Janus-Faced: Molecular Mechanisms and Versatile Nature of Renal Fibrosis

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Abstract
Renal fibrosis is a major hallmark of CKD, regardless of the underlying etiology. In fibrosis development and progression, myofibroblasts play a pivotal role, producing extracellular matrix and interacting with various resident cells in the kidney. Over the past decade, the origin of myofibroblasts has been thoroughly investigated. Emerging evidence suggests that renal myofibroblasts originate from several cellular sources, including resident fibroblasts, pericytes, and bone marrow–derived cells. The contribution of resident fibroblasts is most crucial, and currently available data strongly suggest the importance of functional heterogeneity and plasticity of fibroblasts in kidney disease progression. Resident fibroblasts acquire distinct phenotypes based on their local microenvironment and exert multifactorial functions. For example, age-dependent alterations of renal fibroblasts make a significant contribution to the formation of tertiary lymphoid tissues, which promote local inflammation after injury in the aged kidney. In conjunction with fibrosis development, dysfunction of resident fibroblasts provokes beneficial roles, such as maintaining functional crosstalk with injured proximal tubular cells and supporting their regeneration. These findings provide novel insight into the mechanisms of renal fibrosis, which could be regarded as an adaptive process of kidney injury and repair. Precise understanding of the functional heterogeneity of resident fibroblasts and myofibroblasts has the potential to facilitate the development of novel therapeutics against kidney diseases. In this review, we describe the current perspective on the origin of myofibroblasts and fibroblast heterogeneity, with special emphasis on the dual aspects of renal fibrosis, both beneficial and detrimental, in CKD progression.

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Introduction
Renal fibrosis is a final common manifestation of CKD, regardless of the underlying etiology. Renal fibrosis is characterized by the excessive accumulation of extracellular matrix in the interstitial space, which hinders appropriate tissue repair (1). CKD is a major public health concern worldwide, affecting approximately 10% of the global population, which substantially increases the risks of cardiovascular disease, ESKD, and mortality (2). In spite of its high morbidity, however, specific therapy for CKD has yet to be established. Herein, we describe the current understanding of the mechanisms of renal fibrosis, with special emphasis on the function of resident fibroblasts and their heterogeneous characteristics. We also discuss the dual aspects of fibrosis, both beneficial and detrimental, in kidney injury and repair.

The Origin of Myofibroblasts
Although various types of cells participate in fibrosis development and progression, the contribution of myofibroblasts is most crucial. Myofibroblasts possess characteristics of both fibroblasts and smooth muscle cells, expressing α-smooth muscle actin (αSMA) which enables them to connect to extracellular matrix (1) (Figure 1). Although myofibroblasts are not present under physiologic conditions, myofibroblasts emerge de novo in the interstitium after injury and promote tissue deformation by secreting matrix proteins including collagens, fibronectins, and proteoglycans (3). Over the past decade, the origin of myofibroblasts has been intensively investigated (4). It remains controversial whether myofibroblasts are derived from mesenchymal cells (resident fibroblasts and pericytes), circulating bone marrow–derived cells (fibrocytes), epithelial cells, or endothelial cells. Comprehensive cell fate mapping experiments have been conducted to clarify the origin of myofibroblasts.

Resident Fibroblasts and Pericytes
Fibroblasts are spindle-shaped cells of mesenchymal origin, embedded in the extracellular matrix and stroma of connective tissues and organs (5). In general, fibroblasts are identified by their morphology, their localization, the expression of representative markers such as PDGF receptor β (PDGFRβ) and CD73, and the absence of markers for other cell lineages (e.g., CD45 for differentiated hematopoietic cells) (4,6). Although fibroblasts exist in almost all tissues, they exert distinct...
functions based on their microenvironment. In the kidney, fibroblasts reside in the interstitial space and communicate with various types of cells, including epithelial cells, endothelial cells, and circulating cells. Additionally, certain subpopulations of renal fibroblasts are specialized to produce erythropoietin (EPO) in response to hypoxia (8). The origin and function of EPO-producing cells will be described in detail later.

Several studies have suggested that resident fibroblasts are the major source of myofibroblasts in renal fibrosis. By using myelin protein zero (P0)–Cre transgenic mice, which label migrating neural crest cells and neural crest-derived Schwann cells (9), we demonstrated that P0-Cre lineage–labeled cells are observed in the renal interstitium with the expression of CD73 and PDGFRβ (10). Interestingly, >98% of fibroblasts in the renal cortex and outer medulla are labeled with P0-Cre. In response to injury, these P0-labeled cells transdifferentiate into αSMA-positive myofibroblasts, which contribute to fibrosis development and progression.

Other groups have also reported the possible contribution of pericytes to the myofibroblast pool. Pericytes are mesenchymal-derived cells that reside around the microvessels and control microcirculation (11,12). Humphreys et al. (13) used FoxD1-Cre knock-in mice to label renal stromal cells including pericytes and demonstrated that αSMA-positive cells in the renal interstitium after injury originate from FoxD1-labeled pericytes. They also revealed that glioma-associated oncogene homolog (Gli1)–positive cells are observed in the pericyte niche around microvessels in the kidney (14,15). After injury, Gli1-positive cells transdifferentiate into myofibroblasts, and the genetic ablation of Gli1-positive cells ameliorates renal fibrosis. Intriguingly, only a small fraction of Gli1-positive cells express NG2, a marker of mature pericytes (11), suggesting that Gli1 expression defines immature perivascular cells. These findings indicate that pericytes also transdifferentiate into myofibroblasts in renal fibrosis.

Although both resident fibroblasts and pericytes contribute to the myofibroblast pool, however, it is difficult to differentiate between resident fibroblasts and pericytes because both of them reside in the tubulointerstitial space and express common cellular markers, such as CD73 and PDGFRβ. During kidney development, P0-Cre–labeled cells migrate into the embryonic kidney at embryonic day 13.5, and some of them transiently express FoxD1 (10). Moreover, FoxD1 is also expressed in the migrating neural crest (16). These results indicate that the P0-Cre–labeled and FoxD1-Cre–labeled populations largely overlap. Further study is necessary to clarify the relationship between these two cell populations.

**Fibrocytes**

Whereas renal fibroblasts and pericytes make a major contribution to the myofibroblast pool, the contribution of fibrocytes has also been reported. Fibrocytes are bone marrow–derived cells producing collagen type I and other extracellular matrix and they express hematopoietic markers CD45, CD11b, and CD34 (17–20). Reich et al. (21) reported that renal fibrocytes, detected in the kidney after unilateral ureteral obstruction (UUO), do not originate from infiltrating monocytes, but develop outside the kidney, migrating into the kidney as predifferentiated collagen-producing cells. Additionally, depletion of renal fibrocytes attenuates fibrosis, indicating that fibrocytes make a contribution to renal fibrosis. Bone marrow transplantation from αSMA-RFP mice also revealed that 35% of αSMA-positive myofibroblasts are derived from bone marrow lineage–derived cells (22), although the reliability of transgenic reporters is questioned (23).

In contrast, Lin et al. (24) reported that only a small number of collagen-producing cells (<0.1%) originate from fibrocytes in UUO by using ColI-GFP reporter mice and bone marrow transplantation. By using parabiosis models and single-cell RNA sequencing (scRNA-seq), Kramann et al. (25) suggested that most myofibroblasts are derived from mesenchymal cells, such as resident fibroblasts and pericytes, but not from hematopoietic lineage–derived cells. Interestingly, myeloid-derived fibrocytes express proinflammatory cytokines instead of extracellular matrix genes or other myofibroblast-specific genes, indicating that these circulating myofibroblast progenitors contribute to renal fibrosis by paracrine rather than direct mechanisms. Additional research should be conducted to further determine the contribution of fibrocytes in renal fibrosis.

**Epithelial-to-Mesenchymal Transition and Endothelial-to-Mesenchymal transition**

Previous reports suggested that epithelial cells transdifferentiate into myofibroblasts through epithelial-to-mesenchymal transition (EMT), mainly based on the colocalization of epithelial and mesenchymal markers (26,27). However, detailed cell fate tracing studies argued against this hypothesis, questioning the contribution of EMT in vivo (13,28,29). A recent study reported that phenotypic transition through EMT accounts for <5% of the myofibroblast pool,
suggesting that EMT is not a major mechanism in fibrosis progression (22).

Endothelial-to-mesenchymal transition (EndoMT) is a similar mechanism to EMT, in which endothelial cells undergo phenotypic transition into mesenchymal cells. Although some studies suggest that EndoMT contributes to the myofibroblast pool (30–32), the fidelity of cell fate mapping is questioned (1,33). Furthermore, the contribution of EndoMT appears to be less significant than the transition from other cell populations such as pericytes/fibroblasts and macrophages (22). Further studies are needed to clarify the function of EndoMT in renal fibrosis.

Fibroblast Heterogeneity

Fibroblasts exhibit different functional identities based on their developmental origins, residing tissues, and their microenvironment (34). Fibroblast heterogeneity is intensively investigated in various fields of research. In skin wound-induced regeneration, fibroblasts can be classified into 12 clusters by scRNA-seq, and some clusters likely represent differentiation states toward a contractile phenotype (35). One subset of fibroblasts originated from myeloid-lineage cells, which contribute to adipocyte regeneration as well as the myofibroblast pool. In breast cancer, four subsets of carcinoma-associated fibroblasts (CAFs) have been identified, which accumulate differentially according to cancer subtypes (36). One specific subset of CAF, CAF-S1, enhances regulatory T cell capacity and promotes an immunosuppressive microenvironment in breast cancer. Hence, fibroblast heterogeneity can significantly affect the tissue microenvironment and even modify the pathophysiology of various diseases.

In the kidney, functional and regional heterogeneity of fibroblasts has also been investigated. Although the above-mentioned P0-Cre-labeled fibroblasts and myofibroblasts are observed in the renal cortex and outer medulla, but not in the inner medulla (10), Wnt4 is expressed exclusively in medullary myofibroblasts, but not in cortical myofibroblasts (37). These findings indicate that the origin of myofibroblasts might differ depending on their localization in the kidney, and the regional heterogeneity of myofibroblasts could affect the process of renal fibrosis. To specifically label inner medullary fibroblasts, the tenascin-C-CreER2 knock-in mouse line was developed that could be used to further elucidate the heterogeneous characteristics of fibroblasts (38).

Fibroblast heterogeneity is also investigated in the context of aging-related pathologic conditions (39). We reported that aged mice, but not young mice, develop tertiary lymphoid tissues (TLTs) in the kidney after injury (Figure 2), and the size of TLTs is closely associated with impaired renal function and increased expression of proinflammatory cytokines (40). TLTs are ectopic lymphocyte aggregates induced by chronic inflammation, which are supported by fibroblasts within TLTs both functionally and structurally (41,42). Fibroblasts outside TLTs express retinaldehyde dehydrogenase 2 (RALDH2), a rate-limiting enzyme in retinoic acid (RA) synthesis. Interestingly, fibroblasts within TLTs are negative for RALDH2; instead, they express p75 neurotrophin receptor (p75NTR) and produce homeostatic chemokines, including CXCL13 and CCL19, which regulate lymphocyte recruitment in TLTs (40,43). RA significantly upregulates p75NTR expression in fibroblasts in vitro, suggesting that RA derived from fibroblasts outside TLTs promotes p75NTR expression in fibroblasts inside TLTs. p75NTR is expressed in the fibroblasts of neonatal kidneys but disappears in adult kidneys (10). Therefore, it appears that fibroblasts within TLTs restore the phenotype of fibroblasts in neonatal kidneys. Importantly, all of these TLT-associated fibroblasts are labeled with P0-Cre, indicating that resident fibroblasts in the kidney acquire various phenotypes and exert distinct functions based on their microenvironment (40). Overall, the functional heterogeneity and plasticity of resident fibroblasts play crucial roles in renal fibrosis and TLT formation, although it is still unclear whether heterogeneous characteristics of fibroblasts are determined by their intrinsic mechanisms, including transcriptional and epigenetic modifications, or their surrounding microenvironment.

To comprehensively investigate the heterogeneous cellular characteristics in the kidney, scRNA-seq could be
Effective (44,45). However, the application of scRNA-seq on fibrotic kidneys is limited because the dissociation procedure damages fragile cells and generates stress-induced transcriptional artifacts. Recently, the superiority of single-nucleus RNA sequencing (snRNA-seq) over scRNA-seq in investigating fibrotic kidneys has been reported (46). snRNA-seq, although achieving comparable gene expression quantification to scRNA-seq, offers several advantages including the detection of rare or fragile cell types, the elimination of dissociation-induced transcriptional alterations, and compatibility with frozen samples. Given the important roles of fibroblasts and myofibroblasts in renal fibrosis, a deeper understanding of fibroblast heterogeneity in the kidney by using effective techniques such as snRNA-seq might provide important clues for developing a novel strategy to treat kidney diseases.

Functional Crosstalk between (Myo)Fibroblasts and Renal Tubular Cells

Although the transition of resident fibroblasts into myofibroblasts is regarded as an important process in renal fibrosis, the trigger of this transition remained unclear. We and another group previously reported that proximal tubular injury after AKI promotes the transdifferentiation of fibroblasts into myofibroblasts, leading to fibrosis development and reduced EPO production (47,48). The severity and frequency of proximal tubular injury is closely associated with the progression of several pathologic features of CKD, including interstitial fibrosis, glomerulosclerosis, and atubular glomeruli. Additionally, coculture with injured tubular cells upregulates extracellular matrix genes in fibroblasts, indicating possible crosstalk through secretory factors. These findings exemplify functional crosstalk between resident fibroblasts and proximal tubular cells in fibrosis development. Recently, an in vitro model of Gli1-positive mesenchymal cells, the Kidney-Gli1 (KGli1) cell line, was established that maintains a similar marker expression to its counterpart in vivo (49). KGli1 cells transdifferentiate into myofibroblasts in response to TGFβ and stabilize vasculogenesis in vitro, consistent with their characteristics in vivo. By scRNA-seq and ligand-receptor analysis, the upregulation of nerve growth factor on KGli1 cells and nerve growth factor receptor Ntrk1 on tubular cells have been identified, illustrating the functional crosstalk between these cells. Various paracrine mediators possibly regulating functional crosstalk between fibroblasts and tubular cells have been reported, including TGF-β1, PDGF, Wnt ligands, sonic-hedgehog (Shh), angiotensin II, hepatocyte growth factor (HGF), and connective tissue growth factor (37,50–55). Recently, Liu et al. (56) revealed the involvement of tubule-derived exosomes in renal fibrosis, indicating that injured tubular cells release exosomes shuttling Shh ligand, which promote fibroblast activation. Overall, bidirectional crosstalk between (myo)fibroblasts and tubular cells with multiple mechanisms plays a crucial role in fibrosis development and progression.

The Consequence of Renal Fibrosis

As noted, renal fibrosis is the final common manifestation of CKD regardless of its etiology. Indeed, renal fibrosis in the cortex is regarded as the best histologic predictor of renal dysfunction in CKD (57). In accordance with fibrosis progression, renal anemia, one of the most common complications of CKD, prevails. Renal anemia is mainly caused by the relative deficiency of EPO, a principal regulatory hormone of red blood cell synthesis. Unlike other hematopoietic factors, which are produced in the vicinity of their target cells, EPO is mainly produced by the interstitial fibroblasts in the deep cortex and the outer medulla of the kidney (58,59). We previously demonstrated that EPO-producing fibroblasts transdifferentiate into myofibroblasts and contribute to renal fibrosis, with concomitant loss of EPO production (10). The administration of dexamethasone and neurotrophins restores EPO-producing capacity in transdifferentiated myofibroblasts, suggesting the reversibility of EPO production. Moreover, Souma et al. (60) demonstrated that the activation of NFκB and Smad signaling pathways in EPO-producing cells represses EPO-producing capacity and promotes myofibroblast transformation in UUO, and reversing UUO restores the EPO-producing capacity and physiologic phenotype of these cells. These findings indicate that EPO-producing cells possess functional and cellular plasticity, and their phenotypic transition to myofibroblasts induced by the inflammatory microenvironment underlies renal anemia in CKD.

EPO production is induced under hypoxic conditions and is mainly regulated by hypoxia-inducible factors (HIFs), which are heterodimeric complexes composed of α and β subunits (61,62). Three isoforms of HIFα subunits (HIF1α, HIF2α, and HIF3α) have been identified and all of them are hydroxylated rapidly under normoxic conditions by HIF-prolyl hydroxylase domain-containing proteins (PHDs). The hydroxylation of the HIFα subunit by PHD promotes binding with the von Hippel–Lindau tumor suppressor, leading to their proteosomal degradation (63–65). Indeed, the activation of HIFs by PHD inhibition restores EPO production in myofibroblasts, and the PHD2-HIF2α axis is the major regulatory cascade for hypoxia-inducible EPO gene expression (61). Additionally, Kobayashi et al. (66) revealed that EPO-producing cells are derived exclusively from FoxD1-expressing stromal progenitor cells, which comprise distinct subpopulations that differ in their responsiveness to PHD2 inactivation, regulation of HIF2 activity, and EPO production. These findings also exemplify the importance of fibroblast heterogeneity in EPO production. Recently, small molecule inhibitors of PHD have been developed and are expected to be effective therapeutic options for renal anemia (67,68).

Peritubular capillary loss, frequently observed in advanced CKD, also contributes to renal fibrosis by reducing oxygen supply to tubular and interstitial cells (63). The severity of renal injury is associated with the extent of peritubular capillary loss, and capillary rarefaction is an important component of CKD progression (69). Under physiologic conditions, peritubular capillaries are surrounded by pericytes, which contribute to vascular stability. After injury, however, these pericytes/fibroblasts detach themselves from the capillaries and migrate toward the site of injury, surrounding injured tubular cells instead (52,61). These pericytes/fibroblasts might provide mechanical support for the exposed epithelial basement membrane during tubular cell regeneration, while peritubular capillaries
become structurally unstable, leading to capillary regression and rarefaction. These findings also indicate that pericytes/fibroblasts play a multifactorial role in CKD progression.

The Beneficial Aspect of Renal Fibrosis

Although renal fibrosis is generally considered detrimental to renal outcome, renal fibroblasts and myofibroblasts also exert beneficial effects. Schiessl et al. (52) revealed that resident fibroblasts after injury migrate to the site of tubular cell loss to provide mechanical support, and the inhibition of PDGFRβ signaling compromises their migration and tubular regeneration. Migration of myofibroblasts around injured tubules after AKI has also been observed, suggesting that interstitial fibrosis might support injured nephrons (70). In fact, Zhou et al. (71) reported that HGF, which is upregulated in renal fibroblasts by Shh in vitro, attenuates renal injury by activating c-met, a HGF receptor, that is predominantly induced in tubular cells after injury (72). The administration of pharmacologic doses of EPO, which is endogenously secreted from fibroblasts in vivo, also alleviates ischemic renal injury (73), although the production of endogenous EPO is reduced during the transition from fibroblasts to myofibroblasts (10). These findings indicate that (myo)fibroblasts, considered deleterious in general, could play beneficial roles in tubular cell injury and repair. Kaisling et al. (74) proposed that renal fibrosis could be regarded as a healing process of injury, supporting tubular recovery and regeneration. The beneficial roles of fibroblasts are also reported in the fibrosis of other tissues such as cardiac fibrosis, in which fibroblasts promote an adaptive response to injury by paracrine signaling or mesenchymal-to-endothelial transition (75,76), suggesting their importance in the repair process of various organs.

Recently we revealed that genetic depletion of fibroblasts attenuates tubular regeneration after injury and exacerbates tubular injury (77). Intriguingly, in the transition to myofibroblasts after injury, fibroblasts acquire the expression of RALDH2, whereas RALDH2 is conversely downregulated in injured proximal tubular cells (67). Previously we demonstrated that injured proximal tubular cells promote the transition of fibroblasts into myofibroblasts (46). Taken together, injured proximal tubules possibly enable myofibroblasts to produce retinoic acid, which supports tubular regeneration. These findings suggest the novel functional crosstalk between (myo)fibroblasts and proximal tubular cells, illustrating the beneficial aspect of renal fibrosis.

Figure 3. Functional crosstalk between (myo)fibroblasts and proximal tubular cells underlies tubular injury and regeneration. In healthy kidney, proximal tubular cells express retinaldehyde dehydrogenase 2 (RALDH2). In the transition to myofibroblasts after injury, fibroblasts acquire the expression of RALDH2, whereas RALDH2 is conversely downregulated in injured proximal tubular cells (67). Previously we demonstrated that injured proximal tubular cells promote the transition of fibroblasts into myofibroblasts (46). Taken together, injured proximal tubules possibly enable myofibroblasts to produce retinoic acid, which supports tubular regeneration. These findings suggest the novel functional crosstalk between (myo)fibroblasts and proximal tubular cells, illustrating the beneficial aspect of renal fibrosis.
myofibroblasts. RA receptor γ (RARγ) is expressed in proximal tubular cells and αβ-crystallin, the product of an RAR target gene, is induced in proximal tubular cells after injury, suggesting the possible function of RA signaling in proximal tubules after injury. Additionally, in vitro administration of an inverse agonist for RARs attenuates tubular cell proliferation. Together with our previous work indicating that proximal tubular injury induces the transition of fibroblasts to myofibroblasts (47), these results suggest that proximal tubular cells, renouncing their RA-producing ability after injury, enable fibroblasts to produce RA by inducing their transition to myofibroblasts, which promotes tubular regeneration (Figure 3). Overall, fibroblasts play beneficial roles in tubular regeneration, at least in the early phase of injury, and their transition to myofibroblasts could be regarded as an adaptive process of kidney injury and repair.

Dual aspects of renal fibrosis could be also explained by the timing and duration of (myo)fibroblast activation. Whereas fibroblast activation in the early phase of injury appears to exert reparative effects on the kidney as discussed above, persistent activation of fibroblasts predisposes fibrosis development and could be detrimental. Several epigenetic mechanisms, such as DNA methylation and micro-RNA regulation, contribute to sustained activation of myofibroblasts and aggravate renal fibrosis (82,83). The involvement of a specialized microenvironment within the kidney, the fibrogenic niche, has also been reported, which serves as the initiation site for fibroblast proliferation and expansion. Recently, tenascin-C has been suggested to play a pivotal role in the formation of the fibrogenic niche, promoting fibroblast activation and exacerbating fibrosis (84). Persistent activation of fibroblasts by several mechanisms might predispose the survival of deleterious fibroblasts, leading to the loss of fibroblast plasticity.

Conclusion
Numerous studies over the past decade have provided us with tremendous knowledge concerning the mechanisms of renal fibrosis, including the origin of myofibroblasts and fibroblast heterogeneity. Importantly, current evidence supports the notion that renal fibrosis, considered detrimental in nature, rather exerts beneficial effects that promote renal regeneration after injury. Indeed, a therapeutic strategy to inhibit TGFβ, an important regulator of fibrosis, has not yielded favorable results (85), suggesting the importance of clarifying the dual aspects of fibrosis, both detrimental and beneficial, in tissue injury and repair. Hence, with profound knowledge of fibroblast heterogeneity, specifically suppressing deleterious fibroblasts or enriching beneficial ones is a promising strategy for treating fibrosis, as reported in a treatment for dermatosis (86,87). These findings have prompted us to change our mindset toward the therapeutic strategy against renal fibrosis. Further understanding of the mechanisms of renal fibrosis will facilitate the development of novel therapeutics against CKD.

Author Contributions
H. Arai wrote the original draft; and M. Yanagita conceptualized the study and reviewed and edited the manuscript.

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