Cellular Mechanisms of Tissue Fibrosis. 3. Novel mechanisms of kidney fibrosis

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Submitted 26 December 2012; accepted in final form 12 January 2013

Chronic kidney disease (CKD), affecting as many as 8% of the population, can be initiated by many insults to the kidney including toxic, ischemic, infectious, paraneoplastic, congenital, genetic, and immunological diseases (93). Regardless of the initial insult(s), chronic disease is characterized by stereotyped kidney injury responses seen pathologically as glomerulosclerosis (glomerular fibrosis and associated capillary loop destruction), interstitial fibrosis, tubular atrophy, peritubular capillary rarefaction, and inflammation (36, 87). Even when the initial insults to the kidney have abated or been treated, the pathological syndrome of CKD frequently does not heal but becomes self-sustaining, stimulating further kidney injury, resulting in progression of CKD. Although CKD is widespread, there are no specific therapies except inhibitors of the angiotensin receptor and its ligand, and these have not stemmed the tide of CKD progression to organ failure (73).

Pathologic deposition of fibrillar collagenous matrix, i.e., fibrosis, results when tissues are damaged and normal wound-healing responses persist or become dysregulated, usually in response to sustained or repetitive injury. In addition to the excessive deposition of fibrillar collagens (mainly collagen types I and III but can include V, XI, and others), fibrotic lesions can include other extracellular matrix proteins, including laminins, fibronectin, secreted protein acidic and rich in cysteine (SPARC), type IV collagen, elastin, vitronectin, thrombospondin, heparan sulfate proteoglycans including perlecan, and chondroitin sulfate proteoglycans including versican and decorin (25). Most of these proteins/proteoglycans are essentially identical to the same components laid down in normal basement membranes and interstitial matrices. In the glomerulus and arteriolar wall, there is frequently an accumulation of hyaline, a poorly defined material that may result from extravasated plasma proteins. Biochemical modification of several of these matrix proteins, particularly the fibrillar collagen, by covalent cross-linking by lysyl oxidases and transglutaminases occurs as fibrosis progresses, rendering them resistant to proteolysis (25). Extracellular proteases, including matrix metalloproteinases (MMPs) and disintegrin and metalloproteinases (ADAMs), as well as their inhibitors including tissue inhibitor of metalloproteinases (TIMPs) are upregulated in kidney disease, but their roles in matrix turnover are much less well established. Each of these proteases has multiple functions, and studies of gene-targeted mice suggest that many MMPs and ADAMs do not directly affect matrix turnover but rather serve regulatory roles in related or ongoing processes, such as angiogenesis and inflammation (77).

In other organs, MMP14, whose main function is in bone homeostasis, has been shown to function as an effective but limited collagenase and contribute to matrix degradation, but this role has not been tested in kidney (32, 94). Probably more important in matrix turnover is the role of fibrillar collagen receptors and endocytic receptors, such as discoidin domain receptors (DDRs), leukocyte-associated Ig-like receptor 1 (LAIR1), mannose receptor, and Endo180, in phagocytosis of matrix and disposal via the lysosomal pathway in macrophages (see below) and possibly in myofibroblasts (13, 22, 88). TIMP1 has been shown to be an important cell survival factor in myofibroblasts and this may explain some of the biology associated with its expression, whereas TIMP3 plays inhibitory roles in the activation of myofibroblasts (51, 88).

In CKD, there is an intense accumulation of myofibroblasts in interstitial space, which are the primary cells to synthesize and deposit pathologic components of fibrillar matrix. Accumulation of fibrotic matrix progressively destroys the normal kidney tissue architecture by contraction and increased stiffness, resulting in disrupted blood flow supply and nephron function, as well as by increasing the space between vascular and tubular structures (103). Fibrosis, however, is not just matrix, but is also the cells that make matrix. New studies indicate that myofibroblasts are an important inflammatory cell...
in their own right, producing an array of chemokines and innate inflammatory cytokines as well as toxic oxygen radicals. Therefore the myofibroblast is not only a cell that deposits matrix but also contributes directly to parenchymal and microvascular demise.

The origin of myofibroblasts in the kidney has been intensely studied for the past 20 years and remains somewhat controversial (108). Part of the problem with the study of myofibroblasts has been the lack of uniform markers, and inter-organ and inter-species differences in the existing markers. Until very recently, it was widely thought that injured epithelial cells served as a primary source of myofibroblasts through a transdifferentiation process known as epithelial-to-mesenchymal transition (EMT) (50, 110). The concept that EMT explained the appearance of kidney fibroblasts/myofibroblasts evolved primarily from in vitro studies of cultured epithelial cells, which showed a proclivity to acquire mesenchymal features and lose epithelial features in conditions of serum exposure or treatment with profibrotic factors such as transforming growth factor-β (TGF-β) (45, 109). This in vitro EMT signature was proposed as indirect evidence that renal tubular epithelial cells are one of the major sources of myofibroblasts during kidney fibrosis; however, several independent lines of evidence have opposed the hypothesis that the in vitro phenotypic transition occurs, or contributes to myofibroblast appearance, in vivo (5, 42, 57, 59).

Recent use of reporter mice that track fibrillar collagen-producing cells has helped to define myofibroblasts with greater certainty. State-of-the-art fate mapping studies of pericytes and resident fibroblasts, both of which are poorly appreciated mesenchymal but surprisingly extensive progenitors in the kidney, in combination with fate mapping studies of tubular epithelial cells, have provided strong evidence that epithelial cells do not become myofibroblasts. Rather, it has been found that pericytes and resident fibroblasts are the major, if not the only, source of myofibroblasts in animal models of chronic kidney disease (21, 42, 64). These findings in the kidney have been complemented by independent fate mapping studies in skin, muscle, lung, liver, pancreas, and central nervous system, which all have identified pericytes as the major source of myofibroblasts in these organs, suggesting a unified model of fibrogenesis across our organs (9, 54).

In this review, we describe current knowledge of cellular and molecular mechanisms by which pericytes transdifferentiate into myofibroblasts during kidney disease, the consequence of pericyte detachment from capillaries for capillary integrity, as well as discuss how endothelial cells, epithelial cells, and myeloid leukocytes play an important role in fibrogenesis, predominantly by indirect mechanisms that involve cell-to-cell signaling, rather than differentiation into myofibroblasts. We also discuss the contribution of perivascular cells to fibrosis in the glomerulus.

**Defining Kidney Myofibroblasts**

Until recently there has been debate about the identification of myofibroblasts in interstitial kidney disease. Myofibroblasts were originally defined by electron microscopy (EM) in the skin as contractile cells showing characteristic cytoplasmic features including dense rough endoplasmic reticulum (ER) and moderate stress fibers of the actin cytoskeleton, and a paucity of lysosomes (31). Myofibroblasts can be readily identified in CKD specimens by EM, but this has limited uses. Instead, many investigators have used the intermediate filament α-smooth muscle actin (αSMA) as a marker of myofibroblasts. This is an imperfect marker because it is not specific (it also labels vascular smooth muscle and neonatal pericytes), and because in vitro many cells express this protein. Moreover, αSMA is not a matrix protein and is therefore at best an indirect marker of pathological matrix-forming cells. Finally, commonly used antibodies to detect αSMA are prone to artifact when the protein is detected in mouse tissues since the antibodies are raised from mouse hybridomas and therefore have high affinity for leukocytes (22). These artifacts can be overcome by the use of directly conjugated antibodies and stringent blocking methods (22). A better marker of myofibroblasts is the production of fibrillar collagen protein, but such proteins are predominantly extracellular, and inflammatory leukocytes frequently internalize collagen matrix via collagen receptors, rendering such a protein marker imprecise. We therefore generated a reporter mouse model in which cells generating collagen Iα1 protein express intracellular green fluorescent protein (GFP) (hereinafter referred to as Coll-GFP<sup>‡</sup>) and can be readily detected (64). This mouse has been shown to faithfully report collagen protein producing cells (16, 29, 64). In mouse models of kidney disease that have been progressing for more than 4 days, collagen Iα1-producing cells all express αSMA, but not all αSMA-positive (αSMA<sup>+</sup>) cells produce collagen Iα1 protein (Fig. 1A). Therefore in the interstitium of chronically diseased kidney, αSMA does detect myofibroblasts and these are the cells that make fibrillar collagen. When myofibroblasts were characterized using this reporter mouse they were restricted to the interstitium and lacked epithelial, endothelial, or leukocyte markers and S<sub>100</sub>A4 (Fig. 1B) (64). Stringent analysis of collagen-Iα1-producing cells using flow cytometry of kidney single-cell digest identified rare leukocytes that produced modest amounts of collagen Iα1 protein. They were, however, exceptionally rare (Fig. 1D). In bone marrow chimera mice that carried the Coll-GFP<sup>‡</sup> only in bone marrow cells, the extent of collagen Iα1-producing leukocytes could be more easily defined and characterized. These cells, also known as “circulating fibrocytes”, could not be detected in the circulation and were not detected in kidneys or lymphoid organs of healthy mice, but they were identified rarely in bone marrow and spleen in response to kidney disease (64). In the kidney, however, they were again exceptionally rare, amounting to fewer than 1:1,000 myofibroblasts, and they did not express αSMA (Fig. 1, E and F). Moreover, they were never seen in the interstitium but were restricted to perivascular areas (Fig. 1, E and F) and were not present in the kidney until 7 days after the commencement of injury (64). When systemic LPS was delivered concomitantly with kidney disease, splenic and bone marrow fibrocytes increased in number but there was no impact on the rare kidney fibrocytes (Fig. 1F). These studies indicate that in animal models of kidney disease, cells of leukocyte origin are not a significant source of fibrillar matrix-forming cells or myofibroblasts (14, 64–66). Importantly, similar findings have been reported using stringent experiments in liver, lung, skin, and muscle (82, 86).
Renal Mesenchymal Cells Are the Source of Interstitial Myofibroblasts

Smooth muscle actin protein-α (αSMA) is only expressed in the vascular smooth muscle of arterioles in normal adult mouse kidney. However, in adult human and rat kidney it is expressed at low levels and to a variable extent by very discrete perivascular cells (Fig. 2A). Moreover, in neonatal mouse kidney, similar discrete perivascular cells can be seen to express αSMA weakly and these cells are identical to collagen-producing cells seen in the in the Coll-GFP<sup>Cre</sup> reporter mouse (64). In adult Coll-GFP<sup>Cre</sup> reporter mice, an extensive network of discrete collagen-producing cells in perivascular locations can be identified by fluorescence microscopy (Fig. 2B). These cells lack epithelial, endothelial, and leucocyte markers (64) and are therefore mesenchymal cells. However, the majority are attached to peritubular capillaries and have processes in capillary basement membrane and may therefore be called pericytes (2, 64) (Fig. 2). In cases where the cells are not attached to capillaries they may be called resident fibroblasts. Surprisingly, these discrete cells are extensive, particularly in the medulla of the kidney and overall represent as many as 5% of all adult kidney cells.

These collagen-I<sub>α1</sub>-producing cells in normal kidney express a number of typical pericyte or mesenchymal stem cell markers including platelet-derived growth factor receptor-β (PDGFRβ), PDGFRα, CD73, CD44, and variable levels of CD105 and CD90. They also express a number of other mesenchymal markers including CD248 (91, 92). In neonatal kidney they express NG2 and P75NGR, but expression is downregulated in adult kidney (102).

To determine whether collagen-I<sub>α1</sub>-producing perivascular cells of the kidney were the progenitors of scar-forming myofibroblasts, Humphreys et al. (42) developed Cre/Lox reporter mice. Cre is an enzyme that recombines genomic DNA permanently and can therefore permanently activate a silenced gene reporter in a cell lineage-restricted manner (71). They generated mice in which Cre was under the regulation of transcription factors that specify either epithelial or mesenchymal (not endothelial and not leucocyte) fate in kidney development. Using this system they permanently labeled and fate mapped all epithelial or mesenchymal cells in the kidney. When the epithelial compartment was permanently labeled, none of the injured epithelial cells expressed αSMA weakly and these cells do not generate collagen-I<sub>α1</sub> protein whereas a minority (<0.1%) do generate this protein weakly.

Fig. 1. Characterization of cells that generate fibrillar collagen-I protein in models of kidney disease. A: split panel low-power confocal image of kidney cortex from Coll-GFP<sup>Cre</sup> reporter mice showing green fluorescent protein (GFP) nuclear and cytoplasm fluorescence of cells that are making collagen, colabeled with directly Cy3-conjugated anti-α-smooth muscle actin (α-SMA) antibodies. Note almost complete overlap (a, arteriole; g, glomerulus). B and C: high-power images of diseased kidney cortex from Coll-GFP<sup>Cre</sup> mouse showing CD45<sup>−</sup> leukocytes or S100A4<sup>−</sup> cells. Note there is no overlap with collagen-producing cells. D: high-power image of single cells from a digested fibrotic Coll-GFP<sup>Cre</sup> kidney showing that >99.9% of CD45<sup>−</sup> cells do not generate collagen-I<sub>α1</sub> protein whereas a minority (<0.1%) do generate this protein weakly.

E and F: images and graphs relating to bone marrow chimera mice in which only bone marrow cells harbor the Coll-GFP<sup>Cre</sup>. E: dispersed kidneys from these mice show rare bone marrow-derived cells restricted to perivenular sites that generate collagen protein (i.e., “circulating fibrocytes”) and lack the myofibroblast marker αSMA. F: number of fibrocytes/whole sagittal section (top) in mice with kidney disease in spleen and kidney, ± systemic injection of LPS (6 μg/g body wt). Percentage fibrocytes compared with leukocytes or myofibroblasts (bottom) in diseased kidney sections. Note that kidney circulating fibrocytes are exceptionally rare and are not increased after LPS whereas splenic fibrocytes are also rare, but more abundant and increased by LPS treatment. Bars, 25 μm.
in interstitial kidney disease epithelial cells do not become myofibroblasts (10, 57). The mesenchymal compartment of the kidney was permanently labeled using Cre under the regulation of the forkhead family transcription factor FoxD1. FoxD1 is activated in developmental nephrogenesis in metanephric mesenchymal progenitors fated to become the kidney stroma.

FoxD1 metanephric mesenchyme overlies epithelial-specified metanephric progenitors that expresses the transcription factor Six2 in the developing kidney (42, 61). The FoxD1-Cre allele was bred to fluorescent or enzymatic reporter mice, and the fate of FoxD1-derived mesenchymal cells was mapped (Fig. 2, C and D). The FoxD1-derived cells are fated to become pericytes,
perivascular fibroblasts, vascular smooth muscle cells, and mesangial cells (Fig. 2C) (42). In fact, when FoxD1-derived cells were compared with Coll-GFP pericytes, they were found to be identical (Fig. 2C). When interstitial kidney diseases were induced in these reporter mice, there was an abundance of myofibroblasts in the interstitial space and all cells in the interstitium expressing αSMA were seen to be derived from FoxD1 mesenchymal progenitors (42) (Fig. 2D). Similar results were obtained when FoxD1-CreERT2 allele was used in FoxD1 mesenchymal progenitors (42) (Fig. 2D). When interstitial kidney diseases were induced in these reporter mice, there was an abundance of myofibroblasts in models of interstitial kidney disease. Studies by Yanagita’s group mapped mesenchymal cells even earlier in development and identified a population of mesenchymal cells expressing a myelin protein P-zero that completed migration from the neural crest to the kidney metanephric mesenchyme by embryonic day 13.5 (5). These cells populated the nephrogenic interstitial mesenchyme zone of metanephric mesenchyme, which is identical to the FOXD1⁺ mesenchymal progenitor area, before migrating into the kidney stroma. The investigators showed that these cells became PDGFRβ⁺, CD73⁺ mesenchymal cells of the adult kidney and showed that these cells, not epithelial or endothelial cells, became the kidney myofibroblasts using similar techniques to Humphreys and colleagues. Although the authors named them as resident fibroblasts of the adult kidney, rather than pericytes, they showed that they are attached to capillaries and can generate erythropoietin. It is very likely therefore that Yanagita’s group mapped the same cells as Humphreys et al. (5, 42), but provide strong new evidence that mesenchymal cells may arise before nephrogenesis commences as neural crest progenitors.

Potential Mechanisms of Glomerulosclerosis

It is thought that mesangial cells, which are a glomerular pericycle and originate from FOXD1⁺ embryonic mesenchymal cells (Fig. 2), similar to pericytes of kidney peritubular capillaries, are a major source of glomerular fibrosis (48). In particular in diabetic kidney disease, mesangial nodules of fibrotic tissue are frequently seen and the mesangial extracellular matrix is frequently expanded. However, in recent years there has been increased focus on the role of the podocyte in loss of the glomerular filtration barrier and glomerular scarring. Podocytes are contractile and highly specialized mesenchymal cells restricted to the glomerular capillaries. They are attached to the abluminal side glomerular capillary basement membrane (CBM) and synthesize basement membrane proteins. Between their highly interdigitated processes, they express cell-type restricted proteins that contribute to a second barrier to plasma and cells known as the slit diaphragm. Mutations in these slit diaphragm proteins or cytoskeletal proteins result in increased permeability across the glomerular capillaries. In addition, podocytes express a number of vascular growth factors that are critical for maintaining survival and differentiation of the highly fenestrated glomerular endothelial cells (1, 11, 47). It is currently thought that a reduction in podocyte coverage of glomerular capillary loops is not only a marker of glomerular disease but also functionally critical in disease (46, 80). Increasing evidence indicates that although the podocyte has epithelial characteristics and expresses epithelial genes and transcription factors during nephrogenesis (56), it subsequently acquires pericyte characteristics and functions (26). Like pericytes, podocytes synthesize CBM, stabilize endothelial cells, maintain fenestrations, and regulate capillary permeability. It was interesting therefore that in the normal glomerulus, podocytes, but not mesangial cells, produce collagen Iα1 protein (Fig. 2E) (44, 64). One explanation for this is that nonfibrillar collagen-I protein is an important minor constituent of glomerular basement membrane. Another explanation is that collagen I protein is generated but not secreted. It is possible therefore that in disease states such as CKD, the diseased podocyte is a significant source of pathological fibrillar matrix production. In mice exposed to nephrotoxic serum to induce crescentic glomerulonephritis with focal and segmental glomerulosclerosis (FSGS), it was surprising to find that after 21 days of disease, podocytes remained the only cell type to generate collagen-I protein (Fig. 2F). These findings implicate the diseased podocyte (rather than podocyte loss) directly in the fibrogenic process in glomerular tufts. Clearly, new studies are required to determine the extent to which diseased podocytes are a type of myofibroblast in chronic glomerular diseases, and the extent to which pericyte/myofibroblast gene-targets (see below) will benefit podocyte functions.

Structure and Function of Pericytes

Pericytes are contractile cells of mesenchymal origin that wrap around and support the microvasculature. In the kidney, pericytes are found attached to peritubular capillaries (42, 52). Pericytes and endothelial cells are embedded within the same basement membrane that they contribute to formed by synthesizing laminins and collagens (96, 97). Pericytes have an intimate communication with endothelial cells by direct physical contact in a number of cytoplasmic regions including specialized invaginations of cytoplasm called “peg and socket” where direct signaling is believed to occur (4, 8). Pericytes can be distinguished from perivascular fibroblasts (also known as fibrocytes or adventitial cells), which surround arterioles and have no connection with endothelium (83). They can also be
distinguished from VSMCs, which surround larger blood vessels, by their location, since VSMCs are separated from endothelial cells by an internal elastic lamina and do not completely share molecular marker expression (83, 87). Several recent reviews provide a list of validated markers to identify pericytes including those mentioned above (83, 87).

Pericytes are critical to the stability and integrity of the microvasculature and are necessary for basement membrane synthesis and content (52, 88). They regulate vascular tone, blood flow (41, 76), and capillary diameter (78). In the kidney, medullary pericytes have been studied in greatest detail (74, 76, 111, 112). Medullary pericytes line the descending vasa recta in greatest density, and relatively spare the ascending vasa recta. Medullary pericytes regulate the distribution of blood flow to the medulla, which is critical to the regulation of salt and water homeostasis. Vital signals between the thin descending limb of the loop of Henle and medullary pericytes are critical to this regulation, and a number of signaling factors including physiological levels of NO and H₂O₂ have been shown to be important in this process. More recently, it has become appreciated that pericytes are sensors of oxygen tension generating constitutively high levels of hypoxia-inducible factor-2 transcripts and are the likely source of erythropoietin production in the kidney (5).

A failure of integration of pericytes into the vascular wall during angiogenesis leads to collapse, aneurysm, or hemorrhage of the vessel (90). Likewise in response to sustained tissue injury or inappropriate activation, pericytes detach from the vessel wall. This process may initially be beneficial, since it permits endothelial activation, angiogenesis, and leukocyte migration (79), but also leads to increased capillary permeability and instability, which ultimately leads to capillary destruction (66).

Transcriptional Consequences of Pericyte-to-Myofibroblast Transdifferentiation

To explore the transcriptional changes in pericytes that become myofibroblasts, Schrimpf et al. (88) purified pericytes from normal adult kidney and activated pericytes 48 h and myofibroblasts (derived from pericytes) 7 days after initiation of kidney disease. The RNA from these cells was subjected to differential microarray analysis and provided the first insight into global transcriptional changes (88). Importantly, almost 2% of the total transcriptome was significantly regulated and the vast majority of this change occurred within the first 48 h of injury (Fig. 3A). This extent of transcriptional perturbation is in keeping with a cellular transdifferentiation rather than simply a disease-induced cellular activation. As well as the expected changes in genes associated with matrix protein turnover and migration, it was striking that the greatest change in pericytes that become myofibroblasts was the activation of functionally related groups of genes involved in innate immunity, inflammation, wounding, and cytokine response (Fig. 3A).

To discover putative orchestrators of this transcriptional response, we applied an unbiased, computational transcription factor analysis to the differentially expressed genes and identified several overrepresented candidates (Fig. 3A) (33, 99). A notable finding was the enrichment of Rela (NF-κB p65), a master regulator of inflammatory response and immunity. Our systematic approach also provided an in-depth view on regulatory regions of differentially expressed genes, as shown for

Fig. 3. Transcriptomic analysis of pericytes in kidney disease. A: pericyte transdifferentiation into myofibroblasts during kidney injury is characterized by profound changes in gene expression with over 860 differentially regulated genes (false discovery rate < 0.01, 549 upregulated, 313 downregulated). Normalized gene expression values are depicted as pericytes (day 0) become myofibroblasts (days 2 and 7), with the bulk of the transcriptional changes occurring by day 2. These temporal expression patterns are highly enriched in distinct functional pathways, most prominently, those involved in immunity (P value 3 × 10⁻⁶⁸) and inflammatory response (P value 7 × 10⁻⁶⁰) among others. Furthermore, many of the differentially expressed genes share common, overrepresented transcription factor (TF) binding sites (Bonferroni-adjusted enrichment P < 0.01), implying coordinated regulation by a limited repertoire of TFs. B: our global computational approach is amenable to more detailed data-mining analyses. For example, the cytokine interleukin-6 (IL-6) is highly upregulated when pericytes transdifferentiate into myofibroblasts, functionally maps to immune and inflammatory pathways, and has a Rela (NF-κB p65) binding site upstream of its transcription start site. Interestingly, Rela expression itself doubles when pericytes become myofibroblasts in this animal model of kidney injury.
the proinflammatory and highly upregulated gene interleukin-6 (IL-6) (Fig. 3B). Another transcription factor highly overrepresented among genes upregulated during pericyte transdifferentiation into myofibroblasts was *Etv4*, a critical modulator of kidney development as *Etv4*-null mice were recently reported to suffer from renal hypoplasia or agenesis (68). Collectively, these findings implicate myofibroblasts not merely as matrix-producing cells but also as a major innate inflammatory cell of the kidney. It is striking therefore that recent studies of pericytes in brain and skin have shown that in an activated state they lose pericyte functions and become a potent source of innate inflammatory cytokines (58, 75, 95). Furthermore, in studies of lung, disease-associated fibroblasts are a major source of oxygen radical production that plays a pathogenic role in lung disease (40, 55). It appears therefore that the myofibroblast (or disease-associated fibroblast) is a new innate immune target in kidney disease. Understanding the mechanisms of immune activation in these cells is paramount.

**Molecular Mechanisms of Pericyte Transdifferentiation Into Myofibroblasts**

Pericyte detachment from capillaries, transdifferentiation into myofibroblasts, and regulation of myofibroblast activation or survival appear as attractive and novel therapeutic strategies to treat inflammation, fibrosis, and parenchymal destruction in CKD. Although this area of research is in its infancy, several important cell pathways have been identified that may rapidly lead to the identification and development of drug targets (Fig. 4).

**PDGFR pathways.** PDGFRα and β are expressed by kidney pericytes at rest, and in short-term disease models, these receptors remain restricted to pericyte-derived myofibroblasts. PDGFs are generated by endothelial cells, epithelial cells, and macrophages in kidney disease models. Blockade of these receptors by antibodies or soluble receptors, which act as ligand traps (16, 66), profoundly attenuates pericyte detachment and differentiation into myofibroblasts and even has the capacity to reverse fibrotic disease. Moreover, these receptor-blocking strategies markedly attenuate immune cell recruitment and microvascular disease (66). Strategies to attenuate activation at these receptors may be useful strategies in human CKD, but further studies are required. Importantly, several kinase inhibitors in clinical trials (Novartis and Boehringer Ingelheim) to treat fibrosis in several organs block PDGFR phosphorylation and may impact some signaling pathways at these receptors (22).

**VEGF pathway.** VEGF receptor 2 (VEGFR2) is restricted to endothelial cells in the kidney. Deletion of VEGF in podocytes leads to unstable glomerular capillaries and microthrombi formation (26, 70), but attenuated VEGFR2 signaling in interstitial kidney disease is beneficial (66). This paradox is complex to understand but may hinge around the fact that in disease states the type of VEGFA ligands produced by the kidney change markedly, which may lead to deleterious signaling at VEGFR2 and its coreceptors, neuropilins. Excessive VEGFR2 signaling activates endothelial cells, which may stimulate pericyte activation and recruit leukocytes. Although VEGFR2 signaling is also an attractive therapeutic target in CKD, further understanding of the distinction between glomerular and peritubular capillary signaling is required in order that signaling in once compartment can be targeted separately from the other.

**TGF-β pathway.** TGF-β pathological signaling in fibrotic diseases including that of the kidney is well established, and therapies to block TGF-β or its receptor are in clinical trials for fibrosing diseases. Like the PDGF pathway, most cells in the

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**Fig. 4. Schema showing candidate receptors and pathways involved in pericyte differentiation into myofibroblasts.** Endothelial cell is shown in red and pericyte is shown in green. Factors in orange promote myofibroblast differentiation and activation, whereas factors in shades of blue inhibit differentiation and activation. PPAR, peroxisome proliferator-activated receptor; FA, fatty acid; ROS, reactive oxygen species; miRNA, microRNA; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; ADAMTS1, a disintegrin and metalloproteinase with thrombospondin motif; TIMP3, metalloproteinase inhibitor 3; VEGFA, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor; EphB, ephrin receptor B; GLI, glioma-associated oncogene homolog; LRP, low-density lipoprotein receptor-related protein; WNT, wingless/int1; CTGF, connective tissue growth factor; TGF-βR, transforming growth factor-β receptor; PDGFR, platelet-derived growth factor receptors.
kidney generate the ligands and the receptor is widely expressed. Recent studies have shown that the receptor is expressed by pericytes and signaling from the receptor is sufficient to trigger myofibroblasts transdifferentiation in vivo and in vitro (102). Recent studies suggest that noncanonical signaling pathways via MAPKs may be dominant in myofibroblasts and their progenitors, and further studies are required to understand the significance of canonical signaling in these cells.

**CTGF pathway.** Connective tissue growth factor (CTGF), also known as CCN2, is well recognized as a fibrogenic growth factor in kidney and other diseases (106). However, it is highly produced by pericytes and podocytes and plays important roles in angiogenesis and maintenance of the vasculature. Stimulation of pericytes by CTGF also directly triggers a migratory and myofibroblast phenotype, which may be independent of TGF-β signaling. This also is dependent on MAPK signaling in the pericytes (81). It appears therefore that CTGF can also signal pathologically to stimulate myofibroblast appearance and activation, but that the nature of this signaling is distinct from its effects on kidney epithelial cells (81). Currently, antibodies that bind to CTGF are in clinical trials as antifibrotic therapies (22) (FibroGen).

**WNT/β-catenin**. Recent studies demonstrated that disease-activated pericytes and myofibroblasts show marked up-regulation of the WNT/β-catenin pathway. The soluble protein inhibitor of WNT/β-catenin, which binds to the WNT ligand receptor LRPs-6, is known as Dickkopf related protein 1 (DKK-1) and it blocks pericyte activation in vitro (Fig. 4). When delivered systemically it remarkably attenuates inflammation, fibrosis capillary disease, and epithelial injury. A major target for DKK-1 is the activated pericyte or myofibroblasts (81). Intriguingly, the WNT receptor LRP-6 appears necessary for PDGFR, CTGF, and TGF-βR signaling in pericytes and myofibroblasts that results in MAPK pathway activation in myofibroblasts, suggesting that the WNT pathway and these other fibrogenic signaling pathways converge at the plasma membrane to effect pathological signaling (39, 81).

**Ephrin B pathway.** Recent studies in skin and cancer have implicated Eph/ephrinB signaling in maintenance of normal pericyte endothelial interactions, and disruption of this homeostatic signaling in fibrogenic and pathologic microvascular disease (30, 85). The Eph:ephrinB bidirectional signaling pathway is active in kidney microvascular endothelium and in pericytes (53). When disrupted, it affects endothelial behavior and the ability of pericytes to maintain microvascular stability (53). This pathway may be an important homeostatic pathway that could be targeted to stimulate normal repair processes.

**Hedgehog pathway.** This signaling pathway plays important roles in vasculogenesis and in bidirectional signaling from endothelium to pericytes. In adult kidney disease the tubules are an important source of Hedgehog ligands, but Hedgehog pathway downstream transcription factors Gli-1 and Gli-2 are restricted to pericytes and perivascular fibroblasts (27). There is currently controversy about the importance of this signaling pathway in pericyte activation in response to tissue injury and further studies are required (20, 27).

**Metalloproteinase activity.** Understanding how pericytes detach from capillaries in response to sustained injury may lead to new therapies. Several of the above signaling pathways have been implicated in the activation process that leads to detachment, but if pericytes did not detach after an injury then cumulative fibrogenesis would likely be reduced. The understanding of the detachment process is only beginning to be understood, but several studies have implicated metalloproteinases in this process, including the ADAM and ADAMTS families (Fig. 4). These metalloproteinases cleave specific proteins, including capillary basement membrane proteins, but also regulate migration and VEGF signaling and inhibit normal angiogenesis (6, 49, 88). In kidney, ADAMTS1 is highly upregulated in pericytes early after injury and its natural inhibitor, TIMP3, is highly expressed normally but is rapidly downregulated after injury (88). Targeting specific ADAM or ADAMTS activity may be beneficial.

**Pathogenic MicroRNA.** MicroRNAs are regulators of gene expression, but they act to silence gene expression at the posttranscriptional stage, and therefore may silence beneficial genes. Recent studies have investigated dysregulated microRNAs in interstitial kidney disease (15, 34, 107). From these studies a number of pathogenic microRNAs have been discovered. One of these is miRNA21 (miR-21). Although miR-21 has a number of important targets, a major effect of its activity is to silence peroxisome proliferator-activated receptor-α (PPARα) activity and fatty acid oxidation (Fig. 4). This gene regulation results in enhanced reactive oxygen species (ROS) production by cells, reduced metabolism of biologically active lipids, and reduced energy production. Collectively, these effects serve to amplify disease responses. Furthermore, as in skin and liver, these studies implicate adipogenic signaling in regulation of the differentiation of pericyte progenitors into myofibroblasts. Blocking the effects of miR-21 represents an attractive new therapeutic target because specific oligonucleotides with complementary sequences can be delivered as drugs readily with high distribution to intracellular compartments and silence the effect of miR-21 (15).

**Contribution of Other Cells to Fibrogenesis**

**Monocyte-derived cells.** Recent studies including the fate mapping and bone marrow chimera studies described above suggest that cells arising in the bone marrow are a minor contributor to the cells that deposit fibrillar matrix. This may vary between organs and species, but the most comprehensive studies indicate that fibrillar matrix-producing cells arising from bone marrow in models of kidney disease are exceptionally rare (Fig. 1). Regardless of these findings, macrophages and dendritic cells derived from monocytes are abundant in kidney injury and play an important role in inflammation, tissue repair, and fibrosis. Ablative studies in several models of kidney interstitial and glomerular disease nevertheless indicate that macrophages significantly contribute to the development of fibrosis (24, 28, 37, 63, 67, 69, 101). It is most likely therefore that monocyte-derived cells in chronic kidney disease drive fibrosis by indirect mechanisms, involving cell-to-cell signaling, although the deposition of nonfibrillar matrix proteins such as fibronectin may contribute in some way to pathological matrix, but this has not been assessed rigorously. Indeed, myofibroblasts and macrophages are in very close physical contact, making cell-to-cell signaling feasible (Fig. 1).

Several explanations have been proposed to explain monocyte/macrophage function. One is that the way monocytes are activated upon entering the diseased kidney dictates their

Themes

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differentiation and function. Another is that monocytes exist as functionally discrete subsets. Evidence for both exists, but it is most likely that the initial and continued exposure of monocytes to signals from the kidney results in a profibrotic phenotype that persists. Studies from animal models of progressive interstitial fibrosis have shown that monocytes infiltrate the injured kidney and differentiate into discrete populations of kidney macrophages based on the levels of cell surface markers such as Ly6C or TREM1 (14, 65, 100). One subpopulation generates proinflammatory factors including IL1β, TNF-α, and chemokine (C-X-C motif) ligand 2 (CXCL2), which may stimulate fibrogenesis potentially by worsening inflammation and tissue injury, whereas another population predominantly generates factors that include TGF-β and PDGF, which may stimulate myofibroblast activation. Some texts call this latter population alternatively activated or M2α macrophages. Increasing evidence points to danger-associated molecular patterns (DAMPs), released from injured parenchymal and endothelial cells, as critical factors that contribute to the activation of monocytes in the injured kidney via pattern recognition receptor (PRR) binding and subsequent activation of NF-kB, MAPK, and inflammasome signaling (3, 84). Such “M2α”-like macrophages have been purified from the fibrosing kidney and shown to generate cytokines including PDGF-B, PDGF-C, TGF-β, IGF-1, IGFBP5, CCL18, and galectin-3. In other tissues, profibrotic macrophages generate factors including C1q and IL13 (72). On the other hand, a number of studies in models of repair and regeneration of the kidney show that monocytes and monocye-derived macrophages function predominantly to stimulate repair and regeneration. Such macrophages are characterized by high-level IL10 production. IL10 is an anti-inflammatory cytokine that also downregulates activation of myofibroblasts (14). A part of the reparative process involves phagocytosis and clearance of dead cells, debris, and oxidized matrix. Reparative macrophages express high levels of scavenger receptors and lysosomal pathway proteins, including the transmembrane glycoprotein gpNMB (60, 62), which serve this clearance purpose. In addition, such reparative macrophages produce growth factors, which stimulate tubular cell and microvascular regeneration. Reparative macrophages are poorly characterized in chronic kidney disease, and it may be that their phenotype and functions have been suppressed in such protracted disease states. Several lines of evidence suggest that monocyte-derived macrophages retain plasticity and can change functions. To that end, several lines of research suggest that therapeutic delivery of factors to macrophages may promote this reparative phenotype, which would be beneficial to the kidney (14, 19, 38). In other organs, including the liver, the presence of reparative macrophages that degrade and clear away fibrotic matrix has been clearly demonstrated, but such a phenotype has not been clearly demonstrated in models of kidney disease.

Epithelial cells. Although epithelial cells do not become scar-forming myofibroblasts during kidney fibrosis, they still play a crucial role in fibrogenesis. Injured epithelial cells undergo profound phenotypic changes, which include the acquisition of a migratory phenotype (98). This activated migratory phenotype in response to injury may be termed EMT, since injured epithelial cells activate a program of gene expression, including the transcription factor SNAIL and the intermediate filament vimentin, and acquire a more mesenchymal and migratory appearance. These epithelial phenotypic changes certainly contribute to fibrogenesis indirectly. Increasing evidence shows that epithelial cell stress responses are sufficient to activate intracellular signaling programs conferring a profibrotic phenotype on the epithelial cells without necessarily activating the developmental EMT genes. Examples of epithelial stress are ER stress, dysregulation of energy metabolism, G2/M cell cycle arrest (104), autophagy apoptosis, and necrosis (Table 1). Recent studies support the idea that the diseased epithelial cell alone is sufficient to drive fibrosis and CKD (35, 105). By using genetic tools to express the human diphtheria toxin receptor only on tubule epithelial cells (35), the investigators showed that repeated selective proximal tubule cell ablation led to interstitial fibrosis, glomerulosclerosis, and capillary rarefaction.

Injured epithelial cells are a major source of cytokines, activating integrin receptors, and soluble mediators that may act as soluble factors to drive fibrogenesis (102). There are a number of mechanisms by which epithelial cells contribute to fibrosis: 1) signaling to pericytes and perivascular fibroblasts, thereby stimulating their differentiation into myofibroblasts; 2) signaling to endothelial cells, affecting the cross talk between pericytes-endothelial cells; 3) directly signaling to myofibroblasts, to stimulate extracellular matrix deposition; or 4) recruitment and activation of innate immune cells such as the macrophage, which signals to myofibroblasts. Recent studies indicate that active TGF-β, Hedgehog ligands, Notch ligands, PDGFs, and CTGF produced by tubular epithelial cells during kidney injury are all factors that may stimulate pericyte detachment from capillaries and differentiation to myofibroblasts (10, 27, 102).

Clinical Trials in Fibrogenesis

To date there have been few trials to counteract kidney fibrosis. However, a small number of human trials are ongoing or at early stages, mainly focused on retarding or preventing diabetic nephropathy. The original clinical trials that used angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor antagonists (12) were designed to lower blood pressure and glomerular hyperfiltration, although newer evidence suggests that a major role for the angiotensin receptor, which is widely expressed by the kidney vasculature, perivascular cells, epithelium, and inflammatory leukocytes, is to stimulate inflammatory pathways (18). It is likely therefore that current use of ACEI and angiotensin receptor inhibitors

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ER, endoplasmic reticulum; EMT, epithelial-to-mesenchymal transition; TGF-β, transforming growth factor-β.
to treat kidney disease is effective at least in part by blocking proinflammatory and profibrotic pathways (7). Recent studies using pirfenidone to treat stage 3 diabetic CKD have had limited success (17, 89). Pirfenidone may function by reducing p38 MAPK activation, and further more definitive studies are ongoing. Blocking antibody therapies against CTGF and TGF-β are currently in clinical trial in diabetic nephropathy, and a recent trial focused on blocking the formation of reactive oxygen species by stimulating the transcription factor NRF2-dependent antioxidant enzymes. Although promising, it was terminated as a result of the side effects of the new therapy.

**Future Directions**

With the identification of myofibroblast progenitors and the advent of new molecular tools to study these cells in animal models of CKD it will be possible to develop therapies to specifically target kidney myofibroblasts and their progenitors. Indeed, several therapies targeting myofibroblast and progenitor cell surface receptors are in clinical trial in human chronic kidney disease, and therapies targeting macrophages that drive fibrogenesis are also in trial for diabetic kidney disease (22, 23).

Nevertheless, a major gap in our current knowledge is the phenotype of myofibroblasts in human kidneys that have had CKD for months or years. Most of our current knowledge of myofibroblasts comes from animal models of CKD that are shorter in duration than human disease. Increasing evidence suggests that diseased myofibroblasts have undergone significant epigenetic and microRNA changes, such as microRNA21 activation, (15) that may render receptor blockade less efficacious in long-term disease. Understanding the key changes should be a high priority.

Another area that requires study is the fate of myofibroblasts. We currently know little about the longevity of myofibroblasts in scar tissue, whether their functions change with time and to what extent myofibroblast reversion to pericytes can occur, particularly after weeks or months of disease.

We currently treat anemia from CKD with recombinant erythropoietin. Tantalizing evidence now indicates that when pericytes become myofibroblasts they become insensitive to hypoxia and no longer able to generate erythropoietin. An understanding of why this occurs and how to prevent the silencing of erythropoietin production might enable novel strategies to treat anemia.

Evidence suggests that pericytes are central in normal salt and water homeostasis and therefore in blood pressure homeostasis. When pericytes become myofibroblasts they lose vascular regulation. Strategies to prevent pericyte detachment in response to injury may prevent the loss of control of salt and water balance observed in chronic kidney disease.

**Conclusions**

Mesenchymal cells are the major progenitors of scar-forming cells myofibroblasts in the kidney. During injury, differentiation of pericytes into myofibroblasts not only contributes to deposition of pathological extracellular matrix, but also plays a role in capillary rarefaction and inflammation, which leads to CKD. Although myofibroblasts play a central role during kidney fibrosis, epithelial cells, endothelial cells, and leukocytes have an important role during fibrogenesis by paracrine signaling mechanisms. Here, we have illustrated some of these mechanisms, but further studies are urgently needed to elucidate the mechanisms by which all these cells interact together.

**ACKNOWLEDGMENTS**

The authors thank Dr. William Parks (University of Washington, Seattle) for insight and advice.

**GRANTS**

The Duffield Lab is funded by National Institutes of Health Grants DK-93493, DK-94768, DK-87389 NCATS IUH2 TR000504, AHA Grant 12040023, and the Nephcure Foundation.

**DISCLOSURES**

J. S. Duffield is on the Scientific Advisory Board of Promedior Inc. and of Regulus Therapeutics. He is a co-founder of Muregen LLC. He has consulted with Takeda, Boehringer Ingelheim, Bristol-Myers Squibb, GlaxoSmithKlein, Biogen Idec, and Gilead Pharmaceutical Companies, and has Research Agreements with Regulus and Eli Lilly.

**AUTHOR CONTRIBUTIONS**

G.C., G.L., S.A.G., and J.S.D. prepared the figures; G.C. and J.S.D. drafted the manuscript; G.C., G.L., S.A.G., and J.S.D. edited and revised the manuscript; G.C., G.L., S.A.G., and J.S.D. approved the final version of the manuscript.

**REFERENCES**


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