blockade of MAPK signaling by overexpressing dominant negative ERK1/2 and p38 or pre-treating cells with their specific inhibitors. All these studies reveal that activation of Smads under disease conditions is complicated and that targeting the TGF-β signaling at the receptor levels may not be an optimal therapeutic approach due to the existing intracellular crosstalk pathways.

**1.1.5 Role of Erythropoietin (EPO) in ischemia-reperfusion injury**

Kidneys produce a hormone that regulates red blood cell production in response to anemia i.e Erythropoietin (EPO). it is a glycoprotein hormone. In previous studies it has been reported that the role of EPO in ischemia-reperfusion renal injuries ameliorated and inhibit the apoptosis of TGF-β by cisplatin- without affecting hemoglobin. EPO treatment has also been found to induced EMT and reduce renal fibrosis by inhibiting TGF-β1in a murine model of unilateral ureteral obstruction (UUO), this phenomenon of EPO signifying that inhibition of EMT by EPO administration could be a potential therapeutic strategy to inhibit or slow CKD progression.

**2.0 Synthesis of TGF-β**

TGF-β is a cytokine with diverse physiological functions which include regulation of proliferation, differentiation, apoptosis and immune function. There are three isomers of TGF-β known to date; these consist of TGF-β1, TGF-β2, and TGF-β3. They share a similar biologically active region and bind to the same type I and type II TGF-β receptor complex. In humans, TGF-β1 was found to be the most abundant isoform that is widely expressed by most cells (ong et al 2021).

**2.1. TGF-β targeting approaches:**
Anti-TGF-β-drugs have been difficult to create due to the pleiotropic nature of TGF-β activity and safety concerns. Patients treated with 10 and 80 μM dosages of trabedersen, an ASO that targets TGF-β2 mRNA, had a greater rate of side events linked to neurological problems in a clinical trial compared to normal chemotherapy. Only one patient undergoing standard chemotherapy experienced drug-related significant side events, compared to three patients receiving trabedersen treatment (Bogdahn et

**2.2. Role of TGF-β1 in kidney disease**
TGF-β1 reduce the degradation of the formed extracellular matrix (ECM), evidence shows that the pathogenesis of renal fibrosis highly controlled by stimulating extracellular matrix (ECM). The transformation of tubular epithelial cells (TECs) to myfibroblast play potential role through epithelial-mesenchymal transition (EMT) in renal fibrosis. (EMT) have an ability of blocking with neutralizing TGF-β antibodies. Direct evidence comes from studies shown that the role of TGF-β1 in renal fibrosis by observing the overexpressing an active form of TGF-β1 in liver develop progressive liver and renal fibrosis in mice Smad Signaling In Renal Fibrosis
Intracellular signalling highly upregulaated by the SMAD proteins. The SMAD proteins can be divided into three major groups, i.e.

**Table 1 types of SMAD proteins and their pathway**

| S.no | group | Type | Pathway |
|---|---|---|---|
| I | Receptor-regulated Smads | (Smad2, Smad3, , ( (ii) | TGF-β/activin-pathway; |
| | | Smadl, Smad5 and Smad8 in the) | BMP-pathway |
| II | Inhibitory Smads | (Smad6 and Smad7), and | |
| III | Common mediator Smad | Smad4) (Heldin et al., 1997, Massagué, 1998). | |

in chronic kidney diseases, TGF-β and lots of mediater are is not a sole molecule to activate Smads. Many mediators can activate Smad2 and Smad3 independent from TGF-β1 because Smads act as signal integrators and interact with other signaling pathways such as It should be pointed out that within the TGF-β super family, TGF-β/Smads also interact with the BMP/Smads to counter-regulate each other to maintain the balance between two pathways in the pathophysiological process. It is well known that Smad1, Smad5 and Smad8 transduce BMP action, whereas Smad2 and Smad3 mediate TGF-β1 activities and the interactions between these two pathways can be at multiple levels including receptors and individual Smads. It has been reported that TGF-β-activated Smad2/3 to mediate epithelial-mesenchymal transition (EMT) is reversed by addition of human recombinant BPM-7 via the Smad1-depdnent mechanism. However, a subsequent study fails to show this counter-regulating activity, suggesting the complexity between the TGF-β/Smad and BMP/Smad pathways under disease conditions.

**3.0. Various roles of Smad2 and Smad3 in, EMT, and angiogenesis in kidney disease**
**3.1.** Epithelial-mesenchymal transition (EMT) has been long considered as a process leading to renal fibrosis. Many studies have demonstrated that Smad3 plays a critical role in the EMT process in the kidney as well as in other disease conditions. The previou tuie h been reporte tht Smad2 and Smad3 play a ivers role in attenuates Ang II-induced EMT blocks AGE and angiotensin II-induced CTGF expression, renal fibrosis by disruption of Smad3, and Smad2 repectivey. Smad3, directly binding to the DNA sequences to regulate the target genes..

**3.2. Roles of Smad4 in renal fibrosis and inflammation**
It is now well accepted that TGF-β1 mediates fibrosis by causing the phosphorylation of Smad2 and Smad3, which forms a complex with the common Smad4 and then translocates to nuclei to bind and regulate the target genes. Although Smad4 has been known as the common Smad in the signal transduction pathway of the TGF-β family, its functional role in TGF-β1-regulated fibrosis and inflammatory responses remains largely unclear. This may be largely attributed to the lethality of Smad4 KO mice . Recently, we generated conditional Smad4 KO mice by crossing the Smad4 floxed mouse to the kidney specific promoter driven Cre transgenic mouse in which Smad4 is deleted from most tubular epithelial cells upon Cre recombination. We found that disruption of Smad4 from the kidney enhanced renal inflammation as evidenced by a greater CD45+ leukocyte and F4/80+ macrophage infiltration and up-regulation of IL-1β, TNF-α, MCP-1, and ICAM-1 in the obstructed kidney and in IL-1β-stimulated macrophages . Further studies showed that the loss of Smad4 repressed Smad7 transcription, therefore leading to a loss of Smad7 functional protein. This, in turn, inhibited IκBα expression but enhanced NF-κB activation, thereby promoting renal inflammation. In contrast, deletion of Smad4 inhibited progressive renal fibrosis such as collagen matrix expression in the obstructive nephropathy and TGF-β1-induced collagen I expression by fibroblasts. Interestingly, the mechanism of deletion of Smad4 to suppress renal fibrosis is not associated with inhibition of Smad2/3 activation because disruption of Smad4 does not alter phosphorylation levels of Smad2/3 nor phosphorylated Smad2/3 nuclear translocation. This is consistent with the previous finding in Smad4 null cancer cell lines. However, deletion of Smad4 influences Smad3-mediated promoter activities and the binding of Smad3 to the COL1A2 promoter. It has been reported that Smad3 binding sequences are located in the promoter regions of COL1A2, COL2A1, COL3A1, COL5A1, COL6A1, and COL6A3, therefore, disruption of Smad4 may influence the binding activity of Smad3

to the collagen promoter, thereby inhibiting the fibrotic response Moreover, we also found that deletion of Smad4 impairs Smad7 transcriptional regulation in inhibition of NF-κB signaling. It has been shown that Smad7 transcription is regulated by TGF-β1 through the direct binding of Smad3 and Smad4 to the Smad7 promoter . Thus, disrupted Smad4 results in a loss of Smad7 expression transcriptionally and impairs Smad7 promoter activities functionally. Because Smad7 is capable of inducing IκBα expression, an inhibitor of NF-κB, disruption of Smad4 reduces renal Smad7, thereby promoting NF-κB-dependent renal inflammation and the inhibitory effect of TGF-β1 on interleukin-1β-induced inflammatory response in macrophages in vivo and in vitro. Taken together, Smad4 may be a key regulator for the diverse roles of TGF-β1 in inflammation and fibrogenesis by interacting with Smad7 and Smad3 to influence their transcriptional activities in renal inflammation and fibrosis. Smad4 binds phosphorylated Smad2/Smad3 to form the Smad complex that translocates into the nucleus to regulate target genes related to fibrogenesis including Smad7. Upregulation of Smad7 prevents NF-κB/p50/p65 from phosphorylation and nuclear translocation by inducing IκBα expression. Therefore, Smad4 acts as a fine turner to promote Smad3-mediated fibrosis while inhibiting NF-κB-driven inflammation. Blue lines (symbols) indicate protective or negative regulation pathways, while red arrows (symbols) represent pathogenic or positive regulation pathways.

**3.3. Inhibitory role of Smad7 in renal fibrosis and inflammation**

Smad7 is an inhibitory Smad and negatively regulates Smad2 and Smad3 activation by its negative feed-back mechanism. Expression of Smad7 is induced by TGF-β1, which, in turn, exerts its negative feedback mechanism by causing degradation of TβRI and Smads. In chronic kidney diseases, TGF-β1 and angiotensin II are able to induce Smad7 mRNA expression and also activate the Smurfs and arkadia-dependent ubiquitin-proteasome pathways that, in turn, degrade Smad7 protein via a post-transcriptional modification mechanism. Smurf1, Smurf2, and arkadia are E3 ubiquitin ligases for Smad7 and have been shown to physically interact with Smad7. As shown in Figure Smad7 acts as an adaptor protein to recruit E3 ubiquitin ligases such as Smurf2 and arkadia to the TGF-β receptor complex to promote its degradation through proteasomal-ubiquitin degradation pathways. Once Smad7 is degraded, activation of Smad2/3 and renal fibrosis is enhanced. This is clearly demonstrated by the recent finding that up-regulation of renal Smurf2 causes an ubiquitin-dependent degradation of renal Smad7, resulting in enhanced TGF-β/Smad signaling and progressive renal fibrosis. Furthermore, Smurf2 can interact with Smad2 and selectively target Smad2 and Smad transcriptional corepressors Ski, SnoN, and TG-interacting factor (TGIF) for degradation in tubular epithelial cells, leading to progressive renal fibrosis and EMT in a mouse model of obstructive kidney disease. Thus, ubiquitin-mediated degradation of Smad7 and Smad transcriptional corepressors Ski, SnoN, and TGIF promotes further activation of TGF-β signaling and progressive renal fibrosis as evidenced in a number of animal models. This is further supported by the findings that Smad7 KO mice develop more severe renal fibrosis in both obstructive nephropathy and diabetic kidney disease .Overexpression of Smad7 prevents Smad2/3 from phosphorylation by degrading the TβRI as well as Smads via the ubiquitin degradation pathway (Ub), thereby inhibiting Smad3-dependent renal fibrosis in response to TGF-β1, AGEs, and angiotensin II (Ang II). In addition, overexpression of Smad7 can induce IκBα, an inhibitor of KF-κB, therefore inhibiting NF-κB-driven renal inflammation. Thus, Smad7 acts as a therapeutic agent for treatment of kidney diseases. Blue lines (symbols) indicate protective or negative regulation pathways, while red arrows (symbols) represent pathogenic or positive regulation pathways. Loss of renal Smad7 not only enhances TGF-β/Smad3-mediated renal fibrosis, but also enhances renal inflammation by activating the NF-κB-dependent inflammatory pathway. It is well recognized that activation of NF-κB/p65 is associated with the renal inflammation in crescentic glomerulonephritis, diabetic nephropathy, and obstructive nephropathy. Overexpression of Smad7 substantially inhibits DNA binding activity, nuclear translocation, transcriptional activity of NF-κB/p65, as wells as NF-κB-dependent inflammatory responses induced by IL-1β and TNFα , implying a functional link between the Smad7 and NF-κB. As shown in Figure 1, Smad7 is able to induce IκBα expression, an inhibitor of NF-κB, suggesting that TGF-β may act by stimulating Smad7 to induce IκBα expression to suppress NF-κB activation. The ability of overexpression of renal Smad7 to inhibit NF-κB activation and renal inflammation in remnant kidney disease, autoimmune crescentic glomerulonephritis, and diabetic nephropathy confirms the potential role of Smad7-NF-κB crosstalk pathway in renal inflammation. Evidence for the anti-inflammatory role of Smad7 also comes from the findings that Smad7 KO mice develop more severe kidney inflammation in diabetic kidney disease and UUO models because these mice have significantly higher phosphorylation levels of NF-κB/p65 along with higher levels of renal inflammation including upregulation of IL-1β, TNFα, MCP-1, ICAM-1, and the development of more macrophage infiltration whine the kidney when compared to wild-type mice. In contrast, mice that transgenically express Smad7 on T cells are protected against crescentic glomerulonephritis. Therefore, inhibition of the NF-κB signaling pathway by inducing IκBα expression may be the major mechanism by which Smad 7 inhibits renal inflammation.The therapeutic potential for Smad7 on renal inflammation and fibrosis have been examined in rat models of obstructive nephropathy, remnant kidney disease, diabetic nephropathy and in a mouse model of autoimmune crescentic glomerulonephritis. In these studies, Smad7 was transferred into the kidney using the ultrasound-microbubble-mediated gene therapy technique. Overexpression of Smad7 not only inhibits Smad3-mediated renal fibrosis such as collagen matrix expression including epithelial-mesenchymal transition, but also blocks NF-κB-driven renal inflammation including accumulation of macrophages and T cells in both glomeruli and tubulointerstitium and upregulation of renal IL-1, TNFα, ICAM-1, and iNOS. Thus, restored the renal Smad7 rebalances the TGF-β/Smad and NF-κB signaling pathways and, therefore, inhibits progressive renal functional injury. All these studies demonstrate that Smad7 not only plays a negatively regulating role in renal fibrosis and inflammation, but also acts as a therapeutic agent and has therapeutic effect on renal fibrosis and inflammation.

### 3.4. Regulation of TGF-β/Smad3-dependent microRNAs in renal fibrosis

Recent studies in microRNAs have demonstrated that TGF-β regulates specific microRNAs to influence renal fibrosis in kidney diseases. Recent reports from our laboratory and others describe several microRNAs to be regulated by TGF-β1 during kidney diseases as detailed in a recent review article. Indeed, TGF-β1 is able to up-regulate miR-21, miR-93, miR-192, miR-216a, miR-377, but down-regulates the miR-29 and miR-200 families. We have recently identified that, among these TGF-β-dependent miRNAs, miR-21, miR-192, and the miR-29 family expression during renal fibrosis is tightly regulated by TGF-β1 via the Smad3, but not Smad2, dependent mechanism, which is also illustrated in. Indeed, in vitro studies reveal that deletion of Smad3, not Smad2, inhibits expression of miR-21 and miR-192, but enhances the miR-29 family expression in response to TGF-β1 in both mouse embryonic fibroblasts and kidney tubular epithelial cells. Evidence supporting the interaction of Smad3 with miR-21, miR-192, and miR-29 also came from the findings that there are conserved Smad3-binding sites in the promoter region of all three miRNAs and that Smad3 is able to interact with their individual promoter region as detected by the Chip assay. Interestingly, expression of miRNAs can also interact with Smad3 to influence the Smad3 activity and functions. It is reported that overexpression of miR-200a could decrease Smad3 activity and attenuate TGF-β1-induced fibrosis. However, although it remains undetermined if this interaction is direct or indirect, such an observation indicates the complexity between TGF-β/Smads and miRNAs under pathophysiological conditions. Smad3-dependent miRNAs in renal fibrosis. TGF-β1 acts by stimulating Smad3 to positively regulate miR-21 and miR-192, but negatively regulate the miR-29 or miR-200 families, to mediate renal fibrosis. Blue lines (symbols) indicate protective or negative regulation pathways, while red arrows (symbols) represent pathogenic or positive regulation pathways.

The regulating role of TGF-β/Smad3 in expression of miRNAs during renal fibrosis is also detected in an experimental mouse model of obstructive nephropathy by miRNA microarray and real-time PCR. Mice null for Smad3 are protected against renal fibrosis along with the inhibition of miR-21 and miR-192 expression. In contrast, while severe renal fibrosis in the obstructive nephropathy in Smad3 wild-type mice is associated with a loss of miR-29, prevention of renal fibrosis in Smad3 knockout mice is largely attributed to an increase in expression of renal miR-29. Smad7 also plays a role in regulating Smad-dependent miRNA expression in response to TGF-β1. For example, In vitro, overexpression of Smad7 in tubular epithelial cells abolished TGF-β1-induced miR-192 expression. In vivo, deletion of Smad7 enhanced Smad3 signaling, thereby promoting miR-192 expression and fibrosis in obstructive kidney disease. In contrast, overexpression of Smad7 blocks TGF-β/Smad signaling and thus inhibits miR-192 expression and renal fibrosis in the rat 5/6 nephrectomy model.

The important role of TGF-β/Smad3-dependent miRNAs in renal fibrosis is demonstrated by the in vitro findings that overexpression of miR-21 and miR-192 enhances, but knockdown of miR-21 or miR-192 inhibits, collagen matrix expression in response to TGF-β1. In contrast, knockdown of miR-29 enhances fibrosis, whereas overexpression of miR-29 blocks collagen I expression in response to TGF-β1. More excitingly, we recently demonstrated that the ultrasound-microbubble-mediated gene transfer technique can also be used to deliver the miRNAs into the kidney and have proved that the ultrasound-mediated miRNA therapy is a novel and effective therapeutic approach for kidney disease. Indeed, ultrasound-mediated overexpression of miR-29b or knockdown of miR-21before or after the established mouse model of obstructive nephropathy is capable of preventing or halting the progression of renal fibrosis. These findings suggest that specific targeting the Smad3-dependent microRNAs related to fibrogenesis such as miR-21 and miR-29 may represent a novel and specific anti-fibrosis therapy for renal fibrosis.

Renal fibrosis is a complex and irreversible pathological process that involves the activation and interaction of multiple pro-fibrotic signaling pathways. It is a late-stage feature of all types of chronic kidney disease (CKD), affecting more than 10% of the world's population and posing a major public health challenge [72]. Without alternative treatment, such as dialysis or kidney transplantation, CKD can progress to end-stage renal disease (Zhu et al., 2020). Although scientists have repeatedly studied the idea of reversing CKD over the past decades, existing treatments to prevent CKD progression and CKD-related complications are quite limited (Holden et al., 2020). Current interventions include angiotensin-converting enzyme inhibition, angiotensin receptor block, optimal blood pressure control, and sodium bicarbonate for metabolic acidosis (Onuigbo and Agbasi, 2015). However, none of these treatments improved renal function, and the patient maintained poor renal function. Therefore, it is urgent to find effective drugs to treat CKD.

TGF-β/Smads signaling is the primary pathway of fibrosis formation, according to numerous studies [56] Activation of TGF-β/Smads signals leads to extracellular matrix synthesis and deposition, podocyte depletion, mesangial dilation, renal tubular epithelial fibrosis transformation, and myoblast fibroblast activation. TGF-β1 activation recruits and activates type II TGF receptors (TβRII) and downstream receptor-associated Smads (R-Smads), Smad2, and Smad3. Phosphorylated Smad2/3 then forms oligomeric complexes with Smad4 [12]. Subsequently, the Smad2/3/4 complex is translocated to the nucleus to regulate the transcription of target genes and induce α -smooth muscle actin (α-SMA), Collagen I, and inhibitory Smad7 [33]. Interestingly, Smad7 antagonizes a variety of diseases, including TGF-β-mediated fibrosis, cancer, and inflammation [[18] Smad7 negatively regulates TGF-β/Smad signaling by competing with R-smad and binding to TβRI (Yan et al., 2016). Ubiquitin-mediated proteasome degradation pathway is an evolutionarily conserved cascade that strictly regulates TGF-β superfamily signal transduction (Nakamura, 2018). Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 are HECT (homologous to the C-terminal of E6 co-protein) E3 ubiquitin ligases that regulate TGF-β and BMP signaling (Zhu et al., 1999; Kavsak et al., 2000; Lin et al., 2000; Ebisawa et al., 2001). Smad7 recruits Smurf1 and Smurf2, forms a complex with Smurfs, translocates from the nucleus to TGF-β membrane receptors TβR-I and Smad2, and degrades the complex through the proteasome pathway (Lin et al., 2000; Ebisawa et al., 2001). These studies indicate that

TGF-β/Smads signaling plays a major role in renal fibrosis (Yan et al., 2009; Troncone et al., 2018; Zhou et al., 2018). Smad7 negatively regulates TGF-β/Smad signaling by competing with R-smad and binding to TβRI (Yan et al., 2016). The ubiquitin-mediated proteasome degradation pathway is an evolutionarily conserved cascade that tightly controls TGF-superfamily signal transduction (Nakamura, 2018). Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 are HECT (homologous to the C-terminus of E6 co-protein) E3 ubiquitin ligases that control TGF and BMP signaling (Zhu et al., 1999; Kavsak et al., 2000; Lin et al., 2000; Ebisawa et al., 2001). Smad7 recruits Smurf1 and Smurf2, forms a complex with Smurfs, translocates from the nucleus to the TGF membrane receptors TR-I and Smad2, and degrades the complex via the proteasome pathway (Lin et al., 2000; Ebisawa et al., 2001). These findings suggest that TGF-/Smad signaling is important in renal fibrosis. Studies have shown that the TGF-β/Smads signaling pathway is closely related to metabolic disorders. TGF-β/Smads signaling regulates the expression of genes involved in fat formation and fatty acid β-oxidation, resulting in increased triglyceride synthesis and lipid accumulation in hepatocytes (Tan et al., 2011). It also regulates the expression of genes involved in fat formation and fatty acid oxidation (Yang et al., 2014). Up-regulated expression of extracellular matrix (ECM) components TGF-β1, connective tissue growth factor (CTGF), fibroblastic growth factor (bFGF), and collagen I was observed in patients with hepatic fibrosis, accompanied by changes in lipid metabolism, amino acid metabolism, purine metabolism, and taurine metabolism (Bianchi et al., 2000; Zhang et al., 2006; Zhao et al., 2014). Therefore, the transmission of the TGF-β/Smads signal pathway is closely related to the disorder of lipid metabolism pathway, amino acid metabolism pathway, and purine metabolism pathway, and the regulation of TGF-β/Smads signal transduction can improve the level of disordered metabolites.

**4.0. Shenkang injection (SKI)**

Shenkang injection (SKI) is one of the representative Chinese patent medicine preparations. SKI is developed by renowned and experienced Chinese medicine practitioners and consists of four herbal extracts: Radix Et Rhizoma Rhei Palmati, Radix Astragali Mongolici, HerbaSalviae Japonicae, and Flos Carthami. SKI, as a modern proprietary Chinese medicine intravenous injection (Z20040110), was approved to be marketed by the State Food and Drug Administration of China in 2004. After more than ten years of clinical use, certain clinical research evidence has been accumulated, and it can be used to intervene in strengthening factors, cytokines, chemokines, and fibrosis related pathways[70], control the inflammatory response ([51], alleviate renal oxidative stress[45], improve glomerular filtration [67], and prevent glomerulosclerosis [65] so as to achieve the effect of chronic kidney disease. It is unclear whether SKI can improve metabolic disorders in UUO mice or promote ubiquitination and degradation of the TGF-/Smad signaling pathway. In this study, the anti-fibrosis, metabolite alteration, and EMT phenomena of SKI were evaluated in HK-2 cells and UUO mice, with molecular mechanism studies focusing on ubiquitination of the TGF-β/Smad signaling pathway.

**5.0 Renal Fibrosis**

Renal fibrosis is a consequence of progressive kidney diseases such as chronic infection, obstruction of the ureters, hypertension, and diabetic nephropathy [1, 2]. Renal fibrosis is characterized by tubulointerstitial fibrosis and glomerulosclerosis, both caused by the excessive deposition of collagens and extracellular matrix (ECM) components. Although the kidney can regenerate after acute injury, renal fibrosis is usually irreversible [3]. Transforming growth factor-beta (TGF-β)/Smads signaling plays a pivotal role in renal fibrosis [4–6]. Elevated TGFβ1 and downstream Smad3 and Smad2 in the kidney activate profibrotic genes and mediate renal fibrosis [5]. On the other hand, Smad7 suppresses renal fibrosis by altering the expression of TGF-β/Smad3 through microRNAs [7]. Inhibition of the TGF-β1/Smad2/3 pathway alleviates kidney injury and renal fibrosis [8, 9].

The traditional Chinese medicine (TCM) is based on a personalized approach aiming to restore the balance between Yin and Yang [10, 11]. There are hundreds of TCM formulation against chronic kidney diseases (CKD) [12, 13]. Some TCM components were studied (including Astragalus, Angelica sinensis, Rheum plantarum, Radix bupleuri, Cordyceps sinensis, and Tripterygium wilfordii [11]) and their effects include anti-inflammation, antioxidation, antifibrosis, anticoagulation, and immune system modulation [13, 14]. On the other hand, some TCM components can be toxic to the kidney [11, 15], hence the importance of characterizing them carefully.

**5.1.Smad pathways in chronic kidney disease**

In CKD, Smad2 and Smad3 are highly activated and TGF-β is not a sole molecule to activate Smads [3], [21]. As shown in Fig. 1, many mediators including advanced glycation end-products (AGE) and angiotensin II (Ang II) can activate Smad2 and Smad3 and mediate renal fibrosis including connecting tissue growth factor (CTGF) expression via both TGF-β-dependent and independent mechanisms [22], [23], [24], [25]. The later involves the mitogen-activated protein kinase (MAPK)-Smad crosstalk pathway (Fig. 1). This is supported by the findings that deletion of TGF-β1 or TGF-β receptor II is unable to prevent AGE-induced Smad2 and Smad3 activation and fibrosis [22], [23]. By contrast, blockade of the engagement of AGE to its receptor (RAGE) with the soluble RAGE or ERK/p38 MAP kinases with the specific inhibitors or dominant negative adenovirus can prevent AGE-induced Smad2/3 activation and renal fibrosis [22], [23], identifying the RAGE-ERK/p38 MAPK-Smad2/3 crosstalk pathway in the development of diabetic complications. Similarly, under the hypertensive conditions, Ang II can activate Smad2/3 to stimulate ECM production and EMT via the AT1-ERK/p38 MAPK-Smad2/3 crosstalk pathway in addition to the TGF-β-dependent mechanism [24], [25], [26]. The important role for the MAPK-Smad crosstalk pathway in AGE and Ang II-mediated renal fibrosis is further demonstrated by the ability of Ang II and AGE to induce Smad3-mediated fibrosis, including CTGF expression and EMT in kidney cells lacking

TGF-β1 gene or TβRII, but not in those with a blockade of ERK/p38 MAP kinases [23], [25], [26]. Therefore, in CKD, many mediators like AGE and Ang II can bind to their own receptor and then activate the Smad pathway via the TGF-β-independent mechanism through the ERK/p38 MAPK pathway in addition to the TGF-β-dependent mechanism (Fig. 1). All of these studies reveal the complexity of the activation of Smads under disease conditions. These results may also implicate that targeting the TGF-β signaling at the receptor levels may not be an optimal therapeutic approach due to the existing of the intracellular crosstalk pathways.

### 5.2. Distinct roles of Smads in chronic kidney disease

#### 5.2.1. Pathogenic role of Smad3 in renal fibrosis

Smads have distinct roles in renal fibrosis and inflammation. In the context of renal fibrosis, Smad2 and Smad3 are strongly activated in both experimental and human kidney diseases, including diabetic nephropathy [21], [22], [23], [27], [28], [29], obstructive kidney diseases [30], [31], [32], [33], remnant kidney disease [26], [34], drug-associated nephropathy [35], and immunologically mediated glomerulonephritis [20], [36]. Many fibrogenic genes, such as ColIa1, ColIa2, ColIIIa1, ColVa2, ColVIa1, and ColVIa3 and the tissue inhibitor of MMP-1 (TIMP-1), are the downstream targets of TGF-β/Smad3 signaling [37] suggesting that Smad3 may be a critical mediator of TGF-β/Smad signaling in fibrosis. An essential role for Smad3 in collagen matrix synthesis is confirmed by the findings that deletion of Smad3 from mice suppresses fibrosis in a number of rodent models, including diabetic nephropathy [27] obstructive nephropathy [30], and drug toxicity-related nephropathy [35]. Furthermore, the use of a Smad3 inhibitor to block TGF-β1-induced endothelial-myofibroblast transition in vitro and renal fibrosis in a type 1 diabetic kidney disease demonstrates a therapeutic potential for kidney disease by targeting Smad3 signaling [38].

#### 5.2.2. Protective role of Smad2 in renal fibrosis

By contrast to Smad3, our recent study demonstrated that Smad2 is renoprotective in fibrosis [39]. In conditional Smad2 KO mice in which Smad2 is specifically deleted from tubular epithelial cells by crossing the Smad2 floxed mouse to the kidney specific promoter (Cadherin 16)-driven Cre transgenic mouse [39]. Unexpectedly, we found that deletion of Smad2 from the kidney significantly enhances renal fibrosis in a mouse model of obstructive nephropathy. This may promote Smad3 signaling, including Smad3 phosphorylation, nuclear translocation, promoter activities, and the binding of Smad3 to the collagen I promoter (COL1A2) [39]. Thus, although it is commonly believed that Smad2 and Smad3 bind together physically and work in a nonredundant manner in embryonic development, Smad2 may function to competitively inhibit Smad3 activation and the subsequent binding to its target genes under pathophysiologic conditions. Thus, the loss of Smad2 enhances Smad3-mediated collagen matrix expression in response to TGF-β1 and other fibrotic mediators, including Ang II, AGE, and nephrotoxin [23], [24], [25], [26], [35]. Smad 2 and Smad3 also play a distinct role in EMT. Many studies have demonstrated that Smad3 plays a critical role in the EMT process in the kidney under various disease conditions [26], [30], [35]. Our recent study found that disruption of Smad3 – but not Smad2 – attenuates Ang II-induced EMT as identified by the loss of an epithelial marker E-cadherin and the gain of mesenchymal phenotype α-SMA [26]. This may be mediated by CTGF because the knockdown of Smad3 – but not Smad2 – blocks AGE and Ang II-induced CTGF expression and renal fibrosis [23], [26]. The ability of blocking Smad3 to inhibit EMT and EndoMT in a variety of animal models confirms a critical role for Smad3 in the pathogenesis of CKD [10], [38], [40], [41].

#### 5.2.3. Role of Rosa roxburghii in renal fibrosis

Rosa roxburghii is a member of the rose family and is a wild plant from the Guizhou Province and distributed in south China. In TCM, the fruit of Rosa roxburghii (named Cili or CL) improves immune response, enhances digestive ability, and possesses antiaging effects [16]. Chemical analysis revealed that Cili is rich in antioxidants including superoxide dismutase (SOD), vitamin C, vitamin E, and flavonoids [17, 18]. Cili has a long history in China and has been proven to possess antioxidant [19, 20], antiatherosclerosis [21], antitumor [22], and radioprotective [23] effects. Nevertheless, the exact molecular mechanisms responsible for these beneficial effects are not well understood. preliminary data showed that freeze-dried Cili powder improved renal fibrosis and indexes of oxidative stress in 90 patients with stages 3-4 renal failure [24], but the exact mechanisms responsible for these effects of Cili on kidney diseases are unknown. We hypothesized that Cili could prevent the development of renal fibrosis through mediating TGF-β/Smads signaling, which is known to be involved in renal fibrosis [4–6]. Therefore, this study established a renal fibrosis rat model induced by unilateral ureteral obstruction (UUO) in order to confirm the effects of freeze-dried Cili powder on renal fibrosis and examine TGF-β/Smads signaling. TGF-β/Smads and crosstalk pathways in renal fibrosis and inflammation. After binding to TβRII, TGF-β1 activates the TβRI-kinase, which phosphorylates Smad2 and Smad3. The phosphorylated Smad2 and Smad3 then bind to Smad4 and form the Smad complex, which translocates into the nucleus and regulates the target gene transcription, including Smad7. Smad7 is an inhibitory Smad that functions to block Smad2/3 activation by degrading the TβRI and Smads and to inhibit NF-κB-driven inflammatory response by inducing IκBα, an inhibitor of NF-κB. Note that Ang II and AGEs can activate Smads independent of TGF-β1 via the ERK/p38/MAPK crosstalk pathway. Red arrow lines (symbols) indicate positive regulation and blue lines (symbols) indicate negative regulation. AGE, advanced glycation end-products; Ang II, angiotensin II; ERK, extracellular-signal-regulated kinases; IkBa, IκBα; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappaB; TGF-β, transforming growth factor-β. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) In CKD, TGF-β/Smad signaling is deregulated, resulting in overactivation of Smad2 and Smad3 but a loss of Smad7. Thus, the imbalance of Smads may be a key mechanism leading to renal scar formation and end-stage kidney disease.

### 5.2.4. Role of curcumin in Renal Fibrosis

In short, curcumin has been demonstrated to exhibit anti-inflammation properties in different kidney diseases models by reducing inflammatory molecules release (MCP-1, NF-κB, TNF-α, IL-1β, COX-2, and cav-1) and inducing the expression of anti-inflammation factors (HO-1, M6PRBP1, and NEDD4), suggesting that it could play a contributing role in preventing the initiation of renal fibrosis

#### 5.2.4.1. role of Curcumin in Activation Stage of Renal Fibrosis

At the activation stage, profibrotic cytokines and factors are released from injured tubular cells, which stimulate the myofibroblasts to produce ECM. In addition, EMT further contributes to transdifferentiating endothelial and tubular cells to myofibroblasts [45]. An experiment performed by Sun et al. demonstrated that administration of curcumin (100 mg/kg for 12 weeks, oral gavage) prevented EMT through increasing the expression of epithelia cadherin, synaptopodin, and reducing expression of mesenchymal a-smooth muscle actin (α-SMA), fibroblast-specific protein 1 in the diabetic rats [21]. The possible mechanisms underlying these effects might be involved in suppressing the phosphorylation of cav-1 at Tyr14 and increasing stabilization of cav-1 and β-catenin. In addition, β-catenin favors EMT and renal fibrosis [46–48]. Curcumin inhibited high glucose induced dissociation of β-catenin from cav-1 and decreased active β-catenin expression [21]. In our group we also found that curcumin could inhibit the occurrence of EMT in renal tubular epithelial cells via regulating several sites of the TGF-β/Smads signal transduction pathway in UUO rats [49]. The inhibitory effect of curcumin on EMT was also demonstrated in cisplatin-induced renal fibrosis rats [31]. However, concerns are rising regarding the efficacy of curcumin in the management of renal fibrosis owing to its inherent low bioavailability. In most of the studies, curcumin was administrated by oral gavage. Further investigations are needed to explore the real active ingredients of curcumin after administration. Fortunately, some of curcumin derivatives with good bioavailability (such as C66 and B06) and new formulations of curcumin have been developed in recently years. However, the efficacy and safety of these new analogs and formulations remain largely unexplored. Taken together, as a food derived compound with golden-yellow fluorescence, curcumin may offer a new option in the treatment of renal fibrosis and also provide a new druggable chemical structure for chemists in designing new antifibrosis drug candidates. Curcumin and diabetes mellitus Diabetes mellitus is estimated to currently affect almost half a billion people worldwide as this number is expected to reach 700 million by 2045 (Cho et al. 2018). Although conventional treatment regimens remain a priority, natural compounds provide an attractive alternative as supplemental therapy in terms of their pleiotropic actions and low side effects. The biological activities of turmeric (Curcuma longa, L.), used for centuries in culinary and traditional medicine for treating conditions of the cardiovascular, pulmonary, digestive, renal, and nervous systems as well as its anti-bacterial and anti-pathogenic properties, are well known (Gupta et al. 2013a, b; Maheshwari et al. 2006). Main bioactive compounds in turmeric are curcuminoids (curcumin (1), desmethoxycurcumin (2) and bisdemethoxycurcumin (3), Fig. 1), sesquiterpenoids, and turmerones. Among these compounds, curcumin is the most abundant and active that has been shown to possess anti-inflammatory actions, reduce insulin resistance, decrease glucose and insulin levels, and increase adiponectin release (Anand et al. 2008). Additionally, curcumin reduces the levels of resistin, leptin, IL-6, IL-1β, and TNF-α in patients with type 2 diabetes (Marton et al. 2021; Parsamanesh et al. 2018; Hajavi et al. 2017). The research carried out indicates that curcumin can exert an influence on glucose homeostasis and may help to alleviate the vascular risk in diabetic patients (Pivari et al. 2019). Also, some studies have shown that treatment with curcuminoids improves the lipid profile and increases the overall antioxidant capacity in the blood (Panahi et al. 2017a, b; Altobelli et al. 2021).

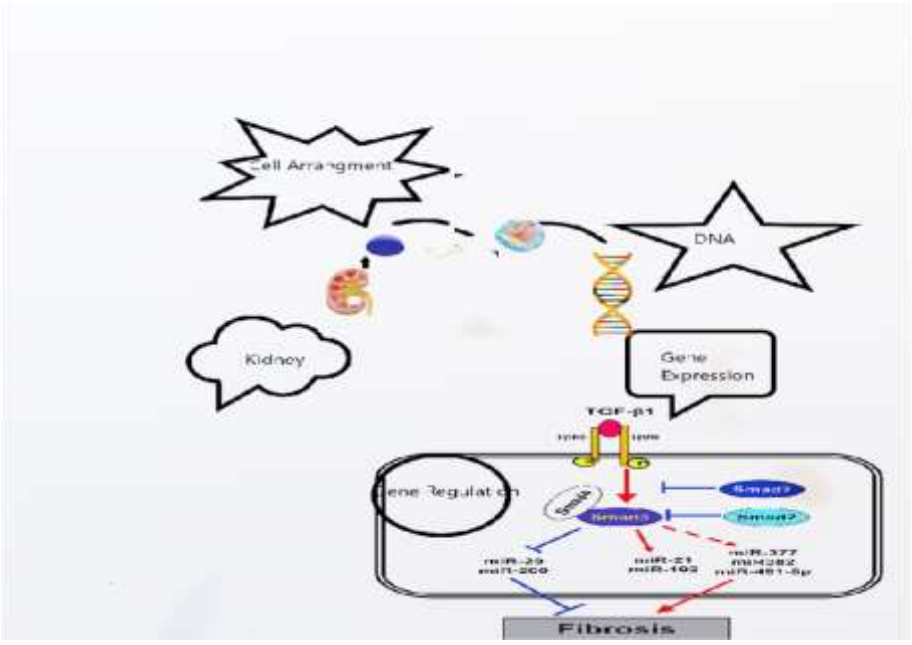


**Fig 1 TGF mechanism in the renal fibrosis**

**6.0. Diabetic nephropathy (DN)**

Diabetic nephropathy (DN) is the most serious end-stage renal disease which characterized by renal glomerular sclerosis including glomerular hypertrophy, glomerular basement membrane (GBM) thickening, mesangial expansion and renal fibrosis. TGF-β/Smads signal pathway plays a crucial role in the development of renal fibrosis. In this study, we found that GdCl3 which was an agonist of Calcium-sensing receptor (CaSR) could repress the activation of TGF-β/Smads signal pathway induced by TGF-β1 or high glucose and then alleviated the accumulation of extracellular matrix (ECM) in mesangial cells and the kidney of type1 diabetic rats. Further study indicated that GdCl3 could induce the binding of CaSR and TβR II and then both of these two receptors translocated from cell membrane to cytoplasm, in this case, TβR II on the cell membrane was decreased and then desensitized to the stimulation of its ligand TGF-β1, so that the activation of its downstream factors such as Smad2 and Smad3 were blocked, finally, ECM expression in mesangial cells were inhibited. We concluded that GdCl3 could alleviate the accumulation of ECM in mesangial cells via antagonizing TGF-β/Smads signal pathway in diabetes mellitus. Renal fibrosis, characterized by the accumulation of myofibroblasts and extracellular matrix (ECM), is a major cause of the end-stage renal disease. However, treatment of renal fibrosis remains non-specific and ineffective clinically. Increasing evidence shows that TGF-β1 is a key mediator of renal fibrosis [1-5]. Many studies have attempted to develop anti-fibrosis therapy by inhibiting the upstream of TGF-β signaling, including antisense TGF-β oligodeoxynucleotides, neutralizing antibodies, and inhibitors to TGF-β receptor (TβR) kinases in a variety of kidney disease models [2, 6-8]. However, treatment of puromycin aminonucleoside nephropathy with a high dose of anti-TGF-β antibody produces no renoprotective effect [9], which is consistent with a recent finding that disruption of TGF-β type II receptor decreased renal fibrosis but enhanced renal inflammation [10]. Results from these studies suggest that inhibiting the upstream of TGF-β signaling may not be an optimal therapeutic approach due to the diverse roles of TGF-β1 in inflammation and fibrosis. Although blockade of the upstream TGF-β signaling inhibits fibrosis, it may also promote inflammation. Therefore, we hypothesized that treatment of renal fibrosis should target the downstream TGF-β/Smad signaling associated with fibrosis, rather than to block the general effect of TGF-β1.

Increasing evidence shows that TGF-β/Smad signaling is a central pathway leading to renal fibrogenesis [1]. It is now clear that TGF-β1 promotes fibrosis positively by activating its downstream molecule Smad3, not Smad2, but negatively by an inhibitory Smad7 [2, 11]. In the context of fibrosis, Smad3 is pathogenic, which is supported by the finding that mice lacking Smad3 are protected against tissue fibrosis in chronic kidney and cardiac diseases [12-15]. In contrast, Smad7 is protective during fibrosis as deletion of Smad7 promotes, but overexpression of Smad7 inhibits, tissue fibrosis [16-19]. Thus, the imbalance of TGF-β/Smad signaling may be a major cause of renal fibrosis and rebalancing this pathway by inactivating Smad3 while upregulating Smad7 may produce a better therapeutic effect on renal fibrosis.

**6.1. C66 and diabetes-associated complications**

Effects of C66 on the prevention of diabetes-associated vascular damages Hyperglycemia is the key link between diabetes and high oxidative stress that is implicated in the pathophysiology of vascular abnormalities caused by diabetes (Maritim et al. 2003), (Fiorentino et al. 2013). Excessive generation of reactive oxygen species (ROS) has been identified as an early pathogenic component in diabetic aortic damage, which can be mitigated by enhanced endogenous antioxidant capacity (Li et al. 2018a, b, c). Chronic inflammation, as one of the main factors in the progression of diabetes and its complications, causes tissue damage and leads to the generation of new vascular structures via oxidative stress, apoptosis, endothelial dysfunction, and fibrosis (Wang et al. 2014a, b, c), (Liu et al. 2014; Li et al. 2018a, b, c) reported elevated levels of proinflammatory (TNF-1α, MCP-1), aortic fibrosis (CTGF, TGF-β1), apoptosis (caspase-3), and oxidative stress (3-nitrotyrosine (3-NT), 4-hydroxynoneal (4-HNE)) markers in the aorta of diabetic mice. Treatment with C66 or deletion of JNK2 (JNK2−/−) in diabetic mice resulted in a reduction of the increased expression of inflammatory and fibrosis markers (Li et al. 2018a, b, c). In fact, treatment with C66 had no additional effect on JNK2−/− diabetic mice, suggesting that C66 protection is based on the suppression of JNK2 (Parsamanesh et al. 2018), (Li et al. 2018a, b, c). As a member of the mitogen-activated protein kinase family, JNK2 influences multiple cellular stress responses, including inflammatory responses, oxidative stress, cell death, cell survival, and protein expression, in various tissues of diabetic animals (Liu et al. 2014; Zhou et al. 2016; Jiao et al. 2015; Fan et al. 2012). JNK was found to be inhibited by curcumin, which is a potent protector against cardiovascular disease (Fiorillo et al. 2008; Stamenkovska et al. 2021; Pan et al. 2013) found that C66 has a high affinity for JNK2 binding, which potentiates its anti-inflammatory effects. In addition, Li et al. (2018a, b, c) have found that JNK2 deletion is associated with reduced aortic inflammation, oxidative stress, apoptosis, and fibrosis, caused by diabetes, but significant changes in the aorta of diabetic JNK2−/− mice were not reported after C66 treatment. These data imply that protection against aortic damage caused by diabetes is mediated by C66-induced JNK2 inhibition. Hence, reducing JNK2 activity by C66 can be suggested as an effective supplementary technique in the treatment of diabetes. Furthermore, Huang et al. (2020) showed that when human umbilical vein endothelial cells (HUVECs) were incubated in high glucose (HG) (25 mM) medium, C66 caused dose-dependent (0–5 μM) suppression of the phosphorylated p-65 (p-p65)-induced expression. Interleukin 1 receptor-associated kinase (IRAK1), as a key adapter downstream kinase of the toll-like receptor (TLR) superfamily, mediates HG-induced NF-kB activation by phosphorylation and degradation of the IkB protein in the proteasomes (Hayden and Ghosh 2004; Huang et al. 2020) also reported that treatment with HG (25 mM) caused a significant reduction in microRNA miR-146a expression, which was restored by the administration of C66. Additionally, the authors suggested that pre-treatment with hsa-miR-146a antagonist, completely abolishes C66-induced miR-146a upregulation in HUVECs incubated with HG [46] C66 at doses up to 5 μM caused a significant limitation of the HG-amplifying effect of IRAK1 and p-p65 expression in HUVECs, but

these effects were reversed with anti-miR-146a treatment [23], Therefore, it is apparent that C66 can counteract HG-induced NF-κB activation in HUVECs by stimulating miR-146a expression (Fig. 5).

**6.2. Role of Asiatic Acid (AA) and Naringenin (NG)**

Asiatic Acid (AA), a triterpenoid component extracted from Centella asiatica [20], has been shown to have a variety of pharmacological effects including anti-inflammation [21], anti-oxidation [22] and anti-fibrosis [23, 24]. We recently found that AA exerts its anti-fibrotic effects on liver fibrosis by inducing Smad7 [23]. Naringenin (NG) is a flavonoid from grapefruit and citrus fruits with anti-inflammatory properties in a number of disease conditions including atherosclerosis and diabetes [25-27]. NG is also found to inhibit TGF-β/Smad3 signaling and tissue fibrosis including epithelial-mesenchymal transition in a number of disease models [28, 29]. Based on these observations, we therefore hypothesized that the combination of AA and NG may produce a better therapeutic effect on renal fibrosis by more effectively correcting the imbalance of TGF-β/Smad signaling, namely upregulation of Smad7 while suppressing Smad3. The hypothesis was examined in the present study in vitro and in a well-characterized mouse model of unilateral ureteral obstruction (UUO). Combination of AA and NG produces a better effect on restoring the balance of TGF-β/Smad signaling and inhibiting TGF-β1-induced fibrosis in vitro We first determined an effective and safe dosage of AA and NG in vitro, in which a better effect on restoring the balance of TGF-β/Smad signaling and inhibiting fibrosis without cytotoxicity was achieved. As shown in Figure Figure1A,1A, phosphorylation of Smad3 was significantly induced in renal tubular epithelial cells (TECs) at 30 minutes after addition of TGF-β1 (2ng/ml), which was significantly inhibited by over-night pre-incubation with AA at dosages of 20 μM and 30μM (Figure (Figure1a).1a). Similarly, addition of NG was also able to block TGF-β1-induced activation of Smad3 in a dosage-dependent manner, with significant dosages over 50μM (Figure 1). We then examined the cytotoxic response to AA or NG in cultured TECs. As shown in Figure 1c and 1d, lactate dehydrogenase (LDH) releasing assay and MTT assay detected that AA at concentrations over 30μM caused significant cytotoxicity and largely decreased cell viability of TECs. In contrast, the cytotoxic and anti-proliferation effects of NG were undetectable with the concentration range from 25μM to 400μM (Figure 1c and 1d). Taken together, AA at 20μM and NG at 50μM were selected as an effective and safe dosage for the in vitro study. Furthermore, the combination of AA (20μM) and NG (50μM) also produced no cytotoxicity to TECs.

**6.2.1. Combined effect of AA and NG**

Effect of AA, NG, or their combination on TGF-β1 (2ng/ml)-induced mRNA expression of collagen I and α-SMA detected by real-time PCR. b. Effect of AA, NG, or their combination on TGF-β1 (2ng/ml)-induced mRNA expression of collagen I and α-SMA detected by Western blot analysis. Data represent the mean ± SEM for at least 3 independent experiments. The combination of AA and NG also produced a better inhibitory effect on renal fibrosis in a well-established mouse model of UUO. We first determined an optimal dosage of AA and NG and their combination for a better anti-fibrosis effect without toxicity in a mouse model of UUO. As shown in Figure Figure4,4, daily intraperitoneal injection (i.p.) of AA for 7 days was able to inhibit collagen I expression in the UUO kidney in a dose-dependent manner, with a better inhibitory effect at dosages of 5 and 10 mg/kg body weight (BW). In addition, i.p. injection of NG also dose-independently inhibited collagen I production in the UUO kidney, with an optimal dosage at 50 mg/kg BW. Thus, AA at 5mg/kg BW and NG at 50 mg/kg BW were selected as a single or combination treatment for renal fibrosis in a mouse model of UUO.

**6.3. Mechanisms of AA, NG, and their combination treatment in renal fibrosis**

The mechanisms of AA, NG, and their combination in anti-renal fibrosis, treatment with AA or NG alone partially inhibited upregulation of TGF-β1 and phosphorylation of Smad3 and phospho-Smad3 nuclear translocation, which was further suppressed by the combination of AA and NG. Consistent with the findings in vitro, the inhibitory effect of AA on Smad3 signaling in the UUO kidney was correlated with a significant upregulation of renal Smad7 at both mRNA and protein levels without altering Smad3 mRNA and protein expression (Figure (Figure7).7). In contrast, blockade of Smad3 signaling with NG was associated with downregulation of Smad3 at both mRNA ad protein levels without altering Smad7 mRNA and protein levels (Figure (Figure7).7). The combination treatment with AA and NG produced a better inhibitory effect on TGF-β/Smad signaling by further suppressing Smad3 signaling while inducing Smad7 mRNA expression as well as preventing Smad7 protein from degradation by decreasing Smurf2.

**7.0 Discussion**

Increasing evidence shows that TGF-β1/Smad signaling is a central pathway leading to tissue fibrosis [1, 2]. It is now clear that Smad3 is a key fibrogenic mediator and is highly activated during renal fibrosis. In contrast, Smad7, an inhibitor of TGF-β/Smad3 signaling, is lost in the scarring kidney [2]. In the present study, we identified that AA, a purified compound from Centella asiatica [20], is a Smad7 agonist, whereas NG, a flavonoid from grapefruit and citrus fruits [28], functioned as a Smad3 inhibitor. The combination of these two purified Traditional Chinese Medicine compounds significantly enhanced the inhibitory effect on TGF-β/Smad signaling and renal fibrosis in vitro and in vivo. Outcomes from this study suggested that restoring the balance of downstream TGF-β/Smad signaling with AA (a Smad7 agonist) and NG (a Smad3 inhibitor) may represent a new therapeutic approach for chronic kidney disease associated with progressive renal fibrosis. The identification of NG as a Smad3 inhibitor and AA as a Smad7 agonist was an important finding in the present study. Consistent with the consensus that Smad3 stimulates tissue fibrosis in a number of chronic kidney diseases [12, 13], inhibition of Smad3 transcription and phosphorylation was a mechanism through which NG

inhibits fibrosis in vitro and in vivo. This finding was consistent with other studies in which inhibition of Smad3 with SIS3 (a Smad3 inhibitor to block Smad3 DNA binding and phosphorylation) and GQ5 (a small compound isolated from the dried resin of Toxicodendron vernicifluum that blocks the binding of Smad3 to TGF-β type I receptor) attenuates renal fibrosis [30, 31]. Interestingly, addition of NG blocks Smad3 mRNA and protein expression without altering levels of Smad7 expression, indicating a specific effect of NG on inhibiting Smad3 without influencing Smad7. In the present study, we also found that addition of AA was capable of blocking Smad3 phosphorylation and renal fibrosis by upregulating Smad7 without altering expression of Smad3, identifying AA as a Smad7 agonist to inhibit Smad3 signaling by inducing Smad7 transcription. This finding was consistent with our previous study that treatment with AA inhibits liver fibrosis by inducing Smad7 [23]. It is well accepted that Smad7 is an inhibitor of Smad3 signaling and protects against tissue fibrosis in a number of diseases [17, 32-35]. However, Smad7 is lost in the fibrosing tissue, which is induced by Smurf2, an E3 ligase that targets Smad7 for ubiquitin degradation [36, 37]. It is reported that TGF-β1 induces Smurf2 expression via a Smad3-dependent mechanism [37]. Thus, the combination treatment with AA and NG produced a better inhibitory effect on Smad3 signaling via direct and indirect mechanisms.We have previously shown that TGF-β/Smad3 mediates renal fibrosis by upregulating miR-21 but downregulating miR-29b in the UUO and diabetic nephropathy [38-41]. In the present study, we mainly focus to the role of TGF with different with AA or NG also produced an additive effect on inhibition of miR-21 expression. However, treatment with AA and NG did not alter the expression of miR-29b in vivo and in vitro. Thus, inhibition of miR-21 expression may also account for a mechanism whereby AA, NG, and their combination treatment inhibited renal fibrosis.The combination of AA and NG also allowed using lower dosages of AA and NG, which prevented the drug cytotoxicity while enhancing the therapeutic efficacy on renal fibrosis. Indeed, a single use of AA or NG induced an inhibition of Smad3 signaling in a dosage-dependent manner; however, it also caused the anti-proliferation effect and cytotoxicity when higher dosages were used. The combination of AA and NG allowed lowering the dosages of individual drugs to produce a safer but effective therapy for renal fibrosis without cytotoxicity. This is important since the cytotoxicity has been a major problem for the clinical application of the Traditional Chinese Medicine [42].

**8.0 Concluding Remarks**

The TGF-β/Smad signaling pathway is a crucial cellular signaling pathway involved in various cellular processes such as growth, differentiation, apoptosis, and immune response. Here's a brief overview of how it works, TGF-β Activation: The pathway begins with the activation of transforming growth factor beta (TGF-β), which can be triggered by various stimuli such as cell-cell interactions, growth factors, or environmental cues. Receptor Activation: Active TGF-β binds to TGF-β receptors on the cell membrane, leading to the formation of a receptor complex. In humans, there are two types of TGF-β receptors: type I (TGFBR1) and type II (TGFBR2). Phosphorylation of Smad Proteins: Upon receptor activation, TGFBR2 phosphorylates TGFBR1, which in turn activates downstream effector proteins known as Smads. Smads are a family of intracellular proteins that transmit signals from the cell surface to the nucleus. Smad Activation and Nuclear Translocation: Once phosphorylated, receptor-regulated Smads (R-Smads), such as Smad2 and Smad3, form complexes with the common-mediator Smad (Co-Smad), Smad4. These complexes then translocate into the nucleus, Gene Regulation: In the nucleus, the Smad complexes interact with transcription factors, co-activators, and co-repressors to regulate the transcription of target genes. This regulation can either promote or inhibit gene expression, depending on the context and cellular conditions. Feedback Regulation: Several feedback mechanisms exist within the TGF-β/Smad pathway to tightly control its activity and ensure proper cellular responses. These mechanisms include the regulation of receptor expression, Smad ubiquitination and degradation, and the expression of inhibitory Smad proteins (I-Smads). Biological Effects: The activation of the TGF-β/Smad pathway can lead to various cellular responses depending on the cell type and context. These responses include cell proliferation, differentiation, migration, apoptosis, extracellular matrix production, and immune regulation. Dysregulation of the TGF-β/Smad pathway has been implicated in numerous diseases, including cancer, fibrosis, autoimmune disorders, and developmental defects. Therefore, understanding the intricacies of this signaling pathway is crucial for developing targeted therapies for these conditions. The TGF-β/Smad signaling pathway plays a significant role in the pathogenesis and progression of chronic kidney disease (CKD). Here's how: In CKD, there is often excessive deposition of extracellular matrix (ECM) proteins, leading to fibrosis and scarring of the kidney tissue. TGF-β signaling is a key driver of this fibrotic process. Activation of TGF-β stimulates the production of ECM proteins such as collagen and fibronectin, while inhibiting the breakdown of ECM, thereby promoting fibrosis. Epithelial-to-Mesenchymal Transition (EMT): EMT is a process in which epithelial cells lose their characteristic features and acquire a mesenchymal phenotype, allowing them to migrate and contribute to fibrosis. TGF-β signaling has been shown to induce EMT in renal tubular epithelial cells, thereby promoting renal fibrosis. Inflammation: TGF-β signaling can modulate the immune response in the kidney by regulating the production of pro-inflammatory and anti-inflammatory cytokines. Dysregulation of TGF-β signaling can lead to chronic inflammation, which contributes to the progression of CKD. Renal Hypertrophy: TGF-β has been implicated in the development of renal hypertrophy, which is a common feature of CKD. Activation of TGF-β signaling can stimulate the proliferation of renal cells, leading to hypertrophy of the kidney. In summary, the present study demonstrates that AA functions as a Smad7 agonist and inhibits Smad3 signaling by inducing Smad7; whereas NG is a Smad3 inhibitor and blocks Smad3 signaling directly by inhibiting Smad3 phosphorylation and transcription. Thus, the combination of AA and NG produces an additive effect on inhibition of renal fibrosis by inhibiting Smad3 while upregulating Smad7 and may represent as a novel and effective therapy for chronic kidney disease including diabetic and hypertensive nephropathy. The current understanding of the molecular mechanisms of TGF-β/Smads in renal fibrosis and inflammation in CKD has enabled us to develop specific therapeutic strategies for CKD. In general, Smad3

is a downstream key mediator of TGF-β/Smad signaling and plays a pathogenic role in renal fibrosis by upregulating miR-21 and miR-192, but downregulating miR-29 and miR-200 families to mediate renal fibrosis (Fig. 2). By contrast, Smad2 is renoprotective and suppresses renal fibrosis by competitively inhibiting Smad3 signaling. Smad4 is the common Smad and plays a diverse role in promoting Smad3-mediated renal fibrosis but suppresses NF-κB-driven renal inflammation by transcriptionally stimulating Smad7 expression. Most importantly, Smad7 is an inhibitor of both Smad3-mediated renal fibrosis and NF-κB-driven renal inflammation. In CKD, TGF-β/Smad signaling is imbalanced with highly activation of Smad2 and Smad3 but at a loss of Smad7. Therefore, therapies aimed to restore the balance of both Smad and NF-κB signaling pathways by overexpressing Smad7 or target Smad3-dependent miRNAs related fibrosis such as overexpression of miR-29 while inhibiting miR-21 may represent novel and effective therapeutic strategies for CKD. Given the central role of the TGF-β/Smad pathway in the pathogenesis of CKD, targeting this pathway has emerged as a potential therapeutic strategy for the treatment of CKD and associated complications. However, due to the complexity of the pathway and its involvement in various physiological processes, targeted therapies must be carefully designed to avoid adverse effects. Additionally, further research is needed to fully elucidate the role of TGF-β signaling in CKD and to develop effective therapeutic interventions.

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