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EMT and Interstitial Lung Disease: A Mysterious Relationship

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Abstract

Purpose of review—Pathogenesis of interstitial lung diseases (ILD) has largely been investigated in the context of the most frequent ILD, idiopathic pulmonary fibrosis (IPF). We review studies of epithelial to mesenchymal transition (EMT) and discuss its potential contribution to collagen-producing (myo)fibroblasts in IPF.

Recent findings—Endoplasmic reticulum (ER) stress leading to epithelial apoptosis has been reported as a potential etiologic factor in fibrosis. Recent studies further suggest EMT as a link between ER stress and fibrosis. Combinatorial interactions among Smad3, β -catenin and other transcriptional co-activators at the α -smooth muscle actin (α -SMA) promoter provide direct evidence for crosstalk between transforming growth factor- β (TGF β) and β -catenin pathways during EMT. Lineage tracing yielded conflicting results, with two recent studies supporting and one opposing a role for EMT in lung fibrosis.

Summary—Advances have been made in elucidating causes and mechanisms of EMT, potentially leading to new treatment options, although contributions of EMT to lung fibrosis *in vivo* remain controversial. In addition to EMT providing a direct source of (myo)fibroblasts, expression of mesenchymal markers may reflect epithelial injury, in which case inhibition of EMT might be deleterious. EMT-derived cells may also contribute to aberrant epithelial-mesenchymal crosstalk that promotes fibrogenesis.

Keywords

interstitial lung disease; IPF; EMT; ER stress; lineage tracing

Introduction

Interstitial lung diseases (ILD) refer to a collection of disorders characterized by varying degrees of inflammation and fibrosis in the lung interstitium. The cause of ILD is sometimes known (e.g., environmental factors, drugs, collagen vascular disease, and sarcoidosis). When the cause is unknown, it is labeled as idiopathic and further classified into the currently recognized single disease entities [1]. Research on pathogenesis has been conducted mostly in the context of the most frequent of idiopathic ILD, idiopathic pulmonary fibrosis (IPF). In the currently accepted paradigm, a major factor in IPF pathogenesis is thought to be recurrent alveolar epithelial cell (AEC) injury [2, 3]. Initial injury leads to aberrant activation of AEC, creating a profibrotic environment with accumulation of collagen-producing fibroblasts and myofibroblasts. To date, (myo)fibroblasts have been suggested to accumulate via three mechanisms: (1) proliferation and differentiation of resident lung fibroblasts, (2) epithelial-to-mesenchymal transition (EMT), in which AEC undergo transition to (myo)fibroblasts, and (3) transition of bone marrow-derived fibrocytes or

circulating progenitors to fibroblasts [4]. The significance and relative contribution of each pathway to fibrosis have not been definitively established, hampering development of novel therapies. We first review the latest advances on causes and underlying mechanisms of EMT and then focus on the ongoing controversy of whether and how EMT contributes to lung fibrosis and whether inhibition of EMT would be of benefit therapeutically.

Potential causes of EMT

Historically, disruption of cell contacts, hypoxia and inflammation have been proposed as potential triggers of EMT [5]. In recent years, progress has been made in understanding the relationship between endoplasmic reticulum (ER) stress and IPF [6]. The ER is an organelle where secreted and membrane proteins fold and mature, and ER stress refers to activation of signaling pathways in response to relative insufficiency in protein folding capacity [7]. In the lung, mutations in genes encoding surfactant proteins SP-A2 and SP-C are associated with interstitial lung diseases [8–11], and ER stress due to mutant protein accumulation has been the proposed underlying mechanism leading to apoptosis and fibrosis [12, 13]. Recently, there have been two papers that both link ER stress to EMT [14, 15]. Induction of ER stress by either tunicamycin or overexpression of mutant SP-C resulted in loss of epithelial markers, induction of α -smooth muscle actin (α -SMA), and transition to a spindle-shaped morphology indicating EMT. Src-dependent signaling was implicated as the link between ER stress and EMT. In addition, Zhong *et al.* [14] found that mild and extensive ER stress resulted in EMT and apoptosis, respectively, indicating that different levels of ER stress may have different consequences for cell survival.

Two papers have reported on interactions between epithelial cells and extracellular matrix, which in turn lead to EMT. It had been known that matrix metalloproteinase (MMP), a group of proteolytic enzymes that degrade extracellular matrix, could paradoxically promote collagen deposition. Yamashita *et al.* [16] used intratracheal adenoviral MMP-3, MMP-3-null mice, and *in vitro* studies to show that MMP-3 promotes lung fibrosis, possibly through EMT involving β -catenin nuclear translocation. DeMaio *et al.* [17] found that cells cultured on collagen I acquired spindle-shaped morphology and expressed α -SMA. Smad signaling was activated by collagen I, indicating transforming growth factor- β (TGF β) receptor-dependent signaling.

Oxidative stress from cigarette smoke extract [18], radiation [19], and hydrogen peroxide [20] (in H358, RLE-6TN, and A549 and human primary bronchial epithelial cells, respectively), β -galactosidase binding lectin galectin-3 (using primary AEC from galectin-3 $-/-$ mice) [21], mechanical stretch (in mice and primary mouse alveolar epithelial type II cells) [22, 23], Epstein Barr virus protein LMP1 (in A549 and HPL1D cells) [24], interleukin (IL)-17A (in MLE-12 cells) [25], and mutant ATP-binding cassette transporter A3 (ABCA3) (in A549 cells) [26] have also been reported as inducers of EMT. Of note, instillation of galectin-3 inhibitor beginning 18 days after bleomycin instillation [21] and intravenous administration of IL-17A-neutralizing antibody beginning 14 days after bleomycin or silica instillation [25] attenuated fibrosis, suggestive of possible novel therapies. The role of these pathways awaits further mechanistic studies.

Mechanisms underlying EMT

EMT converts epithelial cells into migratory and/or invasive mesenchymal cells, and is well-established during development and carcinogenesis with its role in fibrosis being more controversial [27]. TGF β signaling is a powerful inducer of EMT in all three contexts mostly through its canonical Smad-dependent pathway [28, 29]. When TGF β binds type I and type II TGF β receptors, the type I receptor phosphorylates Smad2 or 3, which associates with

Smad4 to enter the nucleus to activate or inhibit target gene expression [29]. Recently, several papers have implicated interactions between TGF β and β -catenin signaling pathways in EMT. Masszi *et al.* had proposed a two-step EMT model in which Smad3 mediates EMT, but inhibits further transition from the mesenchymal phenotype to α -SMA-expressing myofibroblasts by binding to myocardin-related transcription factor (MRTF) and preventing MRTF from activating the α -SMA promoter [30]. In a follow-up study, Charbonney *et al.* [31] found that in pig kidney epithelial LLC-PK1 cells, β -catenin binds Smad3 to release MRTF from Smad3, allowing MRTF to activate the α -SMA promoter. Zhou *et al.* followed up on their previous result showing that bleomycin-induced lung fibrosis could be reversed by the small molecule inhibitor ICG-001, which inhibits the interaction between β -catenin and cAMP response element-binding (CREB)-binding protein (CBP) [32]. Using rat alveolar epithelial RLE-6TN cells, they showed that TGF β 1 activates β -catenin-dependent signaling, and that both pathways synergize to induce α -SMA expression through direct binding of Smad3, β -catenin and CBP at the α -SMA promoter [33]. Ulsamer *et al.* followed up on previous papers showing that integrin α 3 β 1 modulates the interaction between Smad2 and β -catenin to mediate EMT [34, 35]. They found that integrin α 3 β 1 is required for TGF β - β 1-mediated Src kinase activation, which in turn activates β -catenin [36]. In addition, they used a small molecule tankyrase inhibitor to increase expression of axin1, a negative regulator of Wnt/ β -catenin signaling. Administration beginning 10 days after bleomycin instillation improved survival, attenuated fibrosis and lowered expression of α -SMA and collagen I, suggestive of a possible novel therapy. These papers point to the importance of the β -catenin-dependent signaling pathway in mediating TGF β -dependent effects in EMT/fibrosis.

Other papers have investigated the role of Smad3 in relation to other pathways. Aoyagi-Ikeda *et al.* [37] showed that activation of the Notch pathway induces EMT *in vitro*, perhaps through phosphorylation of Smad3, and a TGF β receptor kinase inhibitor suppressed Notch-induced α -SMA expression. Van der Velden *et al.* [38, 39] followed up previous studies linking c-Jun N-terminal kinase 1 (JNK1) and TGF β in lung fibrosis and EMT. They showed that JNK1 directly interacts with Smad3 to phosphorylate the linker region, which enhances interactions between Smad3 and Smad4 and activates canonical TGF β signaling [40].

Two groups have pursued the role of ezrin/radixin/moesin (ERM) proteins in the rearrangement of actin filaments which leads to changes in cell morphology, adhesion and migration in EMT. Haynes *et al.* [41] showed in mammary epithelial cells that expression of moesin increases during TGF β -induced EMT, co-localizing with F-actin at cell protrusions. This increased expression led to morphological changes and actin filament remodeling. The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors. Expression of RAGE is high in the lung compared to other organs, and it has been suggested to have a protective role in lung fibrosis [42, 43]. Buckley *et al.* [44] showed in A549 cells that direct interaction between RAGE and ERM proteins is disrupted by inflammatory cytokines, resulting in phosphorylation of ERM and localization with adhesion receptor CD44 and actin stress fibers. Song *et al.* [45] showed that advanced glycation end products (AGE) decreased TGF β -induced Smad2 phosphorylation and EMT by increasing Smad7 expression. These papers show how ERM proteins and RAGE could be linked to EMT and fibrosis. Other recently reported potential mechanisms of EMT in lung epithelial cells include cadherin-11, miR-200 and sphingosine-1 phosphate [46–48].

Does EMT contribute to lung fibrosis *in vivo*?

We now turn to the controversy surrounding EMT in the context of lung fibrosis.

Evidence for EMT

There are two main reasons that EMT is considered to contribute to pulmonary fibrosis: results of lineage tracing studies in mouse models and co-expression of epithelial and mesenchymal markers in human lung samples with histologic manifestations of usual interstitial pneumonia (UIP), the pathologic correlate of IPF. Lineage tracing is a strategy used to identify progeny of a specific cell or a group of cells by permanently tagging them to express a reporter gene [49]. To show EMT, an enzyme with recombination capacity, usually Cre recombinase, is expressed in one mouse under control of a promoter that drives expression specifically in alveolar epithelium. When these mice are crossed with reporter mice, Cre activates expression of the reporter gene, usually green fluorescent protein (GFP) or β -galactosidase (β -gal), in cells originating from alveolar epithelium. To date, there have been seven lineage tracing studies evaluating the contribution of EMT to pulmonary fibrosis, with six in support of EMT (Table I) [17, 34, 50–53]. Degryse *et al.* [53], using the SP-C promoter to express Cre, labeled lung AEC with β -gal. After administration of bleomycin, confocal microscopy revealed that deletion of TGF β receptor 2 in lung epithelium resulted in fewer fibroblasts expressing β -gal and S100 calcium binding protein A4 (S100A4, also known as fibroblast-specific protein 1, or FSP1). However, α -SMA expression, a marker for myofibroblasts, was rarely seen in EMT-derived cells expressing β -gal, suggestive of EMT but not further transition to myofibroblasts. DeMaio *et al.* [17] utilized the Nkx2.1 promoter to generate mice in which AEC express GFP. Twenty-one days after administration of bleomycin, they co-localized GFP with mesenchymal markers collagen I, vimentin and α -SMA by confocal microscopy. Vimentin and α -SMA were expressed in approximately 4% of AEC-derived cells identified by fluorescence-activated cell sorting (FACS) analysis of GFP-expressing cells. These two recent studies reinforced previous lineage tracing studies indicating that EMT contributes to the pathogenesis of pulmonary fibrosis *in vivo*, at least in the bleomycin model.

Human IPF studies also support the significance of EMT in pulmonary fibrosis. Immunohistochemistry has revealed co-localization of epithelial and mesenchymal markers in the fibrotic lung [28, 50, 54]. Recently, Marmai *et al.* [55], in addition to immunohistochemistry, performed laser capture microdissection to isolate RNA from epithelial cells in the IPF lung. Quantitative reverse transcription real-time PCR showed expression of mesenchymal markers (e.g., calponin 1, α -SMA and collagen I) which was confirmed by FACS of the epithelial (E-cadherin-positive) and hematopoietic (CD45-negative) populations. Co-localization of epithelial and mesenchymal markers in fibrotic lung has also been shown in mouse models in which inhibition of the let-7 microRNA led to fibrosis [56]. Co-localization of SP-C and mesenchymal markers α -SMA and S100A4 was noted in approximately 40% of epithelial cells.

Evidence against EMT

Recent lineage tracing studies carried out in mouse models of kidney and liver fibrosis have failed to provide evidence of EMT, contradicting earlier studies [57–60]. Of note is the report by Humphreys *et al.* [57], in which perivascular fibroblasts (pericytes) were found to be the major source of myofibroblasts. Rock *et al.* [61] reported the first lineage tracing data that were negative for EMT in lung, finding that type II alveolar epithelial cells originally expressing SP-C did not convert to α -SMA-, S100A4- or vimentin-expressing cells after bleomycin challenge. Cells expressing Scgb1a1, a marker for bronchiolar Clara cells and possibly alveolar progenitor cells, also did not convert to mesenchymal cells, nor did pericyte-like cells expressing NG2. They inferred that the majority of myofibroblasts must derive from resident stromal cells.

Resolving the discrepancy

The recent study by Rock *et al.* [61] argues against a role for EMT as a direct source of collagen-producing (myo)fibroblasts in the lung. On the other hand, a number of other studies have shown evidence for EMT by lineage tracing studies or co-localization of epithelial and mesenchymal markers in human IPF lung. Several issues may account for these apparent discrepant results, some of which may also explain the widely varying results with regard to estimates of numbers of cells shown to undergo EMT in different studies: (1) precise evaluation with confocal microscopy cannot be performed with X-gal staining, hampering co-localization, and immunodetection with anti- β -gal antibodies may be difficult, making it preferable to use a fluorescent reporter [62]; (2) mesenchymal markers lack specificity, so that more than one mesenchymal marker should be evaluated concurrently; (3) resolution of immunohistochemistry and fluorescent microscopy are low and prone to artifact; confocal microscopy or evaluation of isolated cells is therefore necessary to show co-localization; (4) changes in morphology and migration across the basement membrane have not always been examined; presence or absence argues strongly for or against EMT, respectively; (5) the specific Cre driver used for recombination may affect results depending on extent and timing of labeling of epithelial cell populations; (6) the bleomycin model, while the most accepted model of IPF, is flawed; results of lineage tracing may be very different in different injury/fibrogenic models [52]; and, (7) humans and mice likely exhibit disparate lung cell biology.

Consideration should also be given to the possibility that EMT represents a spectrum of phenotypic changes in response to injury. Rather than undergoing full transition to *bona fide* (myo)fibroblasts, expression of mesenchymal characteristics may represent an attempt at wound repair and cell survival, since EMT has been shown to confer resistance to apoptosis [63]. These injured cells may therefore express mesenchymal markers or exhibit migratory capacity without necessarily becoming matrix-producing fibroblasts. Intrinsic to this concept would be the ability of these cells to revert back to an epithelial phenotype through the process of mesenchymal-epithelial transition (MET). In addition, significance of EMT to the fibrogenic process may go beyond direct generation of fibroblasts and may include indirect effects related to release of cytokines by injured epithelial cells, thereby creating a profibrogenic environment (Figure 1).

Conclusion

EMT is one of several proposed mechanisms through which fibroblasts and myofibroblasts are generated. Advances have been made towards elucidating causes and mechanisms of EMT in lung fibrosis, potentially leading to new treatment options. Although human IPF studies have clearly shown co-localization of epithelial and mesenchymal markers, the number of fibroblasts arising from epithelial cells was small in some lineage tracing studies, suggesting effects of EMT beyond direct phenotypic conversion to matrix-producing cells. Two other possible implications of EMT beyond direct conversion to a fully differentiated functional fibroblast phenotype are: (1) EMT may be part of a response to injury and an attempt to escape apoptosis and, if so, EMT may serve as a marker of epithelial injury, in which case inhibition of EMT may in fact have adverse consequences in IPF; and, (2) EMT may be involved in aberrant epithelial-mesenchymal interactions, and AEC expressing mesenchymal markers may release factors that promote a fibrogenic microenvironment. Both of these hypotheses await further evaluation.

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Key points (3 to 5 sentences that summarize the article)

- Potential candidates for IPF therapy have arisen through research on EMT, including galectin-3 inhibitor, IL-17A neutralizing antibody, tankyrase inhibitor, and ICG-001.
- Recent studies suggest EMT as a link between ER stress and lung fibrosis.
- β -catenin binds TGF β downstream protein Smad2 or Smad3 and other co-activators at the α -SMA promoter to induce EMT.
- One recent lineage tracing study failed to show EMT in a bleomycin model, in contrast to six other lineage tracing and human IPF studies that support EMT.
- Significance of EMT to the fibrogenic process may go beyond direct generation of fibroblasts and include an attempt to escape apoptosis in response to injury and/or release of cytokines to promote a fibrogenic microenvironment.

Figure 1. Causes and possible implications of EMT

Mutant proteins, protein trafficking defects, viral infections, and reactive oxygen species have been associated with alveolar epithelial cell injury and ER stress. There are several possible implications of EMT: (1) as a direct source of fibroblasts and myofibroblasts, (2) as an escape from apoptosis in response to injury or ER stress, and (3) as a source of profibrotic mediators that promote the proliferation of fibroblasts and the (trans)differentiation of fibroblasts to myofibroblasts.

Table 1

Summary of lineage tracing experiments to date in mouse lung fibrosis models

Reference	Promoter for Cre	Reporter	Injury model	Marker of mesenchymal phenotype	% of reporter and mesenchymal marker positive cells
Kim 2006 (50)	SP-C	β -gal	Adenoviral TGF β 1	α -SMA, vimentin	33% of vimentin (+) cells were β -gal (+)
Kim 2009 (34)	SP-C	GFP	Bleomycin	α -SMA, vimentin, collagen I	4–9% of mesenchymal marker (+) cells were GFP (+)
Tanjore 2009 (51)	SP-C	β -gal	Bleomycin	S100A4	33% of S100A4 (+) cells were β -gal (+)
Degryse 2010 (52)	SP-C	β -gal	Bleomycin (repetitive)	α -SMA S100A4	Rare α -SMA, β -gal double (+) cells 50% of S100A4 (+) cells were β -gal (+)
Degryse 2011 (53)	SP-C	β -gal	Bleomycin	S100A4	25% of S100A4 (+) cells were β -gal (+)
Rock 2011 (61)	SP-C	Tomato	Bleomycin	α -SMA, S100A4, vimentin	No mesenchymal marker (+) cells were Tomato (+)
DeMaio 2012 (17)	Nkx2.1	GFP	Bleomycin	α -SMA, vimentin	4% of α -SMA or vimentin (+) cells were GFP (+)