Published in final edited form as:
*J Mol Med (Berl)*. 2013 May ; 91(5): 549–559. doi:10.1007/s00109-012-0983-z.

# Molecular targets for treatment of kidney fibrosis

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## Abstract

Renal fibrosis is the culmination of processes driven by signaling pathways involving transforming growth factor (TGF)-β family of cytokines, connective-tissue growth factor, nuclear factor κB, Wnt/β-catenin, Notch, and other growth factors. Many studies in experimental animal models have directly targeted these pathways and demonstrated efficacy in mitigating renal fibrosis. However, only a small fraction of these approaches have been attempted in human and even fewer have been successfully translated to clinical use for patient with kidney diseases. Drugs with proven efficacy for treatment of kidney diseases and tissue fibrosis exert some of their effects by interfering with components of these pathways. This review considers key molecular mediators of renal fibrosis and their potential as targets for treatment of renal fibrosis.

### Keywords

kidney; fibrosis; HIPK2; signaling pathways; treatment

## Introduction

Renal fibrosis is considered the final convergent pathway for progressive kidney diseases due to a wide range of pathophysiologically distinct processes [1]. The extent of renal fibrosis is not only a marker of injury, but also predicts the loss of function and progression of damage in the kidney [2–4]. Renal fibrogenesis involves cells from all compartments of the kidney (tubulointerstitial, glomerular, and vascular) as well as bone marrow-derived cells recruited to the kidney (i.e. mononuclear cells and fibrocytes). Although much has been learned of the molecular mechanisms underlying renal fibrogenesis, there is still a paucity of success in translating this knowledge to clinical application. Reviews of therapeutic targets in renal fibrosis have been undertaken in the past [5, 6]. The scope of this review is to summarize key molecular pathways driving renal fibrogenesis and to highlight novel molecular mediators of renal fibrosis. For detail description of cellular and molecular mechanisms of renal fibrosis readers are referred to recent reviews [7, 8].

## Molecular mediators of renal fibrogenesis

Fibrosis is considered an abnormal wound healing response to tissue injury where the balance between self-limited repair and exuberant accumulation of extracellular matrix (ECM) has been tipped in favor of the latter. The pathogenesis of renal fibrosis has been depicted as a continuum of four overlapping phases: *priming, activation, execution and progression* [8] (Figure 1). In the *priming* phase, tissue injury triggers an inflammatory response at the site of injury to recruit lymphocytes, monocytes/macrophages, dendritic

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cells, and mast cells. Nuclear factor (NF) κB is a key driver of this inflammatory response. NFκB signaling in tubular epithelial cells is triggered by CTGF [9], angiotensin II [10], aldosterone [11], or proteins from tubular fluid [12]. Activation of NFκB signaling drives the production of pro-inflammatory molecules such as plasminogen activator inhibitor (PAI)-1 [13], interleukin (IL)-1 [14], IL-6 [15], chemokine (C-C motif) ligand 2 (CCL2; also known as monocyte chemotactic protein 1) [16, 17], CCL5 [17], and tumor necrosis factor (TNF) α [17] by the injured tubular epithelial cells. Injured tubular cells also release Danger Associated Molecular Pattern molecules, which exert their effects on neighboring tubular epithelial cells and inflammatory cells through toll-like receptors to promulgate innate immune response by increasing the production of pro-inflammatory mediators and recruitment of leukocytes [18]. A profibrotic role has been ascribed to infiltrating $CD4^+$ lymphocytes [19], $CD3^+$ lymphocytes [20], M1-type macrophages [21, 22], and fibrocytes [23]. However, not all infiltrating cells are profibrotic: regulatory T cells [24], M2-type macrophages [22], and mast cells [25] have been shown to mitigate renal fibrosis.

In the *activation* phase, profibrotic cytokines generated by injured tubular cells and inflammatory cells contribute to the activation of matrix-producing cells. Although multiple cell types are capable of producing extracellular matrix (ECM), renal interstitial fibroblast is considered the principal source of matrix production. A subpopulation of activated fibroblasts, called myofibroblasts, display increased proliferative activity and acquire the expression of α-smooth muscle actin (α-SMA) [26]. In renal fibrosis, cells from different origins contribute to the pool of myofibroblasts: renal interstitial fibroblasts [27]; bone-marrow-derived fibrocytes [28]; vascular pericytes [29]; and endothelial [30] and tubular [31] cells that had undergone transdifferentiation and acquired a mesenchymal phenotype (Figure 2). Tubular epithelial cells have the capacity to acquire a mesenchymal cell phenotype (i.e. epithelial-to-mesenchymal transdifferentiation, EMT) in the injured kidney [32], but whether tubular cells with mesenchymal marker expression can fully differentiate into interstitial myofibroblasts *in vivo* and how the process contributes to the pathogenesis of renal fibrosis has been debated [7, 33].

In the *execution* phase, myofibroblasts produce ECM. Although the accumulation of matrix proteins, such as fibronectin, and type I and III collagen, is a prominent feature of fibrosis, it is probably not the sole factor contributing to the progressive loss of renal function associated with renal fibrosis. Tubular cell apoptosis and atrophy, lymphocytes and macrophages infiltration, tubular epithelial cells and endothelial cells transdifferentiation, and peritubular vasculature rarefaction are also present in the fibrotic kidney and could also contribute to the progressive loss of renal function [7, 34].

In the *progression* phase, there is a shift from normal wound healing to over-exuberant inflammatory response resulting in the undesirable consequence of fibrosis and functional loss. Although the molecular tipping point that dictates this shift is still unclear, the duration and severity of injury are likely major determinants whether a successful repair is possible and dysregulated tissue fibrosis is averted.

## Molecular targets for treatment of renal fibrosis

Several key mediators of kidney fibrosis were identified based on their contribution to fibrosis in other tissue types and/or involvement in EMT during development. These mediators have been targeted for treatment of renal fibrosis in human (Table 1). Here, we summarize key molecular pathways of renal fibrosis and highlight new molecular mediators of renal fibrosis (Figure 3).

## Transforming Growth Factor (TGF)-β and bone morphogenetic protein (BMP) Signaling

The TGF-β family of structurally related cytokines includes TGF-βs, activins and BMPs [35]. Several lines of evidence suggest that TGF-β-induced activation of downstream Smad signaling contributes to kidney fibrosis [36, 37]: the expression of TGF-β and TGF-β receptor is increased in human and experimental models of chronic kidney disease [38, 39]; overexpression of active TGF-β in mice promotes the development of glomerulosclerosis and tubulointerstitial fibrosis [40]; and inhibition of TGF-β activity attenuates renal disease in experimental models of renal fibrosis [41–45]. TGF-β released from injured tubular cells or inflammatory cells promotes tissue fibrosis by increasing ECM gene transcription, tubular cell apoptosis, ECM turnover, myofibroblast differentiation, and EMT depending on the cellular context of TGF-β action [39].

TGF-β exerts its action by promoting dimerization of type I and type II TGF-β receptors and subsequent phosphorylation of Smad2 and Smad3 [46]. Phosphorylated Smad2 and Smad3 complex with Smad4 in the cytosol then translocate into the nucleus to regulate gene expression. Alternatively, phosphorylated Smad2 and Smad3 can also complex with a subgroup of inhibitory Smads (Smad6 and Smad7) to recruit Smad ubiquitination regulatory factor ubiquitin ligases to the ligand-receptor complexes and initiate receptor degradation [47]. The role of TGF-β/Smad signaling in renal fibrosis, however, is likely more complex than the canonical TGF-β/Smad pathway described above. For instance, Smad2 and 3 can be directly activated by advanced glycation endproducts and angiotensin II through ERK/p38 mitogen-activated protein kinases in a TGF-β-independent manner [48–51]. Furthermore, TGF-β can control the action of several microRNAs (-192 [52–55], -29 [56], -216a [57], -21 [58], -377 [59]) that are involved in the regulation of ECM gene expression and/or EMT [60, 61].

Multiple approaches to block TGF-β-mediated response by blocking TGF-β directly or intracellular mediators of TGF-β-signaling have been investigated in animal models and small clinical trials. Approaches to abrogate TGF-β-mediated responses include direct targeting of TGF-β using antibodies, soluble receptors, or natural TGF-β-binding proteins (e.g. decorin); inhibition of TGF-β receptor using small molecular inhibitors; activation of inhibitory Smads (e.g. Smad7); enhancement of BMP signaling; and targeting of microRNAs that are regulated by TGF-β [5, 61]. In addition to its profibrotic action, TGF-β also possesses anti-inflammatory properties [62–64]. Therefore, direct and complete blockade of TGF-β could have serious effects on the immune system making it undesirable as a therapeutic approach. However, it has been suggested that the complexity of TGF-β regulation could afford specific antifibrotic targets without affecting immune-regulatory functions of TGF-β [6].

Several drugs with unintended anti-TGF-β activity have been studied in human kidney diseases. Two antifibrotic drugs with anti-TGF-β activity, tranilast [65, 66] and pirfenidone [67, 68], have demonstrated modest benefit in animal models of renal fibrosis and small pilot studies of diabetic kidney diseases. Tranilast treatment of rats with subtotal nephrectomy, a model of renal mass reduction leading to progressive renal fibrosis, resulted in an improvement of proteinuria, renal function, and histologic features of renal fibrosis [69]. A study of nine patients with advanced diabetic nephropathy, who demonstrated a progressive decline in renal function despite receiving renin angiotensin aldosterone system (RAAS) blockade, when treated with tranilast had a slower decline in renal function than before treatment [65]. Another study of ten patients with early diabetic nephropathy treated with tranilast for a year found that urinary albumin and type-IV collagen excretion after tranilast treatment were significantly reduced compared to pre-treatment values [66]. Interestingly, the PRESTO trial, a large randomized trial on effects of tranilast in preventing major cardiovascular events and restenosis after percutaneous coronary intervention,

reported elevation in serum creatinine as an adverse event [70]. Although results from small studies are encouraging, studies with a focus on renal outcomes are needed to ascertain tranilast's efficacy in renal fibrosis.

Pirfenidone belongs to a class of drugs, called the pyridones, which exhibit anti-TGF-β activity and have established safety and efficacy for treatment of pulmonary fibrosis and cirrhosis. Pyridones is effective in preventing the development of fibrosis in unilateral ureteral obstruction [71] and subtotal nephrectomy [72] animal models. In a single open-label study of 18 patients with refractory focal segmental glomerulosclerosis who received pirfenidone for 12-months, the rate of renal function decline before enrollment was significantly more than when on pirfenidone [67]. More recently, in a randomized, double-blinded, placebo-controlled trial of 77 patients with diabetic nephropathy, pirfenidone improved renal function at 1 year when compared to placebo [73]. However, no significant reduction in proteinuria was observed in the pirfenidone group. Studies with longer follow up and clinically relevant endpoints are needed to confirm the benefit of pirfenidone in fibrotic renal diseases.

Drugs that inhibit the renin angiotensin and aldosterone system (RAAS) also exhibit anti-TGF-β activity. These drugs have demonstrated efficacy for diabetic and non-diabetic kidney diseases when studied in well-conducted, long-term, randomized trials. RAAS blockade mitigates the progression of chronic kidney disease and proteinuria [74–78]. Inhibitors of RAAS abrogate pro-fibrotic effects of angiotensin-II [79], which include induction of TGF-β and TGF-β receptor levels in the kidney [80–82], enhancement of CTGF expression [83–85], and augmentation of pro-inflammatory mediators through NFkB signaling in renal tissue [86]. The salutary effects of RAAS blockers on renal fibrosis, however, are not solely attributable to and likely extend beyond their anti-TGF-β action [87].

BMP belongs to the TGF-β superfamily of cytokines. BMP7 counteracts TGF-β-induced EMT and kidney fibrosis [88]. BMP7 exerts its action by binding to activin-like kinase (ALK) 3 and ALK 6 type I serine-threonine kinase receptors and activating intracellular Smad-dependent signaling pathway [85]. Activation of ALK3 receptor using a small peptide agonist of BMP signaling suppressed inflammation, apoptosis and EMT program and reversed renal fibrosis in models of acute and chronic renal injury [89]. Targeting of BMP7-ALK3 signaling in human with renal fibrosis has not been reported.

## Connective-tissue growth factor (CTGF) signaling

CTGF is a heparin-binding glycoprotein of the CCN (CYR61/CTGF/NOV) family of intercellular signaling proteins. CTGF antagonizes BMP signaling and enhances TGF-β signaling by preventing BMP binding to BMP receptors and facilitating TGF-β binding to TGF-β receptors, respectively [90]. Since TGF-β is a potent inducer of CTGF and correlative studies in human and animals with tissue fibrosis support a link between CTGF and TGF-β, most models postulate that CTGF acts either downstream or cooperatively with TGF-β [91, 92]. CTGF expression is upregulated in kidneys of human renal disease and not expressed in normal kidneys. Furthermore, the level of expression correlates with the severity of renal fibrosis [93, 94]. Reduction of CTGF in murine models of kidney disease prevents renal fibrosis [95–97]. FG-3019, a human monoclonal antibody against CTGF, was studied in a small phase-I clinical trial on patients with diabetic nephropathy and found to significantly reduce albuminuria [98]. A phase 2 study of FG-3019 in subjects with type 2 diabetes and kidney disease was recently terminated by the sponsor due to suboptimal study design (ClinicalTrials.gov identifier NCT00913393).

Pentoxifylline is a nonselective phosphodiesterase inhibitor known to inhibit cell proliferation, inflammation, and ECM accumulation [99]. Pentoxyfiylline treatment of rat models of remnant kidney and obstructive nephropathy attenuated renal tubulointerstitial fibrosis, myofibroblast accumulation, and the upregulation of CTGF [100, 101]. Small trials of human subjects with chronic kidney disease treated with pentoxifylline have also shown to reduce proteinuria [102, 103] and significantly decrease the rate of renal function decline [104]. A recent systematic review including 991 patients from 17 trials guardedly concluded that pentoxifylline use may reduce proteinuria in diabetic patients with chronic kidney disease [105].

## NFκB Signaling

Excessive, prolonged inflammation is a major driver of fibrosis [106, 107]. NFκB regulates the expression of multiple genes that play a key role in the inflammatory response during kidney injury. NFκB inhibition tend to attenuate inflammation and tubulointerstitial injury in some, but not all disease models [108]. Although some of the beneficial effects of RAAS blockade in kidney disease have been attributed to the inhibition of angiotensin II-mediated and/or aldosterone-mediated NFκB activation [11] [87], no data exists to support direct NFκB inhibition for treatment of renal fibrosis. Since knockout mice with deficiency of the p65 subunit of the NFκB family member develop liver degeneration and reduction in lymphocyte proliferation [109], complete and direct inhibition of NFκB signaling will likely have serious side effects.

## Wnt/β-catenin Signaling

Wnt proteins are secreted lipid-modified glycoproteins that bind to cell surface receptors to initiate outside-in signaling. Wnt signaling regulates mesenchymal-to-epithelial transdifferentiation that occurs during normal kidney development, which mirrors EMT of tubular cells in the process of renal fibrogenesis [110]. Wnt/β-catenin signaling is active in the developing kidney, silenced in normal adult kidney, and reactivated in diseased adult kidney [111]. In canonical Wnt/β-catenin signaling, binding of Wnt to its transmembrane receptors Frizzled and low density lipoprotein receptor-related protein (LRP) results in activation and phosphorylation of LRP. Activated LRP6 recruits Dishvelled and Axin and inhibits glycogen synthase kinase-3β-mediated phosphorylation and proteosomal degradation of β-catenin. In the presence of Wnt, β-catenin accumulates and regulates gene expression through transcription factor T cell factor (TCF) and/or lymphoid enhancer factor (LEF).

In the obstructive nephropathy model of renal fibrosis, several members of the Wnt family are upregulated in the fibrotic kidney and β-catenin accumulates in the cytoplasm and nuclei of renal tubular epithelial cells [112]. Targeted inhibition of β-catenin-mediated gene transcription using a peptidomimetic small molecule ICG-001 in obstructive nephropathy attenuated fibrotic lesions and abolished TGF-β1-induced expression of profibrotic markers without disrupting Smad Signaling [113]. Targeted inhibition of the Wnt/β-catenin pathway has not been studied in human kidney fibrosis. Although strategies for indirect inhibition of Wnt signaling in renal fibrosis by targeting β-catenin and TCF/LEF interaction or at the receptor level have been proposed [114].

## Notch Signaling

Notch signaling is required for normal kidney development. In a wide variety of kidney diseases, the expression of certain components of Notch signaling pathway correlated with the degree of proteinuria, glomerulosclerosis, tubulointerstitial fibrosis, and estimated glomerular filtration rate [115]. Inhibition of Notch signaling by either pharmacologic or genetic approach reduced the development of fibrosis in experimental animal models of

renal fibrosis [116]. Tubule-specific over-expression of the Notch intracellular domain (NICD) in mice triggered the development of tubulointerstitial fibrosis without affecting expression of key drivers of EMT [116]. Efforts to abrogate Notch signaling in tubular epithelial cells by either genetic deletion or pharmacologic inhibition of Notch activation ameliorated tubulointerstitial fibrosis in murine models [116]. In addition, Notch signaling also plays a role in acute kidney injury and glomerular diseases [117]. Even though suppression of Notch signaling using inhibitors of γ-secretase, which is required for the activation and processing of NICD, have been studied for treatment of solid tumors, their role in human kidney diseases has yet to be explored.

### Other growth factors

Fibroblast growth factor (FGF) -1 and 2 have cell proliferative effects on epithelial cells and fibroblasts. FGF-1 is expressed in infiltrating lymphocytes and macrophages; and the expression of FGF receptor-1 is upregulated in tubules of kidneys with inflammatory disease [118]. FGF-2 is able to induce EMT of tubular epithelial cells [119], which could be the causal link for the correlation between FGF-2 expression and the degree of renal fibrosis observed in human kidneys [120]. However, direct causal relation between FGF2 and renal fibrosis *in vivo* has yet to be demonstrated.

Platelet-derived growth factor (PDGF) isoforms stimulate the replication, survival and migration of myofibroblasts in fibrotic processes including renal fibrosis [121]. Antagonism of the PDGF-D isoform prevented renal fibrosis in an experimental model of glomerulonephritis [122, 123] and anti-PDGF-C treatment reduced fibrosis in obstructive nephropathy [124]. No clinical data exists currently to support using anti-PDGF treatment in renal fibrosis.

Epidermal growth factor receptor (EGFR) signaling mediates fundamental cellular functions such as proliferation, growth, and differentiation. Inhibition of EGFR protected against the development of renal fibrosis in models of hypertensive renal injury and renal mass reduction [125]. EGFR signaling crosstalks with angiotensin II-mediated pathway [126] and is crucial for sustained TGFβ expression [127]. Genetic or pharmacologic blockade of EGFR inhibited the development and progression of renal fibrotic in the rodent model of obstructive nephropathy [128]. Although anti-EGFR pharmacologic agents have demonstrated efficacy in the treatment of certain malignancies, they have not been studied in patients with kidney disease.

Hepatocyte growth factor (HGF) exerts antifibrotic properties in several murine models of chronic kidney disease by binding and inactivating Smad2 [129]. However, other studies suggest that HGF overexpression or its presence in the glomerular ultrafiltration is pro-fibrotic and could contribute to tubular injury [130, 131]. Since HGF also promotes metastasis and tumor growth [132], it is not likely that HGF signaling will be targeted in long-term clinical trials of renal fibrosis.

### Homeo-domain interacting protein kinase (HIPK) 2 signaling

We recently found that HIPK2, a previously unrecognized kinase in the context of kidney disease, is upregulated in murine models of kidney injury and fibrosis as well as human with human immunodeficiency virus associated nephropathy (HIVAN), focal segmental glomerulosclerosis, diabetic nephropathy and IgA nephropathy [133]. HIPK2 is known to interact with transcription factors and functions as both a corepressor and a coactivator depending on the transcription factor and its subcellular localization [134]. HIPK2 interfaces with multiple profibrotic pathways that are known to mediate renal injury and fibrosis (i.e. Notch, Wnt/β-catenin, TGF-β, NFκB and p53) (Figure 3). Kidneys of HIPK2$^{-/-}$ mice

subjected to experimental injury developed less up-regulation of mesenchymal cell markers and activation of Notch, TGF-β, p53, and NFκB signaling. Furthermore, HIPK2$^{-/-}$ mice were protected against the development of renal fibrosis in models of obstructive nephropathy, folic acid nephropathy, and HIVAN, suggesting that HIPK2 regulates the expression of genes engaged in kidney injury and fibrosis. We believe that HIPK2 is a potential therapeutic target for renal fibrosis since HIPK2 signaling impinges on multiple pro-fibrotic pathways and protein kinases as a group are important drug targets. Since HIPK2$^{-/-}$ mice did not exhibit significant phenotype at baseline, we do not anticipate pharmacologic inhibition of HIPK2 to cause significant side effects. However, long-term studies to monitor effects of HIPK2 suppression on immunity and carcinogenesis will need to be performed.

## Conclusion

An overwhelming number of studies targeting key fibrogenic pathways have demonstrated efficacy in mitigating renal fibrosis in experimental models. Only a small fraction of these approaches, however, have been studied in human and even fewer have been successfully translated to clinical use. Medications with proven anti-fibrotic effects impact on these molecular mediators of kidney fibrosis. Complete and direct targeting of these profibrotic mediators, which also regulate other fundamental cellular processes, could have undesirable consequences. However, the complexity of regulation and crosstalk between pathways could afford a therapeutic window. Future efforts to better understand and identify novel molecular regulators of these profibrotic pathways could yield targets with improved therapeutic profile and efficacy.

## Acknowledgments

Due to space limitations, the authors could not individually acknowledge work of many investigators in the field of renal fibrosis. We have cited articles that review multiple publications on a topic and we encourage the readers to use them as sources for further study.

JCH is supported by NIH 1R01DK078897 and 1R01DK088541-01A1 and a Veterans Affairs Merit Review Award 1I01BX000345; PYC is supported by NIH 5K08DK082760.

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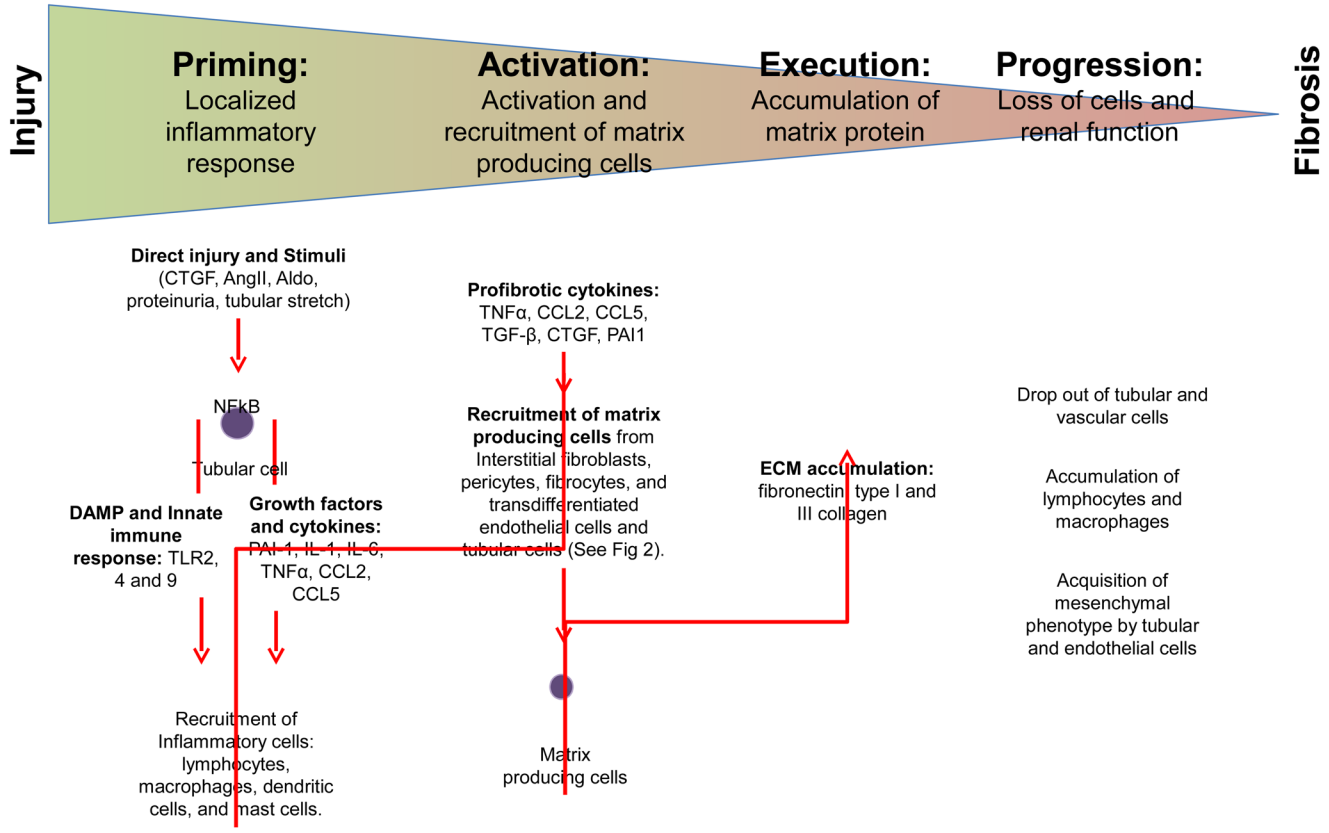


**Figure 1.**

Four overlapping phases of renal fibrosis: priming, activation, execution, and progression. Direct tubular epithelial cell injury or cellular stimuli triggers a pro-inflammatory response involving activation of the innate immune response and production of growth factors and cytokines, which result in the recruitment of inflammatory cells. Localized accumulation of profibrotic cytokines promotes activation and recruitment of matrix-producing cells from different sources. Accumulation of extracellular matrix proteins is observed in renal fibrosis in conjunction with loss of tubular and vascular cells, accumulation of lymphocytes and macrophages, and acquisition of mesenchymal cellular phenotype by tubular and endothelial cells, which are associated with loss of kidney function. CTGF: connective tissue growth factor, AngII: angiotensin II, Aldo: aldosterone, AGEs: advanced glycation endproducts, NFκB: nuclear factor kappa B, TLR: toll-like receptors, DAMP: danger associated molecular pattern molecules, ROS: reactive oxygen species, IL: interleukin, TGF: transforming growth factor, TNF: tumor necrosis factor, CCL: chemokine C-C motif ligand, PAI: plasminogen activator inhibitor, and ECM: extracellular matrix.

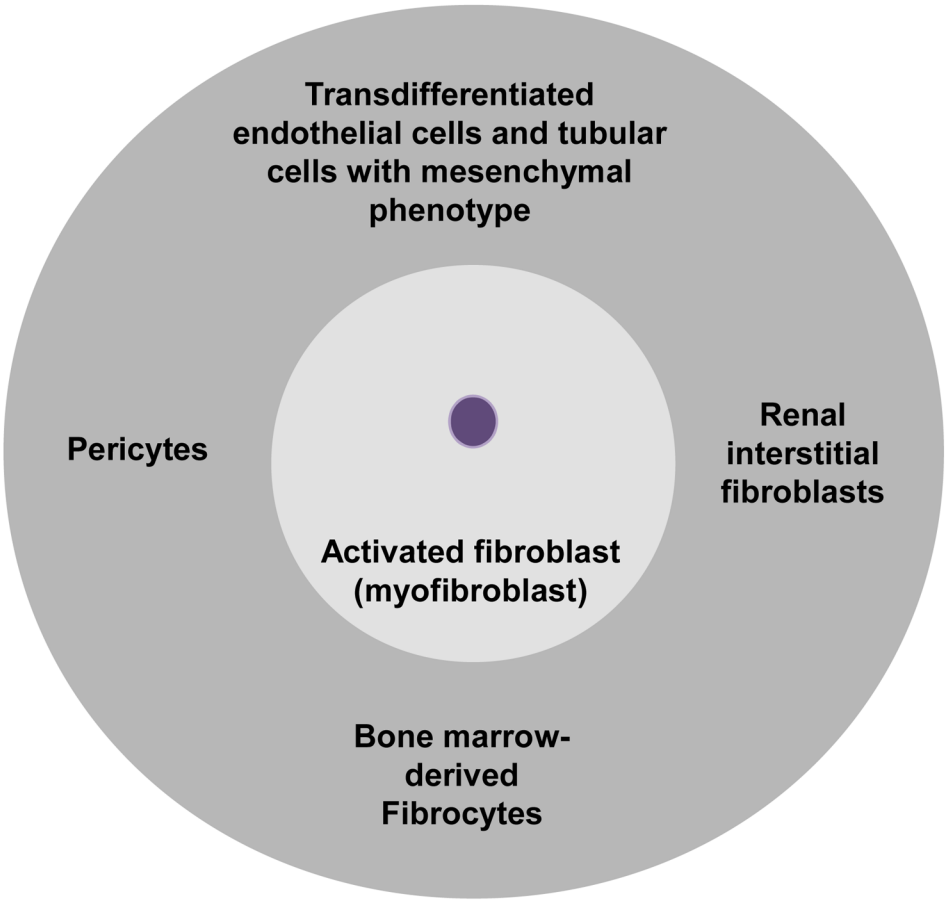


**Figure 2.**
Multiple origins of myofibroblasts in renal fibrosis. Renal tubular interstitial fibroblasts, bone-marrow-derived fibrocytes, vascular pericytes, and transdifferentiated endothelial cells and tubular cells with mesenchymal phenotype have been shown to contribute to the population of myofibroblasts in the fibrotic kidney.

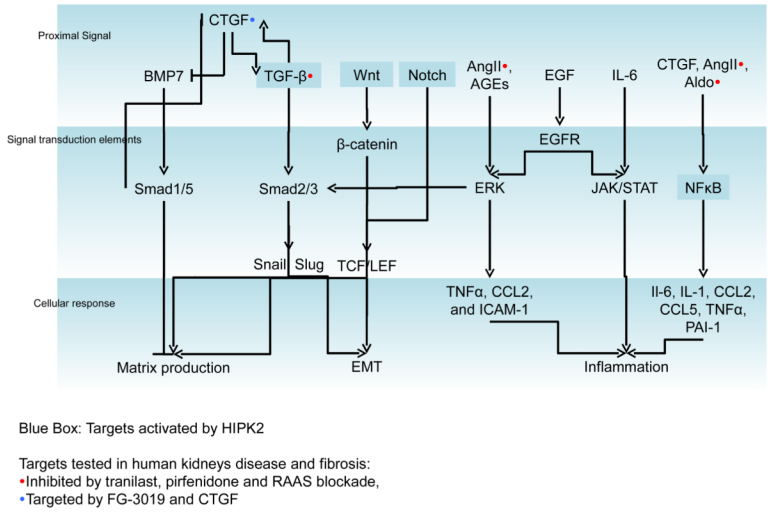


**Figure 3.**

Schematic of key mediators of kidney fibrosis. Proximal signals from growth factors, cytokines and signaling molecules activate cellular signal transduction elements in tubular cells as well as other cell types to promote profibrotic cellular responses that include extracellular matrix production, epithelial-mesenchymal transdifferentiation, and inflammation. Signaling transduction elements are activated by different proximal signals. Homeo-domain interacting protein kinase (HIPK) 2 interfaces with nuclear factor (NF)-κB, Wnt/β-catenin, and Notch signaling components to promote renal fibrosis (highlighted in blue boxes). Approaches to antagonize CTGF, TGF-β, and renin-angiotensin-aldosterone signaling in renal disease and fibrosis have demonstrated clinical efficacy.

## Table 1

Drugs with efficacy in human with renal diseases and fibrosis.

| Drug class/Drug | Human renal diseases | Outcomes studied in human trials | Experimental Models of Renal fibrosis |
|---|---|---|---|
| Tranilast | DKD [65, 66] | UACR, GFR decline, Urinary Type IV Collagen excretion | Sub-total nephrectomy [69] |
| Pyridone: Pirfenidone and fluorofenidone | FSGS [67], DKD [73] | UACR, GFR decline, Urinary TGF-β excretion | UUO [71], Subtotal Nephrectomy [72] |
| Renin-Angiotensin-Aldosterone system blockade | Diabetic, Non-diabetic, and Glomerular kidney diseases [74–78] | UACR, GFR decline | Subtotal Nephrectomy [135, 136] |
| Anti-CTGF monoclonal antibody (FG-3019) | DKD [98] | UACR | UUO [96, 97], DKD[95] |

DKD: Diabetic kidney disease, FSGS: focal segmental glomerular sclerosis, CKD: chronic kidney disease, IgAN: IgA nephropathy, GFR: glomerular filtration rate, UUO: unilateral ureteral obstruction, UACR: urinary albumin to creatinine ratio.