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Fibroblasts emerge via epithelial-mesenchymal transition in chronic kidney fibrosis

Michael Zeisberg, Raghu Kalluri

Division of Matrix Biology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215

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1. ABSTRACT

Our current understanding of epithelial-mesenchymal transition (EMT) in the setting of tissue fibrosis is largely based on pioneering studies in the kidney. Evidence is emerging that EMT is a key component of chronic kidney disease, contributing to both destruction of the tubular epithelial compartment and accumulation of interstitial fibroblasts. While knowledge regarding EMT was previously based on experimental rodent studies, in recent years the evolving evidence demonstrates a role for EMT in human kidney diseases with chronic fibrosis.

2. INTRODUCTION

While in the embryo epithelia give rise to mesenchyme via an epithelial-mesenchymal transition (EMT) at specific times and places, the epithelial and mesenchymal phenotypes in adult tissue were long believed to be stable (1). The concept that adult epithelial cells may undergo EMT under pathological conditions, originates from studies by Greenburg and Hay in which primary adult lens epithelium explants gave rise to migrating mesenchymal cells when cultured on type I collagen gels (2). Madine-Darby canine kidney (MDCK) epithelial cells behaved similarly when cultured on type I collagen gels, giving rise to fusiform-shaped cells that lost cell apical basal polarity, develop a typical actin cytoskeleton and display migratory activity (3). Such EMT was not considered a completed process, as the spindle-shaped cells did not lose keratin and laminin expression. The acquired mesenchymal phenotype however was relatively stable and cells did not revert back to their epithelial phenotype when they were removed from the type I collagen gel and cultured on their regular basement membrane matrix (4). Such initial studies led to many questions such as: 1) how much of a phenotypic change is required to classify as "EMT"?; 2) how stable should these changes be to justify

the term "EMT" ?; and 3) how closely should the epithelial cell culture mimic the *in vivo* epithelial cell phenotype?

3. EMT INVOLVING RENAL TUBULAR EPITHELIAL CELLS *IN VITRO*

Current *in vitro* systems to study EMT involving renal tubular epithelial cell commonly utilize tubular epithelial cell lines that are cultured on plastic. These cell lines (commonly used cell lines include murine MCT and NP1 cell line, human HK-2 cells and canine MDCK cells) appear as cobble stone-like monolayers *in vitro* and form tight junctions (3; 5; 6). In these systems, EMT is generally defined as the acquisition of a spindle-shaped morphology, associated with a decrease of characteristic epithelial marker genes (i.e. e-cadherin and ZO-1, see table 1) and *de novo* expression of typical mesenchymal markers (i.e. FSP1 and αSMA) associated with increased cell motility. Additional common features associated with EMT include increased resistance to pro-apoptotic stimuli and decreased proliferation as compared to activated epithelial cells.

As of now, it is not entirely clear if such *in vitro* systems lend themselves to study the complete conversion of epithelia to fibroblasts. Time course EMT studies in cell culture systems corroborate that EMT has to be considered as an active ongoing continuous process associated with various intermediate stages in which the cells display features of both epithelial cells as well as of fibroblasts – and it is likely that such intermediate stages are just as relevant as the complete conversion into fibroblasts in the fibrosis setting.

4. EVIDENCE FOR CONTRIBUTION OF EMT TO FIBROSIS IN EXPERIMENTAL MODELS OF CHRONIC KIDNEY DISEASE

Demonstration that EMT occurs *in vivo* in association with kidney fibrosis was only made possible when markers to distinguish between epithelial cells and mesenchymal cells became available. In this regard, fibroblast-specific protein1 (FSP1) was identified by subtractive hybridization comparing renal fibroblasts and tubular epithelial cells (7). Using antibodies specific to FSP1, Strutz and co-workers demonstrated positive FSP1-staining in tubular epithelial cells in a mouse model of anti-tubular basement membrane (TBM) nephritis (7). Using this approach of identifying EMT by means of immunostaining for mesenchymal markers in tubular epithelial cells (FSP1 or αSMA) evidence for EMT has been demonstrated in various mouse models of renal fibrosis, including the mouse model of unilateral-urethral obstruction (UUO) (8), the mouse model of nephrotoxic serum nephritis (NTN) (9), a mouse model of chronic allograft nephropathy (10; 11) and the remnant kidney model of 5/6 nephrectomy (12). EMT predominantly affects proximal and distal tubular epithelial cells (which derive during embryonic development via a mesenchymal-to-epithelial transition) but can also occur collecting ducts (13). While evidence for early stages of EMT has been demonstrated in the most prevalent mouse models of renal fibrosis. the prevalence of such early EMT and its contribution to fibrogenesis has not been systematically investigated as yet. The matter is further complicated as different markers have been employed in these studies (most studies used either antibodies to fibroblast specific protein1 (FSP1) or α-smooth muscle actin (αSMA) to detect EMT. Overall, it appears that the UUO model is particularly prone to EMT, while EMT is less abundant in mouse models of NTN, CAN and the remnant kidney (9-12). In this regard, it appears that EMT is confined to renal fibrosis, whereas it does not occur in mouse models of acute kidney injury (unpublished observation).

Evidence for EMT associated with renal fibrosis was corroborated by electron microscopy studies which demonstrated acquisition of mesenchymal actin bundles, preceding detachment of cells undergoing EMT from the tubular basement membrane and their neighboring cells and which documented acquisition of a mesenchymal phenotype associated with disruption of TBM in later stages of EMT (12) (Figure 1). Evidence that epithelial cells indeed contribute to interstitial fibroblasts via EMT was made possible by the availability of transgenic reporter mice. Iwano and co-workers performed UUO in *GGT-Cre;R26Rosa-STOP-LacZ* double-transgenic mice (8). In these mice GGT-cre$^+$ tubular epithelial cells are irreversibly tagged by LacZ expression when the STOP cassette that separates the R26Rosa promoter from the LacZ reporter gene is removed, irrespective of its phenotype. In this study Iwano and co-workers demonstrated that up to 36% of interstitial FSP1$^+$ fibroblasts were *LacZ*$^+$, demonstrating their epithelial origin.

5. EVIDENCE FOR CONTRIBUTION OF EMT TO PROGRESSION OF CHRONIC KIDNEY DISEASE IN HUMANS

The contribution of EMT to fibrosis in human patients is still debated. One issue that has not been resolved in human patients is lineage tracing of epithelial cells that have become fibroblasts, as was done in *GGT-Cre;R26Rosa-STOP-LacZ reporter mice*. Hence, analysis of EMT in patient biopsies is limited to early stages of EMT in which cells are still within the tubular compartment and co-express epithelial cell and mesenchymal markers. Despite these limitations, evidence is accumulating that EMT contributes to fibrogenesis in patients.

S100A4 (human FSP1 analog) and/or αSMA expression by tubular epithelial cells is a common feature of tubulointerstitial fibrosis associated with various underlying diseases in human kidney biopsies, including IgA Nephropathy (14; 15), Lupus Nephritis (15), Diabetic Nephropathy (16) and Chronic Allograft Nephropathy (17). Vongwiwatana and co-workers demonstrated that incidence of EMT (identified by S100A4 and HSP47 staining associated with decreased cytokeratin staining) correlated with increased serum creatinine levels in patients with renal allografts (18). Similarly Hertig and co-workers reported that EMT (identified by cytoplasmic translocation of beta-catenin and vimentin staining) was a regular feature of chronic allograft nephropathy (17). Interestingly, such early

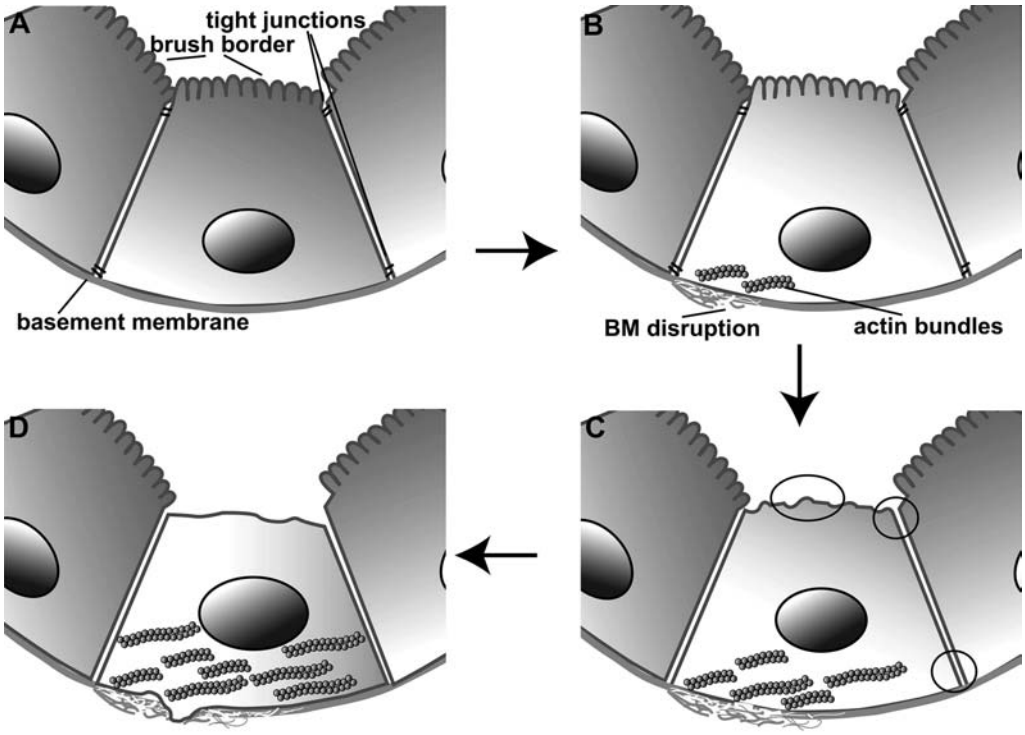

Figure 1. Early stages of EMT. The schematic illustration summarizes the sequence of ultrastructural changes which are associated with early stages of EMT involving tubular epithelial cells in chronic kidney disease. a. In the normal kidney tubular epithelial cells display an apical-basal polarity with a brush border facing the apical lumen. The basal side is tightly connected with the underlying tubular basement membrane. b. Initial stages of EMT are associated with formation of actin bundles which are localized along discrete lesions of the tubular basement membrane. Brush border and tight junctions are initially intact. c. While actin bundles become more abundant, the brush border at the apical side is decreasing and tight junctions are dissembled.d. Brush border is lost in cells. At the basal sides protrusions can be detected in areas of disrupted tubular basement membrane.

stages of EMT correlates with impairment of excretory renal function, suggesting that EMT in human disease is relevant (17).

6. MEDIATORS OF EMT

The list of molecules that can induce EMT involving tubular epithelial cells is constantly growing. Such mediators of EMT include growth factors, altered cell-cell interactions, cell-ECM interactions and environmental factors such as hypoxia, reactive oxygen species, cyclosporine A or hyperglycemia (19; 20; 21; 22). Among the numerous stimuli that can induce EMT in cell culture, we focus here on the most prominent mediators of EMT in the renal fibrosis setting (TGF-β1, proteases that degrade basement membrane, integrin-linked kinase and hypoxia), which have been validated in genetic mouse models. Of the various known signaling pathways that mediate biological activity of these stimuli, we will highlight pathways that have been implicated to mediate EMT in the renal fibrosis setting.

6.1. Transforming growth factor-β

Transforming growth factor-β1 (TGF-β1) is the prototypic mediator of EMT. TGF-β1 is a major mediator of renal fibrosis (23). Various studies demonstrated the efficacy of TGF-β1 to consistently induce EMT of renal tubular epithelial cells *in vitro* and *in vivo* (24; 25; 26). TGF-β1 in general signals through an activated heteromeric complex of a type I (ALK5) and type II (TGFR-II) serine/threonine kinase receptors (Figure 2A) (27). Subsequent cytoplasmatic signaling is mediated by the phosphorylation of ligand-specific Smad proteins (Smad2 and/or Smad3) which translocate to the nucleus in association with the common Smad4 (28). The Smad pathway interferes with various molecules that modulate Smad signaling (28). Enhancers of Smad signaling include RhoA, Rac1 and Jun N-Terminal kinase (JNK) and inhibitors of TGF-β1 dependent-Smad signaling include Smad7, RhoB and calmodulin (28). In addition to the Smad pathway, TGF-β1 elicits transcriptional control via the mitogen-activated protein kinase family (MAPK) and potentiation of the phosphotidylinositol 3-kinase (PI3-kinase) activity (28).

Which of these distinct signaling pathways mediate EMT of renal tubular dependent on Smad3 (26; 29)? Smad3-dependent signaling can be inhibited by Smad7 and also by BMP7-dependent Smad signaling involving Smad1 and Smad5 (9; 30). RhoA enhances TGF-β1-dependent EMT in kidney epithelial cells (31; 32). Effectiveness of TGF-β1 to induce EMT in tubular

epithelial cells is enhanced by epithelial growth factor (EGF) (24). Such synergism is mediated by increased PI3-kinase activity (33).

6.2. Proteases

EMT involving tubular epithelial cells is associated with disruption of the underlying tubular basement membrane (TBM) (12; 25; 34). This observation has led to the concept that proteases, which facilitate degradation of TBM, and which are present in the chronically injured kidney, facilitate EMT (34; 35). Of all the matrix metalloproteinases (MMPs), specifically MMP2 plays a prominent role in the EMT of tubular epithelial cells (36). TGF-β1-induced EMT is associated with increased expression of MMP2 (also known as gelatinase A) (36). MMP2 degrades type IV collagen in basement membranes and also activates latent TGF-β1, further enhancing EMT (36). Overexpression of MMP2 in tubular epithelial cells in transgenic mice in which MMP2 was expressed under control of the GGT-promoter causes spontaneous EMT and renal fibrosis, demonstrating that MMP2 can induce EMT *per se (37)*. Inhibition of MMP2 activity *in vivo* can inhibit progression of renal fibrosis and EMT (38). Interestingly, MMP9, which displays similar substrate specificities as MMP2, does not play a role in EMT, but is rather associated with acute kidney injury and tubular cell apoptosis (39). It is not understood yet why MMP-2 mediates EMT and MMP-9 favors apoptosis despite their overlapping substrate specificities.

In addition to matrix metalloproteinases, plasmin is a protease which is critically involved in renal fibrogenesis and EMT (Figure 2B). Active plasmin is derived from plasminogen by proteolytic cleavage. Such cleavage is facilitated either by the tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Both tPA and uPA are inhibited by plasminogen activator inhibitor (PAI). Mice deficient for plasminogen or tPA (both resulting in decrease or loss of plasmin) are protected against EMT and interstitial fibrosis (40; 41). Plasmin, best known for its fibrinolytic activity, not only degrades extracellular matrix, it also activates matrix metalloproteinases and TGF-β1, further facilitating EMT. Additionally, plasmin directly induces EMT via the protease-activated receptor-1 (PAR-1) in an ERK-signaling-dependent manner (41).

6.3. Integrin-linked kinase

How changes of the extracellular matrix microenvironment regulate EMT is only incompletely understood. While specific integrins sense the changes of ECM composition and cause tubular epithelial cells to undergo EMT have not been identified as yet, several studies implicate an involvement of the integrin-linked kinase (ILK) in this process. ILK was originally discovered through its physical interaction with β1-integrin, it also transduces signals from growth factors and cytokines, in addition mediating cell-matrix interactions (42). At sites of integrin attachment, ILK is found in complex with two adapter proteins, parvin and PINCH (particularly interesting Cys-His-rich protein) (Figure 2C) (42). This ternary complex of ILK, PINCH and parvin (IPP) functions as a signaling platform by interfacing with the actin cytoskeleton and by affecting diverse signaling pathways through phosphorylation of downstream targets (43). Most notably, Akt signaling pathway, GSK3β, JNK signaling and small-weight GTPases are known to be controlled by IPP. With regard to tubular epithelial cells, ILK is involved in mediating TGF-β1-induced EMT (43). Li and co-workers demonstrated that TGF-β1-induced EMT is associated with increased ILK expression and that over-expression of a dominant-negative ILK mutant inhibited such TGF-β1-mediated EMT (44). ILK-mediated EMT involving tubular epithelial cells is dependent on formation of the ILK-Pinch-parvin (IPP) complex (45).

6.4. Hypoxia

Rarification of the peritubular microvasculature associated with chronic hypoxia is increasingly recognized as an important facilitator of renal fibrogenesis. Unlike their apoptotic response to acute ischemia, tubular epithelial cells respond to chronic hypoxia by undergoing EMT (46). Hypoxia-induced EMT is mediated by the hypoxia-inducible factor 1 (HIF-1) (47) (Figure 2D). HIF-1 mediates EMT of tubular epithelial cells it least in part by induction of lysil oxidases Lox and LoxL2 (47). Additionally, HIF-1 induces expression CTGF, which possibly mediates EMT of tubular epithelial cells in an autocrine fashion (48). HIF-1 is a heterodimeric transcription factor which consists of a α-subunit (HIF-1α) and a β-subunit (HIF-1β) (49). While HIF-1β is constitutively expressed, HIF-1α is ubiquinated in presence of oxygen. When oxygen is limited (hypoxia), HIF-1α is stabilized, enabling HIF-1 signaling. Because HIF-1α can also be stabilized by pro-fibrotic cytokines such as TNF-α or angiotensin II, it is also conceivable that HIF-1 is involved in mediating EMT independent of hypoxia.

6.5 Snail

The number of environmental factors such as growth factors, extracellular matrix constituents, proteases or hypoxia, which can induce EMT is increasing. Despite the distinctness of these known factors, the EMT-response is relatively uniform. This raises interest for potential master-regulators of EMT which are at the intersection of various signaling pathways and which control the EMT response. The Snail transcription factors are one prominent example of one common downstream target of various signaling pathways which regulates EMT (50). Of the three vertebrate Snail family members of zinc finger proteins, the functionally equivalent Snail1 and Snail2 (formerly known as Slug) mediate EMT (50). In fact, all known EMTs in development, cancer and fibrosis appear to be associated with Snail activation (50).

Snail is most prominently known as a suppressor of E-cadherin expression, but it regulates various aspects associated with EMT such as increased expression of mesenchymal markers (i.e. fibronectin and vitronectin), decreased expression of various epithelial markers (i.e. claudins, occludins and cytokeratins), inhibition of proliferation (via suppression of Cyclins D and CDK4), increased MMP expression and protection from cell death

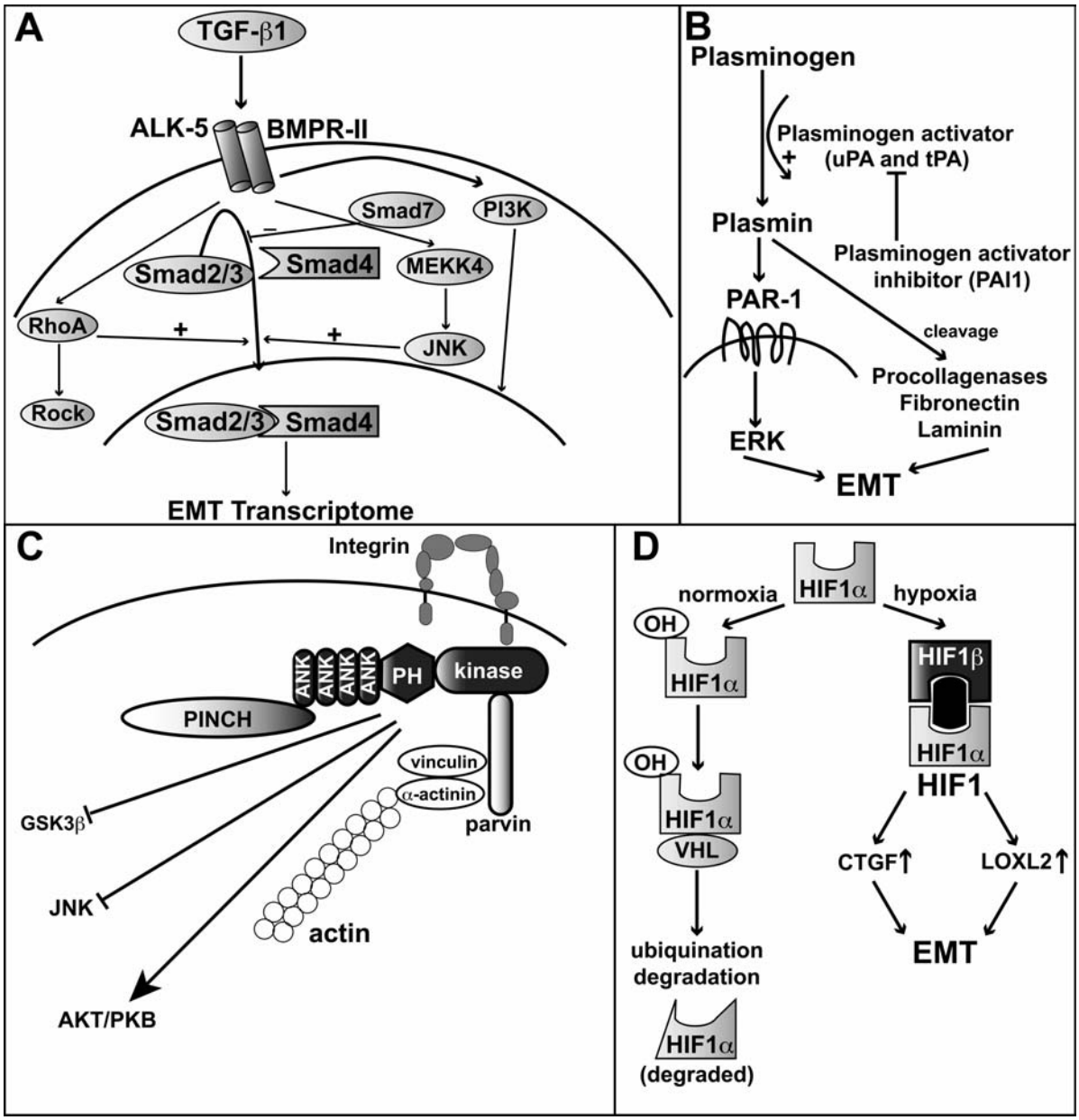

Figure 2. Common pathways associated with EMT in chronic kidney disease. A. TGF-β1-induced EMT. TGF-β1 binds to a heterocomplex of a type I receptor (ALK5) and type II serine/threonine kinase receptors (BMPR-II). TGF-β1 transcriptional control is mediated by various pathways, including the Smad pathway, the MAPK pathway and PI3 Kinase signaling. The Smad pathway is central to EMT involving tubular epithelial cells. Smad2 and Smad3 in complex with the common Smad4 translocate to the nucleus where transcriptional control is elicited. RhoA and JNK enhance this pathway, Smad7 suppresses Smad-dependent EMT. B. The plasminogen system. The precursor plasminogen is converted to the active form- plasmin- by plasminogen activators uPA and tPA. Plasminogen activator itself is regulated by its inhibitor PAI1. Plasmin cleaves the basement membrane constituent and also activates MMPs, enhancing EMT. Plasmin also elicits EMT by binding to the PAR1 receptor. C. Integrin-linked Kinase and EMT. ILK binds to the cytoplasmatic domain of β1 integrin. ILK consists of three domains, N-terminal ankyrin (ANK) repeats, a plekstrin homology domain (PH) and the C-terminal kinase domain. ILK forms with Pinch and parvin the ternary IPP complex, which serves as a signaling platform by interfacing with the actin cytoskeleton and interfering with several signaling pathways, including inhibition of JNK and GSK3β and enhancing AKT/PKB signaling. D. Hypoxia and EMT. In presence of normal oxygen levels, HIF1α is hydroxylated, which allows for binding to the von Hipple Lindau protein (VHL). This complex allows for ubiquinization and degradation of HIF1α. Under hypoxic conditions HIF1α the ubiquitin-proteosome mediated degradation is inhibited and HIF1α rapidly accumulates. HIF1α is phosphorylated and forms a complex with HIF1β. This complex is referred to as HIF1. HIF1 induces EMT via increased CTGF expression and lysyl oxidase-like protein (LOXL2).

(i.e. suppression of caspases, DNA fragmentation factor and Bcl-interacting death agonist).

Snail activity is regulated at the transcriptional level and by control of its subcellular localization. Phosphorylation of Snail causes its export from the nucleus into the cytoplasm resulting in its inactivation as a transcription factor (50). Hence, EMT is associated with nuclear localization of Snail (51).

In human renal biopsies, fibrosis is associated with increased Snail1 expression (51). Snail activation in tamoxifen-inducible Snail1-transgenic mice resulted in EMT and renal fibrosis, demonstrating the prominent role of Snail in EMT (51). Other EMT masterswitches include the CBF-A and KAP-1 proteins binding to FTS1 sites in the genomic DNA and the transcription factor LEF1. However, the role of these EMT regulators has not been established in the renal fibrosis setting yet.

7. EXPERIMENTAL STRATEGIES TO INHIBIT EMT

Relevance of EMT for the progression of renal fibrogenesis is highlighted in studies in which EMT was specifically inhibited. TGF-β1-induced EMT is specifically inhibited by bone morphogenic protein-7 (BMP7) (9). BMPs in general are morphogens that belong to the TGF-β superfamily (52). In the adult, BMP7 expression is highest in the kidney and BMP7-deficient mice die shortly after birth due to severe kidney dysplasia, suggesting a prominent role for BMP7 in the kidney (53). BMP7 signals through an activated heteromeric complex of a type I (ALK3) and type II (BMPR-II) serine/threonine kinase receptors (54). Cytoplasmatic signaling is mediated by the phosphorylation of ligand-specific SMAD proteins (Smad1 and/or Smad5, or Smad8), which translocate to the nucleus in association with the common Smad4. In tubular epithelial cells, BMP7 directly counteracts Smad3-mediated TGF-β1-signaling in a Smad-dependent manner, resulting in reversal of TGF-β1-induced EMT (9). Such antagonism of TGF-β1-signaling in tubular epithelial cells protects the kidney from fibrosis in various animal models of chronic kidney disease.

Hepatocyte Growth Factor (HGF) is another endogenous protein that has been shown to inhibit TGF-β1-induced EMT (55). HGF acts by inducing expression of the Smad-co repressor SnoN, which forms a complex with Smad2/3, making then transcriptionally inactive (56).

8. SUMMARY - THE CURRENT CONCEPT OF EMT IN KIDNEY FIBROSIS

Activated fibroblasts are main mediators of renal fibrogenesis. In addition to proliferation of resident fibroblasts as well as recruitment and differentiation of bone marrow-derived progenitor cells, EMT is a relevant source of fibroblasts in the diseased kidney. In fact, a study by Iwano and co-workers suggests that in the model of UUO ~one third of fibroblasts derive from bone marrow, ~one third from resident fibroblasts and ~one third via EMT (8). However, EMT not only contributes to accumulation of pro-fibrotic fibroblasts, but it also impacts function and integrity of the tubular epithelial compartment. In fact, evidence for the central role of tubular epithelial cell integrity for kidney homeostasis is mounting and it is conceivable that the detrimental effect of EMT on the tubular epithelium is more relevant than its contribution to fibroblast accumulation. Hence, when evaluating the overall impact of EMT on progression of tubulointerstitial fibrosis, early stages of EMT have to be carefully considered. Furthermore, early EMT stages (characterized by loss of the brush border, decrease of tight junctions and intercellular adhesion molecules, acquisition of actin bundles and mesenchymal cell markers and disruption of the tubular basement membrane) are relevant, as they are potentially reversible by pharmacotherapy. The role of later stages of EMT, and the ensuing accumulation of epithelial cell-derived fibroblasts is far less understood. Due to the lack of marker genes, the rate at which early EMT progresses to its final stages is still unclear in human patients. Furthermore, the impact of EMT-derived fibroblasts versus resident fibroblasts and bone-marrow derived fibroblasts has not been functionally dissected as yet. But the vast recent advancements in the EMT field provide optimism that this can be sorted out in the near future.

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Send correspondence to: Michael Zeisberg, Harvard Medical School, Division of Matrix Biology, Department of Medicine, Beth Israel, Deaconess Medical Center, 330 Brookline Avenue, RW 734, Boston, MA 02215, Tel: 617-667-03583, Fax: 617-975-0360, E-mail: mzeisber@bidmc.harvard.edu

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