Resolved: EMT Produces Fibroblasts in the Kidney

ABSTRACT

Epithelial-mesenchymal transition (EMT) is a mechanism for generating primitive mesenchymal cells during gastrulation or mobile tumor cells during cancer metastasis. For 15 years, EMT has also been viewed as a principal source of fibroblasts in tissue fibrosis. Because several recent studies question its role in fibrogenesis, it seems like a good time for debate.

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Epithelial-mesenchymal transition (EMT) was originally described as an early embryonic event enabling primitive epithelia to migrate as mesenchymal cells forming the mesoderm and for primitive neuroepithelia to move as neural crest cells. Without the mechanism of EMT, complex organisms could not exist. It is also without argument that EMT operates in adult cancer cells as a molecular program for tumor invasion and metastasis. The cellular evidence for EMT was first described by Greenburg and Hay in the early 1980s in the context of embryonic development. This same group then reported their original embryonic EMT criteria also applied to adult kidney tubular epithelial cells acquiring a fibroblast phenotype upon immersion in collagen gels in vitro. Today, to address the variations of EMT seen in diverse biologic contexts, EMT involving embryonic epithelia is now classified as type 1 EMT; type 2 EMT refers to adult epithelial and endothelial cells transitioning to fibroblasts, and EMT involving metastatic cancer cells is called type 3 EMT.

The debate question posed here is quite simple: Can the activation of the same pathways that cause EMT in embryonic epithelia and cancer cells also cause adult somatic cells (epithelia and endothelia) to acquire a fibroblast phenotype? In taking the affirmative position in this debate, there is an extensive body of evidence demonstrating EMT in humans or experimental models of tissue fibrosis, the latter issue perhaps initiating this debate.

The notion that adult somatic cells can undergo EMT has evolved greatly in the past 15 years. Although type 2 EMT contributes to fibroblast accumulation in various fibrotic tissues, including lung, skin, eye lens, intestine, liver, and peritoneum, I largely confine my comments to its role in kidney fibrosis. The current view of EMT in renal fibrosis is that stimuli from an inflammatory microenvironment induce conserved signaling pathways in epithelial and endothelial cells that also regulate EMT in embryonic epithelia and cancer cells. Adult tubular epithelial cells respond by acquisition of a mesenchymal phenotype, which in its most complete form leads to formation of a fibroblast.

When detection of EMT in the context of fibrosis is discussed, a few basic principles must be understood to avoid misconception: First, EMT, in its most extreme form, can lead to formation of a fibroblast, but the transition does not necessarily have to go that far. This means that the number of epithelial cells undergoing EMT does not necessarily equal the number of cells that become fibroblasts. In fact, the exact percentage of tubular epithelial cells or renal endothelia that become fibroblasts in a diseased kidney is entirely unknown. Second, there is consensus that fibroblasts within fibrotic tubulointerstitium proliferate—irrespective of their origin. This means, hypothetically, substantial numbers of fibroblasts may originate from only a few EMT-derived fibroblasts over prolonged periods of time. Third, EMT is a dynamic and reversible process, suggesting there are intermediate stages...
whereby epithelial or endothelial cells display features of their parental origin as well as those of an emerging fibroblast, and suggesting there are advanced stages by which point epithelial cell characteristics have been lost. Thus, intermediate stages of EMT precede advanced stages during renal fibrosis, making the timing of tissue analysis critical for interpretation.

To detect EMT in vivo, one would like to view epithelial cells transitioning into fibroblasts and migrating to the interstitium in real time. Unfortunately, this is not feasible with current technology. Instead, one is left with compelling snapshots of the transitional process through histochemical analysis of fibrogenic tissue.20,21 Most studies center around co-immunostaining for markers of epithelial cell lineage, fibroblast markers, and activation of the EMT transcriptional program, particularly nuclear translocation of Snail, Twist, β-catenin, or CBF-A.7–22 Detection of intermediate stages of EMT in injured kidney is straightforward and is the current gold standard; one example is a tubular epithelial cell that expresses the fibroblast marker FSP1, in which Snail signaling is active and E-cadherin is lost.22,23

Detection of near-complete EMT is more complex because cells of epithelial or endothelial origin now look phenotypically like fibroblasts. For proving EMT at this stage, original parental cells must express a stable tag to identify its epithelial or endothelial origin irrespective of current phenotype.24 Ten studies of mice using different lineage-tracing transgenes in epithelial or endothelial cells showed EMT25–34; three of those studies were in kidney.26,27,33 These advanced stages of type 2 EMT cannot be detected within fibrotic human kidneys, because human epithelial cells do not carry obvious lineage tags; an α-smooth muscle actin–positive myofibroblast, for example, provides no known clues about its origin; however, a number of indirect studies suggested that EMT is operative in human tissue.35–37 To use current technology to its best capacity to detect EMT, I suggested previously that a combination of epithelial cell markers, possible lineage tags, fibroblast markers, and transcription signals, best establish the presence of EMT in vivo.7

Adhering to such criteria, hundreds of research articles reported evidence for EMT in various fibrotic tissues, including exquisite detail regarding the biochemistry of transitional signaling.38 Many of these studies identified transcription factors that unequivocally induce EMT in adult epithelial cells (Snail, Twist, β-catenin, CBF-A),22,39 as they do in embryonic epithelia and cancer cells.4 In fact, induction of a Snail transgene alone is sufficient to induce EMT and recapitulate all stages of kidney fibrosis in mice.39 Short of a movie, there is abundant documentation that during renal fibrogenesis, EMT is initiated in single cells within tubular cross-sections.20,21,27 Activation of this EMT program is soon followed by expression of fibroblast markers associated with loss of contact with tubular basement membrane.20 These initial EMT stages have been captured not only in mice27 but also in human renal biopsies.37,40–42 Lineage-tracing experiments also allow for detection of tubular epithelial cell–derived26,22 and endothelial cell–derived fibroblasts within the kidney43 as well as in other organs with fibrosis.26,28,30,31,34 Most important, our advances in understanding EMT have produced several effective strategies to treat experimental fibrosis in rodents. Molecules such as bone morphogenetic protein 719 and hepatocyte growth factor44 inhibit EMT and are leading candidates for clinical development in treating chronic kidney disease.

A few studies, however, recently came to the conclusion that EMT may not be operative in the context of fibrogenesis.11–13 Here, I attempt to understand why studies with almost identical experimental setup lead to opposite results. As it pertains to the kidney, a recent study by Humphreys et al.45 induced renal fibrosis after unilateral ureteral obstruction in kidneys marked with the Six2Cre promoter that should be active in most renal epithelium. Unlike numerous previous publications that unequivocally demonstrated expression of fibroblast markers by intermediate-stage tubular epithelial cells in various models of chronic kidney fibrosis (unilateral ureteral obstruction,5–6 nephrotoxic serum nephritis,19 5/6 nephrectomy,48 streptozotocin-induced diabetic kidneys,49 anti–tubular basement membrane nephritis,50 chronic allograft nephropathy,51 polycystic kidney disease,52 and HIV-associated nephropathy53), this study failed to detect α-smooth muscle actin–positive or FSP1+ immunostaining in a single epithelial cell in vivo. Predictably, this study then also failed to detect advanced stages of EMT forming FSP1+ fibroblasts as assessed by dsRed double-labeling experiments in ACTB-Bgeo, DsRed.MST double-transgenic reporter mice. On the basis of these data, which were collected in one single disease model, this study tried to challenge the existence of both the intermediate and advanced stages of EMT—in contrast to positive findings in more than 100 original publications on various kidney injury models.44 The complete failure to detect EMT markers even in a single tubular epithelial cell in vivo is puzzling and difficult to interpret from the outside. Attempts to find biological explanations for the discrepancies to previous studies are also hindered because no information is provided for determination of whether EMT transcriptional pathways were activated in this particular set of experiments. Furthermore, no technical advances that provide explanation for why so many previous studies would be in error were made.

With regard to expression of fibroblast markers, this group even failed to find collagen I–producing FSP1+ cells in the kidney at all,55,56 which goes against more than 60 articles in the literature and may be the first of its kind; most experimentalists who stain for FSP1 in tissue use antigen retrieval technology before immunohistochemistry, which was not done here. Furthermore, because expression of collagen I was used as their gold standard for defining fibroblasts, this failure to detect was used as further evidence against EMT. I object to this definition of a fibroblast based solely on collagen I expression because not all fibroblasts produce collagen all of the time. Another issue in this study is the collagen I–GFP reporter gene they used was originally optimized to detect collagen–producing stellate cells in the liver. There currently is no collagen I promoter construct available that mirrors type I collagen expression in all cell types,57 and the efficacy of this particular construct to detect type I collagen expression in kidney fibroblasts was not assessed, possibly explaining why they could not detect a single collagen–producing FSP1+ fibroblast.55,56
Despite my concerns about this negative experiment, as well as the others on which I have not commented, I welcome this debate because it not only highlights where misunderstandings of EMT have led to confusion but also pinpoints where further advances can be made. I understand that EMT is sometimes met with skepticism because it cannot be watched to evolve before an observer’s eye; however, neither are leukocytes that undergo diapedesis through vessel walls or cancer cells that metastasize into the bloodstream. Only few will doubt these latter events occur even though they are just as difficult to observe as EMT. Science should evolve through open discussion, but for a fruitful discussion there should be at least some common agreement, in this case, what is EMT, how it should be detected, and what is a fibroblast—questions that have largely been addressed in the past decade using sophisticated genetic and biochemical approaches to which we should adhere.

Conceptually, induction of EMT makes a lot of sense for the kidney as a protective mechanism. Most transcriptional pathways in the EMT program (Snail, CBF-A, and Twist) are also known to be anti-apoptotic and hence provide a way for tubular epithelial cells to avoid imminent death. Because tubulointerstitial injury is associated with disturbed interaction of tubular epithelial cells with their tubular basement membrane,20 watershed cells are also at risk for falling off into their lumens. The migratory capacity provided by EMT enables these transitional cells to invade the basement membrane and evade this threat. Furthermore, EMT from a fibroblast-centric view is a highly efficient way to recruit fibroblasts rapidly to local sites of tubulointerstitial injury as opposed to their recruitment from more distant sites in tissue or even from the bone marrow using fibrocytes that migrate through the bloodstream. The advantageous economy of fibroblast recruitment by EMT is even more obvious at sites with more pronounced surfaces (e.g., skin, epicardium), where EMT is an essential process to seal new lesions. Why would adult kidney epithelial cells not also undergo EMT under duress? As with Occam’s Razor, it just makes sense.

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DISCLOSURES

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REFERENCES


More than 20 years ago, scientists who were studying fibrosis purified hepatic stellate cells (HSCs) from the liver, separating them by virtue of their distinct cell buoyancy.1 These unusual cells store retinoic acid in health and line the specialized fenestrated capillaries of the liver called hepatic sinusoids and have features in common with pericytes, mural cells of capillaries that have been shown in other tissue beds to be important in sprouting angiogenesis and vascular stability by virtue of cell–cell signaling with underlying endothelial cells.2

When cultured in vitro and activated with serum factors, HSCs generate pathologic collagen matrix and activate intermediate genes involved in migration and contraction, including α-smooth muscle actin (αSMA). It became widely accepted that HSCs were progenitors of the liver myofibroblasts.3 In the kidney, similar pericyte-like cells have been much harder to detect or define. Furthermore, other adherent cell types, including epithelial, endothelial, and myeloid cells, generate collagen matrix and activate expression of the intermediate filament αSMA when cultured in vitro, suggesting that pericyte-like HSC myofibroblast precursors in liver may not exist in other organs and that other cell types have a capacity to become myofibroblasts.

Understanding the origin of myofibroblasts (activated spindle or stellate interstitial cells that share features with smooth muscle including expression of αSMA, secreting a pericellular matrix containing collagen and glycosaminoglycans) became more complicated because malignant mammary epithelial cells were shown to migrate through basement membranes and acquire expression of similar intermediate filament proteins to myofibroblasts in vivo.4 The behavior of malignant epithelial cells suggested that epithelium might be a major source of myofibroblasts in nonmalignant diseases.

There are now many examples of cultured, nonmalignant epithelial cells acquiring patterns of gene expression in vitro similar to myofibroblasts, providing rationale for the hypothesis that epithelial cells become myofibroblasts in kidney disease.5 Potential markers of this process, called epithelial-mesenchymal transition (EMT), are αSMA and S100A4 (also known as MTS-1 or FSP1), which are expressed in vitro by epithelial cells in response to exogenous TGF-β. Some have used these markers as surrogates for cell lineage analyses in vivo. Although endogenous lineage markers such as CD45 have proved robust and specific for leukocytes, neither αSMA nor S100A4 is a robust marker of lineage, because they lack cell specificity and constancy of expression.6 Indeed, S100A4 in certain tissue beds including injured kidney is detected in macrophages, not myofibroblasts.7–9 Other possible EMT markers include Snail, Twist, Slug, and 1d transcription factors, but a range of cell types after injury, including epithelial cells, myofibroblasts, and macrophages, express them.8,10 It is likely that although these transcription factors are important in migration, activation, and differentiation, they do not denote lineage in vivo.

Most of the kidney epithelium derives from mesoderm-derived intermediate mesenchyme.11 This process of derivation is termed mesenchymal–epithelial transition (MET), and a reversal of this process has provided a rationale for the hypothesis that epithelium becomes myofibroblasts in kidney injury. In contrast, the epithelium of liver, pancreas, and several other organs derives from endoderm. The absence of developmental MET of other organs, including liver, gives little rationale for EMT events during injury of these organs.

Although the in vitro studies are striking, proof that epithelial cells become myofibroblasts in vivo has been limited.12 Indeed, a number of morphologic and experimental tracing studies have failed to demonstrate that epithelial migration and differentiation into myofibroblasts occurs in vivo.8,13,14 With the advent of genomic recombination technology, it has become possible to trace the fate of cells in vivo in mice with confidence.

The state-of-the-art method to study EMT in vivo is through lineage tracing, or genetic fate mapping, of cells. This provides an advantage that no cell surface or cytosolic marker has—that is, once a heritable marker is activated in vivo, it is permanent. It will always be detectable in any cell that derives from the labeled cell regardless of differentiation, proliferation, migration, or injury state. Fate mapping of cells in vivo, however, is fraught with pitfalls that are not readily apparent: Genomic recombination leading to stable expression of the heritable marker/reporter (LacZ, Alk-Phos, RFP, GFP, or YFP) must occur only in the cell type to be labeled. For example, the gene locus for Tie2, encoding the endothelial receptor, has been used in studies that label endothelial cells, but Tie2 is expressed transiently by all myeloid precursors, and, thus, the locus effectively drives Cre-mediated recombination in monocytes as well as endothelium.15,16 The gene locus driving expression of the recombinase that denotes cell type specificity should preferably not be activated during disease, because the gene locus used to activate the heritable marker could become active in additional cell types. Recombination must occur efficiently to instill confidence that infrequent events can be detected. The reporter must be robust and have the capacity to be actively expressed at sufficient level to be readily detected in all cell types in the body during health and disease. Recombination of genomic DNA during development is preferable because it occurs at very high frequencies, and developmentally active transcription factors frequently exhibit highly restricted expression.

The first studies of epithelial cell lineage after injury were performed in kidney. Iwano et al.12 reported evidence that myofibroblasts in kidney injury derived from proximal tubule cells (PTECs) of the nephron in vivo. They used Cre recombinase driven by a transgene containing part of the γ-glutaryl-

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transferase promoter, which activates in kidney PTECs neon- tally but not developmentally. Mice harboring this transgene were crossed with Rosa26R mice that activate universal bacterial LacZ expression under regulation of the Rosa26 locus promoter after Cre recombination. The authors detected myofibroblasts using antibodies to S100A4 and HSPA4 and detected LacZ using antibodies against the gene product β-gal. These studies concluded that >30% of kidney myofibroblasts derive from injured PTECs.

These conclusions have been cast into doubt by three recently published comprehensive lineage tracing studies that used three different models of kidney disease and found no evidence of epithelial origin of myofibroblasts. One of these recent studies, however, by using similarly rigorous fate mapping of mesenchyme-derived stromal cells of the developing kidney that mature into perivascular pericytes in the adult kidney, demonstrated that a sizable number of these discrete cells persist in the adult kidney, express PDGFRβ and CD73, and are the primary and probably only source of myofibroblasts that appear during disease of the kidney. The new report by Humphreys et al. used the loci of developmentally active transcription factors that play key roles in determining mesenchymal cell fate to the epithelial lineage (Six2) or the stromal lineage (Foxd1) to activate two different heritable markers, LacZ or RFP.

The gene-targeted mice were either knock-in or BAC transgenic, meaning that the expression of the Cre recombinase faithfully reproduces endogenous gene expression. High levels (>95%) of recombination were achieved in all studies, and the reporters used gave high signal-to-noise outputs when analyzed in tissue sections. Furthermore, all positive and negative controls were presented. The authors studied two well-established models of fibrosing kidney injury. Significantly, the authors relied exclusively on expression of S100A4 (FSP1), to detect scar-forming myofibroblasts, the authors concluded that no myofibroblasts originating from epithelium. By contrast, using similar systems, the pericyte origin of myofibroblasts was readily demonstrated. These studies should divert investigation of EMT toward the study of pericyte function and biology in tackling fibrotic disease of the kidney.

Although there is a lack of developmental rationale for epithelial cells undergoing an EMT process because there is no developmental MET in liver, hepatocyte fate in vivo was reported by Zeisberg et al. to include differentiation into myofibroblasts. They used a transgenic model to drive the DNA recombinase Cre under the control of a 2.3-kb fragment of the albumin promoter/enhancer locus to activate the heritable marker LacZ at the Rosa26 locus. Recombination was specific for hepatocytes but was mosaic (approximately 50%). To detect scar-forming myofibroblasts, the authors relied exclusively on expression of S100A4 (FSP1), and to detect the fate marker LacZ, they used antibodies against the gene product β-gal. In a short model of CCL4-liver toxicity and fibrosis, they concluded that as many as 10% of myofibroblasts derive from hepatocytes. Taura et al. recently were unable to confirm those findings, using a similar Alb-Cre transgenic model. They also used the Rosa26R, LacZ expressing reporter mouse, but in addition used the Collagen1a1-GFP reporter to identify simultaneously collagen-producing cells in the injured liver and cells derived from hepatocytes. After detecting cell fate by blue stain on the basis of β-gal enzyme activity, they concluded that no myofibroblasts in the CCL4 models of liver disease derive from hepatocytes. Data reported from our laboratory using a 12-week model of CCL4-liver fibrosis are consistent with those of Taura et al.

Why do similar studies result in such profoundly different interpretations? To the reader, these conflicting reports may be difficult to disentangle. In my opinion, three potential problems exist with the studies of Iwano et al. in the kidney and Zeisberg et al. in the liver that may have inadvertently led to artifacts. First, the genetic system for the kidney studies used a transgenic Cre driver that may lack cell specificity during disease. Second, the investigators relied on antibody detection of the fate marker bacterial β-gal in tissue sections. Carefully performed experiments indicate that the Rosa26 locus drives adequate expression of LacZ in all cells of adult liver and kidney to detect reliably X-gal blue stain triggered by enzyme activity; this should be the gold standard. In our experience, anti-β-gal
antibodies are unreliable and prone to artifacts and need to be compared objectively alongside controls. Third, the marker S100A4/FSP1 has been inappropriately used in the identification of myofibroblasts. Independent studies indicate that S100A4 is expressed in other cells, such as macrophages. In mouse kidney, S100A4 is not detected in any collagen-producing cells.7–9 αSMA, Pdgfrβ, and Coll1a1 all are more reliable markers of the myofibroblast and Pdgfrβ, Coll1a1, and CD73, its precursors.

Current comprehensive analyses of epithelial lineage in three models of kidney injury and in CCl4 liver fibrosis indicate that EMT is unlikely to occur in vivo. Rather, they suggest a new paradigm that vascular pericytes are the source of fibrosis-generating myofibroblasts and that epithelial contribution to fibrosis is by paracrine cell signaling rather than cellular transition. These new studies re-focus fibrosis research on microvascular injury rather than epithelial injury.

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REFERENCES


