Epithelial to Mesenchymal Transition in Renal Fibrogenesis: Pathologic Significance, Molecular Mechanism, and Therapeutic Intervention

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Abstract. Mature tubular epithelial cells in adult kidney can undergo epithelial-to-mesenchymal transition (EMT), a phenotypic conversion that is fundamentally linked to the pathogenesis of renal interstitial fibrosis. Emerging evidence indicates that a large proportion of interstitial fibroblasts are actually originated from tubular epithelial cells via EMT in diseased kidney. Moreover, selective blockade of EMT in a mouse genetic model dramatically reduces fibrotic lesions after obstructive injury, underscoring a definite importance of EMT in renal fibrogenesis. Tubular EMT is proposed as an orchestrated, highly regulated process that consists of four key steps: (1) loss of epithelial cell adhesion; (2) de novo $\alpha$-smooth muscle actin expression and actin reorganization; (3) disruption of tubular basement membrane; and (4) enhanced cell migration and invasion. Of the many factors that regulate EMT in different ways, transforming growth factor-$\beta 1$ is the most potent inducer that is capable of initiating and completing the entire EMT course, whereas hepatocyte growth factor and bone morphogenetic protein-7 act as EMT inhibitors both in vitro and in vivo. Multiple intracellular signaling pathways have been implicated in mediating EMT, in which Smad/integrin-linked kinase may play a central role. This article attempts to provide a comprehensive review of recent advances on understanding the pathologic significance, molecular mechanism, and therapeutic intervention of EMT in the setting of chronic renal fibrosis.

The progression of chronic kidney disease (CKD) is considered to be an irreversible process that eventually leads to end-stage renal failure, a devastating condition that necessitates the patients to be dependent on life-long treatments with dialysis or renal transplantation (1–3). As population of the patients with chronic renal insufficiency is increasing at a rate of approximately 7% per year, the human and economic impact of CKD to the affected individuals and to medical community and society alike is enormous (3,4). Most patients with CKD are diagnosed well before they reach end-stage renal failure; however, no effective treatment can completely halt the progressive decline in renal functions (5,6).

The pathogenesis of CKD is characterized by progressive loss of kidney function and relentless accumulation and deposition of extracellular matrix (ECM), leading to widespread tissue fibrosis. Tubulointerstitial fibrosis is particularly interesting, because the deterioration of renal function is largely determined by the extent and severity of interstitial lesions in many forms of renal disease, both in animal models and in patients. Irrespective of the initial causes, interstitial fibrosis is a remarkably monotonous process characterized by de novo activation of $\alpha$-smooth muscle actin ($\alpha$SMA)–positive myofibroblasts, the principal effector cells that are responsible for the excess deposition of interstitial ECM under pathologic conditions (7–9). In this sense, a possible key to an effective therapy for CKD is to find a strategy that inhibits the activation of renal myofibroblasts in diseased kidney.

While the role of myofibroblasts in renal fibrosis is widely accepted, their origins and activation process in the fibrotic kidney remain largely undefined and controversial. In view of the location of myofibroblasts in vivo, they are often presumed to be derived from local activation of renal interstitial fibroblasts. Pioneering studies of Strutz et al. (10) indicate that tubular epithelial cells could express fibroblast markers in disease states, postulating a possibility of epithelial-to-mesenchymal transition (EMT).

Tubular EMT, by definition, is a process in which renal tubular cells lose their epithelial phenotype and acquire new characteristic features of mesenchyme. Obviously, this phenotypic conversion not only illustrates an incredible plasticity of the differentiated tubular epithelial cells after development; it is also fundamentally linked to generation of the matrix-producing fibroblasts under pathologic setting. It is of interest to point out that the majority of renal tubules in adult kidney except collecting duct are developmentally derived from the metanephric mesenchyme through mesenchymal to epithelial transdifferentiation (MET) (11). Thus EMT, in essence, is a...
process of reverse embryogenesis. In that regard, EMT underlines a conceptual link between the biology of embryonic development and cell dedifferentiation in many disease states.

Although EMT in renal fibrosis was originally postulated as a hypothesis (10,12), growing evidence has implicated this process as a major pathway leading to generation of interstitial myofibroblasts in diseased kidney. During the last several years, substantial progress has been made in providing evidence for the existence and significance of EMT in CKD, and several outstanding reviews and editorial comments have been published in this area (13–18). Therefore, the major focus of this article is to review the most recent advances in the last 2 yr in our understanding of EMT in CKD, with emphasis on its pathologic significance, molecular mechanism, and therapeutic intervention. It should be stressed that the phenomena of EMT is originally described and extensively studied in embryonic development and tumor metastasis. Detailed description of EMT in these areas is clearly beyond the scope and intent of this article; the interested reader is referred to several comprehensive reviews and the vast amount of literature on these topics (19–26).

Evidence for EMT in Renal Fibrosis

The presence of EMT in renal fibrosis was first demonstrated by Strutz et al. (10) in a landmark paper published almost a decade ago. Using the fibroblast-specific protein (Fsp1) as a marker, these authors showed that tubular epithelial cells could express Fsp1, a cytoskeleton-associated, calcium binding protein that is normally expressed in fibroblasts but not epithelia, in a mouse model of anti-tubular basement membrane (TBM) disease. They postulated an EMT, which could serve as a new avenue of generating fibroblasts in fibrotic kidney (10). Subsequent studies from Lan’s group (27) provide morphologic and phenotypic evidence for the existence of EMT in remnant kidney after 5/6 nephrectomy. De novo expression of αSMA as well as actin filaments was detected in tubular epithelia at 3 wk after nephrectomy. As disease progresses, tubular epithelial cells lost their apical-basal polarity and became elongated and migrated into the peritubular interstitium via the damaged TBM (27). In obstructive nephropathy induced by unilateral ureteral obstruction (UOO), we demonstrated that there were abundant cells co-expressing both αSMA and tubular marker, indicating that they are at transitional stage between epithelia and mesenchyme (28,29). These tubular epithelial cells lost epithelial cell marker E-cadherin and acquired mesenchymal features such as αSMA and vimentin and produce interstitial matrix components such as fibronectin and type I collagen (28–30). Active participation of EMT in anti-glomerular basement membrane (GBM) glomerulonephritis, diabetic nephropathy, and nephrotoxic serum nephritis has also been reported in animal models (31–34).

Relatively little is known on whether there is a specific tubular segment that is most susceptible to EMT. Although almost all in vitro studies employ proximal tubular epithelial cells as a model system, it would not be surprising to find out that distal tubular cells also undergo EMT after injurious stimulation. This is specifically authentic, in considering that both proximal and distal tubules are derived from the same embryonic, mesenchymal origin (11). On the other hand, evidence indicates that mouse renal collecting duct epithelial cell line mIMCD-3, which is developmentally originated from ureteric bud epithelium, cannot undergo phenotypic conversion after incubation with TGF-β1 (29). Such observation reminds us of that renal EMT may represent a process of reverse embryogenesis in which the tubular epithelial cells transform back into an embryonic mesenchymal phenotype from which they originate (18,29). Along this line, other renal epithelial cells that originated from the metanephric mesenchyme beyond tubular sites may be capable of committing to phenotypic conversion after injury as well. Indeed, studies showed that glomerular parietal epithelial cells (GPEC) underwent EMT in two rat models of CKD induced by either 5/6 nephrectomy or anti-GBM antibody (34). This glomerular EMT is believed to play a crucial role in the formation and evolution of glomerular crescents in CKD (34).

In accordance with animal studies, EMT is also observed in human renal biopsy tissues (35,36). About a decade ago, Nadasy et al. (37) reported that single cells or loosely organized small cell clusters still positive for epithelial markers could be found in the widened interstitium of human end-stage diseased kidney, consistent with the notion of EMT. Recent studies on human biopsy samples support that EMT has a role in progressive renal fibrosis in human, because tubular epithelial cells underwent phenotypic change as demonstrated by de novo αSMA expression and loss of cytokeratin (35). Rastaldi et al. (36) reported in a study with 133 human renal biopsies of different renal diseases that, independently of histologic diagnosis, EMT was clearly present in human samples with variable degrees. Most significantly, they demonstrated that the number of tubular epithelial cells with EMT features was associated with serum creatinine and the degree of interstitial damage, indicating that EMT participates in the fibrotic process in human (36).

Because EMT often occurs in areas with severe tubular damage, one concern that was raised over the years is that these αSMA-positive tubular cells may represent an infiltration of interstitial myofibroblasts. In an elegant study using sophisticated genetic approaches, Iwano et al. (30) have recently provided compelling evidence showing that interstitial fibroblasts could be derived from tubular epithelium after obstructive injury. By genetically tagging renal proximal tubules, these investigators could track down the fate and movement of the tagged cells with great certainty. They found that LacZ-tagged epithelia exhibited abnormal, degenerated morphology, became disorganized, and moved into the interstitium. Moreover, these transformed cells expressed fibroblast marker Fsp1 and HSP47, a collagen-specific chaperone protein that serve as an indicative marker of the cells producing type I collagen (30).

Dynamic Pattern of EMT in Fibrotic Kidney

EMT typically takes place at the late stage during the pathogenesis of renal fibrosis in diverse animal models. This suggests that tubular epithelial cells are reluctant to undergo phe-
not typic conversion under normal conditions, unless there is a sustained, chronic injury. Indeed, it takes 36 to 48 h to induce αSMA expression, a hallmark of myofibroblast, in tubular epithelial cells after continuous incubation with transforming growth factor (TGF)-β1 (28), whereas αSMA could be induced after only 8 h of TGF-β incubation in interstitial fibroblasts. Using obstructive nephropathy as a model, we recently studied the origins and dynamics of myofibroblast activation in vivo. As shown in Figure 1, αSMA induction displayed a biphasic pattern in the obstructed kidney. The early wave of αSMA induction took place in the period ranging from day 1 to day 3. The characteristic features of this early phase include: (1) that all αSMA-positive cells are localized in the interstitial compartments; (2) that αSMA induction is not associated with alteration of tubular epithelial cell phenotype, as epithelial cell adhesion molecule E-cadherin is intact; (3) that the magnitude of αSMA induction is relatively low. However, a robust αSMA induction followed at 7 d after surgery. The induction of αSMA at this late stage was closely associated with loss of epithelial marker E-cadherin expression (Figure 1A). Double immunofluorescence staining exhibited co-localization of αSMA and tubular lectin, suggesting cells at a transitional stage. As disease progresses, tubular marker largely disappeared in kidney section at 14 d after obstruction; instead, the area was repopulated by αSMA-positive myofibroblasts (38). These results suggest that myofibroblast activation in the obstructive kidney is a dynamic process, in which myofibroblasts mainly come from local activation of residential fibroblasts at the earlier stage (3 d), whereas they are predominantly derived from tubular epithelia via EMT at the later stage (7 d) in obstructive nephropathy. Hence, both interstitial fibroblasts and tubular epithelial cells contribute to the accumulation of myofibroblast cells at different phases with distinct dynamics.

Pathologic Significance of EMT in Renal Fibrogenesis

At this stage, perhaps there is little dispute regarding the existence of EMT in the fibrotic kidney, thanks to the beautiful demonstration using genetic tagging of renal tubules in whole-animal (30). However, the fundamental question remains as to the importance of EMT in renal fibrosis. In other words, is EMT essential for renal fibrogenesis?

At first glance, the relative contribution of EMT to renal fibrosis may be anything but important. This is because (1) the frequency of EMT in fibrotic kidney is low in most studies, (2) the late onset of EMT in diseased kidney suggests that it may not be a causative factor in renal fibrogenesis, and (3) EMT is only one of the many pathways leading to generation of myofibroblasts in diseased kidney; other sources of myofibroblasts certainly include interstitial fibroblasts, circulating precursor cells, and perhaps perivascular smooth muscle cells. However, two recent studies using genetic models provide indisputable evidence for a crucial role of EMT in renal fibrogenesis.

By quantitatively determining the contribution of EMT in the Fsp1-positive fibroblast pool in the fibrotic kidney induced by UUO, Iwano et al. (30) found that a large proportion of Fsp1-positive fibroblasts (36%) co-expressed LacZ that had been tagged to renal proximal tubules, suggesting their epithelial origin. This surprising finding underscores that the contribution of EMT pathway to the fibroblast pool and renal fibrosis is much greater than previously thought. Of note, the contribution of epithelial cells to fibroblast pool may still be underestimated in that study, because only cortical tubular epithelium was tagged and studied (30). Other tubular segments may also contribute to fibroblast generation via EMT.

The importance of EMT to renal fibrosis was also underlined through selective blockade of EMT pathway in a whole-animal model. This approach was once difficult to carry out in vivo, because one cannot separate the myofibroblasts derived from tubules via EMT versus those from fibroblasts in vivo. We recently found that mice lacking tissue-type plasminogen activator (tPA) were largely protected from development of interstitial fibrosis after obstructive injury (39). Further studies revealed that ablation of tPA selectively blocked tubular EMT, but did not affect myofibroblastic activation from interstitial fibroblasts. This is because lack of tPA caused a reduced matrix metalloproteinase-9 (MMP-9) induction in the obstructed kidneys of tPA−/− mice, which led to a dramatic preservation of the structural and functional integrity of TBM (39). The finding on selective blockade of EMT in tPA−/− mice after obstructive injury suggests a unique model system for understanding the role of EMT in the pathogenesis of renal fibrosis. Our results showed that in the absence of EMT, the progression of myofibroblast accumulation was not only blunted, but also reversed (39). This observation provides unambiguous attestation for a definite role of EMT in the pathogenesis of renal interstitial fibrosis in whole-animal.

Figure 1. Origin and dynamics of renal myofibroblast activation. (A and B) Western blot and graphical presentations demonstrate the time-course of α-smooth muscle actin (αSMA) induction and E-cadherin suppression in the obstructed kidney. A biphasic induction of αSMA was observed, in which the second wave of αSMA induction at day 7 was coincided with the loss of E-cadherin. (C through E) Kidney cryosections at 3 (C), 7 (D), and 14 (E) days after ureteral ligation were double-stained with αSMA (red) and tubular marker (lectin from T. purpureas, green). Arrowheads indicate the transitional cells co-expressing both αSMA and tubular marker (yellow). Epithelial-to-mesenchymal (EMT) was evident at day 7, but not at day 3 after surgery. At day 14, tubular marker largely disappeared, presumably due to EMT. This figure is composed from the published works (28,29,38,39) and used with permission.
The pathologic importance of EMT in renal fibrosis is also consistent with an active role of tubular epithelial cells in renal fibrogenesis, which has been increasingly recognized (40). Over the years, the primary focus of tubulointerstitial fibrosis studies is on interstitial fibroblasts and infiltrated mononuclear cells for the obvious reasons (1,18,41). However, fibroblast activation after injury, in essence, is a wound-healing response by which injured kidney attempts to repair and recover from the injury. Lessons learned from the tPA−/− mice have taught us that myofibroblast activation from fibroblasts alone, without participation of EMT, could not sustain and prevail (39). Therefore, fibroblast activation at most may be necessary but certainly not sufficient for development of a full scale of renal interstitial fibrosis. Likewise, the pathologic significance of mononuclear cell/macrophage recruitment and infiltration in fibrotic kidney is being challenged. Once this process was thought to promote renal fibrogenesis, several recent studies suggest that macrophage infiltration may actually be beneficial, at least at some stages during the disease progression (42,43).

The potential role of tubular epithelial cells in renal fibrosis is often concealed, partly because there was no direct connection between tubular cells and the production and deposition of ECM, a hallmark of interstitial fibrosis. However, molecular analyses of gene expression have constantly reminded us of a potential importance of tubular epithelia in fibrotic process. For instance, while it is well known that TGF-β1 expression is increased in almost all of the CKD models studied, the expression of TGF-β1 receptors, which determine the specificity of TGF-β action, are often upregulated predominantly in renal tubular epithelium (29), indicating that tubular epithelial cells are the in vivo natural targets of this pro-fibrotic cytokine. Hence, EMT helps to reconcile the disparity between molecular analysis and pathologic findings in fibrotic kidney.

There are possibly several reasons why EMT has been overlooked in the past studies. First, most studies using αSMA expression in tubular epithelial cells as an indicative marker for EMT. However, for some unknown reasons, αSMA is really not a reliable marker and its expression in tubular epithelial cells displays a tremendous heterogeneity (30,44). Even in a homogenous cell population that originated from a single cell clone, only a small fraction of cultured tubular cells (about 5 to 10%) typically express αSMA protein in response to TGF-β1 stimulation. Therefore, EMT may be greatly underestimated in diseased kidney in previous studies. Second, EMT is an extremely dynamic process that seems to occur in a wavelike fashion. This implies that the timing is an important issue in the detection of EMT. As shown in Figure 1, an examination of the obstructed kidney at 3 d (pre-EMT) or 14 d (post-EMT) after UUO would give rise to an incorrect impression about the prevalence of EMT in this model. Third, most EMT studies heavily rely on positive identification of the cells at transitional stage co-expressing both epithelial and mesenchymal markers at the same time. However, nobody knows exactly how long this transitional stage would keep on. The sudden loss of E-cadherin and immediate induction of αSMA within a short period of time from day 3 and day 7 in the obstructed kidney, as illustrated in Figure 1A, indicate that the transitional phase of EMT may be very short, making it difficult to identify those transition-in-action cells. Finally, our understanding on the fate of transformed cells after EMT is scant. Studies from our laboratory suggest that these cells may be more susceptible to death challenge than normal epithelial cells (45). It is therefore possible that these transformed cells would have a high turnover rate. This would also make EMT underappreciated.

### Key Cellular Events during EMT

Tubular epithelial cells and interstitial myofibroblasts dramatically differ in their morphology and phenotypes and are located in different tissue compartments that are separated by TBM within the kidney. One can envision that there would have to be alterations in the expression of many sets of genes to make this phenotypic conversion possible. Indeed, gene expression profiling using microarray technology has identified hundreds if not thousands of genes with altered expression patterns during tubular EMT (46–48). However, challenges remain to define the cause-effect relationship of these changes and to identify key events during EMT.

In view of that several obstacles have to be overcome to make EMT possible, we have recently proposed that tubular EMT at cellular level is likely a step-wise process involving four crucial events that eventually lead to the completion of the entire course (28). As illustrated in Figure 2, these four key events include: (1) loss of epithelial adhesion properties; (2) de novo expression of αSMA and actin reorganization; (3) disruption of tubular basement membrane; and (4) enhanced cell migration and invasion.

Tubular epithelial cells under normal conditions are tightly connected to each other to form an integrated epithelial sheet through various cell adhesion mechanisms. E-cadherin, the well-characterized adhesion receptor found within adherens-type junctions, plays an essential role in maintaining the structural integrity of renal epithelia and its polarization. One of the earliest changes in TGF-β1–induced EMT is the suppression of E-cadherin expression (28). The expression of ZO-1, a component of tight junction between epithelial cells, is also suppressed during EMT in numerous studies (33,49). Such alterations would consequently allow destabilization of the structural integrity of renal epithelium and makes cells to dissociate from their neighbors and lose polarity. E-cadherin is linked to the actin filament network by catenins, a family of intracellular adhesive junction proteins. The importance of E-cadherin for development of normal epithelium has been established by knockout of its gene in mice and by its role in embryonic epitheliogenesis during kidney development (11).

The reorganization of actin cytoskeleton, and induction of αSMA, may provide a structural foundation not only for defining the morphology of the transformed cells, but also for them to migrate, invade, and even acquire the capacity for contractility. Besides actin cytoskeleton, cytoplasmic intermediate filaments also undergo conversion from epithelial type of cytokeratin to mesenchymal vimentin (28,49,50).

Because tubular epithelial cells and myofibroblasts are separated by TBM in vivo, its disruption will be of fundamental importance in clearing the path for transformed cells to migrate.
toward interstitium. TBM also prevents the contacts between tubular epithelial cells and interstitial matrix components such as type I collagen, which has been shown to induce EMT (49,51). Induction of EMT in vitro and in vivo is accompanied with an increased expression of MMP-2 and MMP-9, which would specifically break down native type IV collagen and laminin, the principal proteins found in the TBM, thereby destructing TBM structural and functional integrity. In accordance with this, incubation of matrigel, a reconstituted TBM, with conditioned media from the transformed cells or tissue lysates from the diseased kidneys results in drastic destruction of its structural and functional integrity as evidenced by bacterial translocation assay (28,39).

The transformed cells have to finally enter into interstitial compartments. Therefore, it is essential for them to acquire the invasive capacity to eventually migrate into peritubular interstitium. The observation that the transformed cells are more motile suggests that the enhanced motility could readily allow them to migrate through the damaged TBM toward the interstitial compartment. The transformed cells could, in reality, combine the efforts of simultaneous destruction of TBM and migration. Such a notion is experimentally confirmed by matrigel invasion assay, in which transformed cells grown on top of matrigel have the ability to destroy and migrate through a reconstituted TBM matrix (28,32). Of note, myofibroblasts retain αSMA expression and potentially have the ability to contract. Thus contraction could potentially be another powerful force leading the transformed cells toward interstitium.

This model of EMT process is largely based on detailed studies of phenotypic alterations in cultured tubular epithelial cells after stimulation with TGF-β1 and in renal tubular epithelia after obstructive injury (28). Despite that, it only represents an initial attempt to understand the complex process of EMT and is certainly subjected to any modifications. Nevertheless, it appears clear that EMT is an orchestrated process that depends on many intricate interactions between extrinsic regulators and intracellular mediators.

**Extrinsic Regulators of EMT**

EMT is regulated by numerous growth factors, cytokines, hormones, and extracellular cues in different ways. Table 1 lists several known EMT regulators that are relevant to fibrotic process in CKD. Of the many factors identified, the chief one perhaps is profibrotic TGF-β1. TGF-β1, as a sole factor, initiates and completes the entire EMT course that consists of

Extrinsic Regulators of EMT

- TGF-β1 (28,52)
- HGF (29)
- EGF (49)
- BMP-7 (33)
- FGF-2 (50)
- IL-1 (52)
- Ang II (38)
- AGEs (31)
- CTGF (104)
- MMP-2 (53)
- Type I collagen (49)

Table 1. Extrinsic factors that regulate EMT

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<th>Factors that Induce and/or Promote EMT</th>
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<th>Factors that Suppress EMT</th>
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*AGE, advanced glycation end products; CTGF, connective tissue growth factor.*

Figure 2. Schematic illustration shows the key events during tubular EMT and therapeutic interventions. The diagram illustrates four key events essential for the completion of entire EMT course at cellular level, which include: (1) loss of epithelial adhesion properties; (2) de novo αSMA expression and actin reorganization; (3) disruption of tubular basement membrane (TBM); and (4) enhanced cell migration and invasion capacity. Transforming growth factor–β1 (TGF-β1) as a sole factor is capable of inducing tubular epithelial cells to undergo all four steps (28). Angiotensin II (AngII) promotes EMT by potentiating TGF-β1–initiated αSMA expression, although it fails to induce αSMA by itself (38). Strategies to block any steps during EMT would have major impact on EMT and thereby on renal fibrosis. For instances, HGF and BMP-7 could antagonize TGF-β1 and consequently inhibited the initiation of EMT (step 1) (29,33). Blockade of AngII by losartan abolished its activity as an EMT promoter and attenuated renal fibrosis (step 2) (38). Preservation of TBM integrity in tPA+/− mice selectively blocked EMT in obstructive nephropathy (step 3) (39). Finally, pharmacologic inhibition of ROCK kinase inhibited cell migration and reduced renal fibrosis (step 4) (101). The figure is modified from the published work (28) with permission.
four key steps (28,51). This extraordinary ability of TGF-β1 underscores that induction of EMT may be a major pathway of TGF-β1 that leads to interstitial fibrosis under pathologic conditions.

Many other factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF)-2 can induce EMT at least extent when they are individually exposed to cultured tubular epithelial cells. In addition, they exhibit a dramatic additive or synergistic effects with TGF-β1 to promote EMT (49,50). Some factors may regulate EMT through modulation of TGF-β1 expression or activity. For instance, interleukin-1 (IL-1) could induce tubular epithelial cells to undergo EMT (52). Such action of IL-1 was abolished by incubating with anti-TGF-β1 neutralizing antibody. A recent report suggests that MMP-2 is both necessary and sufficient for induction of EMT (53), and this action of MMP-2 is thought to be related to its ability to activate TGF-β1 activity. Thus TGF-β1 may function as a common downstream effector that mediates action of other factors to induce EMT.

Studies from our laboratory demonstrate that angiotensin II (AngII), a central component of renin-angiotensin system (RAS), acts as a strong promoter that dramatically potentiates the ability of TGF-β1 to induce EMT in tubular epithelial cells (38). AngII by itself at a concentration as high as 10^{-6} M failed to induce EMT, suggesting that it is not an EMT inducer that initiates the conversion process. However, it markedly promoted TGF-β1–mediated EMT when tubular epithelial cells were incubated with both of them simultaneously. Hence, AngII represents a new class of EMT regulators that merely potentiates the action of other EMT inducers.

Besides soluble factors, other extracellular cues such as collagen may play an important role in regulating EMT. It is reported that type I collagen promoted EMT (49,51), while type IV collagen suppressed it (54). Because type I and type IV collagen are major components of interstitial matrix and TBM, respectively, this suggests that microenvironmental cues are instrumental in conferring phenotypic conversion. It is also shown that TBM integrity is one of the determinant factors for EMT in vivo. Disruption of TBM composition and integrity induces EMT in vitro (54). Preservation of TBM structure and function in tPA−/− mice selectively blocks EMT in obstructive nephropathy (39).

There are endogenous regulators that negatively modulate EMT. HGF is a potent inhibitor of EMT that can dramatically block the phenotypic conversion of tubular epithelial cells induced by TGF-β1 (29). Likewise, recent studies indicate that bone morphogenetic protein (BMP)-7 also suppresses tubular EMT both in vitro and in vivo (33).

It is worthwhile to point out that most EMT regulators are identified by using in vitro cell culture system in which tubular epithelial cells are bombarded with purified factors at high concentrations. This approach is necessary to define the potential role of each individual factor in regulating EMT; however, it may not imitate the real situation in vivo. In reality, tubular cells are more likely to be exposed to a cocktail of many factors in which each individual factor is present at low concentration. Hence, an EMT in vivo may result from an integration of diverse signals triggered by multiple players.

### Intracellular Signal Pathways that Mediate EMT

Our current understanding of the molecular mechanism underlying EMT is incomplete. Multiple intracellular signal transduction pathways have been implicated in mediating EMT in different model systems (22,24,26,55–58). Several general principles should be taken into consideration when we discuss the potential mechanism of EMT. First, the molecular mechanism of EMT may be extrinsic regulators-dependent. There are diverse types of EMT regulators (Table 1); each of them may be unique in conferring its regulation of EMT. It would not be surprising to find out that the intracellular signal pathways of TGF-β1–initiated EMT may be quite different from that induced by FGF-2 and EGF. Second, the signal pathways leading to EMT could be cell content–specific. For instance, it is well known that E-cadherin suppression during carcinoma cell mesenchymal conversion is primarily mediated by Snail transcription factor (25,59,60), whereas its suppression in renal tubular epithelial cells during TGF-β1–induced EMT is clearly Snail-independent (32). Third, EMT is a dynamic, complicated process that may require the participation and integration of multiple signal pathways at different stages (25,26). Consistent with this, EMT typically requires persistent incubation with the inducer for a long duration ranging from 2 to 5 d (28,51). It is conceivable that many pathways may be required, but few of them alone are truly sufficient for completing the entire EMT course. Finally, a single mediator may be involved in multiple cellular events in EMT; conversely, multiple mediators could be required for the regulation of a single cellular process (such as cell migration).

In this article, we mainly focused our discussion of the intracellular signal transduction on TGF-β1–induced EMT in the setting of chronic renal fibrosis. TGF-β1 signals are transduced by transmembrane serine/threonine kinase type I and type II receptors and intracellular mediators known as Smads (55,61,62). Upon TGF-β1 stimulation, Smad-2 and -3 are phosphorylated at serine residues in the carboxyl termini by the type I receptor (63). Such phosphorylation of Smad-2/3 induces their association with common partner Smad-4 and subsequently translocates into the nuclei, where they control the transcription of TGF-β1–responsive genes (55,62). Besides Smad signaling, a comprehensive survey indicates that TGF-β1 is capable of activating several other signal transduction pathways in tubular epithelial cells as well, such as p38 mitogen-activated protein kinase (MAPK), Akt/protein kinase B (PKB), RhoA, and β-catenin (Figure 3) (32,45). However, TGF-β1–induced EMT appears to be primarily dependent on an intact Smad signaling, because overexpression of inhibitory Smad-7 abolishes Smad-2 phosphorylation and tubular cell phenotypic conversion (16,64).

What are the downstream effectors of Smad signaling that mediate EMT is a fascinating question. We recently reported that integrin-linked kinase (ILK) is a potential downstream effector of TGF-β1/Smad that is crucial for EMT (32). ILK is an intracellular serine/threonine protein kinase that interacts
with the cytoplasmic domains of β-integrins and numerous cytoskeleton-associated proteins. ILK has been involved in the regulation of a number of integrin-mediated processes that include cell adhesion, cell shape changes, gene expression, and extracellular matrix deposition. The implication of ILK as a critical mediator for EMT is supported by several lines of observation (32). First, endogenous ILK expression in tubular epithelial cells is induced by TGF-β1, and this induction is dependent on intact Smad signaling. Second, forced expression of exogenous ILK induces numerous key events including loss of epithelial E-cadherin, induction of fibronectin expression and its extracellular assembly, induced MMP-2 expression and secretion, and enhanced cell migration and invasion. This virtually recapitulates the major events during the entire course of tubular EMT induced by TGF-β1 (Figures 2 and 3). Third, ectopic expression of a dominant-negative, kinase-dead form of ILK largely abrogates TGF-β1–initiated EMT, suggesting that ILK signaling is necessary for mediating the action of TGF-β1. Fourth, ILK induction is specifically confined to the basal region of renal tubular epithelium and coincides with tubular EMT in two models of chronic renal fibrosis induced by either obstructive insult or diabetic injury in mice, indicating a spatial and temporal association between ILK and tubular EMT in vivo. Finally, inhibition of ILK induction by HGF blocks TGF-β1–initiated tubular EMT in vitro and attenuates renal interstitial fibrosis in vivo. The role of ILK in mediating EMT was also confirmed in two murine tubular epithelial cell lines after stable transfection (65). Therefore, a linear pathway appears to exist that couples TGF-β1, Smad signaling, ILK, and tubular EMT.

Through multiple interactions by using distinct domains, ILK strategically bridges the integrins and actin cytoskeleton-associated proteins, including PINCH, CH-ILKBP, and paxillin, and transmits signal exchanges between the intracellular and extracellular compartments (Figure 3) (58,66,67). Furthermore, ILK also couples integrins and growth factor receptors to downstream signaling components. It has been shown that activated ILK can directly phosphorylate Akt and glycogen synthase kinase (GSK)-3 (58,68). Phosphorylation of GSK-3 results in its inhibition, leading to stabilization of β-catenin and stimulation of the activity of AP-1 and CREB transcription factors (72). Activation of β-catenin and Akt may lead to induction of EMT (71,73). AP-1 activation by ILK leads to stimulation of matrix metalloproteinase-9 (MMP-9) expression (74). ILK also directly phosphorylates MLC (75), thereby regulating cell motility, contractility, and invasion. Thus ILK is involved, either directly or indirectly, in each and every major step of EMT (32). See text for details.

Figure 3. Simplified model depicts the major intracellular signal transduction pathways that mediate TGF-β1–induced EMT. TGF-β1 activates multiple signal transduction pathways in tubular epithelial cells, including Smad, RhoA, and p38 mitogen-activated protein kinase (MAPK). While p38 MAPK is primarily implicated in TGF-β–induced apoptosis (45), RhoA and its downstream effector kinase ROCK are involved in some aspects of EMT, such as αSMA promoter activation, actin reorganization, and cell migration (57,101). Smad and its downstream effector integrin-linked kinase (ILK) are believed to play a central role in mediating TGF-β1–triggered EMT (32,64). Activated ILK can directly phosphorylate Akt and GSK-3 (58,68). Phosphorylation of GSK-3 results in its inhibition, leading to stabilization of β-catenin and stimulation of the activity of AP-1 and CREB transcription factors (72). Activation of β-catenin and Akt may lead to induction of EMT (71,73). AP-1 activation by ILK leads to stimulation of matrix metalloproteinase-9 (MMP-9) expression (74). ILK also directly phosphorylates MLC (75), thereby regulating cell motility, contractility, and invasion. Thus ILK is involved, either directly or indirectly, in each and every major step of EMT (32). See text for details.
RhoA, a small GTPase that has been implicated in mediating EMT (76,77), and its downstream effector kinase p160ROCK are also activated in tubular epithelial cells after TGF-β1 incubation (57). TGF-β1–induced RhoA activation in tubular epithelial cells is dependent on neither Smad signaling nor ILK (Li et al., unpublished data), suggesting that Smad/ILK and RhoA/ROCK are two parallel signaling pathways initiated by TGF-β1. RhoA is only important in TGF-β–induced stress fiber assembly and cytoskeleton remodeling but not in the disruption of adherens and tight junctions (77). It is therefore plausible to assume that ILK acts as a major intermediate signaling molecule that couples TGF-β1/Smad signaling and tubular EMT.

Besides the signaling mediators described above, other pathways and transcription factors have been implicated in EMT, including Erk-1/2, Snail/Slug, Wnt, and Notch signaling (25,26,55,56,78,79). However, Erk-1/2 are not activated in tubular epithelial (HKC) cells after TGF-β1 stimulation (45), and Snail are not expressed in these cells at any conditions tested (32), suggesting that they are probably not involved in TGF-β1–induced tubular EMT. The contribution of Wnt and Notch signaling in tubular EMT remains largely unknown; however, β-catenin and Jagged-1, the respective components of Wnt and Notch signaling pathways, are activated in TGF-β1–treated tubular epithelial cells and in fibrotic kidney (78).

**Fate of the Transformed Cells after EMT**

Little is known about the final destination of the cells after EMT. Several distinctive fates of the transformed cells can be envisioned, which may include reverting to tubular epithelial cells if the injury is transient; proliferating to expand fibroblast population; committing to suicide by apoptosis; or even re-differentiating into renewed epithelial cells if being exposed to regenerative cues.

EMT is reversible, at least in the early stage. In vitro studies indicate that EMT is dependent on the continuous exposure of tubular epithelial cells to inducer. Acquisition of most mesenchymal markers such as αSMA takes a long time; and the phenotypic conversion is not sustainable if the inducer is removed within a certain period of time (such as 2 d). Gene expression microarray analysis shows that about 90% of gene expression alterations occur after 48 h of incubation with TGF-β1 (47). Therefore, there is probably no such thing as a so-called “master gene” whose alteration would lead to autonomously progressed EMT processes, even though the original inducer is no longer present. We envision that EMT may be one reluctant biological choice that tubular epithelial cells have to take when they face sustained, chronic injury. An alternative option for them may be to die by apoptosis. Along this line, if injury is removed, they would contentedly revert to the original phenotype of epithelia with little hesitation.

Sustained injury would leave the transformed cells with no option to return. These transformed cells certainly possess the ability to proliferate, as demonstrated by the observation that 42% of the proliferating Fsp+ fibroblasts carried the EMT marker in a recent study (30). Such active proliferation would undoubtedly lead to an expansion of matrix-producing fibroblasts in the interstitium of diseased kidney. Therefore, a significant proportion of the interstitial fibroblast proliferation observed in earlier studies (80) is likely contributed by the transformed cells after EMT. On the other hand, evidence suggests that the transformed cells may be more susceptible than normal epithelial cells to commit suicide. It has been reported that TGF-β1 either potentiates or induces tubular epithelial cells to die (45,81). Hence, the transformed cell population may have a high turnover rate with increased proliferation and apoptosis, a common phenomenon seen in diseased kidney.

Possibility also exists that completely transformed cells may undergo redifferentiation to generate renewed epithelial cells if they are exposed to a proper regenerative cue. For instance, HGF could reverse the phenotypic conversion in the tubular epithelial cells that had been previously transformed via EMT (82). This suggests that HGF, a potent regenerative factor, may re-induce a mesenchymal to epithelial transition (MET) that normally occurs during early nephrogenesis. Similar results by using BMP-7 are also reported (33). In support of this view, we recently found that incubation of renal interstitial fibroblasts with HGF could induce E-cadherin expression and a mesenchymal-to-epithelial transition (Yang et al., unpublished data), consistent with a role of HGF in early kidney development (83). Therefore, transformed epithelial cells, even interstitial fibroblasts, can be induced to redifferentiate into renewed epithelial cells, a process that recapitulates embryonic kidney developmental program.

**Therapeutic Intervention of EMT**

To clinical nephrologists, perhaps the most significant recent advance in EMT studies is the identification of endogenous factors that can suppress this phenotypic conversion, thereby suggesting a possible therapeutic intervention of EMT. Two endogenous factors, namely HGF and BMP-7, have been demonstrated to be potent inhibitors that effectively block EMT both in vitro and in vivo (29,33). Retrospectively, these findings are not surprising, because both HGF and BMP-7 play an important role in early kidney development and in the maintenance of tubular epithelial cell phenotypes in adult kidney (84–86). Hence, a new concept is emerging that there are intrinsic renoprotective factors that presumably safeguard tubular epithelial phenotypes by preventing EMT in vivo.

A role for HGF in regulating EMT was first hinted in a HGF neutralization study (87). In rat remnant kidney model, blocking of HGF signaling with neutralizing antibody markedly induced αSMA expression in renal tubules, an indication of phenotypic conversion (87). This suggests that endogenous HGF is crucial in preserving tubular epithelial cell phenotypes. Subsequent in vitro studies confirm that HGF possesses a remarkable ability to block tubular EMT induced by TGF-β1. HGF virtually reversed all phenotypic conversion triggered by TGF-β1 and restored epithelial E-cadherin and suppressed mesenchymal markers such as αSMA, vimentin, and fibronectin (29). Consistently, administration of HGF protein or its gene effectively blocked EMT in vivo and attenuated renal interstitial fibrosis in obstructive nephropathy (29,88), even
when the injury was already established (82). Other groups also demonstrate that HGF is capable of ameliorating renal fibrotic lesions in a variety of models (89–92).

We have recently elucidated the mechanism underlying HGF blockade of EMT induced by TGF-β1 (93). HGF blocks EMT apparently by a mechanism independent of a modulation of TGF-β1 expression. In addition, HGF does not affect TGF-β1–induced Smad-2 phosphorylation or its association with Smad-4 and subsequent nuclear translocation, and it does not influence the expression of inhibitory Smad-7 in tubular epithelial cells. Instead, HGF specifically induces Smad transcriptional corepressor SnoN expression, which in turn physically interacts with activated Smad-2 by forming transcriptionally inactive complex and blocks the trans-activation of Smad-mediated genes, including EMT mediator ILK (32). Of note, Smad transcriptional corepressors SnoN and Ski are markedly downregulated in fibrotic kidney (94). By inducing SnoN expression and restoring its level in vivo, HGF reinstates SnoN transcriptional repressor activity, leading to blockade of tubular EMT and renal fibrosis. Hence, these studies provide a mechanistic insight into understanding the interplay between anti-fibrotic HGF and profibrotic TGF-β1 signaling.

BMP-7 is a member of TGF-β superfamily that can antagonize TGF-β1’s action (95,96); and its expression is reduced in the fibrotic kidney (97). Supplementation of exogenous BMP-7 has been proven to be beneficial in attenuating progressive loss of kidney function and renal fibrosis (33,98–100). Recent studies indicate that BMP-7 could reverse an established renal fibrosis in mice primarily through counteracting TGF-β1–mediated EMT (33).

Intervention of EMT could also help to re-interpret the beneficial effect of the conventional therapies. For instance, pharmacologic inhibition of the activities of AngII has been proven to be effective in the treatments of the patients with chronic renal insufficiency (5,6). The beneficial effects of AngII inhibition are often interpreted by improving systemic and renal hemodynamics. However, because AngII is potent EMT promoter (38), its inhibition would also attenuate EMT, leading to a reduction of renal fibrosis (38). A recent report indicates that administration of ROCK inhibitor, Y-27632, prevents tubulointerstitial fibrosis in mouse model of obstructive nephropathy (101). ROCK inhibition suppressed αSMA expression, cell migration, and interstitial fibrosis in vivo. Because ROCK is the downstream effector kinase of RhoA that plays a critical role in stress fiber assembly and cytoskeleton remodeling during EMT (57), the beneficial effects of ROCK inhibition could potentially be interpreted as an effective attenuation of EMT in diseased kidney.

New therapeutic strategy of renal fibrosis could be developed from the insights of EMT studies. For example, in view of the fact that inhibition of ILK expression by HGF blocks tubular EMT and reduces renal fibrosis, ILK signaling could be exploited as a novel therapeutic target for designing new treatment regimens. It is plausible that new strategies aimed at ILK expression and signaling may be effective for intervening EMT, thereby halting the onset and progression of chronic renal fibrosis. With the availability of small molecule ILK inhibitors (102), it would be interesting to test whether ILK inhibition can block EMT and mitigate fibrotic lesions in vivo.

The fact that EMT is a complicated process with several key steps (Figure 2) provides us a wide window of opportunities for therapeutic intervention. Strategies to disrupt any one of these key steps would potentially have major impact on EMT and renal fibrosis (Figure 2). Recent successful stories on blockade of EMT with HGF (29), BMP-7 (33), ROCK inhibitor (101), and AngII blocker (38), as well as with preservation of TBM in PA–/– mice (39) and ILK inhibition (32), reinforce this notion. Furthermore, a combination of two or more therapeutic strategies aimed at different events during EMT may even be more effective, as recently demonstrated by a combined HGF and AngII blockade therapy (38) and combined anti-TGF-β1 and ACEI therapy (103). Therefore, targeting EMT either by supplementation of endogenous EMT inhibitors (such as HGF or BMP-7) or by specifically disrupting key EMT events (such as by using small molecule inhibitors) would lead to suppression of EMT and ultimate amelioration of the progressive loss of renal function in diseased kidney.

Conclusion

Over the past two years, we have witnessed remarkable advances in our understanding of tubular EMT in the pathogenesis of renal interstitial fibrosis. Initially postulated as a hypothesis, the concept of EMT has revolutionized our comprehension on the biology of renal fibrogenesis. The existence of EMT in fibrotic kidney is increasingly recognized. Studies with genetic models have unambiguously illustrated a definite importance of EMT in progressive renal fibrosis. Although it was largely overlooked in the past, EMT is emerging as a major pathway leading to generation of the matrix-producing effector cells in diseased kidney.

The biology behind EMT is utterly fascinating to many of us. In this respect, we have made substantial progress in dissecting key cellular events during EMT, in identifying its extrinsic regulators, and in elucidating its intracellular signal transduction pathways. Perhaps more importantly, the insights from these studies have evoked novel strategies for therapeutic intervention of EMT and renal fibrosis. Recent success by targeting EMT for blocking renal fibrotic lesions has generated a lot of excitement in this field. It is hoped that better understanding of EMT through intensive investigations will ultimately translate into more effective therapies for the patients with chronic renal insufficiency, a devastating disorder that is otherwise incurable.

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