An integrative view on the role of TGF-β in the progressive tubular deletion associated with chronic kidney disease

Omar García-Sánchez[1,2], Francisco J. López-Hernández[1,2,3] and José M. López-Novoa[1,2]

[1]Unidad de Fisiopatología Renal y Cardiovascular, Departamento de Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain; [2]Instituto Reina Sofía de Investigación Nefrológica, Fundación Íñigo Álvarez de Toledo, Madrid, Spain and [3]Unidad de Investigación, Hospital Universitario de Salamanca, Salamanca, Spain

Transforming growth factor-β (TGF-β) is a cytokine known to participate in several processes related to the development of chronic kidney disease (CKD), including tubular degeneration. This is thought to occur mainly through apoptosis and epithelial-to-mesenchymal transition (EMT) of tubule epithelial cells, which give rise to a reduction of the tubular compartment and a scarring-like, fibrotic healing process of the interstitial compartment. *In vivo* blockade of TGF-β action has been shown to reduce CKD-associated tubular damage. However, a direct action of TGF-β on tubule cells is controversial as the underlying mechanism. On the one hand, TGF-β is known to induce EMT of tubular cells, although its incidence *in vivo* can hardly explain the extent of the damage. On the other hand, a few publications have reported that TGF-β induces a mild degree of apoptosis in cultured tubular cells. This most likely reflects the consequence of the cell-cycle arrest rather than a direct pro-apoptotic effect of TGF-β. The implications of these observations are analyzed in the pathological context, where normal tubular cells do not normally proliferate, but they might divide for repair purposes. Furthermore, renal fibrosis, a TGF-β-mediated event, is integrated as a potential, indirect effect contributing to tubule deletion.

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Correspondence: *José M. López-Novoa, Unidad de Fisiopatología Renal y Cardiovascular, Departamento de Fisiología y Farmacología, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain. E-mail: jmlnovoa@usal.es*

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Chronic kidney disease (CKD) is a condition in which the renal excretory function progressively and irreversibly decreases as a consequence of renal tissue injury and nephron loss. Decreased excretory function gives way to accumulation of metabolic and waste products in the blood and organs, which cause azotemia and multiorgan damage. Eventually, patients may die to secondary conditions, the most important of which being cardiovascular events, or need renal replacement therapy in the form of renal transplant or dialysis.[1] Because of its high incidence and prevalence, and the disproportionate cost of dialysis, CKD represents a heavy human, clinical and socioeconomic burden. It is estimated that 10–20% of the adult population have some degree of CKD, and that dialysis (applied on 0.1% of the population) consumes about 2% of total health expenditure in many developed countries.[2] CKD can be caused by a variety of factors, including diabetes, hypertension, infections, atherosclerosis, renal artery and ureteral obstruction, genetic alterations, and others. Renal function usually declines over a period of years or decades, although some patients become eligible for renal replacement therapy after only a few months.

A critical concept in CKD is that early in the course of the disease, renal tissue injury crosses a no-return point beyond which a malignant scenario of self-destruction ensues independently of the initial insult. Regardless of etiology, a typical pathological phenotype appears where the number of nephrons decreases progressively and fibrosis and interstitial scarring replace the space left by erased nephrons. Transforming growth factor-β (TGF-β) has been recognized as an important mediator of a variety of glomerular, tubular, and inflammatory processes involved in the appearance of this phenotype. Specifically, this review deals with its effects on tubule deletion, which is thought to be the consequence of the death of tubular epithelial cells, their transformation into profibrotic, mesenchymal cells, and a defective repair process leading to fibrosis and scarring. Indeed, inhibition of TGF-β action *in vivo* has been shown to soften tubular damage and afford tissue and functional protection in different models of CKD.

TGF-β SIGNALING AND FUNCTIONS

TGF-β is a group of three ubiquitous cytokines (named 1 to 3) belonging to the TGF-β superfamily. The most abundant form in mammals is TGF-β1. Besides transcription, a key regulatory step of TGF-β action is its activation from its reservoir, a latent protein complex in the extracellular matrix (ECM). Activation of TGF-β receptors in the cell membrane induces intracellular signals that mediate many developmental, physiological, and pathological processes, including CKD. TGF-β membrane receptor complex comprises two families of proteins with serin-threonin kinase activity, namely type II (TβRII) and type I (TβRI) receptors. TβRI includes activin-like kinase (ALK) receptors. TGF-β binds to TβRII, which then recruits TβRI. The complex phosphorylates and activates several intracellular signaling cascades, including (1) the small mothers against decapentaplegic (Smads); (2) mitogen-activated protein kinases, such as extracellular regulated kinase (ERK), p38 and Jun kinase; and integrin-linked kinase (ILK).[3–5] These effectors modulate the expression of target genes involved in physiological as well as CKD-associated events, as for example cell growth, differentiation, apoptosis, and ECM deposition.

TGF-β AND TUBULAR EMT

Through an epithelial-to-mesenchymal transition (EMT) process,[6] under pathological conditions tubule cells may dedifferentiate to mesenchymal, activated fibroblastoid cells called myofibroblasts. Compared to tubule epithelial cells, myofibroblasts bear enhanced motility, increased proliferative and contractile capacity, and overproduce ECM elements contributing to fibrosis. Tubular EMT participates in the processes of tubule degeneration and fibrosis observed during CKD. However, tubular EMT has also been implicated in tubular tissue repair. On tubular injury, some of the remaining epithelial cells undergo EMT to proliferate, migrate, reconstitute the basal membrane in damaged areas, and differentiate again into functional tubule epithelial cells.[7] As such, the pathological EMT observed in CKD is susceptible to be viewed as the result of a skewed repair process.

TGF-β is a strong EMT inducer in renal tubule cells. TGF-β initiates and completes the EMT process *in vitro* and *in vivo* under determined conditions.[8] EMT is not an easy process to study, especially *in vivo*. In most *in vivo* studies, EMT is assessed by the appearance of fibroblastoid markers (that is, fibroblast-specific proteins, α-smooth muscle actin (α-SMA), or vimentin) in tubule cells co-expressing general or specific epithelial markers, actin reorganization, and basement membrane disruption.[8] Still, this is a transition state toward the acquisition of a full phenotype underlying the behavior as a mesenchymal, fibroblastoid cell, which is used as a surrogate marker of the extent of EMT. Three TβRIs have been implicated in TGF-β-induced EMT, namely ALK-4, ALK-5 and ALK-7, of which ALK-5 seems to have the most central role *in vivo*. On binding to the receptor complex, both TGF-β-activated Smad, ILK and ERK signaling pathways have been shown to be crucial for EMT through the modulation of the expression of key genes. On the one hand, expression of mutant TGF-β receptors lacking the ability to activate Smads, as well as inactive mutants of Smad-2, Smad-3, and Smad-4, prevent EMT-related, target gene expression, EMT and the excessive ECM deposition in cultured cells. On the other hand, selective inhibition of components of the Ras-ERK[9] and ILK[5] axis also suppresses EMT in different tumoral cell types. In addition, the lower renal level of myofibroblast markers (α-SMA and vimentin) in an *in vivo* model of obstructive nephropathy after ERK, PI3K/Akt, or ILK inhibition is also consistent with a softened EMT.[5,10] Ras family of proteins mediates TGF-β-induced activation of ERK.[10] Interestingly, on unilateral ureteral obstruction, EMT markers (α-SMA, vimentin, snail-1, and snail-2) are markedly reduced in mice lacking H-ras compared with WT mice.[11] Consistently, the effect caused by the absence of H-ras is not observed early (3 days) after obstruction,[12] coinciding with a time in which EMT is not yet involved in renal fibrosis. Other messengers such as Id proteins, and the gene transcription products slug and snail have also been implicated in or shown to mediate key, specific events of TGF-β-induced EMT, such as α-SMA and e-cadherin gene expression regulation.[6,13]

In mice, TGF-β-mediated EMT is inhibited by endogenous modulators including bone morphogenetic protein-7 (BMP-7),[14] hepatocyte growth factor (HGF),[15] and intercellular contacts,[16] whose role in renal fibrosis in patients needs to be further explored. In the same line, TGF-β cannot induce EMT in an intact confluent cell monolayer. Interestingly, loss of monolayer integrity as by subconfluency, wound, or calcium-mediated attachment disassembly restores TGF-β EMT-inducing capacity.[16] *In vivo*, tubular EMT only occurs after a sustained injury.[8] This suggests that EMT might be induced by TGF-β only on epithelial injury, likely as a repair process distorted within the pathological scenario. Under this scope, EMT would result in further damage rather than in repair, which in turn would further stimulate the repair process though TGF-β-mediated EMT, and other processes, which would get into a vicious circle of exacerbated damage. In these circumstances, inhibition of TGF-β actions would result in a beneficial effect, overall.

However, the real weight of epithelial EMT in the process of tubule degeneration *in vivo* remains to be determined. Many authors assign a central role to EMT in CKD-associated fibrosis independently of the origin of the disease.[6,8] This is based on two concepts: (1) most myofibroblasts, the cells mainly responsible for the excessive ECM deposition in the diseased kidney, come from tubule epithelial cells, whereas only a few result from the activation of resident fibroblasts; and (2) inhibition of EMT significantly reduces fibrosis. This is despite the incidence of EMT *in vivo* being found to be low in most studies.[8] Thus, it is easy to understand how a low EMT can give way to an extensive fibrosis, because dedifferentiated, myofibroblastoid cells

proliferate, migrate, and continuously overproduce ECM components. However, on these grounds it is more difficult to explain an important, direct role of EMT in the disappearance of tubule cells contributing significantly to tubule degeneration. It is possible, notwithstanding, that fibrosis *per se* contributes to tubule cell death (see below).

TGF-β AND TUBULAR APOPTOSIS

The involvement of TGF-β in tubule cell apoptosis has been evidenced by studies showing that inhibition of TGF-β actions *in vivo* reduces the extent of apoptosis seen in the tubular compartment under CKD situations. For example, treatment of mice with an anti-TGF-β antibody reduces tubular apoptosis in a model of kidney damage by ureteral obstruction.[17] This might be the consequence of either direct effects of TGF-β on tubular cells or indirect effects, such as fibrosis or inflammation, which TGF-β might contribute to induce. In this sense, some studies have reported that TGF-β activates apoptosis in cultured tubule epithelial and other cell lines.[18,19] However, the extent of cell apoptosis induced by TGF-β in cultured cells is surprisingly low for an *in vitro* system where purified molecules and optimized conditions are used. Besides, in many other studies, TGF-β lacks a pro-apoptotic effect in different cell lines including renal cells.[20] TGF-β is generally recognized as a homeostatic cytokine, a function of which being to restrict uncontrolled proliferation.[21] TGF-β typically causes inhibition of thymidine incorporation and cell-cycle arrest in the G1 phase.[22] As such, although TGF-β might have a mild, direct pro-apoptotic effect under undetermined circumstances, the apoptosis seen in cultured cells could also be hypothesized to result from the cell-cycle arrest rather than of a direct activation of death pathways.

Despite its general antiproliferative effect, TGF-β does not impede but stimulates the proliferation of myofibroblasts resulting from epithelial EMT, which has been linked to renal tissue repair (see above) and to tumor metastatic potential.[6] The mechanisms of tubule repair on injury are not well understood. Death cells could be substituted by (1) proliferation of remnant epithelial cells; (2) EMT-driven dedifferentiation of epithelial cells, followed by proliferation of myofibroblastoid cells, production of ECM, basal membrane reconstitution, and redifferentiation into epithelial cells; and (3) proliferation and differentiation of resident or blood-borne stem cells. The relative contribution of these processes to tissue repair is unknown. As such, limitation of tubule repair contributing to tubule degeneration might be theoretically ascribed to TGF-β, through the inhibition of differentiated epithelial cell proliferation. In agreement, TGF-β inhibits proliferation and wound healing of confluent tubule cell monolayers.[23] Moreover, TGF-β counteracts the proliferation and motility of epithelial cells, and tubule regeneration induced by HGF.[24]

Angiotensin II (ANG-II) induces apoptosis of cultured rat tubule epithelial cells. Interestingly, either inhibition of membrane death receptor Fas stimulation, or TGF-β neutralization inhibits angiotensin-II-induced apoptosis,[18] indicating that angiotensin-II-induced apoptosis is mediated by these two factors. Because, under normal conditions, epithelial cells including tubule cells are very resistant to apoptosis induced by Fas stimulation (due to Fas signaling uncoupling[25,26]), it might be speculated that TGF-β would directly or indirectly sensitize cells to Fas-mediated cell death, at least under a pathological environment. Interestingly, it has been shown that TGF-β, although lacking a direct effect, reinforces the apoptotic effect of staurosporine *in vitro*, through a Smad-independent and ERK-dependent mechanism.[20] Sensitization to apoptosis may be conceptually viewed as a mechanism by which TGF-β facilitates the death of injured cells under the influence of confusing signals. This might be the case of cells that have lost totally or partially cell–cell interactions and cell–ECM/basement membrane interactions. This might also explain why a mild pro-apoptotic effect has been assigned to TGF-β in some studies with cell cultures, where cell–cell and cell–ECM signaling might not replicate the *in vivo* environment for specific purposes, such as life/death decisions.

Finally, injured tubular cells, as well as immune system cells, are activated by TGF-β and other growth factors to produce inflammatory cytokines and unleash an inflammatory response in a nuclear factor-κB-dependent manner. In addition, TGF-β directly and indirectly stimulates monocyte and macrophage infiltration.[21] In turn, inflammation activates tubule cells, fibroblasts, and myofibroblasts to produce ECM, and amplifies fibrosis and tubular damage.[27,28] Inflammation (1) further activates renal cells to produce TGF-β and cytokines, which activate fibroblasts[29]; and (2) activates macrophages, which damage tubule cells,[30] probably through the production of pro-apoptotic molecules, such as TNF-α, reactive oxygen species, and NO. However, TGF-β is essential for the correct homeostasis of the immune system and its surveillance function.[21] Indeed, TGF-β1 knockout mice exhibit spontaneous multifocal inflammation and deregulated inflammatory responses.[31–32] However, it is likely that, in pathological circumstances, an imbalance between the opposing actions of TGF-β and other cytokines leads TGF-β's net effect from regulated repairing controlling the extent of the inflammatory response, toward deregulated, injuring inflammation.

TGF-β AND INTERSTITIAL FIBROSIS

Tubulointerstitial fibrosis is regarded as a central event in the progression of CKD regardless of etiology. Indeed, even in glomerulopathies, tubulointerstitial fibrosis correlates better than glomerular injury with evolution and prognosis.[33] TGF-β is widely recognized as a strong inducer of fibrosis in renal structures during CKD. Renal fibrosis is thought to be a primary pathological event leading to glomerular and tubular malfunction and degeneration, but also as a mediator of the scarring process that replaces death structures by chaotic ECM in the aftermath of a failed repair process. Tubulointerstitial fibrosis is the result of an increased

deposition of ECM, resulting from an increased production and an altered degradation of ECM components. The profibrotic effect of TGF-β results from a number of actions. Besides stimulating EMT and inflammation, TGF-β activates resident fibroblasts, myofibroblasts, and tubule epithelial cells to produce ECM components, and downregulates ECM degradation. As commented above, these actions are also part of a normal repair process. The transformation of normal production of ECM for basement membrane and tissue repair into a deleterious action is thought to be the consequence of (1) persistence of overstimulation and (2) imbalance of other homeostatic signalers such as other cytokines that counteract TGF-β's effects, such as HGF and BMP-7.[14,34]

A key issue is whether fibrosis, besides skewing cell function, also induces cell death to a significant extent. In other words, is fibrosis a primary inducer of epithelial cell deletion, or is it just the pathological resolution of a defective repair process in which death epithelial cells are not substituted but replaced by scar tissue, or both? It is well known that the normal ECM provides survival signaling to adjoining cells.[35] This is mediated by binding of ECM components, such as collagen IV and laminin to cell membrane integrins, such as β1 integrin. In contrast, fibrosis-associated collagen I and fibronectin fail to activate these survival signals.[36] In addition, when epithelial cells are in contact with an abnormal ECM, they undergo a specific form of cell death called anoikis.[35] It is also known that the ECM is a reservoir of a variety of growth factors including TGF-β. Thus, alterations in the composition or structure of ECM must influence tissue trophic status and homeostasis. This has been studied in glomerular cells, in which ECM alterations sensitize cells to apoptosis, as by serum deprivation, oxidative stress, or NO.[37] Furthermore, genetic ablation of genes related to ECM homeostasis, such as matrix metalloproteinase 9,[38] or tissue inhibitor of metalloproteinases 3,[39] has been shown to be circumstantially related to the modulation of apoptosis in kidney embryonic and mouse kidney, respectively. Besides, basement membrane supports cell differentiation and abolishes apoptosis of epithelial cells, whereas basement membrane degradation leads to apoptosis.[40] Accordingly, it is reasonable to think that fibrosis, basement membrane degradation, and a skewed signaling derived from them have a role in cell sensitization and apoptosis, contributing to an undetermined extent to tubule atrophy and degeneration. Fibrosis also leads to progressive renal tissue destruction through the disruption of the peritubular capillary network and the consequent reduction of renal blood flow and tissue oxygen levels. This, in turn, sensitizes tubule cells to cell death and induces the expression of hypoxia-inducible factor, which also promotes fibrosis in chronic situations.[6,41]

INTEGRATIVE VIEW AND PERSPECTIVES

Under pathological circumstances, TGF-β activation might be viewed as an element of a repair response[6] that, when

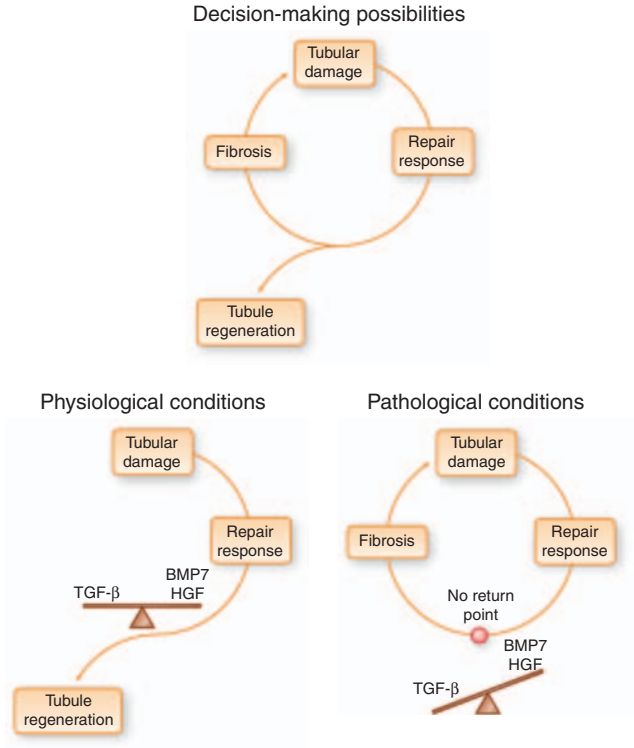

Figure 1 | Schematic representation of two outcomes of tubular injury, namely repair (physiological conditions) and progressive and irreversible degeneration (pathological conditions). Initially, common mechanisms are activated. At some point, the imbalance between the opposing effects of transforming growth factor-β (TGF-β) and of counterregulators, such as hepatocyte growth factor (HGF) and bone morphogenetic protein-7 (BMP-7), impedes critical events of tissue repair, breaks tissue homeostasis, and leads the process toward fibrosis, scarring, and progressive self-destruction.

distorted, gives way to damage progression, understood as an epiphenomenon of repair (Figure 1). Not in vain, this coincides with the critical localization and potential sentry function of pre-active TGF-β in the ECM and basement membranes. The pathological elements that turn the repair process into degeneration are only partially known. Persistence of injuring stimulation has been theoretically invoked to prevail over-repairing mechanisms, whose levels or actions would taper off along the time. Likely, the dominance of the damaging factors makes the renal parenchyma's homeostasis cross a no-return point and install a degenerative process in which progressive damage further accentuates the imbalance. In this scenario, TGF-β might be a passive member in the outcome, which mainly depends on the modulation of inflammatory and fibrotic responses by the relative level of other mediators. Yet, because of the absence of modulation, under pathological circumstances TGF-β turns into a deleterious member and a subject of therapeutic targeting.

As depicted in Figure 2, TGF-β participates in most of the mechanisms initially activated by tubular epithelium injury as a repair response. Surviving tubule cells become activated by (1) the insult itself, (2) death cell remains, (3) absence of appropriate cell–cell signaling, and (4) inflammatory

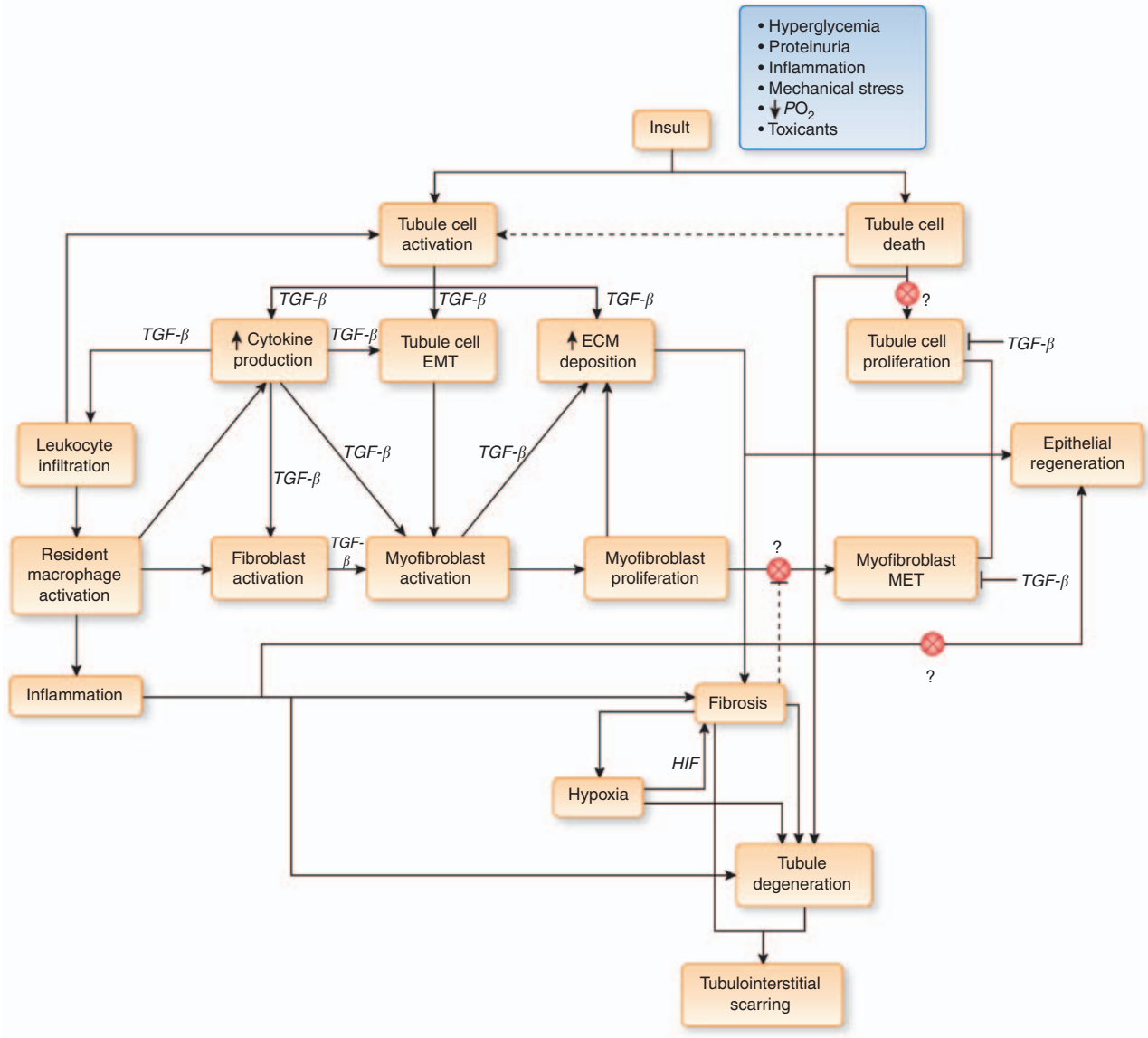

Figure 2 | Depiction of the interplay of tissue and cellular events related to tubular injury, repair, and distortion toward degeneration, in which transforming growth factor-β (TGF-β) is involved. ⊗ Pathways blocked in pathological circumstances, or superseded by them. ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; MET, mesenchymal-to-epithelial transition.

mediators arising from damaged tubule cells or activated resident macrophages (initially) and infiltrated cells (progressively). In this setting, TGF-β is increasingly produced and activated by all these cells, which participates in the inflammatory response and EMT. However, at due time, myofibroblasts should re-differentiate into tubule epithelial cells, which is not observed during progressive tubule degeneration in CKD. Also, after regenerating the intercellular scaffold and basement membranes, ECM deposition, cell activation, and inflammation should cease. However, these events are still observed in CKD (Figure 2).

Renal tissue repair is a tightly controlled process where the intensity and duration of every event must be precisely coordinated, so that cell proliferation, ECM production, and the inception and resolution of inflammation converge appropriately in time and location.[42] Consequently, malfunction of any of these events may send wrong information, wreaking havoc in tissue homeostasis. In this setting, TGF-β appears to have a central pathological role, as a consequence of the absence of appropriate modulation and timely counterbalance of its effects by antagonistic mediators, such as HGF and BMP-7, which converses TGF-β in a surrogate therapeutic target. TGF-β locks the mesenchymal phenotype after EMT-mediated tubule cell dedifferentiation, through the expression of snail, and prevents myofibroblast mesenchymal-to-epithelial transition to the tubule epithelial phenotype. In mice, BMP-7 counteracts this effect as well as the increased ECM deposition[14,34] and production of pro-inflammatory cytokines.[43] Moreover, administration of exogenous BMP-7 reverses moderate fibrosis in experimental

models of CKD. Under inappropriate modulation, the effects of TGF-β prevail, which lock inhibited mesenchymal-to-epithelial transition and tubule regeneration, and endorse deregulated fibrosis that further damages the remnant epithelium and scars unrepaired areas. Clearly, the next step in the immediate future is to translate the knowledge gained on the central role of TGF-β in CKD into therapeutic applications.

DISCLOSURE

All the authors declared no competing interests.

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