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## Cellular and molecular mechanisms of renal fibrosis

**Youhua Liu**

Department of Pathology, University of Pittsburgh, S-405 Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA 15261, USA

Youhua Liu: liuy@upmc.edu

### Abstract

Renal fibrosis, particularly tubulointerstitial fibrosis, is the common final outcome of almost all progressive chronic kidney diseases. Renal fibrosis is also a reliable predictor of prognosis and a major determinant of renal insufficiency. Irrespective of the initial causes, renal fibrogenesis is a dynamic and converging process that consists of four overlapping phases: priming, activation, execution and progression. Nonresolving inflammation after a sustained injury sets up the fibrogenic stage (priming) and triggers the activation and expansion of matrix-producing cells from multiple sources through diverse mechanisms, including activation of interstitial fibroblasts and pericytes, phenotypic conversion of tubular epithelial and endothelial cells and recruitment of circulating fibrocytes. Upon activation, matrix-producing cells assemble a multicomponent, integrin-associated protein complex that integrates input from various fibrogenic signals and orchestrates the production of matrix components and their extracellular assembly. Multiple cellular and molecular events, such as tubular atrophy, microvascular rarefaction and tissue hypoxia, promote scar formation and ensure a vicious progression to end-stage kidney failure. This Review outlines our current understanding of the cellular and molecular mechanisms of renal fibrosis, which could offer novel insights into the development of new therapeutic strategies.

### Introduction

An estimated 13% of the adult population in the USA has some degree of chronic kidney disease (CKD),<sup>1,2</sup> and a considerable proportion of cases eventually progress to end-stage kidney failure, a devastating condition that requires lifelong dialysis or kidney transplantation. Numerous epidemiological studies indicate that the prevalence of patients with end-stage renal disease is increasing worldwide.<sup>1,3,4</sup> As a result, CKD has become a major public health problem on a global scale, which imposes enormous socioeconomic burdens on the affected individuals, families and societies. As the population of patients with diabetes mellitus and obesity continues to grow, it is highly probable that this trend of increasing prevalence of CKD will not come to an end in the foreseeable future. Despite the enormity of this problem, current therapeutic options for CKD in the clinical setting are scarce and often ineffective. An approved treatment specifically targeted to renal fibrosis is almost nonexistent. In this context, an improved understanding of the cellular and molecular

### Competing interests

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mechanisms of renal fibrosis is paramount and essential, not only for gaining novel insights into the pathogenesis of the process, but also for developing rational strategies to treat patients with fibrotic kidney disorders.

Renal fibrogenesis is considered, by and large, to be a failed wound healing process that occurs after the initial insults of various injuries.<sup>5,6</sup> In this regard, the cellular and molecular responses in the injured kidneys are largely dictated by evolutionarily conserved defense programs of wound healing in an attempt to repair and recover from damage. Almost all the cell types in the kidneys, including fibroblasts, tubular epithelial cells, pericytes, endothelial cells, vascular smooth muscle cells, mesangial cells and podocytes, as well as the infiltrated cells such as lymphocytes, macrophages and fibrocytes, are involved and participate in some way in the pathogenesis of renal fibrosis, which illustrates the immense complexity of this process.<sup>7,8</sup> The scope of this Review is limited to cellular and molecular mechanisms of tubulointerstitial fibrosis, which is the final outcome of all progressive kidney diseases. This focus precludes any detailed discussion of the pathogenesis of glomerulosclerosis. However, key events in tubulointerstitial fibrosis also take place in the glomeruli after injury, which include glomerular infiltration of inflammatory cells, myofibroblastic activation of the mesangial cells and epithelial–mesenchymal transition (EMT) of the podocytes.<sup>9–11</sup> Therefore, in many aspects, major fibrogenic mechanisms are common and shared by different tissue compartments in the kidneys.

Major cellular events in tubulointerstitial fibrosis include: infiltration of inflammatory cells; fibroblast activation and expansion from various sources; production and deposition of a large amount of extracellular matrix (ECM) components; and tubular atrophy and microvascular rarefaction (Figure 1). Conceivably, each and every one of these pathologic features could contribute to the relentless progression of fibrosis in its own unique way. Together, they constitute a core set of fibrogenic events that result in the ultimate destruction of renal parenchyma and loss of kidney function. On the basis of the sequence of these destructive events, the pathogenesis of renal fibrosis can be artificially divided into four overlapping phases: priming, activation, execution and progression.<sup>5,12</sup> However, such a division is arbitrary, as in reality renal fibrogenesis is a dynamic process in which many of these events occur concomitantly.

## Priming

Tissue injury provokes inflammation by creating pro-inflammatory niches in which the concentration gradients of chemotactic cytokines provide a directional signal for guiding the infiltration of inflammatory cells to the injured sites.<sup>13</sup> Renal fibrosis is almost always preceded by the infiltration of inflammatory cells, including lymphocytes, monocytes/macrophages, dendritic cells and mast cells. Although inflammation is an integral part of the host defense mechanisms in response to injury, nonresolving inflammation is a major driving force in the development of fibrotic disease.<sup>14,15</sup> Following injury, infiltrated inflammatory cells become activated, produce molecules that damage tissues such as reactive oxygen species (ROS), and induce the production of fibrogenic cytokines and growth factors.<sup>16–19</sup> This series of events builds up sustained profibrotic cytokine pressure within the local microenvironment and primes fibroblasts and tubular epithelial cells to

undergo phenotypic activation or transition and to produce a large amount of ECM components. Therefore, inflammation after a sustained injury serves as a primer that sets up the fibrogenic stage and triggers tissue fibrogenesis.

### Inflammation sets up the fibrogenic stage

The close correlation between inflammation and the extent of fibrosis has long been established and is the subject of several reviews published in the past 2 years.<sup>17–21</sup> A consensus exists that inflammation as a whole has a crucial role in the initiation of renal fibrogenesis after injury, although it is becoming clear that the role of inflammation in the progression of fibrosis is more complicated than previously thought.<sup>19,20</sup> Recruitment and activation of T lymphocytes could be an important early event that mediates the onset of renal fibrogenesis, as it typically precedes the influx of macrophages into the injured kidneys. Indeed, mice that lack mature B and T lymphocytes owing to a deficiency of V(D)J recombination-activating protein 1 (RAG1) are protected against fibrosis after obstructive injury.<sup>22</sup> Similarly, depletion of CD4<sup>+</sup> T cells in wild-type mice considerably reduces the extent of interstitial fibrosis, whereas reconstitution with purified CD4<sup>+</sup> T cells in RAG1-knockout mice restores fibrogenesis after injury.<sup>22</sup> These findings clearly establish a critical role for lymphocytes, specifically CD4<sup>+</sup> T cells, in the onset of renal fibrosis.

The contribution of macrophages to renal fibrogenesis is well documented.<sup>18,20</sup> When monocytes from circulating blood are recruited to the injured site in response to cytokine cues, they differentiate into two broad but distinct subsets of macrophages that are categorized as either classically activated (M1) or alternatively activated (M2).<sup>19,23</sup> In general, M1 macrophages display a typical proinflammatory phenotype, produce a variety of chemokines, as well as ROS, and therefore have pathogenic functions that lead to tissue damage and fibrosis. Accordingly, depletion of macrophages is beneficial and ameliorates renal fibrosis after various injuries, while adoptive transfer of macrophages aggravates the fibrotic lesions, demonstrating their profibrotic roles in renal fibrogenesis.<sup>23–25</sup> In addition to the number of macrophages, the activation status of infiltrating macrophages is another major determinant of injury. For instance, in mice with doxorubicin-induced nephropathy, intravenous infusion of macrophages preactivated by a Toll-like receptor 9 agonist, but not resting macrophages, substantially exaggerates disease progression.<sup>26</sup>

Dendritic cells, which derive from the same bone marrow myeloid progenitors as macrophages, are abundant in normal kidney interstitium.<sup>27–29</sup> As they are antigen-presenting cells, dendritic cells have been shown to create antigenic peptides from albumin through a proteasome-dependent pathway in a remnant kidney model, which consequently activates syngeneic CD8<sup>+</sup> T cells.<sup>30</sup> In a mouse model of proteinuric diseases, renal dendritic cells capture and present filtered antigens to T cells, leading to the production of proinflammatory cytokines. Depletion of dendritic cells is able to attenuate disease progression.<sup>31,32</sup> Altogether, these results indicate that activation of inflammatory lymphocytes, macrophages and dendritic cells is required for the initiation and progression of fibrotic kidney disease, although the role of mast cells in renal fibrogenesis remains controversial and uncertain.<sup>33–35</sup> Consistent with this notion, renal fibrosis can be effectively ameliorated by inhibition of nuclear factor  $\kappa$ B (NF $\kappa$ B) signaling and/or renal

inflammation by a variety of means, such as administration of C–C chemokine receptor type 1 (CCR1) antagonist,<sup>36</sup> CCR2 antagonist,<sup>37</sup> vitamin D receptor activator,<sup>38</sup> peroxisome proliferator-activated receptor  $\gamma$  agonist,<sup>39,40</sup> anti-tumor necrosis factor blocking antibody,<sup>41</sup> hepatocyte growth factor<sup>42,43</sup> or an IL-1 receptor antagonist.<sup>44</sup> Notably, the renal protection elicited by inhibition of angiotensin II is also, at least in part, attributable to its nonhemodynamic, anti-inflammatory effects.<sup>45</sup>

### Mechanistic link of inflammation and fibrosis

The link between inflammation and fibrosis is also consistent with and supported by morphological evidence. As fibrotic foci in the injured kidneys are typically instigated and localized in the tissue surrounding renal blood vessels, an inflammatory microenvironment that is probably facilitated by endothelial dysfunction or activation.<sup>46</sup> Albeit not tested, the concentration of local cytokines in the inflamed area is presumably very high, and as a result resident fibroblasts and neighboring tubular epithelial cells are readily susceptible to fibrogenic activation.<sup>47</sup> The classic view on the connection between inflammation and fibrosis is that they are mediated in a paracrine fashion, whereby inflammatory cells secrete profibrotic cytokines that act on resident fibroblasts and tubular cells to promote fibrogenesis. This assumption is corroborated experimentally, as culture of tubular cells with activated leukocytes *in vitro* induces EMT and matrix production.<sup>48</sup> In addition to the soluble mediators, findings also indicate that direct contact of monocytes and tubular cells might be required to induce tubular EMT by monocytes via an NF $\kappa$ B-dependent pathway.<sup>49</sup>

Evidence is emerging that an intrinsic connection at the molecular level exists between inflammatory signals and fibrosis within the same cells. For instance, activation of NF $\kappa$ B that is dependent on tumor necrosis factor stabilizes Snail1 by blocking its ubiquitin-mediated degradation.<sup>50</sup> As Snail1 is a key transcription factor that promotes EMT, fibroblast migration and renal fibrosis,<sup>51–53</sup> this finding provides a molecular link between inflammatory signaling and fibrosis. Activation of NF $\kappa$ B could directly contribute to fibroblast activation and renal fibrosis, as conditional expression of an inhibitor of  $\kappa$ B dominant-negative transgene in interstitial fibroblasts attenuates renal fibrogenesis.<sup>54</sup> Expression of inhibitor of differentiation 1 (Id1), a dominant-negative transcriptional antagonist, is rapidly and markedly induced in the degenerated kidney tubules after obstructive injury.<sup>55</sup> Although Id1 represses the transcription of E-cadherin and tight junction protein ZO-1, leading to the dedifferentiation of tubular epithelial cells,<sup>55</sup> it also augments NF $\kappa$ B signaling<sup>56,57</sup> and potentiates the expression of CCL5 (also known as RANTES) in tubular cells. The notion that a single molecule such as Id1 promotes both epithelial dedifferentiation and chemokine expression in the same cells illustrates that inflammation and fibrosis are somehow intrinsically connected at the molecular level.

Nonresolving inflammation after chronic injury is a relentless driver of fibrogenesis because it creates a vicious cycle of inflammation, tissue damage and fibrosis. However, in normal wound healing, inflammation is an early beneficial response to injury, and it is spontaneously resolved once the repair and recovery is completed. What factor determines whether inflammation is a physiologic response or a driving force for tissue destruction remains elusive. Nevertheless, studies have begun to shed new light on this issue by

identifying multiple mechanisms that normally ensure the resolution of inflammation.<sup>15,19</sup> As is widely recognized,<sup>19,20,58</sup> inflammatory cells exhibit heterogeneous phenotypes and have incredible functional plasticity, and they also have different roles in the development and recovery of kidney diseases. Unlike M1 macrophages, subsets of M2 macrophages can resolve inflammation and repair injury. In addition, macrophages can switch phenotypes in response to the ever-changing microenvironment and alter their effect from proinflammatory to anti-inflammatory. Along this line, FoxP3<sup>+</sup> regulatory T cells (T<sub>REG</sub> cells) are known to inhibit the inflammatory response and reduce kidney injury,<sup>59</sup> and macrophages modified *ex vivo* by IL-10 or transforming growth factor  $\beta$  (TGF- $\beta$ ) attenuate renal inflammation and structural injury, and preserve kidney function in a mouse model of nephropathy induced by doxorubicin.<sup>60</sup> These new data demonstrating the plasticity of inflammatory cell function call for conceptual and strategic innovations in order to develop effective anti-inflammation therapies for the treatment of patients with CKD. Sorting out 'good' from 'bad' inflammation over the disease models and stages is a daunting task and warrants more and careful investigations.

## Activation

The upsurge of profibrotic cytokine pressure in the inflamed microenvironment after kidney injury inevitably leads to the activation of matrix-producing cells, which is arguably a central event in renal fibrogenesis. Although many cell types in the tubulointerstitium of the kidneys, such as fibroblasts, tubular epithelial cells, vascular smooth muscle cells and a subset of macrophages, are capable of producing ECM, fibroblasts are commonly regarded as the principal matrix-producing cells that generate a large amount of interstitial matrix components, including fibronectin and type I and type III collagens. Activated fibroblasts in diseased kidneys often express  $\alpha$  smooth muscle actin ( $\alpha$ SMA), and are also referred to as myofibroblasts. In this context, one of the fundamental issues in the field is to delineate the origin, activation and regulation of these matrix-producing myofibroblasts.<sup>61–63</sup>

At least five different sources, with diverse mechanisms, have been proposed as contributors to the myofibroblast pool in diseased kidneys (Figure 2). These include activation of interstitial fibroblasts, differentiation of pericytes, phenotypic conversion of tubular epithelial cells and endothelial cells and recruitment of circulating fibrocytes.<sup>64</sup> The relative contribution, and even the very existence, of each particular myofibroblast-generating pathway to renal fibrosis is a matter of intense debate and is highly controversial. This controversy is largely attributable to the inherent difficulty in identifying and tracking fibroblasts owing to the lack of specific markers for this cell type. Two other major problems in determining the contributions of the different pathways are that fibroblasts exhibit enormous phenotypic heterogeneity, probably reflecting their diverse origins, and they change their phenotypes depending on the activation status, localization and stage of renal fibrogenesis.

### Activation of fibroblasts and pericytes

Historically, matrix-producing myofibroblasts were assumed to derive from resident fibroblasts by phenotypic activation after renal injury.<sup>65</sup> Although this concept has lately been challenged, it remains largely authenticated.<sup>62,66</sup> The majority of myofibroblasts could

conceivably originate from local activation and proliferation of interstitial fibroblasts in the injured kidneys.

In normal adult kidneys, fibroblasts are situated in the interstitial space between the capillaries and the epithelia and form a network throughout the renal parenchyma, thereby stabilizing tissue architecture.<sup>27</sup> Morphologically, these cells are stellate shaped and exhibit abundant rough endoplasmic reticulum, collagen-secreting granules and actin filaments. They possess multiple cell processes, which connect them to the tubular and capillary basement membranes.<sup>27</sup> In the resting, quiescent state, interstitial fibroblasts express CD73 (also known as ecto-5'-nucleotidase) in their plasma membrane, and produce erythropoietin.<sup>27,67</sup> They also express platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ),<sup>8,68,69</sup> and fibroblast-specific protein 1 (FSP1; also known as S100A4), a small protein that binds calcium and is associated with the cytoskeleton.<sup>7,70,71</sup> Fibroblasts maintain interstitial matrix homeostasis in physiologic conditions by producing a basal level of ECM components. Upon activation by profibrotic cytokines and mechanical stress *in vivo*, fibroblasts acquire a myofibroblast phenotype by expressing  $\alpha$ SMA and producing a large amount of ECM components. Myofibroblasts also retain FSP1 and PDGFR $\beta$  and express vimentin (an intermediate filament protein) *de novo*. In many respects, myofibroblasts resemble both activated fibroblasts and smooth muscle cells because of their excessive production of matrix components and their expression of  $\alpha$ SMA and contractility, respectively.

Several markers have been used to characterize fibroblasts and myofibroblasts in the kidneys (Table 1). Unfortunately, none of these markers is specific. In addition, they are rarely expressed by all fibroblasts and myofibroblasts or are hardly ever present all the time. Even  $\alpha$ SMA, a classic marker of myofibroblast activation in organ fibrosis of all types,<sup>72,73</sup> is not without problems. The expression of  $\alpha$ SMA is not exclusive to myofibroblasts, as it is also present in vascular smooth muscle cells.<sup>62,66</sup> In addition, not all activated fibroblasts express  $\alpha$ SMA all the time. Although the majority of cells that produce collagen I are shown to also express  $\alpha$ SMA in a collagen I  $\alpha$ 1-green fluorescent protein reporter mouse model,<sup>74</sup> production of  $\alpha$ SMA and matrix components are not always functionally coupled *in vivo*.<sup>75</sup> Despite these problems, extensive studies indicate that the abundance of  $\alpha$ SMA is closely correlated with the severity of renal fibrosis and predicts the decline of kidney function.<sup>65</sup>

Vimentin is another marker for fibroblast activation, as it is expressed *de novo* specifically in the activated but not quiescent fibroblasts of adult kidneys.<sup>27</sup> However, vimentin is also expressed in glomerular podocytes.<sup>76</sup> Similarly, nestin (another intermediate filament protein) could be a useful marker of fibroblast activation,<sup>8</sup> but is also found in glomerular podocytes.<sup>76</sup> Other fibroblast markers have their own drawbacks. For instance, although FSP1 was initially characterized as specific for fibroblasts and myofibroblasts, studies suggest that it also co-localizes with leukocytes.<sup>27,74</sup> With regard to CD73, it is expressed not only in interstitial fibroblasts, but also in several other cell types, including proximal tubular cells, mesangial cells and T cells.<sup>27</sup> PDGFR $\beta$  is found in fibroblasts, myofibroblasts, vascular smooth muscle cells and pericytes.<sup>8,69</sup>

Activated fibroblasts are often characterized by two key features: proliferation and myofibroblastic activation. The latter is illustrated by  $\alpha$ SMA expression and matrix production. Both fibroblasts and myofibroblasts have the capacity to proliferate in response to cytokine cues, which leads to expansion of the fibroblast population and interstitial space in diseased kidneys. Several fibrogenic growth factors, including PDGF, TGF- $\beta$ , basic fibroblast growth factor (FGF2) and connective tissue growth factor (CTGF), are well-known mitogens for fibroblasts.<sup>65,68,77–80</sup> In addition to these classic cytokines, studies indicate that tissue-type plasminogen activator is another critical factor that promotes fibroblast survival, proliferation and myofibroblastic activation.<sup>81–84</sup> The action of tissue-type plasminogen activator is independent of its protease activity, and is mediated by its ability to recruit  $\beta$ 1 integrin.<sup>84,85</sup> Interestingly, two downstream effectors of integrin signaling—focal adhesion kinase and integrin-linked kinase (ILK)—control fibroblast proliferation and matrix production, respectively.<sup>82</sup> Therefore, it seems that cell proliferation and matrix production, two events that characterize fibroblast activation, could be uncoupled at the molecular level.

Studies suggest that vascular pericytes are a major source of myofibroblasts in fibrotic kidneys.<sup>63,74,86,87</sup> Pericytes are a subset of the stromal cells that partially cover capillary walls, thereby stabilizing the endothelium. Following kidney injury, pericytes are detached from the endothelium, undergo migration and proliferation, and differentiate into myofibroblasts (Figure 2).<sup>74,87</sup> The story of pericytes is quite interesting, because pericyte detachment and differentiation into myofibroblasts under pathological conditions not only results in destabilization of the microvasculature, but also contributes to myofibroblast activation, which leads to interstitial fibrosis. However, because the markers for pericytes, such as PDGFR $\beta$ , are not specific and are present in many cell types including fibroblasts, and because fibroblasts are intricately connected to the capillaries via cell processes in renal interstitium, whether the pericytes identified and interstitial fibroblasts are the same entity remains to be elucidated.<sup>27</sup>

### Epithelial–mesenchymal transition

Another source of matrix-producing cells could be tubular epithelium that undergoes EMT, a cell phenotypic conversion process that occurs during embryonic development, tumor metastasis and organ fibrosis.<sup>47,53,88–90</sup> Similarly, fibroblasts and/or myofibroblasts might derive from capillary endothelium by endothelial–mesenchymal transition (EndoMT).<sup>91,92</sup> EndoMT is considered to be a unique form of EMT, as endothelial cells are a specialized type of epithelia. The contribution of EMT to renal fibrosis is controversial and is the focus of several reviews and debates.<sup>86,93–99</sup>

Broad agreement exists that tubular epithelial cells *in vitro* can undergo EMT, characterized by loss of epithelial features and acquisition of mesenchymal markers, under the bombardment of various profibrotic cytokines, particularly TGF- $\beta$ 1.<sup>87,100</sup> However, whether this transition merely represents an *in vitro* artifact or does occur *in vivo* is at the center of the argument. Using a genetic-lineage tracking, fate-mapping technique, an early study demonstrated that over one-third of FSP1<sup>+</sup> interstitial fibroblasts were derived from tubular epithelia in a mouse model of obstructive nephropathy.<sup>101</sup> Likewise, two independent

studies also show that endothelial cells make a substantial contribution to the generation of fibroblasts and/or myofibroblasts via EndoMT in a variety of fibrotic kidney diseases.<sup>91,92</sup> However, these results have been challenged by a number of similar cell-fate-mapping studies in which no epithelial or endothelial origin of fibroblasts is evident.<sup>87,102</sup> Thus far, the reason behind these discrepancies is unsettled.

A large number of studies have demonstrated a potential role of EMT in kidney fibrosis by examining the phenotypic conversion of tubular cells in animal models as well as in renal biopsy samples from patients with CKD.<sup>103–107</sup> Tubular expression of fibroblast markers such as  $\alpha$ SMA, vimentin and FSP1 in fibrotic kidneys is well documented.<sup>8,103,105,106,108</sup> Key mediators of EMT-regulatory signaling such as TGF- $\beta$ 1/Smad, ILK, Wnt/ $\beta$ -catenin and Snail1 are preferentially activated in renal tubular epithelia after injury.<sup>109,110</sup> Furthermore, morphological evidence indicates that epithelial cells do indeed traverse the tubular basement membrane (TBM) into the interstitium after injury.<sup>111</sup>

EMT is a dynamic process in which epithelial cells and fibroblasts represent two extremes of a continual spectrum of intermediate cell phenotypes. The frequency at which epithelial cells complete the entire EMT course and ultimately become fibroblasts is probably limited, and depends heavily on the disease models, stages and persistence of raised cytokine pressure in the inflamed milieu.<sup>112</sup> In most circumstances, tubular cells undergo a partial EMT, in which epithelial cells only change one or two phenotypic markers, although the transcriptional program of EMT is activated. Such a partial EMT, however, is closely associated with poor outcomes and predicts the progression toward interstitial fibrosis in humans.<sup>107</sup> Not surprisingly, blockade of EMT by a variety of agents, such as hepatocyte growth factor,<sup>105</sup> bone morphogenetic protein 7,<sup>106</sup> ILK inhibitor,<sup>110</sup> Wnt antagonists,<sup>109,113</sup> paricalcitol,<sup>114</sup> or induction of endogenous heat shock protein 72,<sup>115</sup> ameliorates renal fibrosis and preserves kidney function.

### Recruitment of circulating fibrocytes

Fibrocytes are a subset of bone marrow-derived, circulating monocytes with fibroblast-like features in the peripheral blood.<sup>116</sup> They are spindle-shaped cells that express hematopoietic cell marker CD45 and are capable of producing type I collagen.<sup>117,118</sup> Fibrocytes also express certain chemokine receptors, such as CCR7. In response to kidney injury, fibrocytes mobilize, infiltrate renal parenchyma and participate in fibrogenesis. The differentiation of fibrocytes is modulated by other inflammatory cells, such as CD4<sup>+</sup> T cells, through secreted cytokines.<sup>119</sup> Profibrotic cytokines IL-4 and IL-13 promote fibrocyte differentiation, whereas antifibrotic cytokines IFN- $\gamma$  and IL-12 inhibit this process, suggesting that an inflamed milieu that contains a complex mixture of cytokines is a major determinant of fibrocyte differentiation.<sup>120</sup> Interestingly, ciclosporin, but not sirolimus, promotes the development of fibrocytes, which could partly explain the nephrotoxicity of ciclosporin (a calcineurin inhibitor) in the clinical setting.<sup>119</sup> In mouse models of renal fibrosis, pharmacological inhibition of angiotensin II type 1 receptor reduces the number of fibrocytes in both the kidney and the bone marrow and ameliorates renal fibrosis.<sup>121</sup> This finding suggests that activation of the renin–angiotensin system could contribute to the



pathogenesis of renal fibrosis by regulating the differentiation and expansion of fibrocytes.<sup>121</sup>

The relative importance of fibrocytes in renal fibro-genesis is another area full of controversy. As specific markers for these cells are lacking, clear discrimination of fibrocytes from monocytes, macrophages, fibroblasts and myofibroblasts is a great challenge. In addition, subpopulations of fibrocytes seem to exist.<sup>118</sup> Thus far, results from experimental studies on the involvement of fibrocytes, and bone marrow-derived cells in general, in renal fibrosis are inconsistent.<sup>74,101,119,122</sup> Although some studies indicate that a considerable percentage of all collagen-producing fibroblasts in a mouse model of obstructive nephropathy originate from fibrocytes or bone marrow-derived cells,<sup>101,119</sup> other studies in the same model show that bone marrow-derived cells hardly contribute to the pool of collagen-producing cells at all.<sup>23,74,122</sup> Clarification of this issue needs further investigation.

The different origins of fibroblasts probably contribute to their phenotypic heterogeneity. The relative contribution of each lineage to the myofibroblast pool might depend on the disease model and specific stage of the disease. Conceivably, myofibroblast activation from fibroblasts, pericytes or fibrocytes is an early event,<sup>27</sup> whereas EMT often takes place at a late stage after a sustained injury (Figure 2).<sup>112</sup> The pathological effects of the early activation of fibroblasts and EMT on renal fibrosis might be different. Although activation of fibroblasts is important for the onset of renal fibrosis, EMT could be a major determinant of fibrosis progression and irreversibility (Figure 2).

## Execution

Regardless of their different origins, activated fibroblasts from all sources produce a large amount of ECM components, leading to excessive accumulation and deposition of interstitial matrix and formation of collagenous fibers that predominantly contain type I and type III collagens and fibronectin. How these fibrotic matrix proteins are synthesized, secreted, properly assembled in the interstitial space and finally modified to resist proteolysis is a central question to understanding the execution phase of renal fibrogenesis.

## Assembly of fibrogenic molecular machinery

The expression and synthesis of ECM proteins by the matrix-producing cells is primarily controlled at the level of gene transcription in response to various extracellular fibrogenic cues. Key fibrogenic factors include TGF- $\beta$ 1, PDGF, FGF2, CTGF and angiotensin II, whereas hepatocyte growth factor and bone morphogenetic protein 7 inhibit the production of matrix components primarily by antagonizing TGF- $\beta$ 1 action.<sup>68,123–127</sup> Through their respective receptors and specific downstream intra-cellular signal cascades, these fibrogenic cytokines activate a host of transcription factors that act on the cognate elements in the promoter regions of the collagen and fibronectin genes to activate their transcription. Such signal transduction cascades and expression of matrix genes are also regulated by a variety of microRNAs.<sup>128–132</sup> These studies have provided important insights into understanding the regulation of matrix genes at the molecular level. However, whether the data obtained from these *in vitro* studies truly reflect the regulation of matrix genes in fibrotic kidneys *in*

*vivo* remains uncertain. As fibroblasts in the fibrotic milieu are typically bombarded with a cocktail of cytokines, how they respond to these stimuli in a coordinated fashion is an unsolved conundrum.

Activated fibroblasts contain stress fibers and display abundant transmembrane connections (also known as fibronexus)<sup>133</sup> between extracellular fibronectin-containing matrix and intracellular actin microfilaments, suggesting that an increased interaction of ECM and integrins occurs.<sup>8</sup> Integrins are a family of heterodimeric transmembrane receptors that consist of  $\alpha$  and  $\beta$  subunits and bind to the ECM and to the cytoskeleton, thereby integrating the ‘outside-in’ and ‘inside-out’ signals between cells and their extracellular environment.<sup>134,135</sup> Integrins transmit their signals by activating the downstream effector kinase, focal adhesion kinase and ILK, as they possess no enzymatic or actin-binding activity. Extensive studies indicate that ILK, a scaffolding or adaptor protein and a serine/threonine protein kinase, is especially suited to serve as a molecular platform that integrates various fibrogenic signals.<sup>136</sup>

Structurally, ILK contains four ankyrin repeats, a pleckstrin homology domain, a kinase catalytic domain and an integrin-binding domain. Whether ILK is a true kinase remains to be resolved,<sup>137,138</sup> but a consensus exists that overexpression of ILK leads to glycogen synthase kinase  $3\beta$  (GSK3 $\beta$ ) phosphorylation and inactivation, which results in stabilization of Snail1 and  $\beta$ -catenin.<sup>11,110</sup>  $\beta$ -catenin directly controls the transcription of fibronectin and plasminogen activator inhibitor 1,<sup>139,140</sup> whereas Snail1 regulates fibroblast motility and tubular EMT.<sup>90</sup> Perhaps more importantly, ILK, as a scaffolding protein, assembles a multicomponent protein complex that contains ILK, PINCH and parvin.<sup>136,141</sup> These features enable ILK to bridge the integrins, actin cytoskeleton and other growth factor signal cascades, as PINCH connects to receptor tyrosine kinase signaling via cytoplasmic protein NCK2 and parvin binds to F actin (Figure 3). Interestingly, either disruption of this complex formation or inhibition of ILK activity blunts fibronectin expression, deposition and extracellular assembly, which suggests that the integrity and activity of this complex are obligatory for matrix production.<sup>110,142,143</sup>

Increasingly, it is becoming clear that integrins, ILK and their associated proteins constitute a fibrogenic molecular machinery that orchestrates the production and assembly of the ECM. Analogous to the concept of the inflammasome,<sup>14</sup> we propose this multicomponent, integrin-associated protein complex as a ‘matrisome’, which is a molecular platform activated as a result of injury that integrates various fibrogenic signal inputs and triggers the production and assembly of matrix components (Figure 3). This view is supported by copious studies demonstrating that integrin signaling is essential for the production and assembly of matrix proteins.<sup>134,144</sup> More importantly, it seems that almost all the key fibrogenic cues somehow promote the production of matrix components by regulating this molecular machinery, either directly or indirectly. For example, TGF- $\beta$ 1 is known to induce the expression of  $\beta$ 1 integrin, ILK and PINCH1, the major components of this complex, and promote their assembly.<sup>134,142,144,145</sup> ILK is also upregulated by many fibrogenic factors, including CTGF and high levels of glucose.<sup>11,145–147</sup> Angiotensin II is known to induce the expression of TGF- $\beta$ 1, and activates Smad signaling in both TGF- $\beta$ -dependent and TGF- $\beta$ -independent manners.<sup>148–150</sup> Intriguingly, angiotensin II also induces the expression of  $\beta$ 1

integrin and ILK,<sup>147</sup> and thus its profibrotic effects might be attributable to facilitating formation of the integrin–ILK complex. PDGF and FGF2 signaling is potentially connected to this machinery through PINCH1.<sup>141</sup> In fibroblasts, tissue-type plasminogen activator promotes collagen I expression by triggering the LDL receptor-related protein 1 (LRP1)-mediated recruitment of  $\beta$ 1 integrin and ILK activation.<sup>84</sup> Of interest, CTGF can bind to both integrins and LRP1, leading to activation of ILK and production of matrix components. In essence, this molecular machinery serves as a platform that integrates diverse ‘outside-in’ fibrogenic signals and controls matrix production in a coordinated fashion (Figure 3).

### Matrix deposition and modification

Assembly and deposition of fibrillar collagens *in vivo* are also an integrin-dependent event. In the early stage of renal fibrosis, deposition of fibronectin precedes the production of fibrillar collagens. Fibronectin activates integrins, co-localizes with procollagen secretion and forms loose scaffolding for fibrillar collagens. This process is followed by the production of a variety of new matrix proteins, such as secreted protein acidic and rich in cysteine (SPARC) and type IV collagen, as well as vitronectin, thrombospondin, decorin and heparan sulfate proteoglycan.<sup>151</sup> In addition to fibronectin, SPARC seems to be particularly important in regulating the assembly of fibrillar collagen.<sup>152</sup> SPARC is a so-called matricellular protein that binds collagen, and its expression in adult tissues is frequently associated with excessive deposition of collagen.<sup>152</sup> SPARC-null mice fail to generate a robust fibrotic response to a variety of stimuli.<sup>152</sup> Interestingly, SPARC is able to bind to  $\beta$ 1 integrin and activates ILK signaling,<sup>153</sup> which suggests a potential connection between the integrin–ILK complex and matrix assembly.

In the early stage of renal fibrosis, the collagen matrix is susceptible to proteolysis, and therefore the fibrosis is potentially reversible, which leads to wound healing. However, as fibrosis progresses, matrix in the fibrotic kidneys is considerably modified compared with normal tissues. Biochemical modifications of the matrix proteins by cross-linking are induced by enzymes such as tissue transglutaminase and lysyl oxidase, rendering them stiff and resistant to proteolysis.<sup>151</sup> In biopsy samples from patients with CKD, the expression of tissue transglutaminase is upregulated and correlates closely with the severity of renal fibrosis.<sup>8</sup> Consistently, both genetic ablation and pharmacological inhibition of tissue transglutaminase in mice substantially reduce renal fibrosis after injury.<sup>154,155</sup>

### Progression

The concept that renal fibrosis is solely the result of excessive accumulation of matrix components is overly simplistic and inaccurate, and might be erroneous. Whether fibrosis itself actually causes kidney failure remains an open question; after all, it is the normal early response to wound healing. Many cellular and molecular events beyond the production of matrix components promote the progression of fibrotic injury and are actually responsible for the progressive loss of kidney function. In many ways, the events that occur in the progression phase determine the reversibility and ultimate outcome of renal fibrogenesis.

## Tubular injury and atrophy

Tubular epithelial cells comprise the bulk of renal parenchyma and are the primary target of a variety of metabolic, immunologic, ischemic and toxic insults. Depending on the severity and duration of injury, tubular cells exhibit a wide range of responses, such as proliferation, autophagy, growth arrest, EMT and apoptosis. In patients with CKD, the histopathological presentation of tubular damage is often characterized as tubular atrophy, presumably as a consequence of apoptosis and EMT.

The potential involvement of tubular epithelia in renal fibrosis is illustrated by the preferential activation of major inflammatory and fibrogenic signaling in renal tubules after injury. In response to cell stress or injuries, NF $\kappa$ B signaling is activated in renal tubular epithelium, which triggers the production and release of inflammatory cytokines, and initiates peritubular inflammation. Tubular epithelial cells are susceptible to TGF- $\beta$ 1, produced by both the injured tubule and the infiltrating cells, and instigate a fibrogenic program that includes EMT. In tubular epithelial cells, sustained injury also activates ILK,<sup>145</sup>  $\beta$ -catenin,<sup>109,156</sup> Notch1<sup>157,158</sup> and hypoxia-inducible factor 1 (HIF1),<sup>159,160</sup> all of which are key intracellular signaling mediators implicated in renal fibrogenesis. In essence, tubular activation of major fibrogenic signaling, such as NF $\kappa$ B, Smad and  $\beta$ -catenin is characteristic of various fibrotic kidney diseases.<sup>38,131,156</sup>

Damage to tubular epithelia often instigates protective and regenerative responses such as autophagy and cell proliferation, at least initially. Autophagy, a process of cell 'self-eating', is regarded as a mechanism of cell survival and protection that mediates adaptation to calorie restriction or hypoxia in renal tubules *in vivo*.<sup>102,161,162</sup> However, excessive autophagy is proposed as the underlying mechanism that leads to total decomposition of tubular cells, as demonstrated in tetracycline-controlled TGF- $\beta$  overexpression transgenic mice.<sup>163</sup> Although cell proliferation is increased in the renal tubules of fibrotic kidneys, the net mass of epithelial cells is decreased, presumably owing to an increased rate of apoptosis and EMT, leading to tubular atrophy. Furthermore, defects in progression of the cell cycle after injury, such as arrest at G2/M phase, cause tubular cells to switch to a pro-fibrotic phenotype with an increased expression of TGF- $\beta$ , thereby promoting fibrosis.<sup>164</sup>

Tubular injury and atrophy undoubtedly impair tubular function and diminish the effectiveness of its endogenous protective mechanisms. For instance, levels of bone morphogenetic protein 7, an antifibrotic factor produced by tubular cells in normal kidneys, are progressively reduced in patients with CKD.<sup>165</sup> Proximal tubules are also the site of active vitamin D synthesis through the action of 1  $\alpha$  hydroxylase. Therefore, tubular atrophy could cause vitamin D deficiency, while defective vitamin D receptor signaling promotes EMT, tubular atrophy and renal fibrosis, establishing a vicious cycle.<sup>156,166–168</sup> Chronic injury not only damages tubular cells but impairs the integrity of the underlying TBM as well. Although a thickened TBM is a major pathological feature of advanced CKD, the integrity of the TBM is impaired and type IV collagen, a major component of TBM, is transiently downregulated in renal fibrogenesis.<sup>111,169</sup> Consistently, matrix metalloproteinase 2 (MMP2) and MMP9, the proteases that degrade type IV collagen and laminin, are induced in the injured kidneys,<sup>111</sup> and transgenic expression of MMP2 or knock out of

MMP9 promotes or ameliorates renal fibrosis, respectively.<sup>169,170</sup> Therefore, MMPs could have a dual function in the pathogenesis of renal fibrosis that is specific to the stage of fibrosis.<sup>171</sup> Although MMPs might protect the kidney by reducing matrix accumulation via promoting degradation of the ECM in the advanced stage, their activation in the early stage is generally pathogenic by impairing the integrity of the TBM and facilitating EMT.<sup>111,169</sup>

### Microvascular rarefaction

Renal fibrosis is typically associated with peritubular microvascular rarefaction, particularly in the advanced stage. Multiple mechanisms could account for the loss of peritubular capillaries in the fibrotic kidneys. As discussed above, the process of EndoMT not only generates the matrix-producing fibroblasts and/or myofibroblasts, but also directly leads to the loss of endothelial cells. Accordingly, blockade of EndoMT by a specific inhibitor of Smad3 (SIS3) can preserve endothelium, reduce renal fibrosis and retard the progression of diabetic nephropathy.<sup>172</sup> In addition, activation of myofibroblasts from pericytes under pathological conditions causes pericyte deficiency and destabilizes the endothelium, eventually resulting in microvascular rarefaction.<sup>63</sup> When PDGFR $\beta$  signaling in pericytes is blocked by circulating soluble receptor ectodomains, capillary rarefaction and renal fibrosis are attenuated in an animal model of progressive CKD.<sup>173</sup> Furthermore, pericyte–myofibroblast differentiation triggers a switch in secretion of vascular endothelial growth factor (VEGF) isomers (from VEGF164 to VEGF120 and VEGF188), leading to endothelial loss and microvascular rarefaction in renal fibrogenesis.<sup>173</sup>

Peritubular vasculature is susceptible to damage induced by apoptosis of endothelial cells in advanced CKD. Ischemia and oxidant stress are the known apoptotic stimuli for endothelial cells, which could be a potential mechanism that leads to rarefaction of peritubular capillaries.<sup>174,175</sup> Likewise, the integrity of peritubular capillaries could be impaired by endothelial dysfunction. In endothelial cells, activation of the receptor for advanced glycation end products, a common finding in advanced renal fibrosis, induces superoxide generation, activation of NADPH oxidase and p38 mitogen-activated protein kinase and nuclear translocation of NF $\kappa$ B.<sup>176</sup> In mice that lack functional Crim1, a protein involved in endothelial maintenance and integrity, the peritubular capillaries are dysfunctional, permeable and encircled by collagen deposition, which results in age-related fibrosis.<sup>177</sup>

### Chronic hypoxia

Chronic hypoxia in renal interstitium is characteristic of advanced CKD, and is a common pathology in patients with this condition.<sup>178–180</sup> Several mechanisms lead to chronic hypoxia in diseased kidneys, including microvascular rarefaction, decreased oxygen diffusion as a result of fibrosis and increased metabolic demands of tubular cells. Hypoxia can lead to tubular EMT or apoptosis, activate resident fibroblasts and impair peritubular capillaries, thereby creating a cycle of chronic hypoxia and progressive kidney failure. HIF1 is a key regulator of cell responses to hypoxia.<sup>181</sup> Although HIF1 controls a battery of genes that mediate adaptive responses against hypoxia, inappropriate activation of HIF1 can be deleterious as it evokes EMT and induces the expression of fibrogenic genes.<sup>159,160</sup>

Chronic hypoxia often coexists with increased oxidative stress and generation of ROS, which incites damage to biologically important macromolecules, including proteins, lipids and carbohydrates, and causes them to undergo structural modifications, leading to generation of advanced glycation end products, advanced oxidation protein products and advanced lipoperoxidation end products.<sup>182,183</sup> These modified macromolecules are not merely a reliable surrogate marker for estimating the degree of oxidant-mediated damage in CKD, they also affect various kidney cells as pathogenic mediators and trigger renal inflammation and fibrogenic responses, thereby promoting the progression of kidney injury and renal fibrosis.<sup>184–187</sup> In addition, the hypoxic and fibrotic microenvironment might lead to perturbed cellular signaling and induce epigenetic modifications, which could contribute to increased fibroblast proliferation, activation and fibrogenesis.<sup>188</sup>

## Conclusions

Renal fibrogenesis is an enormously complex, dynamic process that involves almost all the cell types in the kidneys, as well as the infiltrated cells. Over the past decade, our understanding of renal tubulointerstitial fibrosis, particularly as to how inflammation, fibroblast activation, tubular and microvascular injury contribute to fibrogenesis, has considerably evolved and improved.<sup>7,8</sup> We now have a better appreciation than previously for the diverse origins and phenotypic heterogeneity of the matrix-producing fibroblasts. Furthermore, the newly characterized novel actions of several well-known factors in fibrosis, such as proteases and macrophages, have challenged the established dogma in the field and represent a paradigm shift in our thinking about their roles in renal fibrogenesis and injury repair.<sup>19,189</sup>

Many gaps exist in our understanding of renal fibrogenesis, and the challenges ahead are daunting. The relative contribution of each fibroblast-generating pathway to renal fibrogenesis is difficult to delineate, and is perhaps dependent on the disease context and stage. Another important area that deserves attention is how the matrix-producing cells integrate the innumerable fibrogenic signals in the inflamed microenvironment and produce matrix in a coordinated fashion. New findings on macrophage heterogeneity and plasticity also necessitate conceptual and strategic innovations in the development of future therapeutics. As a variety of strategies that target key pathogenic mediators and pathways to halt renal fibrosis are effective in animal models, some of these remedies should eventually become clinically relevant for patients with CKD in the future.

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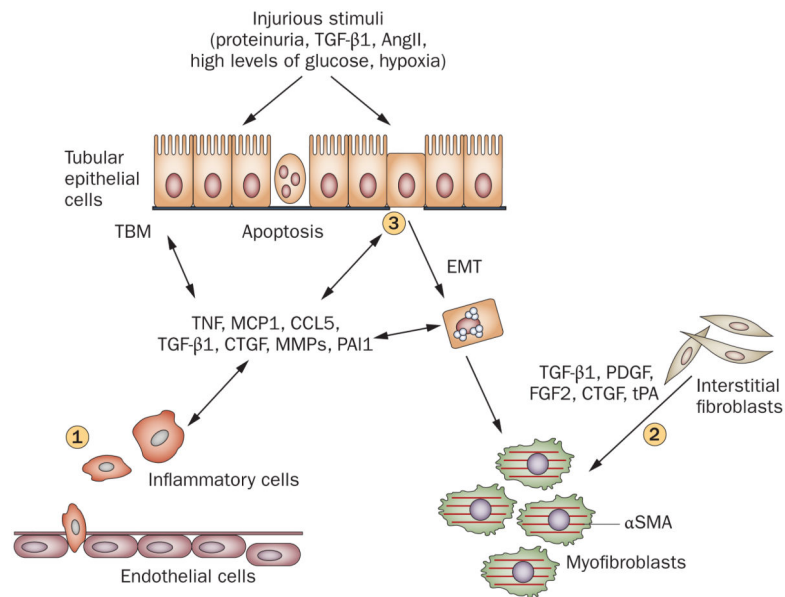
### Key points

- Despite having various initial causes, renal fibrogenesis is a converging and highly dynamic process, which consists of four overlapping phases: priming, activation, execution and progression
- After a sustained injury, nonresolving inflammation sets up a fibrogenic stage (priming) and triggers the activation and expansion of matrix-producing fibroblasts from multiple sources through a range of mechanisms
- Upon activation, the matrix-producing cells build a multicomponent, integrin-associated, ternary protein complex, which integrates various fibrogenic signals and orchestrates the production of extracellular matrix and its assembly
- Many cellular and molecular events, such as tubular atrophy, vascular rarefaction and hypoxia, promote the progressive loss of kidney function and determine the outcome of renal fibrosis



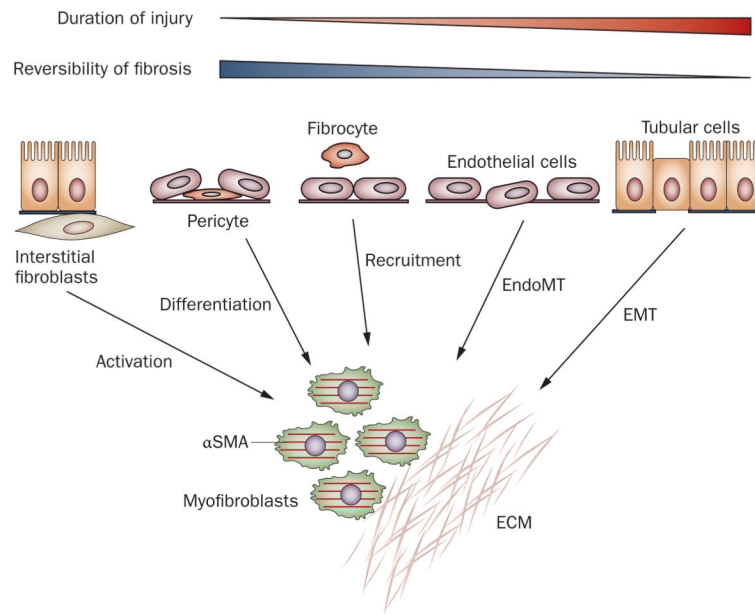
### Review criteria

The PubMed database was searched for English-language articles published up to June 2011, using the following search terms: “renal fibrosis”, “kidney fibrosis”, “myofibroblast”, “epithelial–mesenchymal transition”, “integrins” and “renal inflammation”. This Review is primarily focused on recent literature published after January 2006, although older papers that are very relevant to this topic were also selected.



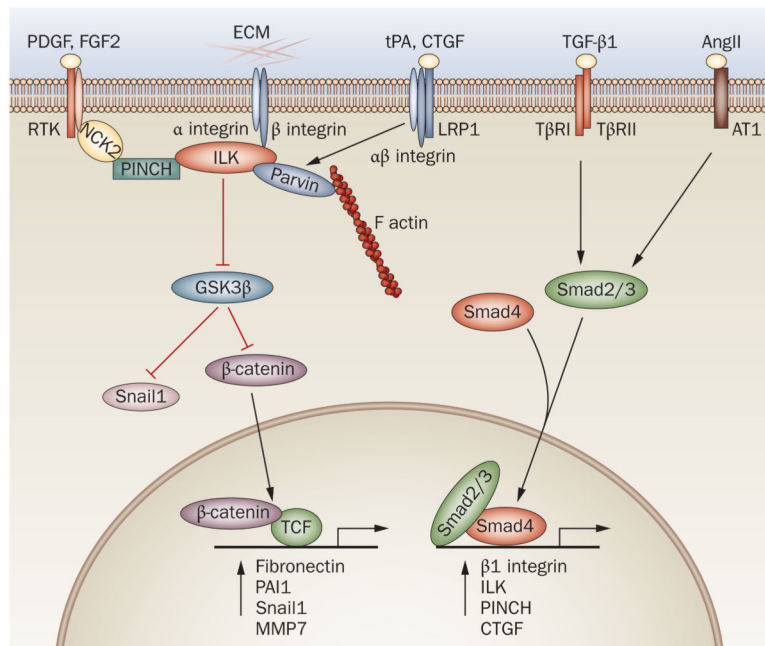
**Figure 1.**

Major events in renal interstitial fibrogenesis. (1) Peritubular infiltration of inflammatory cells, particularly T cells and macrophages, is an early event that sets up a fibrogenic stage. (2) Myofibroblast activation and expansion from various sources. The majority of the matrix-producing myofibroblasts are probably generated from local activation of interstitial fibroblasts. (3) Tubular cell apoptosis and EMT, leading to tubular atrophy. Abbreviations:  $\alpha$ SMA,  $\alpha$  smooth muscle actin; AngII, angiotensin II; CCL5, chemokine (C–C motif) ligand 5; CTGF, connective tissue growth factor; EMT, epithelial–mesenchymal transition; FGF2, basic fibroblast growth factor; MCP1, monocyte chemoattractant protein 1; MMPs, matrix metalloproteinases; PAI1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; TBM, tubular basement membrane; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; TNF, tumor necrosis factor; tPA, tissue-type plasminogen activator.



**Figure 2.**

Multiple origins of myofibroblasts have been proposed in renal fibrosis. Myofibroblasts can be derived from at least five different sources through various mechanisms: phenotypic activation from interstitial fibroblasts; differentiation from vascular pericytes; recruitment from circulating fibrocytes; capillary EndoMT; and tubular EMT. The relative contribution of each source to the myofibroblast pool in renal fibrosis is controversial. Conceivably, local activation of resident fibroblasts remains the major route for the generation of myofibroblasts in diseased kidneys, at least in the early stage. By contrast, EMT could be a late event, and contribute to the irreversible progression of fibrosis. Abbreviations:  $\alpha$ SMA,  $\alpha$  smooth muscle actin; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; EndoMT, endothelial–mesenchymal transition.



**Figure 3.**

A multicomponent, integrin-associated protein complex constitutes the molecular machinery that integrates various fibrogenic signals and orchestrates matrix production and assembly. Integrins and ILK and their associated proteins constitute the core components of this machinery. TGF- $\beta$ 1 regulates the expression of major components of this complex such as  $\beta$ 1 integrin, ILK and PINCH via Smad signaling. AngII activates Smad3 signaling through pathways that are either dependent on or independent of TGF- $\beta$ 1, which leads to upregulation of the components of this complex. PDGF and FGF2 activate their respective receptor tyrosine kinases and influence this complex via cytoplasmic protein NCK2. CTGF and tPA bind to LRP1 and recruit  $\beta$ 1 integrin, and then activate this machinery. This complex also activates  $\beta$ -catenin and Snail1 through inhibition of GSK3 $\beta$ , and thereby directly controls the expression of various fibrosis-related genes. The red lines indicate inhibition, the black lines indicate promotion. Abbreviations: AngII, angiotensin II; AT1, angiotensin II type I receptor; CTGF, connective tissue growth factor; ECM, extracellular matrix; FGF2, basic fibroblast growth factor; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; ILK, integrin-linked kinase; LRP1, LDL receptor-related protein 1; MMP7, matrix metalloproteinase 7; NCK2, non-catalytic region of tyrosine kinase adaptor protein 2; PAI1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; PINCH, particularly interesting new cysteine-histidine rich protein; RTK, receptor tyrosine kinase; TCF, T cell factor; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; T $\beta$ R1, type I TGF- $\beta$  receptor; T $\beta$ R2, type II TGF- $\beta$  receptor; tPA, tissue-type plasminogen activator.

**Table 1**

Markers for renal interstitial fibroblasts and myofibroblasts

Marker	Fibroblasts	Myofibroblasts	Function	Other cell types
$\alpha$ SMA	Negative	Very positive	Stress fiber, cell contractility	Vascular smooth muscle cells
FSP1	Positive	Positive	Actin-binding protein, cell motility	CD45 <sup>+</sup> , leukocytes
Vimentin	Negative	Very positive	Intermediate filament protein, cytoskeleton	Glomerular podocytes
Nestin	Negative	Positive	Intermediate filament protein, cytoskeleton	Glomerular podocytes
CD73	Very positive	Positive	Ecto-5'-nucleotidase, conversion of 5'-AMP to adenosine	Proximal tubular cells, mesangial cells, T cells
PDGFR $\beta$	Positive	Positive	Cell proliferation, matrix production	Vascular smooth muscle cells, pericytes

Abbreviations:  $\alpha$ SMA,  $\alpha$  smooth muscle actin; CD, cluster of differentiation; FSP1, fibroblast-specific protein 1; PDGFR $\beta$ , platelet-derived growth factor receptor  $\beta$ .