TGF-β/Smad signaling in chronic kidney disease: Exploring post-translational regulatory perspectives (Review)

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Abstract. The TGF-β/Smad signaling pathway plays a pivotal role in the onset of glomerular and tubulointerstitial fibrosis in chronic kidney disease (CKD). The present review delves into the intricate post-translational modulation of this pathway and its implications in CKD. Specifically, the impact of the TGF-β/Smad pathway on various biological processes was investigated, encompassing not only renal tubular epithelial cell apoptosis, inflammation, myofibroblast activation and cellular aging, but also its role in autophagy. Various post-translational modifications (PTMs), including phosphorylation and ubiquitination, play a crucial role in modulating the intensity and persistence of the TGF-β/Smad signaling pathway. They also dictate the functionality, stability and interactions of the TGF-β/Smad components. The present review sheds light on recent findings regarding the impact of PTMs on TGF-β receptors and Smads within the CKD landscape. In summary, a deeper insight into the post-translational intricacies of TGF-β/Smad signaling offers avenues for innovative therapeutic interventions to mitigate CKD progression. Ongoing research in this domain holds the potential to unveil powerful antifibrotic treatments, aiming to preserve renal integrity and function in patients with CKD.

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Contents

1. Introduction

Chronic kidney disease (CKD) poses a significant challenge to healthcare systems, affecting an estimated 8-15% of the global population (1,2). This condition is signified by the gradual deterioration of kidney function over time, culminating in end-stage renal disease, which requires treatment through dialysis or kidney transplantation (3). Fibrosis originates from kidney damage stemming from a range of factors such as diabetes, hypertension, glomerular diseases, toxins, infections and autoimmune conditions, resulting in excessive accumulation of the extracellular matrix (ECM), which in turn disrupts the normal tissue structure (4,5). At present, no treatments have received approval to specifically address the fibrotic process in an effort to prevent, arrest or reverse CKD (6). The limited efficacy observed in addressing kidney fibrosis underscores the need for a comprehensive review of its foundational mechanisms and the exploration of emerging therapeutic targets.

Transforming growth factor-β1 (TGF-β1) signaling is intrinsically associated with the advancement of renal fibrosis (RF) in CKD (5). Extensive animal research underscores TGF-β1 as the key pathogenic driver, propelling both glomerular and tubulointerstitial fibrosis (5). TGF-β1 signaling is regulated at several stages to ensure homeostasis (7). This

encompasses TGF-β activation, the creation, activation and breakdown of functional TGF-β receptor complexes, the modulation of Smad both in activation and degradation, and the assembly of Smad transcription complexes (7). These complexes co-operate with various DNA-binding transcription factors and coregulators at gene regulatory sequences (8). During these procedures, the hyperactivation of TGF-β1/Smad3 signaling may occur, which subsequently causes tubular dysfunction, interstitial fibroblast proliferation, inflammation and augmented ECM deposition (8). Reestablishing the balance of TGF-β1 signaling offers a compelling antifibrotic strategy to halt CKD progression (7). In this context, the present study aimed to delve deeper into the complex functions of the TGF-β/Smad pathway in detail, examining its influence on biological activities such as renal tubular epithelial cell apoptosis, inflammation, myofibroblast activation, cellular aging and its involvement in autophagy (Fig. 1). Of particular emphasis in the present review, recent findings regarding the roles of post-translational modifications (PTMs) including but not limited to phosphorylation, ubiquitination, SUMOylation, and acetylation (Fig. 2) in determining the strength and duration of TGF-β/Smad signaling were comprehensively summarized.

2. Pathogenic role of TGF-β signaling in CKD

Activation of TGF-β signaling. TGF-β ligands comprise three distinct isoforms, namely TGF-β1, TGF-β2 and TGF-β3 (9). These isoforms are ubiquitously distributed across various cellular and tissue contexts, with TGF-β1 being the most prevalent (10). TGF-β ligands initially emerge as precursor proteins, which undergo a cleavage process at the N-terminal region (11). The cleavage process results in the formation of the latency-associated peptide, which stays bound to the mature TGF-β homodimer at the C-terminal (Fig. 1). This complex association with the latent TGF-β-binding proteins ensures that TGF-β remains inactive, commonly termed latent TGF-βs (12,13). To become biologically active, these latent forms require the intervention of certain environmental triggers such as specific enzymes or an acidic milieu (5). In the extracellular environment, associations with the ECM and subsequent cleavage by various proteases, including plasmin and specific matrix metalloproteinases, facilitate the liberation and activation of the TGF-β ligands (11).

Upon engagement with the TGF-β1 ligand, the TGF-β receptor (TGF-βR) II triggers the activation of TGF-βRI through phosphorylation (5). This series of activations subsequently culminates in the activation of Smad2/3 transcription factors through phosphorylation, commencing the standard signaling process (5). Integral to this cascade is Smad4, which associates with Smad2/3 after phosphorylation, directing the Smad2/3/4 complex towards the nucleus (7) (Fig. 1). This migration to the nucleus is a pivotal step for transcribing genes, which includes key genes such as NADPH oxidase 4 (NOX4), connective tissue growth factor (CTGF) and others [receptor interacting protein kinase 3 and proto-oncogene tyrosine-protein kinase Src (Src)] involved in tissue repair and cellular regulation (14-21). Furthermore, Smad7, an inhibitory molecule, becomes active in response to Smad3, and engages in competitive binding with TGF-βRI, thereby hindering the phosphorylation process of Smad2 and Smad3 (22). The transcriptional modulation orchestrated by Smad3-containing complexes is influenced by an array of non-Smad co-activators, including the transcriptional coactivator p300, activator protein 1 and yes-associated protein, as well as co-suppressors such as SKI-like proto-oncogene (SKI) and Ski-like oncoprotein N (SnoN) (23-26). The transcriptional effects of TGF-β1 necessitate the involvement of these accessory factors, driving structural alterations in Smad2/3 to interact effectively with target motifs (8). In addition, TGF-β1 activates an array of pathways independent of Smad signaling, which contribute to its biological activities. These routes include TGF-β-activated kinase 1, phosphatidylinositol 3-kinase/Akt and Rho-like GTPase signaling pathways (27,28).

TGF-β and myofibroblast activation. Activation of myofibroblasts and the ensuing accumulation of ECM are pivotal events in RF (29). The activated myofibroblast serves as the key driver of RF, given its significant capacity to produce the majority of the matrix (29). While myofibroblasts are scarce under normal conditions, their numbers surge in fibrotic kidneys (7,29). Proposed precursors for myofibroblasts include pericytes, cells of epithelial and endothelial origin, circulating cells derived from bone marrow and local fibroblasts (30-35). For epithelial cells, research has clarified the fibrosis-promoting influence of TGF-β1, emphasizing the critical roles of key molecules in the Smad signaling pathway, such as TGF-βRI, TGF-βRII and Smad3, in epithelial-to-mesenchymal transition (EMT) (36-39). Furthermore, a number of miRNAs and long non-coding RNAs (lncRNAs) have been identified that are reliant on Smad3 function in different capacities to regulate EMT (7,40-42). For circulating bone marrow-derived cells, mounting evidence indicates that macrophages originating from bone marrow can directly transition into myofibroblasts (MMT) (43,44). In fibrotic kidneys, the recruited Smad3-deficient macrophages do not differentiate into myofibroblasts (44). Additionally, a series of factors transcriptionally regulated by Smad3, such as Pou4f1, P2Y12 and Src, have been proven to be involved in the MMT process (20,45-48). This suggests that the progression of MMT is closely governed by the TGF-β/Smad3 signaling pathway. For endothelium, in models of RF triggered by either folic acid or unilateral ureteral obstruction, the specific reduction of TGF-βRII levels in endothelial cells led to a mitigation of fibrotic remodeling and an inhibition of endothelial-to-mesenchymal transition (49). For fibroblasts, researchers discovered that half of the myofibroblasts arise from the proliferation of local resident fibroblasts, while an additional 35% originate from bone marrow fibroblasts (50). The TGF-β pathway appears instrumental in this process, as evidenced by the fact that specific deletion of TGF-βRII in α-smooth muscle actin (+) cells leads to a marked decrease in fibroblast numbers (50). Furthermore, initiating the conditional deletion of Smad2 in fibroblasts under the influence of the fibroblast-specific protein 1 promoter, diminishes RF in streptozotocin (STZ)-triggered diabetic nephropathy (DN) (51). In summary, the TGF-β/Smad pathway plays a crucial role in guiding cellular dynamics and transitions vital for RF.

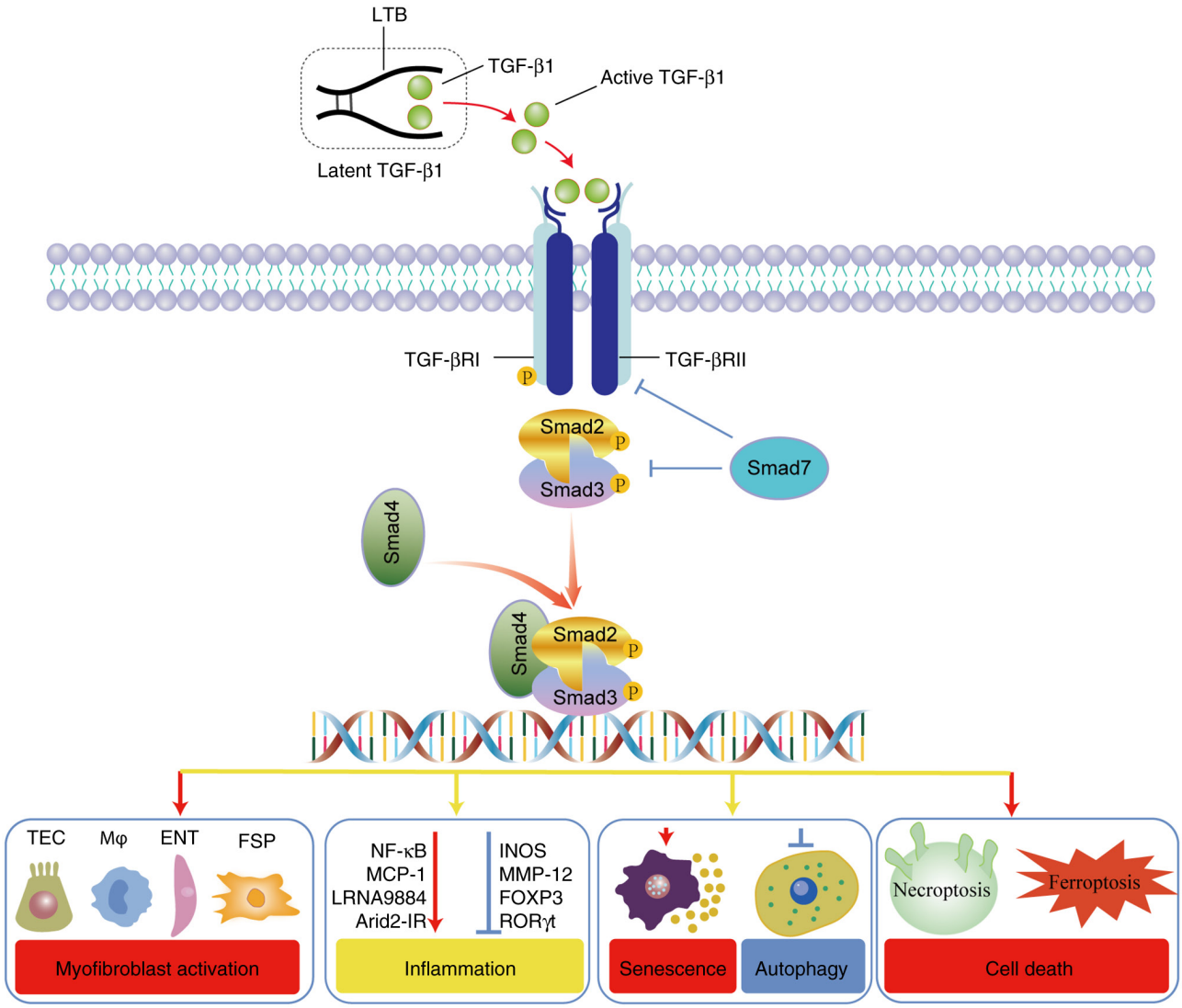

Figure 1. Schematic graph illustrating the TGF-β/Smad signaling pathway and its roles in the regulation of various biological processes during chronic kidney disease. TGF-β ligands, initially synthesized as latent forms, bind to TGF-βRII upon release. This interaction recruits and activates TGF-βRI, leading to the phosphorylation of intracellular Smad2/3. Subsequently, phosphorylated Smad2/3 forms a complex with Smad4, which then translocates to the nucleus to regulate gene transcription. Conversely, Smad7 disrupts TGF-β signaling by competing with Smad2/3 for TGF-βRI and facilitating TGF-βRII degradation. Red arrows or symbols denote pathogenic mechanisms or pathways of positive regulation, whereas blue lines or symbols signify protective mechanisms or pathways of negative regulation. TGF, transforming growth factor; TGF-βR, TGF-β receptor; TEC, tubular epithelial cell; Mφ, macrophage; ENT, endothelial cells; FSP, fibroblast; Arid2-IR, AT-rich interaction domain 2 intronic transcript; MCP-1, macrophage chemotactic protein-1; iNOS, inducible nitric oxide synthase; MMP-12, matrix metalloproteinase-12; Foxp3, forkhead box P3; RORγt, retinoid acid receptor-related orphan receptor γt.

Renal inflammation. Sterile inflammation, characterized by the inflammatory response devoid of any infectious agents or specific immunogens, serves as a key trigger for the development of RF (52). TGF-β is instrumental in the formation, balance, diversification and tolerance of immune cells (53,54). Diminishment of TGF-β1 can result in the hyperactivation of immunocytes and trigger the occurrence of autoimmune diseases, a phenomenon noted in mice lacking either TGF-β1 or its receptors. In such cases, excessive inflammatory responses with massive lymphocyte and macrophage infiltration were observed in many organs, primarily in the heart and lungs (55,56). Consequently, broadly blocking upstream TGF-β signaling might exacerbate renal inflammation.

Smad3 acts as a central regulator in renal inflammation, uniquely modulating its dynamics by either inhibiting or promoting the functions of macrophages and T cells (8). As a crucial effector molecule, Smad3 is involved in the TGF-β1-driven suppression of macrophage activation, as demonstrated by its capability to inhibit the regulatory actions of the inducible nitric oxide synthase and matrix metalloproteinase-12 promoters within these cells (57). Furthermore, Smad3 has a critical function in preserving the equilibrium between Treg and Th17 immune responses. This is verified by the observation that a lack of Smad3 results in diminished forkhead box P3 induction, while simultaneously enhancing Th17 cell generation by inhibition of retinoid acid receptor-related orphan receptor γt transcriptional activity within both controlled (*in vitro*) and living (*in vivo*) contexts (58). Conversely, Smad3 potentially facilitates macrophage recruitment during renal inflammation through its chemotactic effects, as it has been found to engage with macrophage chemotactic protein-1 (MCP-1), in turn amplifying the

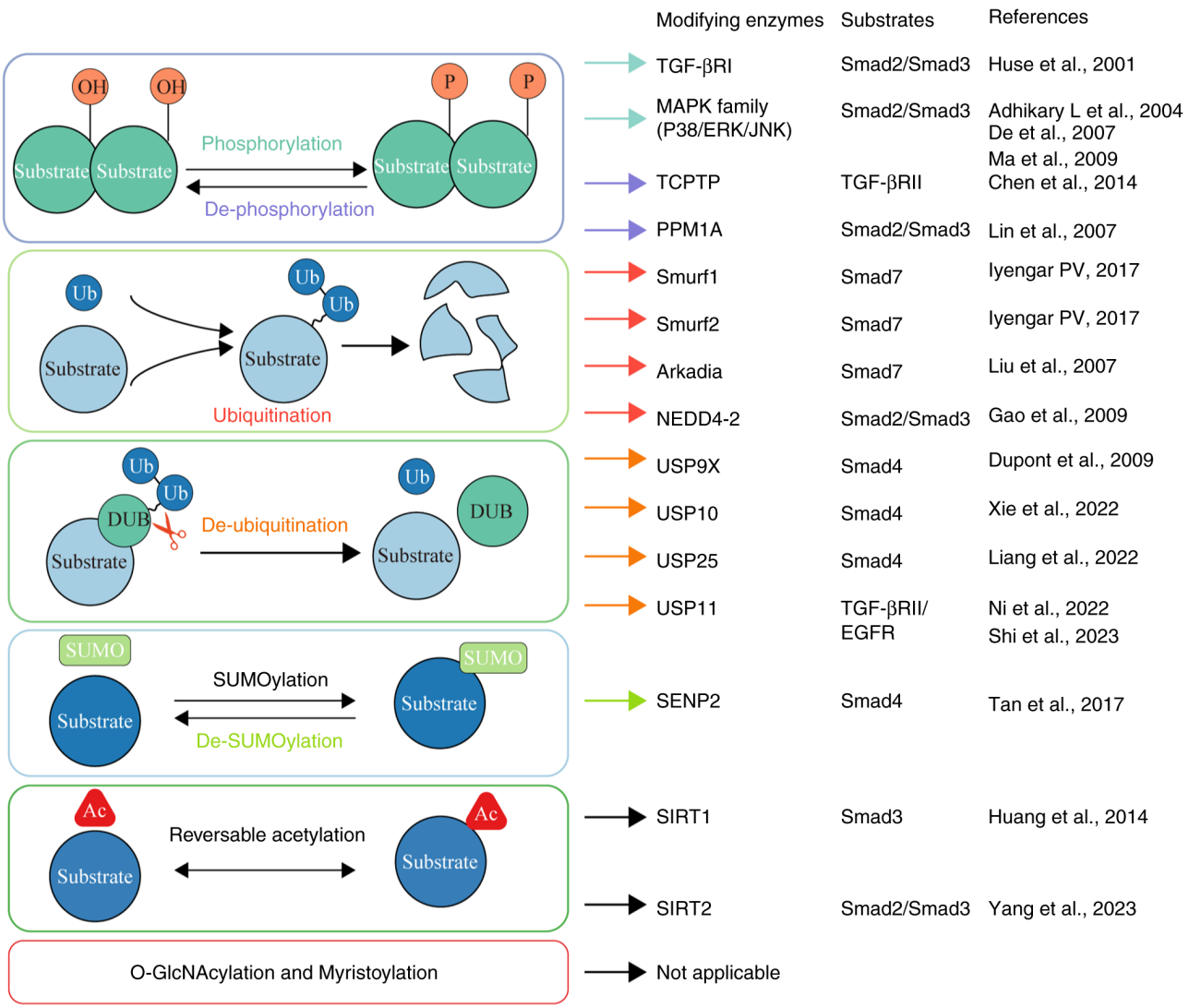

Figure 2. Manner of post-translational modifications in modulating TGF-β/Smad signaling during chronic kidney disease. The figure depicts the key enzymes involved in these modification processes and their respective substrates, which are crucial proteins in the TGF-β/Smad pathway. TGF, transforming growth factor; TGF-βR, TGF-β receptor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; TCPTP, T cell protein tyrosine phosphatase; PPM1A, protein phosphatase magnesium-dependent 1A; Smurf, Smad ubiquitin regulatory factors; NEDD4-2, neural precursor cell expressed developmentally down-regulated 4-2; USP, ubiquitin-specific protease; SENP2, SUMO specific peptidase 2; SIRT, sirtuin deacetylase; EGFR, epidermal growth factor receptor.

renal inflammation driven by macrophages (59). Furthermore, LRNA9884, a lncRNA that is transcriptionally regulated by Smad3 has been shown to influence DN in db/db mice by stimulating the synthesis of MCP-1, further amplifying renal inflammation (60). Additionally, in obstructed nephropathy, AT-rich interaction domain 2 intronic transcript, facilitated by TGF-β/Smad3, enhances the inflammation triggered by IL-1β via the NF-κB pathway, without influencing the fibrosis modulated by the TGF-β/Smad3 process (61). Notably, Smad7 serves as a negative regulator of TGF-β/Smad signaling (62). Previous investigations have further demonstrated that Smad7 functions as a key adjuster by stimulating IκBα, a suppressor of NF-κB, consequently mitigating renal inflammation (63,64). The observed phenomenon suggests that a deficiency in Smad3 inhibits renal inflammation, which is driven by NF-κB in the unilateral ureteral obstruction (UUO) model (65). Presumably, the removal of Smad3 inhibits the breakdown of Smad7 by E3 ubiquitin-protein ligases, such as Smad ubiquitin regulatory factor (Smurf)1/Smurf2 (66). In summary, Smad3 has been identified as a key controller in renal inflammation, orchestrating various molecular interactions and pathways to either amplify or mitigate inflammatory responses.

Cellular senescence and autophagy. Cellular senescence describes the process wherein cells lose their ability to replicate and permanently exit the cell cycle after repeated duplications (67). These senescent cells resist apoptosis and consistently release a diverse secretome, termed the senescence-associated secretory phenotype, which includes pro-inflammatory and pro-fibrotic mediators (67). In recent studies, cellular senescence in renal tubular epithelial cells has been identified as a primary contributor to the onset of RF, and consequently, delaying this senescence presents an effective intervention to curb RF and offers a crucial strategy for decelerating the progression of CKD (67-69). A previous study indicates that the TGF-β/Smad pathway

promotes cellular senescence by reducing histone 4 lysine 20 tri-methylation through miR-29, impacting DNA repair and genome stability (70). Furthermore, a recent study has demonstrated that ubiquitin-specific protease 11 (USP11) promotes cellular senescence and fibrosis regulated by the Smad/P53 complex, by inhibiting the ubiquitination of TGF-βRII (71). In the context of the aging kidney, heightened stimulation of the TGF-β/Smad3 pathway in podocyte-specific TGF-β overexpressing mice leads to cell senescence through processes that involve transference of p16 and initiation of p21 (72).

Autophagy, a preserved lysosomal pathway, facilitates the degradation of cytoplasmic constituents (73). Yet, its role in kidney fibrosis, whether protective or pathological, remains ambiguous (73). Moreover, the exact mechanisms and signaling pathways that govern autophagy responses across various kidney cell types and disease spectra require further elucidation (73). Recent studies highlighted the influence of Smad3 on autophagy and its prospective role as a treatment focus for fibrotic diseases. A study has demonstrated that Smad3 contributes to lysosomal depletion by inhibiting transcription factor EB-mediated lysosome biogenesis, resulting in impaired autophagy during the advancement of DN (74). Moreover, TGFβ, through an epigenetic mechanism that involves the Smad3-mediated decrease of histone acetyltransferase KAT8 (also termed as MYST1), activates autophagy which promotes fibrotic diseases, including dermal and pulmonary fibrosis, suggesting a potential therapeutic target (75).

Cell death. Preventing the death of renal tubular epithelial cells is crucial in halting the progression of CKD (76). It is widely acknowledged that TGF-β is known to facilitate cell death by the interruption of the cell cycle at its G1 phase, orchestrated through the Smad pathway (77-79). A previous study revealed that Smad3 contributes to acute kidney injury (AKI) by directly interacting with cyclin-dependent kinase inhibitor proteins p21 and p27 (78). This interaction results in the death of renal tubular epithelial cells (TECs) due to G1 phase cell cycle arrest (79). Moreover, a recent study has indicated that Smad3 can also transcriptionally activate the receptor-interacting protein kinase 3/mixed lineage kinase domain-like protein necroptosis pathway, subsequently resulting in the death of TECs (21).

Ferroptosis, a regulated form of cell death induced by oxidative stress and dependent on iron-mediated lipid peroxidation, is intricately linked with numerous renal and fibrotic diseases, including AKI, CKD and diabetic kidney diseases (80,81). However, the precise mechanisms driving RF through ferroptosis are yet to be fully understood. Recently published studies demonstrated that Smad3 induces ferroptosis in TECs, primarily through the modulation of NOX4 gene transcription (18,82,83). It also works in conjunction with activating transcription factor 3 (ATF3) to suppress the gene expression of solute carrier family 7 member 11 (SLC7A11), thereby modulating the ferroptosis process (84). In light of these findings, the present research group has made some interesting discoveries. Preliminary investigations have revealed that active natural compounds exhibit specificity towards key components involved in the ferroptosis process. For example, tectorigenin has been found to specifically target Smad3, regulating NOX4 and thereby inhibiting the ferroptosis pathway, suggesting a potential therapeutic role against RF (18). Formononetin, on the other hand, suppresses the binding of the SMAD3/ATF3 complex, promoting the expression of SLC7A11 and highlighting its potential as a novel modulator for ferroptosis in RF (84). Lastly, kaempferitrin has demonstrated the ability to bind with NOX4, leading to an improvement in tubular ferroptosis within the kidneys, proposing a new approach to the treatment of RF through its impact on ferroptosis (83). These promising steps forward provide valuable insights into how natural compounds can potentially be utilized in the modulation of ferroptosis and treatment of RF.

3. PTMs of TGF-β1 signaling

PTMs are chemical alterations essential for regulating protein functions (85). They modulate the activity, localization, stability, and interactions of proteins with other cellular components, including other proteins, nucleic acids, lipids and cofactors (85). PTMs can occur on amino acid side chains or at the C- or N-termini of proteins (85). These modifications enhance the chemical diversity of the 20 standard amino acids, either by altering an existing functional group or introducing novel ones such as phosphate (85). Common PTMs include (de-)phosphorylation, (de-)ubiquitination, (de-)SUMOylation, reversible acetylation and O-GlcNAcylation (86). In the present study, related reports of PTMs in the TGF-β/Smad signaling pathway and their roles in TGF-β/Smad signal transduction are discussed.

Phosphorylation and de-phosphorylation. Phosphorylation is a process occurring after protein translation, characterized by the addition of a phosphate group to certain amino acids in a protein, typically serine, threonine or tyrosine residues (87). Normally, Smad2 and Smad3, which are among the receptor-regulated Smads (R-Smads), undergo activation via ligand-induced phosphorylation at two serine residues within their carboxy-terminal SSXS motif, mediated by TGF-βRI (88,89). Beyond the established TGF-β signaling pathway involving Smad2 and Smad3, the TGF-β/Smad signaling pathway is further influenced by multiple kinases, providing further refinement, expansion or modulation of the signaling output (90). The mitogen-activated protein kinase (MAPK) family, comprising three primary kinases including p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), has been shown to be activated in humans with both acute and CKD, as indicated by a number of studies (91-93). In addition, through employing pharmacological and genetic interventions, the blocking of p38 and/or JNK effectively mitigates RF across diverse animal studies (94-96). Notably, each of the three MAPKs are capable of phosphorylating specific sites within the linker regions of both Smad2 and Smad3 (97). These regions are situated in the interdomain space that separates the mad homology domain 1 (MH1) and MH2 domains within a Smad protein, subsequently altering the transcription of Smad3-dependent genes (97). Hence, besides being activated by TGF-β1, Smad3 activation can be triggered by various stress-inducing agents such as angiotensin II (Ang II), advanced end products (AGE) and C-reactive protein (CRP) through MAPK-dependent

pathways. Specifically, research reveals that Ang II has the capability to directly stimulate Smad3, thereby promoting the upregulation of both CTGF and collagen I via the AT1-ERK/p38 MAPK interaction route (98). In addition, there is a strong link between AGEs and the expression of CTGF, and EMT. Through the Smad3 pathway that functions independently of TGF-β1, introducing AGEs induces CTGF expression in TECs devoid of TGF-β1, evidenced by the swift activation of Smad2/3, ERK1/2 and p38 (99). Furthermore, CRP, reported as an inflammatory marker and mediator, is known to regulate the fibrotic process, primarily by modulating the CD32b-ERK/p38 MAPK pathway, subsequently activating Smad3 (100).

Protein dephosphorylation, mediated by protein phosphatases, serves as a key control process that influences the operation of a number of proteins within signal transduction routes (87). Chen *et al* (101) discovered that loss of integrin α1β1, a regulator for collagen synthesis, exacerbated RF in a UUO model via a TGF-β/Smad-dependent manner. In terms of the mechanism, integrin α1β1 promotes the recruitment of the phosphatase, T cell protein tyrosine phosphatase (TCPTP) to TGF-βRII which results in the dephosphorylation of tyrosine residues in the TGF-βRII cytoplasmic tail, subsequently impairing TGF-βR-dependent fibrotic signaling transmission (101). In addition, protein phosphatase magnesium-dependent 1A (PPM1A) facilitates the dephosphorylation of TGF-β-activated Smad2/3 within their carboxy-terminal SSXS motif, subsequently promoting their nuclear export (102). In obstructive and aristolochic acid-induced nephropathy, a decrease in PPM1A levels within the tubulointerstitium has been noted and this diminution plays a role in enhancing Smad3 phosphorylation, leading to subsequent RF (103). In obstructive nephropathy, maxacalcitol, an analog of vitamin D, enhances the function of the PPM1A/vitamin D receptor complex, resulting in the dephosphorylation of Smad3, thereby reducing tubulointerstitial fibrosis (104). Furthermore, PPM1A and PTEN collaboratively work to diminish the phosphorylation of Smad3 and the activation of genes associated with fibrosis (105).

Ubiquitination and deubiquitination. In all organ tissues, intracellular proteins undergo continuous turnover through degradation and synthesis (106). The ubiquitin-proteasome system (UPS) primarily degrades intracellular proteins (106). This multi-enzyme process covalently attaches ubiquitin to a substrate protein, which the proteasome, a core proteolytic complex of the UPS, then recognizes and degrades (106).

Ubiquitination alters different elements within the TGF-β signaling pathway (87). The Smurfs, identified as the first ubiquitin E3 ligases for Smads, exemplify such ubiquitin E3 ligases (107). Extensive research has focused on these ligases, which specifically act upon R-Smads, Smad7 and certain Smad-associated proteins for proteasomal degradation (108). Smurf1 and Smurf2 are both E3 ubiquitin ligases for Smad7 (87). Additionally, Smurf2 is capable of initiating the polyubiquitylation and degradation of Smad2, as well as the Smad gene silencing cofactors SKI and SnoN within kidneys undergoing fibrosis (107,109). This is evidenced by the fact that the overexpression of Smurf2 has been shown to encourage the activation of the TGF-β-responsive promoter and induce EMT within human kidney cortical epithelial cells (107). Recent research has demonstrated that flavin-containing monooxygenase 2 can enhance the expression of Smurf2 and facilitate its nuclear translocation, resulting in tubular cell fibrogenesis and paracrine secretion (110). In addition, the E3 ligase, Arkadia/RNF111, regulates TGF-β signaling by degrading Smad7 (111,112). This degradation consequently fostered the advancement of fibrosis in a rat model of tubulointerstitial fibrosis (111,112). Overexpression of latent TGF-β1 has been shown to ameliorate DN through the inhibition of Arkadia-induced Smad7 imbalance, which subsequently provoked renal inflammation and tissue fibrosis in type 1 diabetes mice induced by STZ (113). In addition, neural precursor cell expressed developmentally down-regulated 4-2 (NEDD4-2) has been identified as the ubiquitin ligase that facilitates proteasome-mediated degradation of TGF-β-induced phosphorylated Smad2/3 (114,115). Deficiency of NEDD4-2 in mice results in progressive kidney injury, marked by fibrosis, tubular epithelial cell apoptosis and various characteristics of CKD, including dilated/cystic tubules, and elevated expression of kidney injury markers (116). In conclusion, the UPS may either promote or inhibit fibrotic outcomes, depending on the TGF-β signaling elements that undergo degradation.

Deubiquitination, the counterpart to ubiquitination, is a key cellular procedure encompassing the removal of ubiquitin molecules that have been added to proteins (117,118). This dynamic interplay between ubiquitination and deubiquitination ensures the precise regulation of protein function, stability and interactions within the cell (117). Deubiquitinating enzymes, also known as deubiquitinases (DUBs), constitute a vast group of proteases responsible for removing ubiquitin from proteins (117). Moreover, they aid in the creation of independent entities from freshly translated polyubiquitins and repurpose ubiquitins after the breakdown of polyubiquitinated protein substrates (119). While the control of TGF-β signaling through ubiquitination has been widely studied in the past decades, the function of deubiquitination steered by DUBs in the TGF-β signaling pathway, especially in the context of CKD, has only begun to gain attention recently.

PR-619, a comprehensive DUB inhibitor, mitigated fibrosis in mice undergoing UUO and in rat kidney fibroblast cells, namely NRK-49F cells, triggered by TGF-β1 (120). Furthermore, PR-619 demonstrates an inhibitory effect on Smad4 levels, while it does not affect the production of TGF-βR, Smad2 or Smad3 (120). This indicates that DUBs may regulate fibrosis by modulating Smad4 (120). At present, members of the ubiquitin specific proteases family (USPs), which have been reported to regulate Smad4 deubiquitination, include USP9X (121), USP10 (122), USP13 (123), USP17 (124) and USP25 (125). USPs are currently known as the most extensive and predominant family of enzymes associated with deubiquitination (117). In fact, USP9X is documented to suppress fibrosis triggered by the stimulation of AGEs in mesangial cells, as well as EMT in renal tubular cells (126,127). USP10 has been reported to counteract renal impairment caused by sepsis, primarily by reducing apoptosis in TECs and mitigating oxidative stress (128). Furthermore, recent research has confirmed that USP25 is instrumental in advancing hypertensive renal disease (125). Knockout of USP25 in mice has been shown to reduce kidney malfunctions

and fibrotic conditions (125). From a mechanistic viewpoint, USP25 is associated with the regulation of TGF-β signaling activation (125). Specifically, USP25 functions by reducing Smad4 K63-linked polyubiquitination (125). For R-Smads and Smad7, although some DUBs have been reported to regulate their ubiquitination processes (129-133), their functions in CKD remain to be further elucidated.

Additionally, several DUBs, such as 26S proteasome-associated PAD1 homolog 1 (134) and ubiquitin C-terminal hydrolase 37 (131), have been reported to regulate the deubiquitination process of TGF-βRI, thereby promoting TGF signaling. However, only USP11 has been documented to modulate the fibrotic process in CKD (135,136). Ni *et al* (71) conducted an intervention using the USP11 inhibitor mitoxantrone on mice experiencing both UUO and folic acid-triggered RF. They discovered that this intervention could inhibit TGF-βRII expression and associated fibrotic and aging phenotypes. The use of USP11 conditional knockout mice further confirmed this phenomenon (135). It is noteworthy that these DUBs often have multiple substrates. For instance, USP11 can regulate the deubiquitination of both TGF-βRII and epidermal growth factor receptor (EGFR) (135,136).

SUMOylation and de-SUMOylation. SUMOylation, a process where SUMO covalently attaches to specific protein targets, plays a pivotal role in modulating signal transduction through changes in subcellular localization, influencing enzymatic activity and directing the ubiquitin-mediated breakdown of its target substrates (137). This modification process is driven by a series of enzymes requiring ATP, encompassing the E1 activator, the E2 conjugator known as Ubc9 and various E3 ligases (138). Notably, the function of SUMOylation in the TGF-β signaling pathway is attracting more and more interest (139).

To date, researchers have identified five SUMO proteins, labeled SUMO1-5 (140). Given the considerable sequence resemblance between SUMO2 and SUMO3, they are commonly categorized as SUMO2/3. Among these, SUMO1 and SUMO2/3 are expressed ubiquitously, while the distribution of SUMO4 is confined to specific organs, such as the spleen and kidney (137). The ability for SUMOylation to be reversed is maintained by SUMO-specific proteases (SENPs), which can detach SUMO proteins from their targets (108). Currently, seven distinct SENPs, ranging from SENP1-3 to SENP5-8, have been identified (141,142). Apart from reversing SUMOylation, these enzymes also mature pro-SUMO into a conjugatable form (142).

Within the scope of TGF-β signaling, the SUMOylation process of TGF-βR1 amplifies its capability to interact with Smad3, which promotes the phosphorylation of Smad3 (139). SENP2 counteracts this alteration, and an upsurge in SENP2 expression curtails the EMT instigated by TGF-β (143). The role of Smad4 SUMOylation in TGF-β transcription regulation remains contentious. Some researchers posit a detrimental effect of Smad4 SUMOylation on TGF-β signaling, highlighting that the K113R/K159R mutation curtails the polyubiquitination of Smad4 (144). In the context of renal mesangial cells under high glucose conditions, the SUMO2/3-driven SUMOylation of Smad4 triggers the TGF-β/Smad pathway, subsequently elevating fibronectin levels (145). These contrasting perspectives might arise from distinct cellular contexts (146). Conclusively, adjusting the (de-)SUMOylation of the TGF-β/Smad pathway presents a hopeful approach for CKD treatment.

Reversible acetylation. Protein acetylation is recognized as a significant and reversible post-translational modification, underscoring its various cellular and physiological activities (147). Reversible acetylation is orchestrated by two primary enzyme classes: Acetyltransferases (KATs) and deacetylases (KDACs) (147). KATs enable the addition of acetyl groups onto lysine residues and encompass the general control non-derepressible 5, p300 and MYST families, along with other unclassified KATs (147). Although KATs primarily acetylate histones, enzymes such as p300 also influence the TGF-β/Smad pathway, and are also recognized for enhancing TGF-β activity through the acetylation of Smad2 or Smad3 (148,149). Furthermore, the inhibition of p300 with a novel FATp300 inhibitor, L002, mitigates RF caused by hypertension and opposes fibrogenic responses in fibroblasts (150).

Unlike KATs, KDACs are divided into two main categories: Classical histone deacetylases, which are Zn^{2+}-dependent, and sirtuin deacetylases (SIRTs), which rely on NAD^{+} (147). In recent studies, seven mammalian counterparts of the yeast Sir2, specifically labeled as SIRT1 to SIRT7, have been discerned (151). Within the kidney, SIRT1 stands out as the predominant SIRTs under investigation. Primarily localized in the nucleus, it orchestrates the acetylation patterns of nucleosome histones and influences the dynamics of multiple transcriptional regulators (151). As a result, SIRT1 plays a pivotal role in cellular defense by mitigating processes such as apoptosis, inflammation and fibrosis (151). SIRT1 deficiency promotes Smad3 acetylation, subsequently activating TGF-β signaling and exacerbating the progression of CKD (152). Resveratrol intervention facilitates the interaction between SIRT1 and Smad3, thereby attenuating Smad3 acetylation (153). Moreover, elevating SIRT1 levels in tubular cells impedes the transition from AKI to tubulointerstitial fibrosis (151). This also curtails the subsequent accumulation of matrix metalloproteinase-7 in the kidney through the deacetylation of Smad4 (154). Hence, SIRT1 emerges as a promising candidate for therapy in treating CKD (152). Unlike SIRT1, SIRT2 predominantly resides in the cytoplasm and plays a role in hindering fibrosis within renal tubules (155). The specific removal of SIRT2 from TECs aggravates RF, while its deliberate overexpression in these cells reduces RF (155). In terms of mechanism, SIRT2 forms a direct association with Smad2 and Smad3, leading to their deacetylation; this interaction subsequently mitigates the fibrotic effects triggered by TGF-β (155). This highlights the therapeutic potential of SIRT2 in addressing fibrosis. In the context of CKD, there are limited reports on the regulatory roles of other SIRT family members, specifically SIRT3-7, concerning the TGF-β/Smad pathway. To sum up, the complex equilibrium and interaction between these enzymes underscore their potential as therapeutic targets in CKD and fibrotic conditions.

O-GlcNAcylation. O-GlcNAcylation, a fluctuating and reversible type of PTM, entails the addition of a β-D-N-acetylglucosamine (GlcNAc) molecule onto serine or

threonine residues within proteins (156). This modification, predominantly occurring within the cytoplasm and nucleus, is distinct from the conventional N- and O-glycosylations that take place in the endoplasmic reticulum and Golgi apparatus (156). The enzymes O-GlcNAc transferase and O-GlcNAcase are key in controlling O-GlcNAcylation, which is vital for numerous cellular functions such as signal transduction and transcription, as well as maintaining protein stability (156,157). Due to its significance, changes in O-GlcNAcylation are linked to various diseases including diabetes, neurodegenerative conditions and cancer (158-160).

A previous study has indicated that an increase in O-GlcNAcylation levels in kidney tissues following UUO, and that glucosamine-driven O-GlcNAcylation can promote fibrosis in the renal parenchymal cell, HK2 TECs (161). Notably, a previous study observed that Smad4 has been identified as a newly discovered protein undergoing O-GlcNAc modification in human lung cancer cells. In this context, O-GlcNAc hinders the linkage between Smad4 and GSK-3β, interactions that are crucial for the proteasomal breakdown of Smad4 (162). Nevertheless, the precise function of O-GlcNAcylation in fibrosis, along with its interplay with the TGF-β1/Smad3 signaling pathway remains to be further elucidated.

Myristoylation. Myristoylation, a post-translational modification process, involves covalently linking myristate (a 14-carbon fatty acid) to the N-terminal glycine residue of the protein via an amide bond (163). This modification is critical in numerous protein signaling systems as it imparts various effects such as modulating protein stability, facilitating protein-protein interactions and enhancing subcellular localization to organelles or the plasma membrane (163).

There is relatively scant research focusing on the role of myristoylation in the progression of CKD. Notably, a previous study noted that myristoylated TGF-βRI and TGF-βRII can induce transcriptional activation of Smad2, suggesting a potential role for myristoylation in the activation of the TGF-β pathway (164). In addition, myristoylation may exert an indirect influence on the regulation of the TGF-β signaling pathway. For instance, the myristoylation of PPM1A could enhance the phosphatase activity of PPM1A, as promoted by the cellular senescence-inhibited gene, thereby inhibiting TGF-β signaling further (165). This suggests that the modulation of myristoylation states could represent a novel strategy for managing TGF-β-driven processes in CKD.

4. Perspectives

The TGF-β/Smad signaling pathway stands as a primary contributor to RF in CKD (5). Its intricate post-translational regulation dictates both the intensity and temporal specificity of the signaling (90). A myriad of PTMs, encompassing phosphorylation, ubiquitination, acetylation and SUMOylation, govern the stability, activity and interplay of TGF-β receptors, Smad proteins, and associated co-regulators (87,90). This fine-tunes the fibrotic signaling cascade. Delving into these multifaceted post-translational mechanisms offers profound insights, paving the way for innovative antifibrotic strategies to combat CKD progression (87,90).

However, despite these insights, there remain several considerable limitations that hinder a comprehensive understanding. A complete grasp of individual PTMs and how each modification precisely regulates this process is still lacking, necessitating further exploration into each PTM to clarify their specific roles and impacts on overall signaling. In addition, the intricate and complex interactions among various PTMs and their collective influence on TGF-β/Smad signaling presents a challenging aspect that is not yet fully understood due to several reasons. First, the crosstalk between different PTMs can be complex and context-dependent, making it difficult to predict the net effect on Smad signaling (87). Second, many PTMs can target multiple proteins within the pathway, further complicating the overall picture (136,155,166). Finally, technological limitations can hinder our ability to comprehensively analyze these interactions and their dynamic regulation within cells (167). Despite these challenges, unraveling these complexities holds immense promise for developing more targeted therapeutic strategies for fibrotic diseases. More research is needed to elucidate these dynamic relationships and create a more holistic view of the signaling network. Furthermore, despite advances in proteomics techniques, current approaches are limited in their ability to uncover disease-specific regulations in patient-derived samples (168). The development and application of more sophisticated tools could enable a finer resolution of these regulatory intricacies, providing invaluable insight into disease progression and response to treatment (168). Moreover, the current landscape of therapeutic strategies lacks personalization, with most approaches not considering the unique progression patterns of CKD in different individuals (167). This represents a significant gap in effectively managing and treating CKD. Lastly, inefficiencies in existing drug delivery systems, particularly those targeting the kidneys, pose another challenge by reducing therapeutic efficacy and leading to potential side effects (169). Existing kidney-targeting drug delivery systems face challenges related to nanoparticle size. Small nanoparticles (<6-8 nm) can pass through the glomerular filtration barrier but are quickly cleared by urine, limiting their use for sustained drug delivery (170). Larger nanoparticles (350-400 nm) may accumulate in the kidneys but struggle with bioavailability and filtration (170). Additionally, the protein corona on nanoparticles can reduce targeting efficiency, complicating effective treatment (170).

Emerging research horizons include finding ways to translate cellular insights into physiologically relevant disease models, elucidating the interconnections between diverse PTMs, broadening the spectrum of known post-translational regulators and leveraging advanced proteomics techniques to decode previously concealed, disease-specific regulations in patient-derived samples. A more detailed and stage-specific understanding of TGF-β/Smad signaling regulation could pave the way for personalized therapeutic strategies tailored to individual CKD progressions, thus enhancing CKD treatment outcomes and preserving renal architecture and functionality in patients.

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Authors' contributions

The manuscript was initially drafted by JL and YZ. It was then edited and revised by JK, HS, LW and ND. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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