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# Renal Fibrosis: Primacy of the Proximal Tubule

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## Abstract

Tubulointerstitial fibrosis (TIF) is the hallmark of chronic kidney disease and best predictor of renal survival. Many different cell types contribute to TIF progression including tubular epithelial cells, myofibroblasts, endothelia, and inflammatory cells. Previously, most of the attention has centered on myofibroblasts given their central importance in extracellular matrix production. However, emerging data focuses on how the response of the proximal tubule, a specialized epithelial segment vulnerable to injury, plays a central role in TIF progression. Several proximal tubular responses such as de-differentiation, cell cycle changes, autophagy, and metabolic changes may be adaptive initially, but can lead to maladaptive responses that promote TIF both through autocrine and paracrine effects. This review discusses the current paradigm of TIF progression and the increasingly important role of the proximal tubule in promoting TIF both in tubulointerstitial and glomerular injuries. A better understanding and appreciation of the role of the proximal tubule in TIF has important implications for therapeutic strategies to halt chronic kidney disease progression.

### Keywords

Chronic kidney disease; Tubulointerstitial fibrosis; Epithelial de-differentiation; Cell cycle; Proteinuria; Metabolism

## Introduction

Chronic kidney disease (CKD), affecting 13% of the global population, is a growing epidemic resulting from renal insults such as diabetes mellitus, hypertension, inflammatory glomerular diseases, genetic disorders, and toxins[1]. Despite the diverse etiologies of CKD, the progression of CKD follows a common pathway whereby normal renal parenchyma is

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replaced with matrix proteins such as collagens I, III, IV, and fibronectin. The basic functional unit of the kidney is the nephron which filters the blood in the glomerulus. The resulting ultrafiltrate passes through the tubules, composed of highly specialized epithelia, which reabsorb water and electrolytes. Matrix proteins accumulating in the glomerulus is termed glomerulosclerosis, whereas tubulointerstitial fibrosis (TIF) describes the presence of matrix proteins replacing the tubules and/or surrounding interstitium. Although some renal injuries may target the glomeruli versus the tubular compartment, the best predictor of renal survival in CKD of all etiologies is the amount of TIF on kidney biopsy[2, 3]. Therefore, there is great interest in understanding the pathogenesis of TIF progression to facilitate development of new therapeutics for CKD.

TIF development and progression are complex processes that involve many different cell types. There is expansion of the interstitium with myofibroblasts, increased inflammatory cells (e.g. macrophages), tubular atrophy, and microvascular injury. Renal biopsies from humans with CKD offer a snapshot in time and usually show all of these pathologic changes, making it difficult to discern the relative importance of each cellular compartment to TIF progression. Much attention has understandably focused on myofibroblasts and their precursors as this interstitial cell is thought to be the main producer of extracellular matrix (ECM). However, recent data has strengthened the role of the proximal tubule, a specialized tubular segment adjacent to the glomerulus, as not only the target of injury, but also an important mediator of TIF progression. Although it is clear that all of the cellular compartments mentioned above contribute to TIF, this review will make a case for the primacy of the proximal tubule.

## Current Paradigm of Renal Injury and TIF

**Myofibroblasts: Source of Extracellular Matrix—**The myofibroblast is a mesenchymal cell with increased stress fiber formation allowing for contractile properties and increased matrix production. Myofibroblast formation, proliferation, and ECM production are stimulated by growth factors such as TGF-β, Wnt, and PDGF-β produced by injured proximal tubule cells [4–7]. As the myofibroblast is the key ECM producer, there has been considerable interest in and controversy over the origin of these cells. Earlier work described a role for proximal tubule cells transforming into interstitial myofibroblasts through a process of epithelial-to-mesenchymal transformation (EMT)[8]. However, more recent fate-tracing studies have not shown that epithelial cells transform into interstitial myofibroblasts[9, 10]. Most of the data implicate resident fibroblasts and/or pericytes as the likely precursor to the myofibroblast, but some studies suggest that inflammatory cells and bone-marrow derived “fibrocytes” may become matrix-producing interstitial cells as reviewed elsewhere[11–13]. The controversial nature of the myofibroblasts’ origin is likely due to the fact that matrix-producing interstitial cells are a heterogeneous population and extremely difficult to identify as there are no great markers[14]. Often, α-smooth muscle actin (α-SMA), the best currently available myofibroblast marker, is used but recent literature has shown that many collagen-producing cells in the kidney do not express α-SMA[15, 16]. There is a great need to further characterize these interstitial cells and define which markers predict functional differences (e.g. matrix production).

**Myofibroblasts and Fibrosis: Consequence or Cause of Injury?—**As ECM accumulation is the *sine qua non* of TIF, it is reasonable to focus on the cells that synthesize these matrix proteins. However, this does beg the question of whether matrix production per se in renal injury is deleterious. Fibrosis as a response to tubular injury that promotes healing rather than progression of disease has been reviewed elsewhere by Kriz's group[17]. Briefly, this viewpoint suggests that a local fibrotic process is supportive for recovery and provides the structural framework that allows injured nephrons to survive[17]. Consistent with the link between tubular injury and fibrosis, most ECM localizes around injured tubules early in disease. However, recent data shows that myofibroblast-induced ECM is not just the consequence of injury but also promotes fibrogenesis through augmented tissue stiffness. This stiffness accelerates TIF progression by activating profibrotic growth factors like TGF-β in a Yap/Taz-dependent pathway[18]. TIF likely also promotes further tubular injury through increasing the diffusion distance of oxygen, thereby worsening hypoxia. The degree to which peritubular fibrosis promotes proximal tubular hypoxia is difficult to determine as concomitant capillary dropout causes the same effect. In normal tissue repair, myofibroblasts are present but then undergo apoptosis. It is unclear if myofibroblast persistence in CKD is in response to ongoing local injury or if they begin to act autonomously and independent from tubular and/or inflammatory stimuli. More research is necessary to determine whether myofibroblast ECM production may impair the ability of an injured tubule to recover and whether myofibroblasts reach a "point of no return" where they continue to promote fibrosis even after the tubular injury has resolved.

**Inflammatory Cells: Macrophages May Promote Fibrosis in CKD—**Inflammation is an integral part of tissue injury and can either promote repair or stimulate further injury depending upon the cell type and microenvironment. The monocyte/macrophage is the most abundant immune cell in most models of chronic kidney injury, and the presence of macrophages in human CKD biopsies is associated with TIF and poor renal survival[19, 20]. The depletion of macrophages in AKI has different effects depending upon the timing, suggesting that macrophages may be injurious early in AKI and reparative at later stages[21]. In chronic models of renal injury, macrophages appear to play more of a pro-fibrotic role. Ablating macrophages either genetically (CD11b-DTR) or with clodronate protected against fibrosis in the unilateral ureteral obstruction model (UUO), a mechanical injury that induces TIF and heavy inflammation[22, 23]. In addition, an antagonist to CCR1, a chemokine receptor that promotes macrophage infiltration, reduced TIF in a murine model of diabetic nephropathy[24].

Macrophages are a heterogeneous population and have been further sub-classified into many subsets using various surface markers (e.g. Ly6C, F4/80). Macrophage classification can become quite complex, but there are two broad populations: M1 (the classically activated, $Ly6C^{hi}$) macrophage that promotes inflammation and the M2 (alternatively activated, $Ly6C^{lo}$) macrophage that can be reparative but also pro-fibrotic. Rat kidneys had greater expression of genes related to M1 rather than M2 polarization 120 days after $5/6^{th}$ nephrectomy, a renal reduction model of chronic injury [25]. Some investigators have shown a switch in the UUO model from M1 polarization at day 5 to M2 polarization at day 14 after obstruction[26, 27]. There are conflicting reports about M1 versus M2 macrophage

polarization in diabetic nephropathy[28, 29]. Most studies suggest that macrophage infiltration in CKD contributes to TIF progression, but clarifying macrophage polarization in chronic injury requires further investigation.

Macrophages likely promote TIF progression through the production of pro-fibrotic cytokines that have paracrine effects on neighboring fibroblasts/pericytes and epithelial cells. M1 polarized macrophages produce proinflammatory cytokines such as IL-1β and chemokines Mip1α, while M2 macrophages are potent sources of profibrotic proteins such as TGF-β, PDGF, and IGF[22]. Conditional knockout of macrophage-derived TGF-β1, one of three mammalian TGF-β isoforms, did not mitigate TIF, implying that M2 macrophages may have effects on neighboring fibroblasts through TGF-β-independent pathways[30]. One such mediator may be galectin-3, a macrophage-derived lectin, which augmented fibrosis and activation of myofibroblasts in the UUO model of injury[23]. In addition, macrophages are potent sources of matrix metalloproteinases (MMPs). Although some MMPs reduce TIF progression through degradation of ECM components, MMP-2, -9, and -12 are associated with TIF progression[31]. In late-stage UUO, macrophages were one of the cellular sources of MMP-9 and inhibiting MMP-9 reduced TIF[32]. However, the role of macrophage-derived MMPs in CKD progression requires further study as some of these MMP effects vary depending upon the injury model and timing (early versus late) of injury[33].

**Dendritic Cells, T Cells, and NK Cells: Role in TIF is Less Clear—**Macrophages are the most abundant inflammatory cells in CKD, but others may also contribute to TIF progression as well. Dendritic cells (DCs), like macrophages, are myeloid-derived antigen-presenting cells that modulate adaptive immunity but also effector cells that produce local-acting cytokines. In human biopsies, DC infiltration was associated with the degree of TIF in both immune and non-immune causes of CKD[34]. DCs have an important role after renal injury by increasing T-cell-dependent inflammation that promotes TIF[35, 36]. Whether DCs also have an adaptive-independent, direct effect on TIF progression through cytokine production is less clear. DCs, defined as CD11c+ cells, become activated after renal injury and produce cytokines[37]. Their role in promoting TIF is unclear as their deletion (CD11c/DTR) did not alter matrix accumulation in the UUO model[38, 39]. The role of DCs may also depend upon the etiology of injury as these cells have been shown to activate the inflammasome leading to renal fibrosis in crystal induced nephropathies[40].

Other immune cells such as T cells and natural killer (NK) cells may be important in immune-mediated causes of CKD, but their importance in other etiologies such as hypertension and diabetic nephropathy remain unclear. The importance of type 2 immunity, characterized in part by a predominance of T helper 2 ($T_H2$) over $T_H1$ cells in promoting fibrosis, particularly in the lung and liver, has been reviewed extensively by others[41]. Recently, the pro-inflammatory $T_H17$ cells, triggered by the proteoglycan biglycan, were implicated in albuminuria development in diabetic mice[42]. Others have described very few T cells present in murine diabetic nephropathy in contrast to the UUO rodent model where $T_H2$ cells were present and correlated with fibrosis[43, 44]. Mice lacking T cells that were injured by UUO had reduced TIF in some studies but unchanged fibrosis in others, raising the question of how important these T cells are in the pathophysiology of TIF[43, 45]. Similarly, NK cells have been shown to be present in chronically diseased kidneys, and a

recent study correlated the CD56$^{bright}$ NK cells with fibrosis and loss of function in human biopsies of immune and non-immune CKD[46]. However, the only study to deplete NK cells in mice failed to show protection in a model of adriamycin-induced nephropathy[47]. Thus, though DCs, T cells, and NK cells may be associated with CKD, animal models have shown a much stronger role for macrophages in TIF pathogenesis than for these other immune cells. One caveat is that most of these studies were done with the UUO injury model which has a more robust inflammatory response than would be expected in most human CKD biopsies.

**Microvasculature: Impaired in CKD—**The vascular compartment has received increasing attention as not only an important mediator of AKI to CKD conversion but also integral to TIF in other causes of CKD. Severe AKI induced by ischemia/reperfusion leads to TIF and a reduction in peritubular capillary number and size measured by fluorescence microangiography[48]. Increased endothelial cell death as well as pericyte detachment and thus de-stabilization of the peritubular capillaries are thought responsible for the reduced capillary number. In support of this, a recent study marked a subset of pericytes that express Gli1 and showed that the distance between these Gli1+ cells and endothelial cells increased after injury, supporting pericyte detachment[49]. Moreover, ablating these pericytes by expressing the humanized diphtheria toxin receptor in Gli1+ cells induced endothelial injury and loss of cells, suggesting that pericyte loss is sufficient to cause endothelial injury[49]. There is substantial evidence reviewed elsewhere that this capillary rarefaction leads to tissue hypoxia which further promotes TIF progression[50–52].

Many proteins mediate these changes in the microvasculature through both epithelial/endothelial as well as endothelial/pericyte cross-talk. Vascular endothelial growth factor (VEGF) plays an important role in vascular homeostasis and response to injury. Both injured proximal tubule cells and pericytes produce VEGF which stimulates endothelial cell proliferation and sprouting through the VEGFR-2 receptor. VEGF is considered a potent prosurvival growth factor for endothelial cells, and capillary rarefaction in the 5/6$^{th}$ nephrectomy model was ameliorated with exogenous VEGF[53–55]. However, while VEGF protects endothelial cells, there is evidence it may impair pericyte-mediated vessel stabilization in the presence of platelet-derived growth factor (PDGF)[56]. Also, in the UUO model, pericytes switched from producing the usual VEGF164 isoform to the VEGF120 and VEGF188 isoforms which cause vascular instability[57]. Blocking VEGFR in this model improved capillary rarefaction[57]. Thus, the role of VEGF signaling in the injured vasculature is complex and likely depends upon the microenvironment, levels, and isoforms. Other growth factors and angiogenic proteins such as PDGF, angiopoietins 1/2, tissue inhibitor of metalloproteinases (TIMP)-1, and metalloproteinases mediate altered cross-talk between endothelial cells and pericytes to promote rarefaction in CKD as discussed elsewhere[58–60].

**Microvascular Injury May Promote TIF—**Biopsies of patients with CKD show significant capillary rarefaction concurrent with TIF[61]. Although this does not prove causation, there are a number of studies linking capillary rarefaction to hypoxia. Furthermore, many in vitro studies suggest that hypoxia promotes TIF through effects on

injured epithelia, inflammatory cells, and myofibroblasts. Hypoxia may prevent renal tubular recovery and re-differentiation after injury as suggested by the strong association between peritubular capillary rarefaction and tubular de-differentiation, measured by vimentin expression, 4 weeks after injury by I/R[62]. Abundant in vitro data suggests that hypoxia's direct effects on proximal tubule cells promote TIF by augmenting ECM production and changing the type of collagen from IV, a component of the tubular basement membrane, to pathogenic collagen I[63]. Hypoxia can also promote tubular atrophy by inducing mitochondrial damage that leads to proximal tubular apoptosis[64, 65]. Hypoxia may augment TIF indirectly by stimulating proximal tubule cells to produce pro-fibrotic factors like TGF-β, endothelin-1 (ET-1), and VEGF that can have paracrine effects on surrounding myofibroblasts and endothelia. Hypoxia also upregulates fibroblasts' production of collagen I and TIMP-1 in a TGF-β-independent manner in vitro[66]. In addition to these increases in ECM synthesis, hypoxia augments inflammation as shown by administration of a mitochondrial uncoupler (dinitrophenol) to rats for 30 days[67]. Thus, the injured microvasculature plays an important role in propagating TIF by hypoxia-induced changes in neighboring cells. Also, as the injured endothelia have limited regenerative capacities, capillary dropout may be a limiting factor for recovery in CKD.

**Proximal tubules: Target of Renal Injury—**It is well established that the proximal tubule is the main target of acute kidney injury (AKI) due, in part, to its vulnerability to changes in oxygen delivery. Proximal tubule cells are mitochondria-rich to support the energy necessary for this segment to reabsorb over 60% of the filtered water and electrolytes. To facilitate this, the apical surface has a brush border which increases the surface area available for reabsorption. In addition, the innermost part of the proximal tubule, the S3 segment, is supplied by a unique countercurrent vasculature with relatively lower oxygen tensions. Thus, a mismatch of oxygen supply and demand occurs if there is any disruption to the blood supply or drop in blood pressure. Although AKI usually resolves with restoration of renal function, there is increasing recognition that, if severe enough or repetitive, AKI leads to TIF and CKD. In addition, the proximal tubule has multiple transporters that render this segment susceptible to chronic injury from toxins (e.g. lead, aristolochic acid) or filtered proteins (glomerular nephropathies). The injured proximal tubule is an important source of cytokines and growth factors like epidermal growth factor (EGF) that promote regeneration in the acute setting, but may be deleterious in chronic injury[68]. Through these secreted factors, the injured proximal tubule has paracrine effects on surrounding compartments such as the vasculature, inflammatory system, and interstitial fibroblasts.

## Primacy of the Proximal Tubule: Tubulointerstitial Injuries

As described above, renal TIF progression involves many different cell types and growth factors that mediate cross-talk between these different cellular compartments. The proximal tubule has long been regarded as a target of injury, but emerging evidence suggests that it also plays a prominent role in the development and progression of TIF. This review now focuses on proximal tubular responses to chronic injury that, directly or indirectly, promote tubular atrophy and TIF progression in both tubular and glomerular causes of CKD. One of the difficulties in determining the relative role of each cellular compartment in TIF

progression is that most injury models directly affect many cell types. For instance, the I/R model affects the vascular, inflammatory, and epithelial cell compartments in a rapid fashion making cause and effect difficult to parse apart, particularly when most injury is assessed at only one or two time points.

These limitations have been addressed in recent studies suggesting that proximal tubule injury is sufficient to cause TIF and often precedes the involvement of other cellular compartments. Two independent groups developed proximal tubule-specific murine models of injury by crossing a mouse containing the Cre-inducible diphtheria toxin receptor (DTR) with Cre driven by either the Six2 promoter or Ndrg1-Cre(ERT2)[69, 70]. Although the Six2 promoter is active in all cells derived from metanephric mesenchyme (proximal tubules, podocytes), both groups showed that administration of diphtheria toxin (DT) led to proximal tubule-specific injury. One dose of sublethal DT caused reversible AKI, but multiple injections lead to the full spectrum of CKD with inflammation, increased TIF, capillary rarefaction, and even glomerulosclerosis[69, 70]. These data suggest that isolated proximal tubular injury is sufficient to recapitulate the histologic changes in CKD and support the clinical observation that multiple episodes of AKI lead to CKD.

Toxins, drugs, ischemia, and multiple episodes of AKI are some of the renal injuries which primarily target the tubulointerstitium, and more specifically the proximal tubule, and can lead to CKD. The nature of injury (severity and duration) and the proximal tubular response to chronic injury dictate whether repair or TIF progression occurs. Some of these proximal tubular responses to injury are initially adaptive but become detrimental in the context of chronic kidney injury. These maladaptive responses can promote tubular atrophy and TIF both through cell autonomous effects that alter cell survival or ECM production and by paracrine effects on surrounding cells (e.g. endothelium, fibroblast/pericyte, macrophage).

**Proximal Tubule De-differentiation and TIF Progression—**The proximal tubule is a highly differentiated cell that expresses multiple channels and transporters which are necessary for the reabsorption of key proteins and electrolytes. The injured proximal tubule cell de-differentiates and loses expression of typically epithelial proteins such as cadherins and ZO-1. Acutely, this may be beneficial as a de-differentiated cell should require less ATP and energy to maintain, a benefit in hypoxic conditions. In the AKI field, dedifferentiation and proliferation of surviving proximal tubules is considered a critical component of repair. However, increasing evidence suggests that persistently de-differentiated proximal tubule cells may play a role in CKD progression. It can be difficult to distinguish causation from correlation as it is possible that continued de-differentiation merely reflects ongoing renal injury and is an adaptive response that allows the cell to survive[71, 72]. However, tubular deletion of Snail, one of the transcription factors integral to epithelial de-differentiation, reduced TIF in both the UUO and folic acid nephropathy models, suggesting that epithelial de-differentiation per se is detrimental[73]. The one caveat is that Snail has been shown to directly up-regulate inflammation, and so its deletion may be protective through anti-inflammatory effects which are not necessarily related to cell differentiation[74]. Unfortunately, other transcription factors related to de-differentiation (members of the SNAI, ZEB, and TWIST families) also modulate inflammation, making it difficult to discern one effect from the other.

The mechanism whereby persistently de-differentiated epithelial cells promote TIF is likely through production of paracrine factors such as TGF-β which can stimulate ECM production by neighboring myofibroblasts. As mentioned earlier, complete EMT as defined by loss of epithelial-specific markers, expression of mesenchymal markers, and movement across the tubular basement membrane to become myofibroblasts is unlikely. However, it is well-accepted that injured epithelial cells lose some epithelial markers and up-regulate mesenchymal proteins such as vimentin, a process referred to as either de-differentiation or partial EMT. These structural alterations of the injured proximal tubule are associated with a functional change into a potent producer of pro-fibrotic cytokines. These growth factors are mainly thought to induce TIF progression through paracrine actions on pericytes/fibroblasts. However, in the aristolochic acid model of injury, TIF was associated with increased transcription of COL1A2 in injured epithelial cells, suggesting that de-differentiation may promote TIF through cell autonomous production of ECM as well[75]. It is likely that epithelial de-differentiation happens on a continuous spectrum with some cells having a slight change in epithelial/mesenchymal markers while other cells have more dramatic differences. One unanswered question regarding this spectrum of de-differentiation is how it relates functionally to adaptive or maladaptive repair. Not all degrees of de-differentiation may be deleterious, and it remains to be seen if expression of certain proteins (e.g. α-SMA) indicates a maladaptive response and TIF progression.

**Proximal Tubule Cell Cycle and TIF—**Renal injury clearly alters the cell cycle of epithelia, but epithelial cell cycle change may also impact TIF progression and the transition from AKI to CKD. Most proximal tubule epithelia in the uninjured kidney are quiescent (cell cycle stage G0). However, during injury, cells may enter the cell cycle (G1, S, G2, M) to help replace cells lost to apoptosis/necrosis, and some cells become arrested in either G1 or G2. This cell arrest is adaptive to allow time for repair of any DNA damage and prevent the propagation of mutations that occur in injured cells. However, persistence of G2 arrest in epithelial cells leads to increased production of profibrotic cytokines TGF-β and CTGF in a JNK-dependent pathway[76, 77]. Data positively links the percentage of epithelial cells arrested in G2/M after murine AKI with a higher chance of developing CKD[76, 78, 79]. Interestingly, manipulation of the cell cycle inhibitor p21, which affects both G1 and G2 arrest, has had mixed results depending upon the acuity of injury and model[80–85]. Selective blockade of G1 arrest using palbociclib, an inhibitor of cyclin dependent kinase (CDK)4/6 that promotes progression from G1 to S phase, was protective in AKI by reducing proximal tubular apoptosis[86, 87]. The use of a selective G1 inhibitor in CKD has not been tested in CKD though theoretically, this may be beneficial by reducing the cells that progress to G2 arrest. In Jurkat T cells, progression from G1 to S phase was associated with increased $O_2$ consumption, suggesting that G1 arrest may reduce $O_2$ demand in injury though this has not been studied in renal epithelia[88]. In summary, epithelial G2 arrest likely promotes TIF progression through paracrine effects (i.e. secretion of profibrotic factors acting on myofibroblasts), but the role of proximal tubular G1 arrest in CKD is unclear.

**Maladaptive Unfolded Protein Response (UPR) Promotes Tubular Atrophy—**The activated unfolded protein response (UPR), autophagy, and senescence are three proximal tubular responses to injury which have both adaptive and maladaptive effects

depending upon the microenvironment and the duration and severity of injury. Endoplasmic reticulum (ER) stress, hypoxia, and oxidative stress can all activate the UPR which regulates the balance between protein folding and synthesis/degradation. In the adaptive UPR response, the ER chaperone GRP78/BiP binds to various ER stress sensors leading to improved protein homeostasis or proteostasis through reduced protein translation, increased ER chaperone expression, and degradation of misfolded proteins[89]. Knock-in expression of a mutant BiP led to tubular atrophy, TIF, and increased tubular apoptosis in kidneys of aged mice suggesting that BiP plays a protective role[90]. Renal biopsies from patients with diabetic nephropathy showed increased expression of UPR proteins in the proximal tubule, suggesting that the UPR may be particularly important in this cellular compartment[91]. However, when the insult is prolonged or excessive, then activation of the CHOP-mediated pro-apoptotic UPR pathway leads to TIF through enhanced tubular atrophy. This maladaptive UPR pathway causes proximal tubular damage in both hypoxia/reperfusion and age-related proteinuric models of injury[92, 93].

**Autophagy and Senescence: Double-edged Swords in Injury—**Autophagy, a method of degrading intracellular components and organelles in response to external stressors, can be an adaptive way to eliminate abnormal proteins or to recycle energy substrates (e.g. amino acids, sugars) during times of limited nutrient availability. Impairing autophagy by Atg5 deletion worsened the proximal tubular response to injury by cyclosporine in vitro or diabetes and/or high fat diet in vivo[94–96]. However, tubular autophagy has also been implicated in renal TIF progression. In the UUO model, autophagy correlated with epithelial apoptosis and tubular atrophy[97]. Also, tubular over-expression of TGF-β1 led to tubular autophagy and degeneration as well as peritubular fibrosis[4]. Thus, the role of proximal tubular autophagy and renal injury is complex and likely protective early in injury though may promote apoptosis or cell degeneration if injury is too severe.

Epithelial senescence is irreversible growth arrest in the context of growth-stimulating factors and is thought protective against the development of cancer. Senescence can be triggered by aging, reactive oxygen species, or other stressors (e.g. DNA damage). Although senescent epithelial cells do not divide, they are metabolically very active and have a senescence-associated secretory phenotype (SASP) that can recruit inflammatory cells. A study comparing young to aged mice subjected to I/R injury found that the aged mice had higher numbers of senescent tubules (β-galactosidase positive), greater fibrosis, and increased inflammation compared with the younger mice[98]. Some evidence suggests that CKD itself may accelerate the development of senescence through uremic toxins, leading to a vicious cycle promoting injury[99, 100]. Therefore, while epithelial senescence may have a protective anti-oncogenic role, it likely promotes TIF through paracrine effects that augment inflammation. It should be recognized that these proximal tubule responses (UPR, autophagy, senescence) do not necessarily occur in isolation. ER stress can lead to proximal tubular autophagy[101], and proximal tubular autophagy can cause senescence[102]. These responses begin as adaptive responses to injury, but if the injury is severe enough or persistent, may promote TIF.

**Metabolic Changes in the Injured Tubules Contributes to TIF—**In CKD, the number of functional nephrons decreases creating more metabolic work for the remaining tubules. Similar to Brenner's hypothesis about declining renal function and glomerular hyperfiltration[103], the remaining proximal tubules hypertrophy to meet the increased demand of reabsorbing water and solutes[104]. Although total energy expenditure of the CKD kidney decreases, the metabolism of the surviving nephrons increases to support the compensatory changes in reabsorption[105, 106]. Protein intake correlates positively with proximal tubule hypertrophy[104], and augmented protein consumption in the subtotal nephrectomy model in rats led to more oxidative stress[107]. Other studies suggest that increased calories, rather than protein per se, increased the tubular workload and, conversely, that limiting intake protects against TIF[108, 109]. Changes in metabolism after injury may be better understood from the perspective of evolutionary tradeoffs. Natural selection leads to changes that maintain kidney function through the reproductive years but may promote renal decline after this time period[110]. For example, nephron loss leads to hypertrophy and increased energy consumption by the remaining nephrons to maintain renal function in the short-term, but this cannot be sustained and eventually leads to tubular loss and fibrosis. This evolutionary adaption may explain other responses in the CKD kidney that are initially adaptive but lead to dysfunction such as senescence and inflammation.

The energy substrates utilized by proximal tubules also change in the context of injury. Proximal tubule cells normally use fatty acid oxidation as their preferred energy substrate, but recent data suggests that anaerobic glycolysis occurs in acutely injured proximal tubules[111]. Increased glycolysis may help enable tubule survival, especially when mitochondrial function is impaired[112]. However, persistent glycolysis was associated with the development of proximal tubular atrophy, a key component of TIF. Whether this persistent glycolysis continues in chronic injury and how it contributes to TIF progression is unclear. Many CKD models such as the $5/6^{th}$ nephrectomy model, a nephron reduction model, have a reduction in fatty acid oxidation[113]. Levels of peroxisome proliferator-activated receptor (PPAR)α, an inducer of fatty acid oxidation, were reduced in both the $5/6^{th}$ nephrectomy and UUO models of TIF. Genetically restoring PPARα to proximal tubules reduced the expression of collagen I, fibronectin, and TIF after UUO[113, 114]. Thus, the injured proximal tubule has altered metabolic needs and energy substrates and reducing the tubule workload and/or maximizing fatty acid oxidation may be protective in TIF progression.

## Primacy of the Proximal Tubule: Glomerular Diseases

The proximal tubule is also an important mediator of TIF in glomerular injuries though the exact mechanisms are still debated. In a broad sense, glomerular disease may injure the tubulointerstitial compartment either from local injury extending into the proximal tubule or from filtered albumin and associated proteins/lipids which are then reabsorbed by the proximal tubule. In the first mechanism, proximal tubular apoptosis/necrosis driven by glomerular injury leads to tubular atrophy; in the protein overload mechanism, the proximal tubule can facilitate TIF through autocrine and paracrine signaling. These two mechanisms are discussed below.

**Local Extension of Glomerular Injury to Proximal Tubules—**Severe glomerular injury may extend into the proximal tubule and cause tubular injury by misdirected glomerular filtration. Glomerular injury leads to podocyte loss and formation of a tuft adhesion between the glomerular basement membrane and parietal epithelium. Protein-rich ultrafiltrate usually passes from the capillary looks to the Bowman's space and tubular lumen, but tuft formation and breaks in the parietal epithelium lead to misdirected protein ultrafiltrate to the interstitium[115]. Fibroblasts wall off this extra-glomerular filtrate which then extends down in between the proximal tubule and its basement membrane, resulting in tubular atrophy. Tracer studies in rat models of focal segmental glomerulosclerosis (FSGS) demonstrated accumulation of tracer outside the glomerulus and extending down to the proximal tubule[116]. Histologic studies of human FSGS also show a peri-glomerular space filled with matrix that extends to the proximal tubule and is associated with interstitial inflammation[117]. Another mechanism by which glomerular injury affects proximal tubules is through local expansion of the crescent or inflammatory cells. In inflammatory glomerular diseases, crescents appeared to involve the urinary pole and reach the proximal tubule[118]. Either epithelial or inflammatory cell proliferation may obstruct the glomerulotubular junction leading to a block in urinary flow, epithelial deterioration, and local peri-glomerular inflammation. Such atubular glomeruli have also been described in diabetes and even non-glomerular injuries such as the UUO[119]. However, in these situations, the junction may be blocked by proximal tubule epithelia that are damaged by increased proteinuria or injurious growth factors. Proximal tubule cells at the tubuloglomerular junction are particularly sensitive to apoptosis, and atubular glomeruli lead to tubular atrophy and peritubular fibrosis/inflammation.

**Glomerular Injury Injuring the Proximal Tubule by Excessive Filtered Proteins —**There is much recent data supporting the concept that glomerular injury extends to the tubulointerstitium through filtered proteins reabsorbed by the proximal tubule. Megalin and cubulin receptors expressed on proximal tubules' brush border bind filtered proteins, undergo endocytosis and release the proteins for lysosomal degradation. Glomerular injury increases the filtered load of albumin, and this augmented proximal tubular protein reabsorption can have both direct toxic and paracrine effects that may promote TIF. Increased albumin concentrations induced proximal tubular apoptosis in vitro[120, 121]. Similarly, renal biopsies from children with FSGS showed proximal and distal tubular apoptosis[122]. Reducing apoptosis through ouabain treatment in a rat model of proteinuric injury protected cortical tubules from apoptosis, reduced the number of atubular glomeruli, and attenuated apoptosis[121]. A recent study using intravital two-photon microscopy showed that a salt sensitive model of hypertension in rats resulted in increased glomerular filtration of albumin and compensatory augmented protein reabsorption in the proximal tubule[123]. Subsequently, the proximal tubules dilated and developed granular casts and necrosis suggesting that tubular injury plays an important role in the pathophysiology of proteinuric hypertension[123].

There is also compelling data that proteinuria promotes TIF progression through proximal tubular production of proinflammatory and profibrotic cytokines. Earlier in vitro data showed that albumin induced proximal tubular production of proinflammatory factors

monocyte chemoattractant protein (MCP)-1, RANTES, and fractalkine through activation of the NF-κB pathway[124–126]. Proximal tubules exposed to albumin also augmented production of profibrotic TGF-β and its type II receptor as well as accumulation of ECM components collagen IV, laminin, and fibronectin in vitro[127–129]. More recent data has linked protein reabsorption and lysosomal rupture with stimulation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in the proximal tubule[130, 131]. Albumin exposure to the proximal tubule also impairs autophagy in vitro by activating mammalian target of rapamycin (mTOR), a known autophagy inhibitor. The role that albumin-dependent NLRP3 stimulation and autophagy suppression plays in TIF progression in vivo requires further investigation. Concerns over the relevance of these in vitro studies to proteinuric diseases in vivo have been expressed, especially regarding whether the quantity and quality of albuminuria in vitro matches that in disease states. Albumin alone may not be as injurious to the proximal tubule as the compounds bound to albumin such as fatty acids. In humans, a strong association was found between proteinuria, MCP-1 expression, and NF-κB activation in tubular cells but these data are correlative and do not prove causation[132, 133].

Addressing the question of whether this proximal tubular reabsorption of protein drives inflammation and TIF progression in vivo, two independent groups subjected a mosaic megalin knock-out mouse to proteinuric injury models. One group used an anti-glomerular basement membrane (GBM) model of glomerulonephritis and found that megalin+ proximal tubule cells had more TGF-β, intercellular adhesion molecule-1 (ICAM-1), and endothelin-1 expression than those lacking megalin[134]. However, megalin negative proximal tubule cells had more apoptosis than those with megalin expression, and tubulointerstitial inflammation and fibrosis were driven by the degree of crescentic glomerulonephritis and not the proximal tubular expression of megalin[134]. In contrast, another group used a podocyte selective injury to induce higher amounts of proteinuria and found that these megalin expressing proximal tubule cells had greater markers of oxidative stress (HO-1), MCP-1, and apoptosis compared to those without megalin[135]. These divergent results may be explained by the differing models: the anti-GBM is intensely inflammatory with comparatively less proteinuria than the podocyte-selective model. In summary, pathologic levels of filtered proteins are likely injurious to the proximal tubule and stimulate proinflammatory and profibrotic cytokines. However, the extent to which this response promotes TIF progression and the exact mechanisms involved require more investigation in vivo. These differing mechanisms whereby glomerular injury leads to TIF, namely local extension to the proximal tubule and increased protein reabsorption, are not necessarily mutually exclusive. The local extension mechanism may play a larger role in inflammatory crescentic glomerulonephritides, whereas increased protein reabsorption may be the predominant mechanism in diseases with high amounts of proteinuria such as membranous nephropathy, but more research is necessary to further clarify.

## Primacy of the Proximal Tubule and Implications for CKD Treatment

In addition to basic science studies elucidating an important role for the proximal tubule in TIF, a new class of diabetic medications that targets the proximal tubule has been shown to protect against CKD progression in diabetic nephropathy. The sodium-glucose co-

transporter 2 (SGLT2) inhibitors treat diabetes by blocking the reabsorption of glucose in the proximal tubule, leading to glucosuria and reduced blood sugar levels. In EMPA-REG OUTCOME, treating type II diabetics with the SGLT2 inhibitor empagliflozin led to a 39% reduction in relative risk of incident or worsening nephropathy compared to placebo[136]. The exact mechanisms of this renoprotective effect are unknown, but it is postulated that reducing sodium and glucose reabsorption in the proximal tubule increases distal delivery and restores tubuloglomerular feedback. This leads to constriction of the afferent arteriole, the blood supply leading to the glomerulus, which reduces glomerular hyperfiltration, albuminuria, and the metabolic demands of the proximal tubule[137, 138]. Other potentially beneficial effects of the SGLT2 inhibitors include blood pressure lowering effects through natriuresis and reducing toxic effects of glucose reabsorption in the proximal tubule. Thus, a proximal tubule-specific therapy is highly promising as a treatment for CKD caused by diabetic nephropathy.

An increasing appreciation for the role of the proximal tubule in CKD progression is important because many growth factors critical to TIF affect the proximal tubule differently from cells in the interstitium. Several groups have shown that β-catenin activation in fibroblasts or pericytes promotes TIF progression[7, 139, 140]. In contrast, we recently demonstrated that β-catenin activity in the proximal tubule protected against TIF[141]. Although our study explored proximal tubular activity in the context of deleted TGF-β type II receptor, others have shown protective effects of Wnt/β-catenin activity in proximal tubular injury[142, 143]. Thus, a therapeutic approach targeting the proximal tubule may be more effective than systemic alteration of growth factor pathways. Such an approach has been used in research settings by conjugating drugs to lysozyme which is reabsorbed by the proximal tubule[144, 145]. A platinum-based linker was used for conjugation in these studies which may be problematic for human studies given the known nephrotoxic effects of free platinum, but the concept remains useful for more targeted treatment of the proximal tubule.

## Final Considerations

CKD is characterized by TIF, a complex process mediated by several different compartments within the kidney. Many therapeutic approaches have focused on the myofibroblast as this is the main cell type responsible for ECM production[146]. This may be a reasonable strategy in later stages of disease when myofibroblast function and ECM synthesis could be acting independently of tubular injury. However, recent therapies have been disappointing. The proximal tubule has long been recognized as the target of diverse injuries, but increased attention has focused on how its responses to injury may mediate TIF progression. Many of these proximal tubular responses (e.g. de-differentiation, cell cycle and metabolic changes) are initially adaptive, but as the injury persists, these responses may promote tubular atrophy and TIF through autocrine and paracrine signaling pathways. Though other cells (e.g. myofibroblasts, macrophages) are the effectors of kidney injury, the injured proximal tubule is likely the driver. Consistent with this, a proximal tubule-specific drug (SGLT2 inhibitor) has promising effects on renal function and proteinuria in human diabetic nephropathy. Further investigation is necessary to better understand how modulation of these proximal

tubular responses can alter TIF progression and guide much needed new therapeutic options for CKD.

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## Abbreviations used

| | |
|---|---|
| **AKI** | acute kidney injury |
| **α-SMA** | α-smooth muscle actin |
| **CKD** | chronic kidney disease |
| **ECM** | extracellular matrix |
| **CTGF** | connective tissue growth factor |
| **DC** | dendritic cells |
| **DTR** | diphtheria toxin receptor |
| **EGF** | epidermal growth factor |
| **EMT** | epithelial to mesenchymal transformation |
| **ER** | endoplasmic reticulum |
| **FSGS** | focal segmental glomerulosclerosis |
| **MCP-1** | monocyte chemoattractant protein-1 |
| **MMPs** | matrix metalloproteinases |
| **NK** | natural killer |
| **PDGF-β** | platelet-derived growth factor-β |
| **PPARα** | peroxisome proliferator-activated receptor α |
| **NLRP3** | NOD-like receptor family, pyrin domain containing 3 |
| **SASP** | senescence-associated secretory phenotype |
| **SGLT2** | sodium-glucose cotransporter 2 |
| **TGF-β** | transforming growth factor-β |
| **TIF** | tubulointerstitial fibrosis |
| **TIMP-1** | tissue inhibitor of metalloproteinase |
| **I/R** | ischemia/reperfusion |
| **UPR** | unfolded protein response |

| | |
|---|---|
| **UUO** | unilateral ureteral obstruction |
| **VEGF** | vascular endothelial growth factor |
| **ZO-1** | zona occludens-1 |

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## Highlights

- Tubulointerstitial fibrosis is the common pathway to end-stage renal disease.
- Many different cell types contribute to tubulointerstitial fibrosis.
- The proximal tubule is targeted in acute and chronic renal injuries.
- Many proximal tubule responses are initially adaptive but can become maladaptive.
- Proximal tubule responses to chronic injury drive tubulointerstitial fibrosis.
- Understanding proximal tubule responses may inform novel CKD therapies.

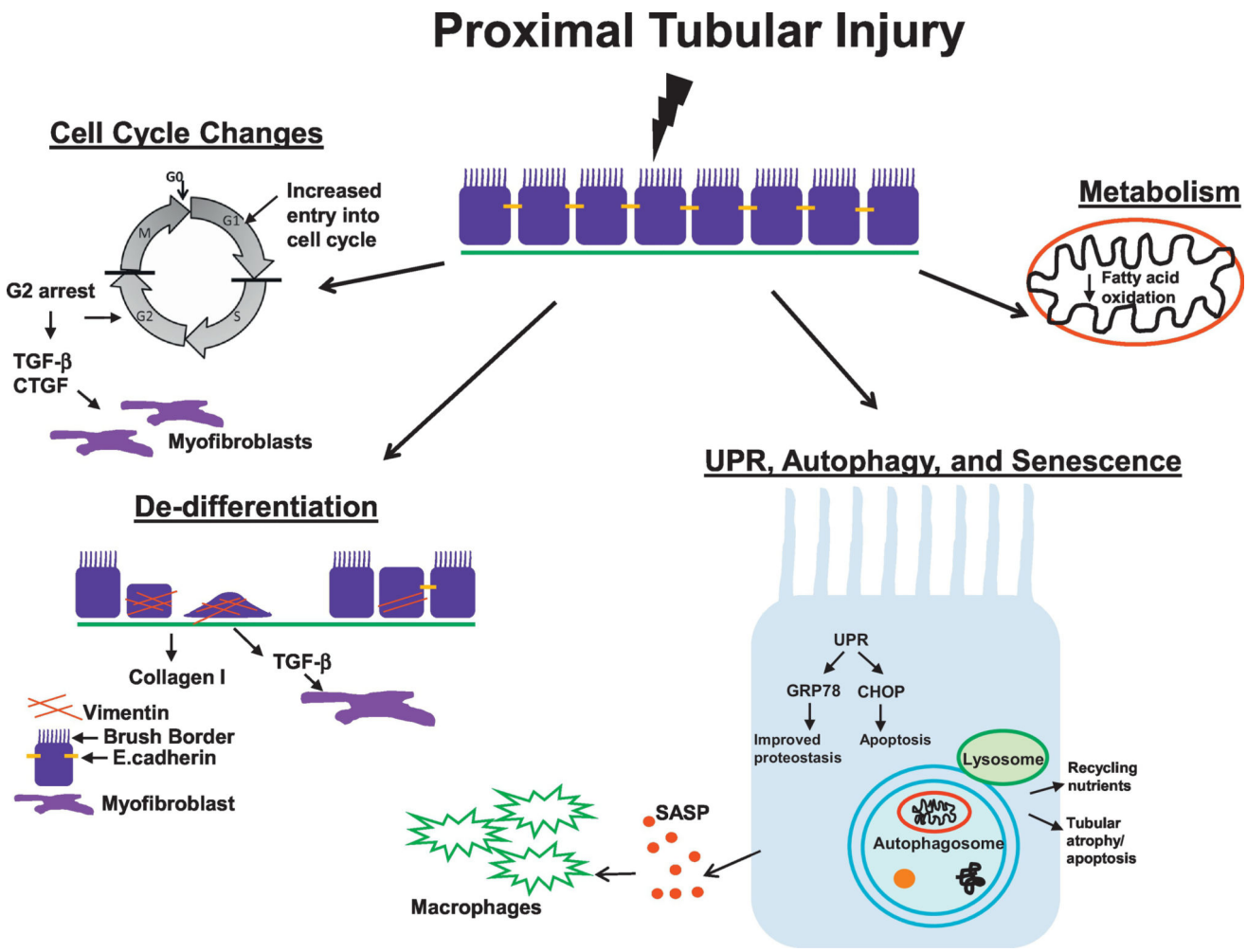


**Figure 1. Proximal tubular responses to chronic injury promote tubulointerstitial fibrosis**

Chronic injury of diverse etiologies leads to proximal tubular responses that can be both adaptive and maladaptive, depending upon the chronicity and severity of injury. Most uninjured proximal tubule cells are quiescent (cell cycle stage G0), but in injury, the surviving cells reenter the cell cycle to proliferate and replace apoptotic/necrotic cells. Some cycling cells will arrest at either G1 or G2 which protects against damaged cells proliferating and potentially introducing DNA mutations to progeny cells. However, epithelial cells arrested in G2 exhibit a profibrotic phenotype with increased TGF-β and CTGF which promote TIF through paracrine actions. Injured proximal tubule cells de-differentiate as shown by loss of brush border, decreased E.cadherin expression, increased vimentin expression and cytoskeletal changes. Although some de-differentiation may reduce oxygen consumption and promote survival, these changes are also associated with increased production of TGF-β and other profibrotic factors that can act on neighboring myofibroblasts to promote TIF. Injured proximal tubule cells also undergo the unfolded protein response (UPR) and autophagy, both of which may have adaptive or maladaptive effects as shown. Senescence inhibits cell proliferation but enhances production of proinflammatory cytokines as part of the senescence-associated secretory phenotype

(SASP). Chronic injury also alters metabolism of proximal tubule cells which normally rely on fatty acid oxidation as the main energy substrate.

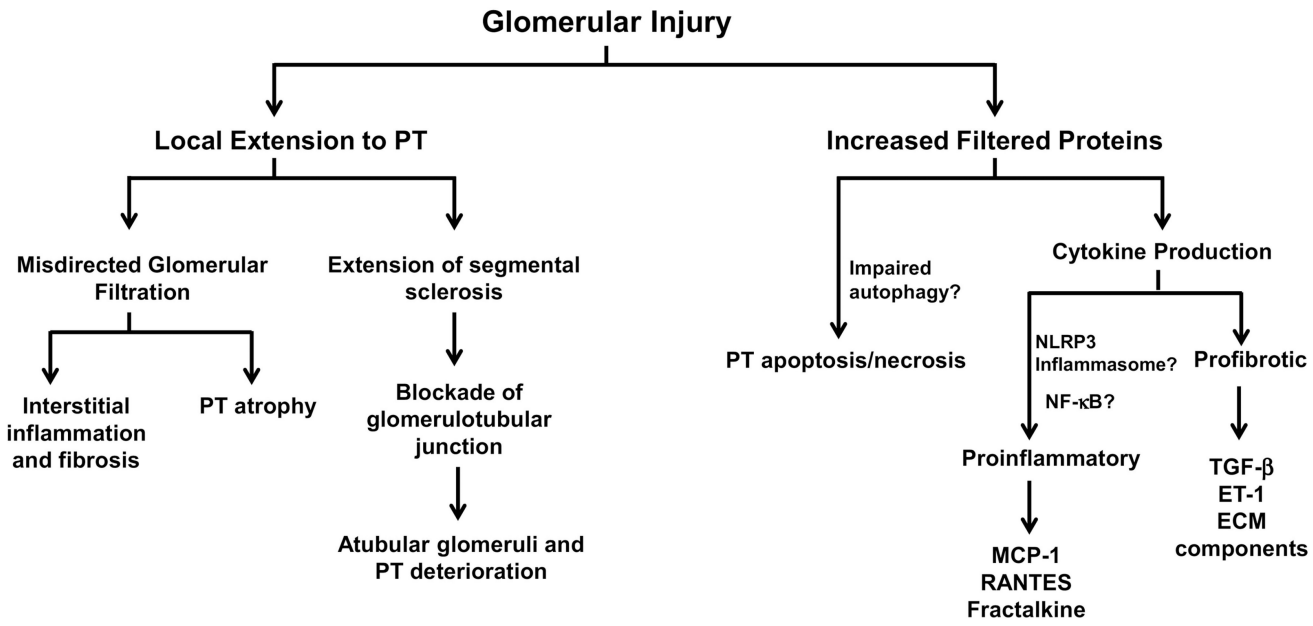


**Figure 2. The proximal tubule (PT) plays a role in mediating tubular atrophy and TIF in glomerular injury**

Glomerular injury can affect the tubulointerstitium by local extension or through increased protein and albumin-bound filtration which is reabsorbed in the proximal tubule. These different mechanisms are not mutually exclusive, and their relative contribution to TIF progression may vary depending upon the etiology of glomerular injury.