The FOXD1 lineage of kidney perivascular cells and myofibroblasts: functions and responses to injury

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Recent studies have identified a poorly appreciated yet extensive population of perivascular mesenchymal cells in the kidney, which are derived from metanephric mesenchyme progenitor cells during nephrogenesis at which time they express the transcription factor FOXD1. Some studies have called these resident fibroblasts, whereas others have called them pericytes. Regardless of nomenclature, many are partially integrated into the capillary basement membrane and contribute in important ways to the homeostasis of peritubular capillaries. Fate-mapping studies using conditional CreER recombinase-mediated tracing of discrete cell cohorts have identified these pericytes and resident fibroblasts as the major precursor population of interstitial myofibroblasts in animal models of kidney disease. Here, we will review the evidence that they are the major population of myofibroblast precursors, highlight some critical functions in homeostasis, and focus on the cell signaling pathways that are important to their differentiation into, and persistence as myofibroblasts.

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Recent studies using state-of-the-art genetic fate-mapping have provided compelling evidence that discrete cells partially attached to kidney peritubular capillaries are the major precursor population for cells that deposit pathological fibrillar matrix in interstitial kidney disease. Controversy has arisen because of the nomenclature of these cells, as some investigators have named them pericytes because of their spatial relationship to capillaries, whereas others have preferred the name resident fibroblasts.[1–4] Nevertheless, the identification and characterization of these cells has led to important new insights into the fibrogenic and regenerative processes and has identified new candidate pathways for potential therapeutic development.

FOXD1+-EXPRESSING NEPHROGENIC PROGENITORS AND THEIR PROGENY

Compelling evidence suggests that the kidney develops from a single mesenchymal progenitor, which expresses the transcription factor OSR1.[5] This progenitor gives rise to distinct mesenchymal progenitors, which form the nephron and the microvascular endothelium as well as the stroma of the kidney. Whereas epithelial progenitors express transcription factors such as SIX2, CITED1 and Wilms tumor-1, stromal progenitors transiently express the transcription factors FOXD1 and TCF21. Endothelial precursors express the vascular endothelial growth factor receptor-2.[3,5,6] FOXD1+ progenitor cells lie on the outer surface of the developing kidney, are maintained by self replication, and are possibly replenished later in development by a second population of mesenchymal progenitors arising from the neural crest where they transiently express the marker protein P-zero[1] (Figure 1). FOXD1+ nephrogenic stromal progenitors highly overlap with P-zero lineage mesenchymal progenitors from 13.5 days post conception (dpc) onward.[1] Collectively, FOXD1+ progenitors give rise to an extensive population of stromal cells, which mature to form vascular smooth muscle cells, glomerular mesangial cells, and an extensive population of highly branched cells throughout the interstitium of the adult kidney (Figure 1a). These cells are variably integrated into the walls of peritubular capillaries where they appear to serve important functions in homeostasis.[2,7] More than 95% of these non-glomerular perivascular cells produce collagen-I protein in the healthy
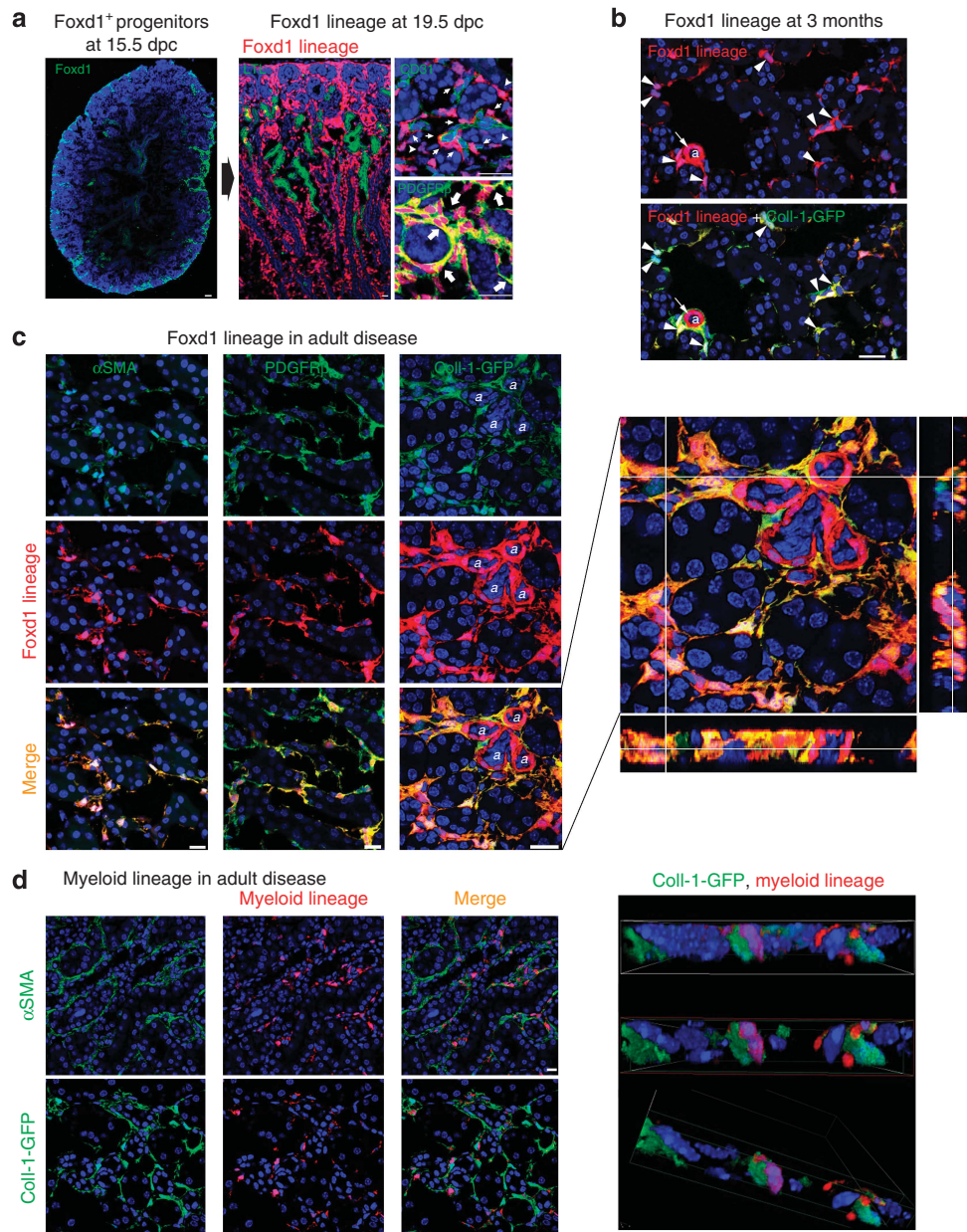
Figure 1 | Fate mapping of FOXD1 and myeloid lineage in kidney disease. (**a**) Confocal images of FOXD1 expression (left panel) restricted to stromal progenitors on the outer surface of the nephrogenic zone during nephrogenesis, whereas offspring of FOXD1 progenitors seen in $FOXD1^{+/Cre}$; $Rs26$-$tdTomato$-R bigenic mice (right panels) form an extensive population of stromal cells that are distinct from lotus lectin + (LTL) epithelium, CD31 + endothelium, but co-express platelet-derived growth factor receptor (PDGFR)-β (large arrows). Many stromal cells are attached to the endothelium (small arrows), whereas a minority are not (arrowheads). (**b**) Confocal images of healthy adult $FOXD1^{+/Cre}$; $Rs26$-$tdTomato$-R; $Coll$-GFP^{Tg} kidney showing FOXD1 lineage cells forming a network of branched cells between the tubules and attached to capillaries. Almost all generate collagen-I protein (arrowheads). They also form vascular smooth muscle of arterioles (**a**), which do not generate collagen-I protein. (**c**) Confocal images including z-stack three-dimensional (3D) reconstruction of unilateral ureteral obstruction (UUO) model (d10) of kidney disease showing FOXD1 lineage of interstitial cells expands, continues to express collagen-I protein (detected by $Coll$-GFP transgene) and PDGFR-β and now additionally co-expresses α-smooth muscle actin (α-SMA). (**d**) Confocal images including z-stack 3D reconstruction showing fate mapping of myeloid lineage in $Lysm^{+/Cre}$; $Rs26$-$tdTomato$-R or $Lysm^{+/Cre}$; $Rs26$-$tdTomato$-R; $Coll$-GFP^{Tg} diseased (d10 UUO) adult kidney, where myeloid lineage and their descendants permanently express red fluorescent protein. Note that, although there is an expansion myeloid lineage cells, and they closely interact with collagen-I-producing cells or α-SMA protein–producing cells, a lineage boundary is maintained between these two cell populations. Scale bar = 50 µm (**a**) and 25 µm (**b**–**d**).

kidney, the majority of which is likely turned over without being deposited, whereas a minority is incorporated into capillary basement membranes or supplies collagen for the loose connective tissue of the interstitial space (Figure 1b). Electron microscopy, three-dimensional (3D) imaging, and 3D cell cultures indicate that these cells form long, branched

processes[8–10] that extend many cell lengths. Although many branches are embedded in the basement membrane of the capillary, some extend to touch the tubular basement membrane.[11] Because of this spatial relationship to the capillary basement membrane, many of these cells may be considered to be pericytes, but others are more clearly located in the interstitial space and are considered to be fibroblasts. At this time, no marker has been identified to discriminate FOXD1 lineage cells attached to the capillary from FOXD1 lineage cells that are only in the interstitium, but it is likely that subpopulations will be identified with further studies.

In the setting of adult kidney disease, FOXD1+ nephrogenic progenitor–derived resident pericytes and fibroblasts have been mapped for their fate.[3,9,12,13] FOXD1 is only active in this lineage during kidney development and is not reactivated in disease (Figure 1a). This observation has been confirmed using the *Foxd1*$^{+/CreER}$ allele crossed to a fluorescent reporter mouse to label any FOXD1-expressing cells by administration of the drug tamoxifen during the unilateral ureteral obstruction model of adult kidney disease (data not shown). Because FOXD1 is not reactivated in adult disease and is only active during development of the nephrogenic stroma progenitor cells, it has proven to be a very useful gene locus to map cell fate from development into adult kidney disease. In disease states, FOXD1 lineage perivascular cells detach from capillary walls and migrate into the interstitial space where they proliferate.[2,7] Here, they continue to express collagen-I protein and lineage-restricted cell surface receptors, such as platelet-derived growth factor (PDGF) receptor-β, PDGF receptor-α, and CD248.[14,15] However, they also now express α-smooth muscle actin (α-SMA) protein, a marker of myofibroblasts and other markers such as P75 nerve growth factor receptor. In quantitative studies utilizing models of adult kidney disease, >98% of cells that co-express both collagen-I protein and α-SMA are of the FOXD1 lineage (Figure 1c).

CONTROVERSIES SURROUNDING THE IDENTITY OF MYOFIBROBLAST PROGENITORS

Previous studies suggested that kidney tubular epithelial cells are the major precursors for interstitial myofibroblasts. This hypothesis was based on cell-phenotype changes observed *in vitro* in tissue culture, but several fate-mapping studies in the kidney using rigorous methods have cast considerable doubt on that assertion.[3,16–20] At the same time, similarly robust studies in lung, liver, and other organs provide no evidence that the epithelium gives rise to myofibroblasts in those tissues during chronic disease.[21–24] A consensus has developed that the kidney tubular epithelium contributes to the fibrosis process predominantly by indirect mechanisms.[13,25] Understanding those mechanisms and cell signaling to FOXD1 lineage cells has become of paramount importance, and factors including metabolic derangements, endoplasmic reticulum stress, cell cycle arrest, senescence, and DNA damage are emerging as important stimuli for profibrotic signaling pathways.[26,27] A competing hypothesis

that endothelial cells differentiate into myofibroblasts has gained some support from lineage-tracing studies using the Cre-Lox DNA recombination method in mice, where the enzyme Cre is under the regulation of transgenic promoters for endothelium-restricted receptors that are expressed in adults. Early studies in this area proposed that nearly all myofibroblasts are derived from the endothelium.[28–30] Unfortunately, endothelial cells have very few truly lineage-restricted receptors. TIE1 and TIE2 are expressed by myeloid lineage, FOXD1 lineage, and other vascular smooth muscle cells.[31,32] Recently, studies using the vascular endothelial-cadherin promoter suggested that perhaps 15% of myofibroblasts are derived from injured endothelium, but definitive fate-mapping studies are still lacking.[33] Another possibility is that injured and activated endothelium drives fibrosis by indirect cell–cell signaling mechanisms, similar to the epithelium, but further studies are awaited.

The role of myeloid lineage cells in fibrogenesis in the kidney remains highly controversial. Although many studies indicate that macrophages and neutrophils can drive fibrotic disease, there is controversy surrounding whether the profibrotic effects of these cells occur by indirect mechanisms or whether some myeloid cells become fibrillar matrix-producing cells (also known as circulating fibrocytes). Several studies have identified rare cells of myeloid lineage that can synthesize collagen-I protein (the major fibrillar collagen of scar tissue), which may perhaps represent a distinct subpopulation of myeloid cells with characteristics of antigen-presenting cells.[2,4,34] Several papers have reported numerous myeloid leukocytes in the diseased kidney that produce low levels of collagen-I, as determined by antibody detection of this protein.[35] Interpretation of these studies is limited due to the recognized role of macrophages in collagen matrix internalization and degradation in disease via surface collagen receptors including Endo180 and CD206. Interpretation is also limited by the inability to distinguish production of collagen-I from internalization. Other studies have identified increased amounts of leukocytes being transformed into myofibroblasts by their production of *Acta2* transcripts using a short transgenic reporter.[33]

Our laboratory recently mapped the fate of myeloid leukocytes using the Cre recombinase enzyme knocked into the lysozyme M gene locus.[36] This gene is widely recognized to be completely restricted to myeloid lineage.[37,38] As expected in mice with kidney disease, there is intense recruitment of myeloid lineage cells into the diseased kidney interstitium, although <2% of these cells appear to produce collagen-I or α-SMA protein. (Figure 1d). As these two cell lineages are intertwined in the interstitium, however, it is difficult to appreciate their clear separation without 3D reconstruction of the interstitial tissue. Such 3D reconstruction of the tissue similarly clearly shows almost no overlap with collagen-I-producing or α-SMA protein–producing cells (Figure 1d). These divergent results between recent bone marrow transplant studies[33] and myeloid lineage fate
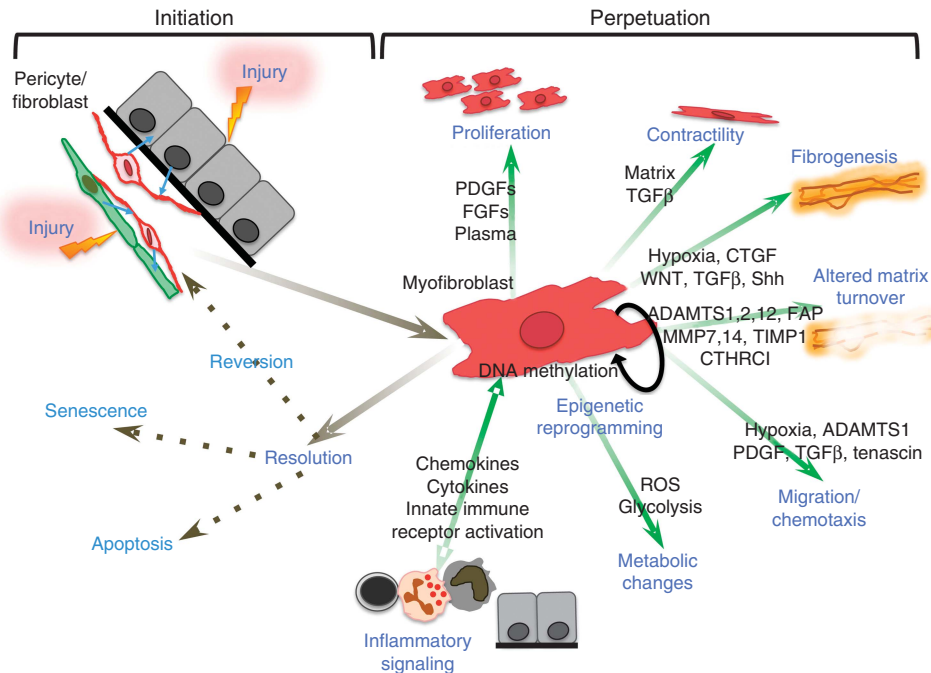
Figure 2 | Schema showing the multiple functions of FOXD1 lineage myofibroblasts in interstitial kidney disease. Pericytes (attached to the endothelium) and resident fibroblasts (red), both derived from FOXD1 + nephrogenic progenitors, transition to become a major source of myofibroblasts in interstitial kidney disease. A poorly defined leukocyte may also contribute to the appearance of myofibroblasts. As myofibroblasts, this cell lineage undergoes major phenotypic changes, acquiring a migratory, highly proliferative phenotype under the early regulation of developmental pathway growth factors including platelet-derived growth factors (PDGFs). Evidence for chromatin remodeling has been documented that serves to enhance the myofibroblast phenotype. Myofibroblasts acquire new actin filament machinery rendering them contractile; they increase synthesis of pathological matrix proteins including fibrillar and non-fibrillar collagens as well as other matrix proteins, processes regulated by the developmental receptor signaling, matrix protein signaling, and inflammatory, hypoxia signaling mechanisms. Factors regulating matrix turnover and stability are highly expressed, and metabolic changes occur, rendering myofibroblasts highly resistant to the hostile metabolic environment. In addition to these roles, myofibroblasts are a major source of inflammatory factors and regulate recruitment of leukocytes. Although resolution of myofibroblast expansion occurs, the mechanisms are currently obscure. ADAMTS, a disintegrin and metalloproteinase with thrombospondin repeats; CTGF, connective tissue growth factor; CTHRC1, collagen triple-helix repeat containing-1; FAP, fibroblast-activated protein; FGFs, fibroblast growth factors; MMP, matrix metalloproteinase; ROS, reactive oxygen species; Shh, sonic hedgehog; TGF-β, transforming growth factor-β.

mapping may be explained by unreliable activity of short transgenic reporters that were used to identify cells generating *Acta2* transcripts. It may be informative to compare the findings in the kidney with those observed in other organs. Although the circulating fibrocyte was initially reported and defined molecularly in skin wounds, recent comprehensive studies of skin wounds in parabiotic mice, in which the blood circulation of one animal is crossed permanently with that of another, provide no evidence for leukocyte transformation to myofibroblasts in the skin.[39–42] Although the precise role of myeloid lineage cells in the fibrogenic process in the kidney continues to be evaluated (Figure 2), transdifferentiation to myofibroblasts in rodents does not appear to occur significantly in the lineage-mapping or bone marrow transplant studies performed in our laboratory.[36]

FACTORS REGULATING FOXD1 – LINEAGE CELL DIFFERENTIATION INTO MYOFIBROBLASTS
Compelling studies from our laboratory indicate that FOXD1 lineage cells purified from the kidney peritubular compartment

migrate to capillaries in 3D gel matrices *ex vivo*, where they perform important tasks in stimulating vessel stabilization.[9,32,43] The pericytes stimulate capillary basement membrane deposition, form gap junctions directly with endothelial cells, regulate vessel diameter, and prevent regression in response to stressors. In other circumstances, they stimulate angiogenic sprouting and coat new capillaries.[32] Moreover, they are the cellular source of erythropoietin.[1] Preliminary studies also suggest pericytes have important roles in local capillary flow and permeability.[32] FOXD1 lineage pericytes attached to the peritubular capillaries spread and detach from capillaries as an early response to injury (Figure 2). This process involves separation from the capillary basement membrane and migration.[2,7,8] Our understanding of this process has been enhanced by identification of the transcriptional changes occurring early in disease. These transcriptional changes are stimulated by factors that regulate matrix turnover, migration, and proliferation including growth factors, morphogens, chemokines and enzymes which regulate matrix turnover.[9]

There is also evidence of activation of cell to cell signaling pathways important in development. These include

angiogenic, tubulogenic pathways. Specifically WNT, transforming growth factor, PDGF, and Ephrin, Hedgehog and Integrin signaling are activated in these cells in adult injury (Figure 2). Investigators who blocked signaling in these pathways in vivo, as well as blocking pathway interactors such as CD248 (a PDGFR interactor) during models of adult interstitial kidney diseases, suggest that these signaling pathways play important roles in regulating detachment, spreading, migration, and proliferation of FOXD1 lineage interstitial cells as well as their production and deposition of a pathological matrix.[7,8,14,43–48] Inhibiting these receptor signaling pathways appears to also regulate fibrosis itself, indicating they have important roles in maintaining the pathological myofibroblast pool of cells once they have formed and accumulated, and suggests such pathways and interactors are important potential targets for therapeutics. Early, but accumulating evidence points to intracellular stress signaling pathways directly under the regulation of these receptors such as JNK, MAPK, Src and reactive oxygen species as critical in differentiation of FOXD1 lineage cells to, and maintenance as myofibroblasts.[8] For example, direct inhibition of WNT and PDGF receptors at the cell surface appears to have profound effects on dampening the fibrogenic response in models of interstitial kidney disease by blocking JNK and MAPK downstream signaling. A recent study of FOXD1 lineage perivascular cells has demonstrated an important role for WNT signaling in differentiation to and maintenance of myofibroblasts. However, the authors also highlighted evidence for multiple receptor signaling pathway convergence at the plasma membrane in this cell lineage, whereby ligands for one receptor transactivate additional signaling pathways. The converging receptors include those for transforming growth factor-β, PDGF receptor-β, WNT, and integrins.[8] One explanation for these observations is that receptor complexes form at the surface of FOXD1 lineage cells, possibly in caveolae, and that complex formation is critical for pathogenic signaling. A better understanding of this receptor convergence is required.

The regulation of matrix turnover as an early event in the transition of FOXD1 lineage pericytes to pathogenic myofibroblasts has been underscored by new studies. mRNA attached to ribosomes that is undergoing translation, has been selectively purified from FOXD1 lineage medullary pericytes in healthy kidney and again from these same cells several days after the onset of interstitial kidney disease when they have migrated to the interstitial space. At this point, these pericytes express α-SMA and are considered to be myofibroblasts.[49] Among the highly upregulated genes in these myofibroblasts are members of the family of ADAMTS (a disintegrin and metalloproteinase with thrombospondin repeats) enzymes (Figure 2). These metalloproteinases have discrete roles in cell function. ADAMTS-1 and -12 have important roles in cell migration and inhibition of angiogenesis, whereas ADAMTS-2 regulates fibril assembly from collagen monomers.[50,51] Another upregulated gene is that for fibroblast-activated protein, which is a gelatinase with collagenolytic activity that has a direct role in collagen internalization and may regulate regenerative

functions of myofibroblasts[52] (Figure 2). Other highly upregulated factors include collagen triple-helix repeat containing-1, which may stimulate myofibroblasts to deposit matrix,[53] and thrombospondin-2, which is known to coordinate collagen fibril formation and inhibit angiogenesis.[54] Overall, a pattern emerges of a cell that not only regulates matrix protein synthesis, but regulates the machinery that processes, deposits, and turns-over fibrillar collagen, and at the same time, generates factors that inhibit angiogenesis.

MYOFIBROBLASTS AS INFLAMMATORY CELLS
Comparison of FOXD1 lineage perivascular cells in the healthy kidney with interstitial myofibroblasts of diseased kidney, indicate myofibroblasts retain a number of characteristics of their perivascular precursor cells and therefore may be thought of as an activated form of the resident precursors. The resident perivascular precursors perform important tasks in homeostasis, maintaining capillary and tubular health and functions, and during nephrogenesis they play critical roles in tubulogenesis and microvascular patterning through bidirectional signaling.[55] One might predict that myofibroblasts in early disease states therefore play roles in tubular and microvascular regeneration. Similar pericyte cells in skin have been shown to be critical innate immune-sensing cells, which regulate leukocyte transmigration and local leukocyte recruitment and thus control inflammation.[56–58] Early studies in the kidney have confirmed a similarly important role for perivascular cells in regulating the leukocyte recruitment into the tissue.[59,60] Surprisingly, FOXD1 lineage pericytes/fibroblasts and myofibroblasts activate NFκB and PU.1 and generate high levels of proinflammatory cytokines and chemokines in response to tissue injury[4,59,60] (Figure 2). Myofibroblasts purified from diseased kidneys spontaneously generate high levels of similar cytokines and chemokines, including IL6, TNF-α and MCP1 and KC. These findings suggest myofibroblasts are not simply cells involved in the deposition of pathological matrix, but are also important inflammatory cells that contribute to tissue destruction and chronic inflammation, as has been shown for myofibroblasts in other organs including the liver and lung.

UNDERSTANDING MYOFIBROBLAST PERSISTENCE IN DISEASE CONDITIONS
Chronic disease is characterized by the persistence of FOXD1 lineage myofibroblasts, whereas acute injury is characterized by transient expansion of the FOXD1 lineage myofibroblasts, which later resolves. Understanding the mechanisms of this myofibroblast persistence may be central to halting the progression of chronic kidney disease. At this time, the turnover of FOXD1 lineage myofibroblasts is poorly understood, and the factors that permit such cells to persist and thrive in a hostile tissue environment are unknown. Ample evidence exists, however, that myofibroblasts proliferate and migrate more than their unactivated precursors in ex vivo experiments, suggesting that some level of reprogramming

has occurred. Moreover, it is clear that myofibroblasts have great capacity to thrive in conditions where other cell types such as epithelial cells lose function and ultimately die. Recent studies suggest myofibroblasts undergo epigenetic changes to the chromatin, resulting in persistent silencing or activation of genes[61] (Figure 2). Such epigenetic changes to cell cycle regulators, intracellular stress signaling pathways, migratory machinery, and metabolism may help explain how myofibroblasts adapt to remain active in diseased tissue.

FOXD1 REGULATES THE FATE OF PODOCYTES AND PARIETAL EPITHELIAL CELLS

FOXD1+ nephrogenic progenitors differentiate into vascular smooth muscle, mesangial cells and pericytes; highly specialized mesenchymal cells, all of which have important functional relationships to the vascular wall. FOXD1 is only expressed in the nephrogenic progenitor cells and is turned off permanently after the progenitors have differentiated, but downstream effects of FOXD1 transcriptional activity persist via activation of important transcriptional targets, which include matrix factors such as collagen-II and collagen-XI, regulators of migration including Tenascin-N, regulators of collagen deposition including collagen triple-helix repeat containing-1, and regulators of endothelial to mural cell crosstalk such as the Netrin receptor, UNC5C. These targets are all implicated in pericyte and fibroblast functions and suggest an upstream role of FOXD1 in cell programming. Further support for this hypothesis is provided by the finding that mutation of FOXD1 causes severe dysregulation of the FOXD1 lineage in development.[62] Recent studies, however, have identified activation of FOXD1 in the undifferentiated epithelium that gives rise to mature podocytes and parietal epithelial cells (PECs) of the glomeruli. This activation occurs at a late time point in nephrogenesis (after 15.5 dpc in mice) once the rudimentary glomerulus has already formed and activation of FOXD1 in these epithelial cells persists well into the neonatal period.[55,63] In addition, ~1% of already-differentiated tubular epithelial cells transiently activate FOXD1 at the late developmental time point of 15.5 dpc, although the significance of this is not clear. Studies from our laboratory suggest FOXD1 has important roles in converting glomerular epithelial precursors to mature podocytes and PECs.[55] It is striking therefore that healthy podocytes generate and turnover high-levels of collagen proteins, have critical roles as pericyte-like cells to the glomerular endothelium, express the mesenchymal protein vimentin, and are implicated as matrix-producing cells in glomerulosclerosis.[2,4,64,65] Moreover, PECs have recently been proposed to be major sources of glomerular myofibroblast progenitors.[66] One interpretation of these observations is that podocytes and PECs, although originating as epithelial cells, share transcriptional and functional similarities with mural (vascular wall) cells and fibroblasts in the healthy kidney, rendering them capable of acquiring myofibroblast characteristics in disease settings. Further studies will be required to understand the role of FOXD1 and other mesenchymal transcription factors in glomerular epithelial cell reprogramming in the late stages of nephrogenesis.

CONCLUSIONS

Pericytes and resident fibroblasts derived from FOXD1 nephrogenic progenitors have recently emerged as important resident precursors of myofibroblasts, the pathogenic cells that deposit fibrillar matrix. Detachment of pericytes from the capillary wall is an important step in this injury response, but this leaves capillaries without the vital functions of pericytes in the maintenance of capillary homeostasis. In kidney development and homeostasis, FOXD1 lineage cells have important roles in regulating both capillaries and tubular cells. Some of these beneficial functions are lost when the cells become myofibroblasts. Many factors are involved in the change of cell function, including persistent activation of developmental signaling pathways, cell cycle activation, and changes not only to the expression of matrix proteins, but also to the expression of molecular factors that regulate deposition and turnover of the matrix.

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