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Mechanisms of Renal Fibrosis

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Abstract

Tubulointerstitial fibrosis is a chronic and progressive process affecting kidneys during aging and in chronic kidney disease (CKD), regardless of cause. CKD and renal fibrosis affect half of adults above age 70 and 10% of the world's population. Although no targeted therapy yet exists to slow renal fibrosis, a number of important recent advances have clarified the cellular and molecular mechanisms underlying the disease. In this review, I highlight these advances with a focus on cells and pathways that may be amenable to therapeutic targeting. I discuss pathologic changes regulating interstitial myofibroblast activation, including profibrotic and proinflammatory paracrine signals secreted by epithelial cells after either acute or chronic injury. I conclude by highlighting novel therapeutic targets and approaches with particular promise for development of new treatments for patients with fibrotic kidney disease.

INTRODUCTION

Kidney fibrosis is the buildup of scar within the parenchyma, and it represents the common final pathway of nearly all chronic and progressive nephropathies. Indeed, cortical interstitial expansion is the best histologic predictor of renal functional decline in chronic kidney disease (CKD), glomerular diseases, and type I diabetic nephropathy (1–3). The deposition of fibrotic matrix after injury may initially aid in the tissue repair process, and after a mild injury, it is subsequently resorbed during tissue repair. However, during chronic injury that occurs in CKD, fibrotic matrix deposition continues unchecked, ultimately disrupting organ architecture, reducing blood supply, and disturbing organ function. Fibrosis reduces the capacity for tissue repair and ultimately causes kidney failure.

CKD affects 26–30 million adults in the United States and remains a major public health problem (4). The US Centers for Disease Control and Prevention project that 47% of 30-year-olds will develop CKD during their lifetime (5). Eleven percent of individuals with stage 3 CKD will eventually progress to end-stage renal disease (ESRD), requiring dialysis or kidney transplantation. CKD is also one of the strongest risk factors for cardiovascular disease (6–8). The costs to care for CKD (US$49 billion) are more than twice as large as ESRD costs ($23 billion). Available treatments to slow CKD progression and prevent CKD-related complications are quite limited (9) and include angiotensin-converting enzyme inhibition, angiotensin receptor blockade, optimal blood pressure control, and sodium bicarbonate for metabolic acidosis. Despite these therapies, outcomes in CKD remain poor (10).

Fibrosis is a pathologic extension of the normal wound healing process characterized by injury, inflammation, myofibroblast activation and migration, and matrix deposition and remodeling. Many of the pathophysiologic principles underlying kidney fibrosis are shared by other fibrotic diseases, for example, cirrhosis (11), the cardiomyopathies (12, 13), and idiopathic pulmonary fibrosis (14). Collectively, fibrotic diseases have been estimated to account for up to half of deaths in the developed world (15).

THE MYOFIBROBLAST AND FIBROSIS

Excess connective tissue accumulation during a reparative or reactive process causes tubulointerstitial fibrosis. All compartments in kidney can be affected, including glomeruli during glomerulosclerosis and arteriosclerosis in the vasculature. Interstitial fibrosis is characterized by expansion of the space between tubular basement membrane and peritubular capillaries through deposition of matrix proteins. The most abundant matrix protein in renal fibrosis is collagen 1, but other collagens accumulate, including types III, V, VI, VII, and XV, as well as the adhesive glycoprotein fibronectin (16). It is generally accepted that the activated myofibroblast is a primary matrix-secreting cell type, though it is by no means the only cell type capable of secreting matrix proteins (17). Indeed, the precise contributions of different cell types to overall matrix mass in renal fibrosis remain undefined and represent a balance between matrix secretion and degradation.

Myofibroblasts combine features of fibroblasts and smooth muscle cells, and they are largely reactive cells present under conditions of injury or pathology such as cancer. The best marker of the myofibroblast is alpha-smooth muscle actin (α-SMA), which forms bundles of myofilaments called stress fibers. These stress fibers are critical in connecting the myofibroblast to extracellular matrix, and when the cell contracts, this exerts mechanical forces on the matrix, causing reorganization and wound closure during healing. Other myofibroblast markers include plasma membrane fibronectin, vimentin, and collagen-1α1 (18). All myofibroblasts express platelet-derived growth factor receptor-beta (PDGFR-β); however, fibroblasts and pericytes also express this marker. The

secretory capacity of myofibroblasts is reflected by their abundant rough endoplasmic reticulum and collagen secretion granules (19). These cells are exquisitely sensitive to transforming growth factor-beta 1 (TGF-β1), a master regulator of myofibroblast differentiation in fibrosis (20–24). Roles for regulation of renal fibrosis by TGF-β have been recently reviewed and are not covered in detail herein (25).

A large body of experimental evidence supports a primary role for the activated myofibroblast in renal fibrosis. Using a PDGFR-β–Cre driver, Henderson and colleagues (26) deleted the αV integrin, an integrin subunit important for transmitting profibrogenic TGF-β signaling. They observed substantially reduced fibrosis not only in the kidney unilateral ureteral obstruction (UUO) model but in lung and liver fibrosis models as well. Recently, the same group reported that pharmacologic targeting of αV integrin on kidney myofibroblasts recapitulates the antifibrotic phenotype (27). Lebleu et al. (28) generated a mouse model in which herpes simplex viral thymidine kinase is expressed in myofibroblasts under control of the α-SMA promoter. Delivery of ganciclovir results in thymidine kinase–mediated phosphorylation of ganciclovir to a product that induces apoptosis only in proliferating cells due to incorporation into synthesizing DNA. Specific ablation of proliferating α-SMA$^+$ myofibroblasts caused a 50–60% reduction in collagen accumulation and interstitial fibrosis in several different renal fibrosis models, strongly implicating the myofibroblast in fibrogenesis.

We have described the zinc finger transcription factor glioma-associated oncogene homolog 1 (Gli1) as specifically upregulated in myofibroblasts during renal fibrosis (29). In order to assess the functional importance of myofibroblasts in the fibrotic process, we crossed a Gli1–CreER^{t2} line, which expresses tamoxifen-inducible CreER^{t2} exclusively in cells that express Gli1, against the inducible diphtheria toxin receptor (iDTR) mouse line (30). Administration of tamoxifen to bigenic Gli1–CreER^{t2} iDTR progeny causes Cre-mediated excision of a STOP cassette and expression of the human DTR, rendering cells sensitive to diphtheria toxin (DT)–mediated cell death. Using this model, we observed a 55–60% reduction in renal fibrosis after DT-mediated ablation of Gli1$^+$ myofibroblasts in UUO (31). Notably, the same genetic model improved cardiac function after ablation of cardiac myofibroblasts in the ascending aortic constriction model of cardiomyopathy and fibrosis, indicating that cardiac myofibroblasts are responsible for loss of organ function (31). The importance of Gli1$^+$ cells in fibrotic disease is further emphasized by their central roles in the pathophysiology of vascular calcification and myelofibrosis (32, 33).

MYOFIBROBLAST PROGENITORS

Although a strong body of evidence implicates myofibroblasts as critical cells in mediating organ fibrosis, controversy remains regarding the cell type(s) that contribute to the myofibroblast pool. Most available evidence indicates that resident stromal cells—kidney pericytes, perivascular fibroblasts, or mesenchymal stem cell–like cells—represent the predominant source of myofibroblasts in CKD (34). However, data also support contributions from other cell types, including bone marrow–derived cells (macrophages and "fibrocytes") and endothelial cells. For some time, it had been concluded that epithelial cells, through a process of epithelial to mesenchymal transition (EMT), made a major contribution to the interstitial myofibroblast pool. Subsequent studies have not supported this theory (35–39), however, and an emerging consensus suggests that epithelial-derived cells comprise a very small portion of the myofibroblast pool, if at all.

Several investigators have used genetic lineage analysis to measure the relative contributions of the various myofibroblast progenitor pools. We recently reported that Gli1 expression marks a perivascular population of mesenchymal stem cell–like cells that form an extensive network from the arterial adventitia in the pericyte niche across organs (40, 41). Genetic fate-tracing experiments

revealed that these perivascular Gli1$^+$ cells are a major source of kidney myofibroblasts and can be targeted therapeutically (40, 42). Quantification of Gli1-derived myofibroblasts revealed that they contributed to approximately 55% of the total myofibroblast pool, and genetic ablation of the myofibroblast progenitors reduced fibrosis by almost the same percentage. Importantly, this leaves 45% of the remaining myofibroblasts that arise from Gli1-negative cells. These might be other resident stromal cells such as fibroblasts, endothelial cells, or bone marrow–derived cells.

Reports also implicate endothelium as a myofibroblast progenitor pool through endothelial to mesenchymal transition. Cultured endothelial cells do undergo a phenotypic transformation toward a myofibroblast phenotype after exposure to TGF-β in vitro, but this does not necessarily mean the same process occurs in vivo. Other studies purport to show colocalization of endothelial and myofibroblast markers, but both cell types are very thin, and the fluorescent imaging techniques utilized cannot reliably resolve whether apparent colocalization is actually partial cell overlay, diffraction, or true coexpression (43). Fewer studies have attempted to validate and quantify this process using lineage analysis in vivo. Two studies used the Tie2Cre mouse strain to genetically tag endothelium and track its fate in kidney fibrosis (44, 45). Resulting estimates ranged between 31% and 50% of α-SMA$^+$ myofibroblasts originating from endothelium. Yet these studies suffer from limitations in specificity of Tie2Cre expression in endothelium. Tie2 is known to be expressed in myeloid lineage cells, in addition to endothelial cells (46, 47). Furthermore, De Palma et al. (46) reported that mesenchymal Tie2-expressing cells may differentiate into vascular pericytes in mammary tumors, and Tie2 is strongly expressed in cultured retinal pericytes (48). Because Tie2 is expressed in the myeloid lineage and may be expressed in pericytes, it remains unclear to what degree endothelium might contribute to the myofibroblast pool, but 30–50% is almost certainly a significant overestimate.

Recently, Lebleu et al. (28) used a Cdh5–Cre mouse line to genetically label and track endothelial cells during renal fibrosis, concluding that 10% of the myofibroblast pool originated from endothelial precursors. Unfortunately, Cdh5–Cre is expressed in roughly half of all hematopoietic lineages, precluding its use as an endothelial-specific Cre driver (49). In the same article, the investigators performed bone marrow transplantation experiments with α-SMA–red fluorescent protein transgenic reporter mice and measured that 35% of renal myofibroblasts after UUO were derived from bone marrow (28). There is a considerable literature implicating bone marrow–derived cells as myofibroblast progenitors, and candidates include mesenchymal stromal cells (MSCs) and fibrocytes (CD45$^+$, CD34$^+$), the latter derived from circulating monocytes in a process of monocyte to mesenchymal transition (50, 51). Reich et al. (52) reported that bone marrow–derived collagen-I$^+$ and CD11b$^+$ fibrocytes are distinct from monocytes and were recruited to injured kidneys after UUO. Depletion of CD11$^+$ fibrocytes using the human DTR resulted in reduced renal collagen I deposition (52). By contrast, Duffield and colleagues (18) have reported exceedingly low numbers of myeloid-derived collagen-I–producing cells in UUO using a ColI–GFP reporter and bone marrow transplantation. At this point, further lineage-tracing studies are required to clearly define which bone marrow–derived cells serve as myofibroblast progenitors in solid organ fibrosis and to elucidate the exact bone marrow cell type involved.

MESENCHYMAL CELL HETEROGENEITY

The emerging consensus that kidney myofibroblasts predominantly come from resident mesenchymal cells has refocused interest on this historically understudied kidney lineage. An important advance in this area has been fate-mapping studies by Kobayashi and McMahon, which were enabled by FoxD1–Cre and FoxD1–CreER^{t2} mouse lines that they generated. These investigators showed unambiguously that FoxD1 expression defines a self-renewing population of progenitors

during nephrogenesis that give rise to cortical and medullary interstitium, mesangium, and pericytes in kidney (53). We and others have shown that in adults, descendants of FoxD1$^+$ progenitors differentiate into myofibroblasts during renal fibrosis (35).

It remains unclear whether FoxD1-derived cells—interstitial cells, mesangial cells, and pericytes—are homogenous populations, or rather heterogeneous cell types with distinct functional properties. Several lines of evidence suggest the latter. For example, during nephrogenesis, FoxD1 derivatives include LIV2$^+$ capsule and Liv2$^-$ medullary interstitium. Conversely, FoxD1-derived medullary interstitium is CDKN1C$^+$, but the capsule is CDKN1C$^-$. Furthermore, all PDGFR-β^+ kidney cells derive from FoxD1$^+$ progenitors, and Gli1$^+$ cells are also PDGFR-β^+ in adult kidney, suggesting that Gli1$^+$ cells also derive from the FoxD1 population (35). However, Gli1$^+$ cells represent a relatively small subset of the PDGFR-β^+ kidney population, enriched in the outer medulla with much less expression in pericytes and perivascular fibroblasts of the outer cortex (29).

Yanagita and colleagues have also provided genetic evidence for the lineage of kidney mesenchymal cells (54). They studied the fate of Myelin protein zero (P0), a marker of migrating neural crest cells in the early embryo (55), as well as in Schwann cells, which also originate from neural crest. Using P0–Cre mice crossed to several different reporter strains, they showed that 98% of renal fibroblasts in both medulla and cortex are PDGFR-β^+ mesenchymal cells (36). They further show that a subset of P0–Cre lineage cells migrates into the embryonic kidney through the capsule, where some of them coexpress FoxD1. In models of kidney fibrosis, P0 lineage fibroblasts also differentiated into myofibroblasts. These observations suggest that the P0- and FoxD1-derived interstitial populations largely overlap, with P0 expressed earlier in development. FoxD1 is also expressed in neural crest, however, so it is also possible that FoxD1–Cre labels cells at that early stage as well, but this has not been investigated (56).

Other evidence from adult kidney that strongly suggests the existence of multiple stromal populations although genetic lineage analysis data is lacking. Ecto-5′-nucleotidase (CD73) is expressed exclusively in cortical mesenchymal cells of healthy kidney but not medullary interstitium (57). Whereas α-SMA expression is induced in both cortical and medullary myofibroblasts during fibrosis, only medullary myofibroblasts upregulate expression of Wnt4 (58). Very recently, the Haase group reported (59) that only a subpopulation of FoxD1-derivative renal cells regulates erythropoietin expression in a prolyl-4-hydroxylase domain-2–dependent fashion. This capacity, they propose, results from the differential ability of interstitial cells to regulate erythropoietin regulator hypoxia-inducible factor (HIF)-2α.

A recent paper from Evans and colleagues (60) illustrates the challenges of defining stromal cell subtypes. These authors sought to test whether pericytes function as mesenchymal stem cells in vivo. Crisan et al. (61) have previously reported that, in human tissue, some pericytes express the typical surface marker pattern of mesenchymal stem/stromal cells and have a trilineage capacity. Moreover, several genetic lineage analyses showed that perivascular cells had a capacity to differentiate into adipocytes in vivo, a property consistent with an MSC. Using an α-SMA–CreER^{t2}, Jiang et al. (62) showed new labeling of adipocytes after a 30-day chase. Previously, a Pdgfr-β–Cre line was used to label mural cells of the adipose vasculature, and descendants included the bulk population of adipocytes, which is also consistent with MSC localization to the pericyte niche in vivo (63).

Evans and colleagues (59) had identified *Tbx18* as a gene expressed specifically in pericytes and vascular smooth muscle cells but not in Pdgfr-α^+ perivascular fibroblasts. They generated an inducible Tbx18–CreER^{t2} mouse strain and compared the labeling pattern with that obtained with the constitutive Pdgfr-β–Cre line (64). They screened multiple organs and concluded that Tbx18–CreER^{t2} exclusively labels interstitial cells that were neither leukocytes (CD45$^-$) nor endothelium

(CD31$^-$). They were PDGFR-β$^+$, CD146$^+$, and NG2$^+$ but not PDGFR-α$^+$. Combined with their localization directly adjacent to endothelial cells, they concluded that Tbx18–CreER^{t2} specifically labeled pericytes. The fact that Pdgfr-α$^+$ cells were not labeled was important and suggested that Tbx18–CreER^{t2} does not label perivascular fibroblasts (also known as adventitial fibroblasts). By contrast, the constitutive Pdgfr-β–Cre labeled not only interstitial mesenchymal cells but a wide range of others, including most hematopoietic cells and parenchyma in kidney, lung, skeletal muscle, and brain. They concluded that the Pdgfr-β–Cre line lacked specificity for lineage tracing of pericytes (60).

Utilizing several pulse-chase designs and disease models, the authors went on to show that Tbx18$^+$ pericytes do not contribute to any other cell lineages (such as adipocytes) during aging or when on a high fat diet. Finally, they report that Tbx18$^+$ pericytes do not give rise to myofibroblasts during the aortic constriction model of cardiomyopathy. This is in contrast to our own results, showing that Gli1$^+$ MSC–like cells did contribute substantially to cardiac fibrosis, and their ablation rescued heart function (40). What can explain these discrepant results? Although still speculative, it seems likely that the MSC properties and myofibroblast progenitor functions may reside within the adventitial fibroblast niche, at least in some vascular beds (no kidney fibrosis models were tested in this study). Other lineage tracing strategies ended up labeling both pericytes and perivascular fibroblasts, such as Pdgfr-β–Cre-, Nestin–Cre- and α-SMA–CreER^{t2}–based strategies. We have shown that Gli1 also is expressed in both pericytes and perivascular fibroblasts, so it is possible that in heart, it is the Gli1$^+$ perivascular fibroblast population that predominantly contributes to the myofibroblast pool.

These studies point to the strong need for better markers to distinguish stromal cell subtypes. New technologies such as single-cell RNA sequencing will likely aid greatly in defining cellular hierarchies in the interstitium, as this powerful approach is very well suited to discovering novel cell types (65). The next challenge will be to define functional differences between these cell subtypes, for example, using genetic lineage analysis. The EMT controversy in renal fibrosis (66) and the pericyte controversy in adipogenesis (67) point to the importance of basing conclusions about cell potential primarily on the in vivo situation and not on ex vivo culture, where artifactual cell phenotype changes may be misinterpreted as reflecting cell biology within the animal.

GENETICS OF CHRONIC KIDNEY DISEASE

The last decade has witnessed an accelerating pace of discovery regarding the genetic basis for CKD. Genome-wide association studies (GWAS) have identified more than 50 genetic loci associated with CKD and estimated glomerular filtration rate (eGFR) (68). In several cases, functional studies of genes identified in these studies have identified novel mechanisms for CKD pathogenesis, confirming the power of this approach. Given ongoing concerns over the translatability of rodent models of CKD to human biology, the expanding knowledge base of the genetics of CKD represents one of the most exciting areas in the field today.

Pattaro et al. (69) have published the largest GWAS meta-analysis to date, which analyzed up to 175,579 individuals of diverse ancestry. The authors examined associations between eGFR and 2.5 million single-nucleotide polymorphisms (SNPs). They confirmed 29 previously identified loci and identified 24 new ones. Because of the large size of this study, the authors had sufficient statistical power using bioinformatic approaches to ask whether genes at associated loci were specifically expressed in kidney. Indeed, many genes at associated loci had enriched expression in kidney and urinary tract. Furthermore, some SNPs were located in areas likely to be transcriptionally active, as assessed by DNase I hypersensitivity. Cell types with DNase I hypersensitivity patterns in eGFR-associated SNPs include proximal tubule epithelial cells, further solidifying the

conclusion that many genetic variants associated with eGFR affect gene regulation in the kidney itself.

Several of the genetic loci identified by Pattaro et al. (69) are of particular interest. Wnt signaling is critical both during nephrogenesis and in fibrotic kidney disease. One SNP associated with eGFR maps to an intron in the *WNT7A* gene. Although this SNP demonstrated only suggestive replication, experimental evidence correlates increased Wnt7a expression with renal fibrosis (70, 71), and experimental manipulations that inhibit Wnt-β–catenin signaling ameliorate fibrosis (72). Therefore, it is tempting to speculate that this SNP might alter *WNT7A* protein levels and thereby either influence kidney development in such a way as to predispose toward the future development of CKD, or to promote fibrosis itself in the adult kidney.

Another identified eGFR-associated SNP falls within an intronic region of the *NFKB1* gene. This is an inflammation regulatory transcription factor that drives transcription of age-associated gene expression in human kidney (73). Furthermore, myeloid differentiation primary response gene 88 (MyD88)—an adaptor protein linking both interleukin-1 (IL-1) and Toll-like receptors (TLRs) to nuclear factor-kappa B (NF-κB)–mediated proinflammatory signaling—regulates proinflammatory and profibrotic signaling in kidney pericytes (74). Finally, mutations in TLR4 or IL-1 associate with kidney injury and ESRD, respectively (75, 76). Although the mechanism underlying the association of the *NFKB1* SNP with reduced eGFR remains unknown, these observations collectively suggest the possibility that this association could impact pericyte-mediated proinflammatory signaling in kidney.

CELL CYCLE ARREST AND TUBULOINTERSTITIAL CROSS TALK

One of the major developments in the study of renal fibrosis over the past five years is a heightened appreciation for tubulointerstitial cross talk as a major driver of progression. Two contributors to this knowledge base include the expanding use of cell-specific gene ablation techniques and the growing field of cell senescence in aging (77). Epithelial injury remains a central event in the pathogenesis of CKD. This may arise through inflammation, for example, from acute interstitial nephritis; alternatively, it occurs through direct epithelial injury, as occurs with albuminuria in diabetic nephropathy. Direct epithelial damage causes dedifferentiation and upregulation of proteins that may themselves orchestrate proinflammatory signaling, such as the phosphatidylserine receptor kidney injury molecule-1 (Kim-1), which is adaptive in the short term because it mediates clearance of dead cells and debris from the tubular lumen (78). However, chronic expression of Kim-1 is maladaptive and sufficient to cause leukocyte infiltration, tubular atrophy, CKD, and ultimately, renal failure (79).

Repetitive acute insults or ongoing inflammation cause epithelial damage that may accumulate over time and ultimately cause cell senescence. As an evolutionarily conserved state of stable replicative arrest, cell senescence is driven by injuries often accumulated during aging, such as oxidative stress, telomere shortening, and DNA damage (77). These injuries stimulate upregulation of cyclin-dependent kinase (CDK) inhibitors, including $p16^{Ink4a}$ and $p53\text{-}p21^{Cip1/Waf1}$, leading to cell cycle arrest (**Figure 1**) (80). These senescent cells then adapt the senescence-associated secretory phenotype, which is a cell state characterized by both resistance to apoptosis and the ongoing secretion of a broad range of proinflammatory and profibrotic paracrine mediators, such as growth factors, TGF-β, cytokines, and proteases (81).

Yang et al. (82) have shown that, in multiple models of renal injury, epithelial cells become arrested in G2/M of the cell cycle and adopt a profibrotic secretory phenotype. Importantly, these G2/M-arrested cells can be targeted pharmacologically to reduce fibrosis (82, 83). $p16^{Ink4a}$ inhibits CDK4 and CDK6, and we have also shown that targeting these cell cycle regulatory proteins can

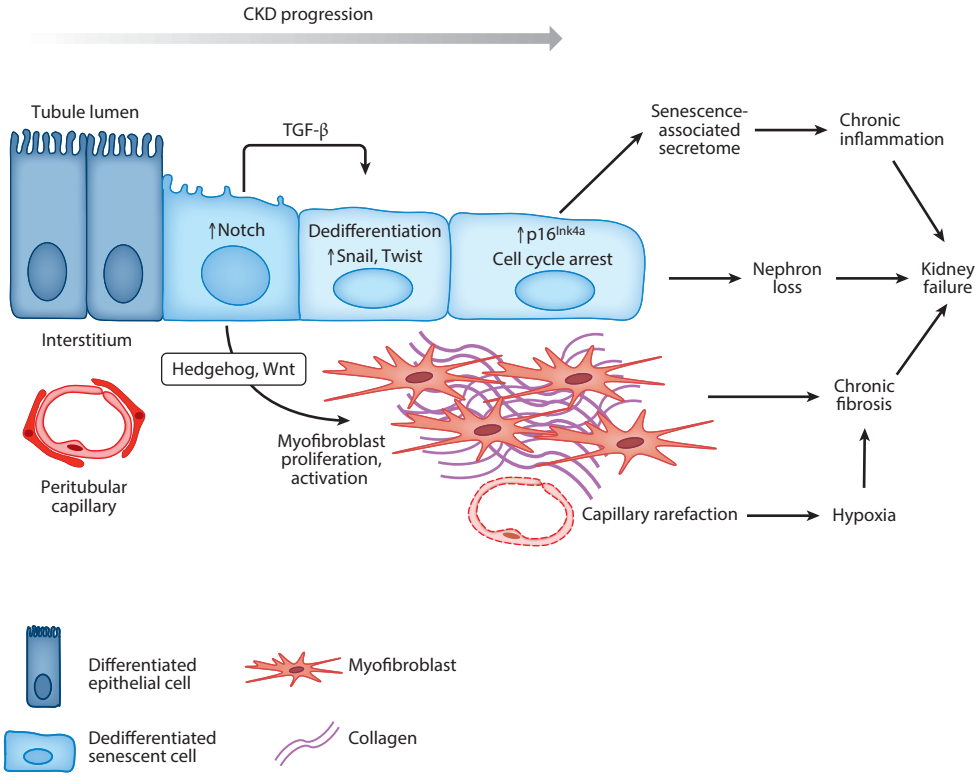

Figure 1

Critical cells and signaling pathways activated in progressive chronic kidney disease (CKD). CKD is initiated by cellular injury, either to the epithelial or endothelial compartment. In the tubulointerstitium, injury leads to epithelial dedifferentiation that may be induced by TGF-β and Notch pathway upregulation. Dedifferentiated epithelial cells secrete paracrine signaling factors, such as hedgehog and Wnt ligands, that act on interstitial pericytes and mesenchymal stem cell-like cells to activate myofibroblast differentiation, proliferation, and matrix secretion. This in turn causes peritubular capillary rarefaction and ongoing hypoxia. Chronic tubular injury leads to epithelial cell cycle arrest and senescence, with accompanying secretion of proinflammatory cytokines that amplify inflammation. These interrelated events ultimately drive nephron loss, ongoing interstitial fibrosis, and kidney failure.

also be renoprotective in acute kidney injury (AKI) (84). Administration of the CDK4/6 inhibitor PD-03332991 caused a transient inhibition of proximal tubule proliferation at both 24 h and 48 h after injury, which was associated with reduced DNA damage, epithelial apoptosis, interstitial inflammation, and improved renal function (84). These results were corroborated by another group (85). The induction of transient proximal tubule cell cycle arrest or "pharmacologic quiescence" by CDK4/6 inhibition may protect cells from DNA damage associated with cell cycle progression in the harsh postischemic inflammatory environment. This allows more time for repair of DNA damage with reduced early apoptosis, as well as reduced senescence and AKI to CKD transition.

Consistent with an important role for tubular cell cycle arrest in renal fibrosis, Braun et al. (86) observed that mice with *INK4a* deletion escaped from p16^{INK4a}-dependent senescence and had less interstitial fibrosis and tubular atrophy, increased tubular proliferation, and reduced senescent tubular cells after unilateral ischemia-reperfusion injury compared to wild-type mice. Van Deursen and colleagues (87) generated an elegant mouse model that allows inducible

elimination of p16^{INK4a}-positive cells upon administration of a drug. Ablation of p16^{INK4a}-expressing cells in the BubR1 progeroid mouse delayed the onset of age-related pathologies in adipose tissue, skeletal muscle, and eye. Very recently, the same group published a follow-up study in which they ablated the cells in wild-type mice. They showed that by ablating the p16^{INK4a}-expressing cells, there was an increase in life span, delayed tumorigenesis, and attenuation of age-related pathologies. Remarkably, in kidney, the senescent cells were localized to the proximal tubule, and ablation of these *INK4a*$^{+}$ senescent tubular cells caused an improvement in age-related glomerulosclerosis (88). Targeting senescent cells—senotherapy—shows promise for treatment of idiopathic pulmonary fibrosis and is under investigation as a novel therapeutic target in CKD (81, 89).

Signaling pathways that are required for nephrogenesis, such as the Wnt, hedgehog (Hh), and Notch pathways, also play important roles during renal fibrosis. Transient upregulation of β-catenin in tubular cells in injured kidney (70, 90, 91) promotes survival of renal tubular cells through inhibition of proapoptotic Bax expression (90, 92). By contrast, tubule-specific ablation of β-catenin aggravates the intensity of AKI (90) but has no effect on interstitial fibrosis in a UUO model (91). In CKD, numerous Wnt ligands are upregulated, resulting in protracted activation of the Wnt/β-catenin pathway in tubular cells (93). Prolonged epithelial β-catenin activation causes epithelial dedifferentiation and interstitial fibrosis (91). Interstitial pericytes and fibroblasts are also important targets of Wnt ligands. Studies from the Liu laboratory (94) have established that tubule-derived Wnts are necessary for myofibroblast activation and interstitial fibrosis, whereas pericyte-derived Wnts do not play an apparent role. This emphasizes that Wnt ligands play a critical role in paracrine signaling between injured epithelia and interstitial myofibroblasts (**Figure 1**). Indeed, tubular secretion of Wnt1 is sufficient to drive spontaneous interstitial fibrosis (95). Remarkably, this genetic model of fibrosis has no inflammatory component whatsoever, indicating that inflammation is not strictly required for the development of renal fibrosis.

We have shown that chronic kidney injury causes induction of Wnt4 in kidney medullary interstitial fibroblasts (58). However, stromal deletion of Wnt4 did reduce the severity of fibrosis. In fact, β-catenin levels were elevated in both wild-type and Wnt4-null kidneys, suggesting that other Wnt ligands compensated for the absence of Wnt4 (70, 96). Consistent with this, constitutive activation of canonical Wnt signaling in renal stroma led to spontaneous myofibroblast differentiation (58).

We and others have defined important roles for Hh–Gli signaling in renal fibrosis. The three Hh ligands—sonic (Shh), Indian (Ihh), and desert—bind to their receptor, patched1, in target cells. In healthy kidney, Hh ligand expression is very low, limited to rare expression of Ihh in the outer medulla and Shh in the papilla (29). During fibrotic injury, Hh ligands are upregulated in the proximal tubule. There is disagreement in the literature concerning whether this ligand is Ihh or Shh (29, 97). Ligand upregulation is accompanied by Hh pathway activation, as reflected by transcription of two Hh target genes, *Ptch1* and *Gli1* (29). Similar to Wnt signaling, the Hh ligand acts in a paracrine fashion to activate interstitial pericytes and peritubular fibroblasts in a tubulointerstitial signaling loop (**Figure 1**). We have shown that genetic deletion of Gli2 in pericytes and perivascular fibroblasts inhibits fibrosis and is associated with a G0/G1 cell cycle arrest of those cells. This pathway can be targeted pharmacologically, and the Gli inhibitor Gant61 is also antifibrotic in several models of renal fibrosis (42).

A growing body of evidence suggests that Hh–Gli activation in MSCs represents a conserved profibrotic pathway across organs. Gli1^{+} MSC–like cells localize to the adventitia of large vessels, where they serve as a local stem cell pool for vascular smooth muscle cells during homeostasis, but they also serve as a progenitor for osteoblast-like cells responsible for vascular calcification during

injury. Triggers consisting of renal failure, hyperphosphatemia, and hypercholesterolemia lead Gli1$^+$ cells to migrate from the adventitia, through the intima, and into the vascular lumen, where they are responsible for calcification of the atheromatous plaque (32). Additionally, Gli1$^+$ MSCs in bone marrow are responsible for myelofibrosis, and administration of Gant61 is therapeutic in this disease context as well (33).

Similar to the Wnt and Hh pathways, the Notch pathway is also required for kidney development. The Notch signaling pathway consists of the Notch1–Notch4 receptors and their ligands, Delta and Jagged (Jag). Pathway activation occurs by expression of a Notch ligand in one cell with a Notch receptor in an adjacent cell. This triggers a series of proteolytic cleavages, ultimately resulting in translocation of the Notch intracellular domain to the nucleus where it activates target genes such as the transcription factors Hes and Hey (98). Notch signaling typically controls cell fates during differentiation. During fibrosis, the Notch pathway is reinduced in both mouse and human CKD. The Susztak lab (99) has shown in elegant studies that epithelial dedifferentiation that accompanies CKD results in Notch induction, which is both necessary and sufficient for tubulointerstitial fibrosis. The mechanism appears to be via Notch-dependent induction of epithelial cell proliferation and inhibition of cell differentiation (**Figure 1**).

It is tempting to speculate that epithelial Notch may mediate fibrosis through activation of the EMT transcriptional program. Twist and Snail are transcription factors that play a critical role in regulating cancer EMT during metastasis. The Nieto and Kalluri groups (100, 101) have both observed that these transcription factors are required for renal fibrosis. Using both genetic and morpholino-based approaches, the Nieto group showed that reactivation of epithelial Snail1 is both required for and sufficient to drive renal fibrosis. Snail1 can also be targeted therapeutically to prevent fibrosis. The mechanism is through a partial EMT, which refers to an epithelial dedifferentiation that triggers secretion of factors (as yet undefined) that mediate interstitial myofibroblast proliferation and inflammation. Of note, "partial EMT" in this context refers to epithelial dedifferentiation with secretion of profibrotic factors that activate interstitial myofibroblasts in a paracrine fashion—but not direct conversion of epithelial cells into myofibroblasts.

VASCULAR DAMAGE AND CAPILLARY RAREFACTION

Excessive collagen production by myofibroblasts reduces peritubular blood flow, causing tubular hypoxia and nephron dropout, ultimately leading to renal failure. Proposed nearly 20 years ago, this "chronic hypoxia hypothesis" posits that tubulointerstitial hypoxia plays a central role in both the initiation and progression of CKD (102). Until recently, a large body of mostly correlative data supported this theory, but evidence of direct causality was lacking (103). Human kidneys damaged by CKD are characterized by peritubular capillary rarefaction (104). Measurements of tissue oxygenation in a number of different experimental models of CKD confirm hypoxia (105, 106). HIF-1α is upregulated in hypoxic tubular epithelial cells, including in human CKD, and it regulates profibrotic signaling pathways (107, 108). The loss of peritubular capillaries through acute or chronic injury triggers local hypoxia, further damage to adjacent tubules, upregulation of profibrotic pathways, and a self-reinforcing vicious cycle.

During kidney injury, Gli1$^+$ perivascular cells detach from the renal vasculature to become interstitial fibrosis–driving myofibroblasts (18, 109), and genetic ablation of GLI1$^+$ cells reduces kidney fibrosis (31). To investigate the physiological role of these resident kidney MSC-like cells during homeostasis, researchers performed genetic ablation of GLI1$^+$ cells in healthy mice that were followed up for 10 or 56 days. Interestingly, GLI1$^+$ cell ablation alone was sufficient to cause endothelial cell damage with capillary rarefaction and hypoxia and with transient tubular epithelial injury (109). Thus, kidney GLI1$^+$ MSC–like cells are critical for kidney homeostasis and stabilize

the renal vasculature. The absence of Gli1$^+$ MSCs alone is sufficient to drive capillary rarefaction. It will be important to follow up these studies by analyzing whether this capillary rarefaction is in turn sufficient to drive renal fibrosis; if so, this would represent strong and direct support for the chronic hypoxia hypothesis (110).

Pericyte–endothelial interactions are also a therapeutic target in kidney fibrosis. The ephrinB2 ligand binds to its receptor EphB4, and this interaction is required for angiogenesis during development (64). EphrinB2 is membrane bound, and upon ligation with EphB4, sends a "reverse signal" though its intracellular PDZ domain. Kida et al. (111) identified a novel role for this signaling pathway in kidney injury. EphrinB2 and EphB4 are expressed on both endothelial cells and pericytes in kidney, which is consistent with known roles for this signaling axis in other vascular beds. Using mutant mice that lack the ephrinB2 intracellular PDZ domain, the authors showed that ephrinB2 signaling was required to prevent capillary rarefaction after injury because mutant mice had worsened angiogenesis and fibrosis after injury. Moreover, pericytes with the mutant PDZ domain failed to stabilize vasculature and exhibit enhanced migration.

These results suggest that the ephrinB2–EphB4 interaction between pericytes and endothelial cells stabilizes vasculature and that this effect may be lost after injury. Whether administration of soluble ephrinB2 might rescue this angiogenic deficiency after injury, prevent capillary rarefaction and interstitial fibrosis, and allow endogenous repair to proceed remain unanswered. Basile et al. (112) have also shown that acute ischemia represses expression of angiogenic vascular endothelial growth factor (VEGF) while enhancing expression of the VEGF antagonist, a disintegrin and metalloproteinase with thrombospondin motif 1 (ADAMTS-1). By contrast, administration of a VEGF fragment (VEGF-121) during repair preserved microcapillary density, indicating that VEGF agonism may be a therapeutic strategy to prevent AKI-induced capillary rarefaction (113). However, Lin et al. (114) recently reported that antagonism of VEGF receptor signaling attenuated capillary rarefaction after injury. They expressed a soluble VEGF receptor to bind and sequester VEGF. It is not yet clear how to reconcile these apparently contradictory results. One possibility is that Basile and colleagues used an acute injury model, whereas Lin and colleagues used a chronic injury model. Further investigation is required to better evaluate the utility of targeting the VEGF pathway in renal fibrosis.

Bijkerk et al. (115) have also shown that vasculogenesis itself can be augmented as a therapeutic strategy. MicroRNA-126 (mir-126) positively regulates angiogenesis, and they hypothesized that overexpression of this microRNA would decrease renal injury by a provasculogenic effect. They generated mice with bone marrow–specific mir-126 overexpression by transplantation and showed enhanced neovasculogenesis of subcutaneous matrigel plugs compared to control mice. The overexpression of mir-126 protected mice from AKI by preserving peritubular capillary density, and these capillaries contained bone marrow–derived progenitors that expressed mir-126. These experiments provide another encouraging proof-of-principle that neovasculogenesis can be induced by manipulating angiogenic pathways and that such an approach can ameliorate kidney injury.

ON THE HORIZON: REPROGRAMMING AND FATE CONVERSION TO TREAT CHRONIC KIDNEY DISEASE

Following development, cellular identity is highly stable and typically maintained for the life span of an organism. The landmark discovery that mature cells can be reprogrammed to pluripotency has refocused attention on cell plasticity, however. Cell conversion from one differentiated cell type to another can occur by either dedifferentiation or transdifferentiation. Dedifferentiation is

reversion to a more primitive cell type encountered within that developmental lineage. The generation of induced pluripotent stem cells by transient expression of certain transcription factors in differentiated cells represents the most dramatic example of experimentally induced dedifferentiation (116). Transdifferentiation refers to direct conversion of one differentiated cell type into another without an intermediary progenitor-like step. An example of transdifferentiation is the direct reprogramming of human fibroblasts to hepatic cells (induced hepatic cells or iHeps) (117, 118).

The ability to directly reprogram fibroblasts into hepatocytes in vitro raised the possibility that such a fate conversion in vivo (from myofibroblast to hepatocyte) might represent a novel therapeutic approach to treat cirrhosis, a fibrotic chronic liver disease. Using similar approaches, two research groups recently did just that. The Sharma laboratory (119) used a p75 neurotrophin receptor peptide–tagged adenovirus to deliver the four iHep transcription factors—FOXA3, GATA4, HNF1A, and HNF4A—to liver myofibroblasts. iHep induction was rare: It was only 1% efficient, in fact, yet this was sufficient to ameliorate fibrosis in the CCL4 model. The iHeps were functional, including demonstrated albumin secretion and urea synthesis. In a study published in the same journal issue, the Willenbring group (120) used an adeno-associated virus (AAV) that is already known to efficiently transduce liver myofibroblasts and that has already been used in human clinical trials; it is known as AAV6. Similar to the Sharma laboratory's study, the Willenbring group's induced iHeps inefficiently, but their induction was antifibrotic, and iHeps exhibited hepatocyte function, including albumin synthesis and urea production.

Can such an approach be envisioned to treat CKD? Indeed, Kaminski and colleagues (121) have recently reported a new method to generate induced renal tubular epithelial cells (iRECs) from mouse or human fibroblasts. They selected a group of 55 transcription factors based on high kidney expression, evolutionary conservation, and essential function in kidney development. Through combinatorial testing, the authors identified four transcription factors—Emx2, Hnf1b, Hnf4a, and Pax8—that were sufficient to reprogram fibroblasts to iRECs. These cells formed tight junctions and had typical transport gene expression, three-dimensional spheres when grown in matrigel, and other characteristics of mature iRECs. This is an exciting advance that suggests that, in principle, myofibroblasts could be directly reprogrammed to tubular cells in vivo as a novel antifibrotic and regenerative strategy. Such an approach is shown in **Figure 2**.

Several barriers to implementation of direct reprogramming as a therapy for CKD remain. First, there have been no reports of successful targeting of kidney fibroblasts, pericytes, or myofibroblasts by a nonintegrating viral vector, for example, adenovirus or adeno-associated virus, in vivo. Second, the iREC protocol generates epithelial cells from multiple nephron segments, and which segment identity a reprogrammed cell takes on appears to be stochastic (121). This represents a major limitation because a cortical myofibroblast reprogrammed into a medullary thick ascending limb epithelial cell will not be in the correct kidney region to integrate into its proper segment. Third, unlike liver, iRECs need to migrate across the tubular basement membrane to take up residence in an existing nephron. It is not clear whether such a process is even possible, particularly because studies of EMT in fibrosis have shown that epithelial cells do not do the reverse—migrate across the basement membrane into the interstitium. Despite these hurdles, attempts to harness the power of transdifferentiation for therapy of CKD remain a promising and exciting new area of investigation.

SUMMARY

The wealth of new knowledge concerning the basic pathophysiology of renal fibrosis gives reason for optimism that new therapies to slow or even reverse CKD may be on the horizon. Targeting the

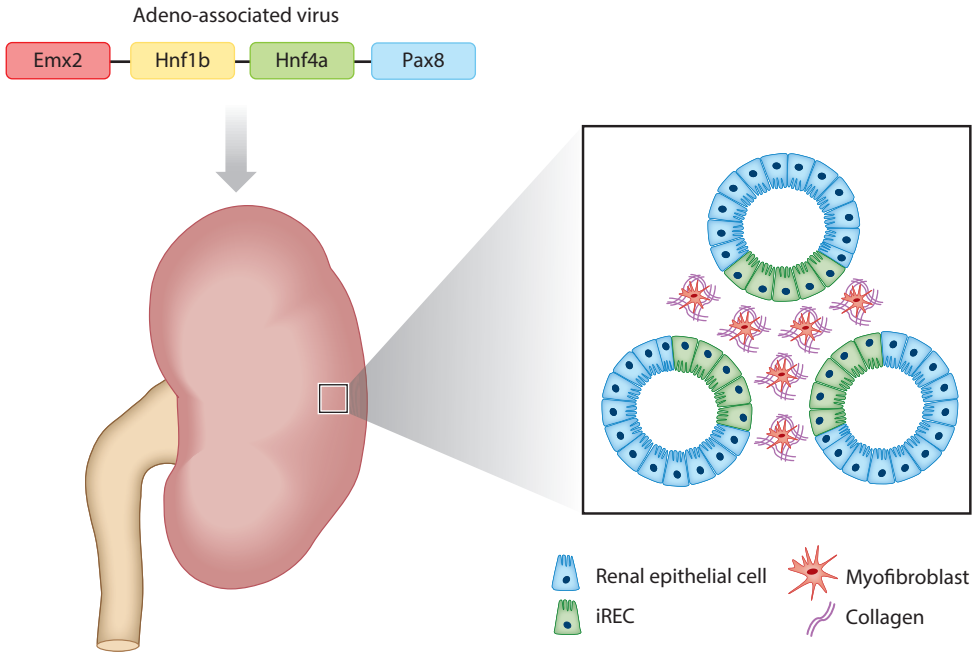

Figure 2

An approach to in vivo reprogramming of myofibroblasts into induced renal epithelial cells (iRECs). It is possible to convert fibroblasts into renal epithelial cells by expression of the transcription factors Emx2, Hnf1b, Hnf4a, and Pax8, albeit at low efficiency (121). Two recent reports have succeeded at reprogramming myofibroblasts into hepatocytes in vivo (119, 120). Such an approach could be applied to kidney and is depicted here. A polycistronic adeno-associated virus (AAV) is engineered to express all four factors. The precise AAV subtype would be determined based on its ability to transduce kidney myofibroblasts. After intravenous injection, kidney myofibroblasts would be reprogrammed into iRECs. These new epithelial cells could integrate into diseased tubules, replacing senescent and dedifferentiated cells with functional iRECs, thereby restoring tubular function.

signaling pathways that mediate tubulointerstitial cross talk appears to be a particularly promising strategy. Although the slow progression of CKD makes designing clinical trials especially challenging, advances in our understanding of the important cell types and molecular pathways that mediate fibrosis should lead to improved diagnostics that will ultimately facilitate translation to human CKD.

DISCLOSURE STATEMENT

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