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Epithelial mesenchymal transition (EMT), a spectrum of states: role in lung development, homeostasis and disease

Running title: EMT in lung development, homeostasis and disease

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Abstract

Epithelial Mesenchymal Transition (EMT) plays key roles during lung development and many lung diseases such as Chronic Obstructive Pulmonary Disease (COPD), lung cancer and pulmonary fibrosis. Here, integrating morphological observations with underlying molecular mechanisms, we highlight the functional role of EMT in lung development and injury repair, and discuss how it can contribute to pathogenesis of chronic lung disease. We discuss the evidence of manifestation of EMT and its potential driving role in COPD, idiopathic pulmonary fibrosis (IPF), bronchiolitis obliterans syndrome (BOS), and lung cancer, while noting that all cells need not display a full EMT in any of these contexts, i.e. often cells co-express epithelial and mesenchymal markers but do not fully convert to extracellular matrix-producing fibroblasts. Finally, we discuss recent therapeutic attempts to restrict EMT in chronic lung disease.

What is epithelial to mesenchymal transition (EMT)?

EMT is a biological process in which epithelial cells lose their traits of cell-cell adhesion and apico-basal polarity, and gain some mesenchymal traits of migration, invasion, and producing extracellular matrix (ECM) components (Kalluri and Weinberg, 2009). These traits often lead to degradation of underlying basement membrane; thus, the fragmented anatomical changes in basement membrane has been observed to be associated with EMT and now considered as a key hallmark of the process (Kalluri and Weinberg, 2009). An often noted hallmark of EMT is the loss of epithelial cell-cell adhesion molecule CDH1 (E-cadherin), and/or a concomitant gain of mesenchymal markers such as CDH2 (N-cadherin), VIM (Vimentin), and/or αSMA (alpha-smooth muscle actin) (Nieto et al., 2016).

EMT was initially described in 1982 as an ‘epithelial mesenchymal transformation’. But, with a better molecular and functional characterization, the term ‘transformation’ has been replaced with ‘transition’ to reflect its reversible nature as observed during embryogenesis (type I EMT), wound healing and fibrosis (type II EMT) and cancer metastasis (type III EMT) (Jolly et al., 2015). Recent investigations in all these contexts have underscored that EMT and its reverse process MET (Mesenchymal to Epithelial Transition) are not binary, i.e. ‘all-or-none’ processes (Nieto et al., 2016). Instead, cells can display a spectrum of hybrid

states ranging from being fully epithelial to fully mesenchymal (Jolly et al., 2015), and a hybrid epithelial/ mesenchymal (E/M) state may be stable phenotype that cells can display, unless forced out from that state due to external factors (Figure 1). Such hybrid state(s) may enable amalgamated epithelial and mesenchymal traits such as adhesion and migration leading to collective cell migration. Cells leading this form of collective migration tend to display mesenchymal features transiently, while still exhibiting cell-cell contact with follower cells, as noted during wound healing, carcinoma dissemination, branching morphogenesis etc. (Revenu and Gilmour, 2009; Nieto et al., 2016). Given the pathological and physiological significance of hybrid E/M phenotype, its functional implications in all these contexts necessitate a detailed investigation.

EMT is known to drive key steps in multiple stages of embryonic development, - gastrulation, neural crest migration, and heart development. Genetic studies have identified many signaling pathways that can induce EMT - such as transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF), and nuclear factor-kB (NF-kB), and Wnt (Nieto et al., 2016). These pathways can activate one or more EMT-driving transcription factors (EMT-TFs) such as SNAIL1, SNAIL2 (SLUG), TWIST1, ZEB1, ZEB2 (SIP1), PRRX1 and Goosecoid – each of which can directly or indirectly repress the cell-cell adhesion molecule E-cadherin (CDH1) – the hallmark of an epithelial phenotype (De Craene and Berx, 2013). On the other hand, signaling pathways such as retinoic acid (RA) (Biddle et al., 2016) that oppose TGF-β during lung development (Chen et al., 2007) can inhibit EMT.

The well-established role of EMT in embryonic development and wound healing has driven investigations into its functional implications in carcinoma initiation and dissemination, and tissue fibrosis (Kalluri and Weinberg, 2009). Besides, EMT can contribute significantly to chemoresistance (Fischer et al., 2015; Zheng et al., 2015). We recently reported that hybrid E/M states, or equivalently, a partial EMT could be potentially more aggressive than a full EMT, and (Jolly et al., 2015). EMT and MET are considered to be reversible, however, reversibility may depend on accompanying epigenetic modifications (Somarelli et al., 2016).

In this review, we discuss the role of EMT in both lung development and lung diseases, highlight that EMT need not be a binary process in multiple contexts, and comment on EMT as a potential therapeutic target.

Lung histology and anatomy

The respiratory system supplies oxygen to cells all over the human body and disposes them of carbon-dioxide, a respiratory waste product. Functionally a single unit, it connects the external environment with the internal extensively laid-out thin alveolar surface where gas exchange occurs. Thus, it coordinates breathing, transport of gases, gas exchange in alveoli, and also internal cellular respiration. This system is comprised of conducting airways that begins from the nose and ends at lung alveoli, following the path from nose to pharynx and larynx to trachea, bronchi, and bronchioles, and finally the alveoli (Scanlon and Sanders, 2007).

The respiratory system constitutes upper respiratory tract (nasal air passages, nasal cavities, pharynx, larynx, and trachea) and lower respiratory tracts (trachea, branched lung bronchial tree (large airways), highly branched bronchioles (<2 mm in diameter, i.e. small airways), and finally the clusters of alveoli) (Kerr, 2010). The mucosa of the upper respiratory tract consists of an outer ciliated epithelium lining, dense connective tissue lamina, a large smooth muscle bundle area, a continuous basement membrane, supporting cartilage structure, and intertwined goblet cells (mucus secretory cells). The cartilage, goblet cells, smooth muscles and the connective tissue gradually diminish as the respiratory system approach the tiny bronchioles, and are completely absent in the alveolus (Figure 2). Ciliated epithelial cells persist in the bronchioles, while goblet cells are replaced by non-ciliated dome shaped clara cells, present intermittently along the bronchiole epithelial lining. The clara cells carry out the important function of secreting surfactant proteins (Kerr, 2010).

The connective tissue across the airway wall mucosal lamina have many resident interstitial cells, mainly comprised of fibroblasts. Fibroblasts produces extracellular matrix protein (ECM) such as fibers, collagen fibrils and varieties of proteoglycans, which are vital to maintain lung elasticity and their mechanical function (Burgess et al., 2016). Thus, there lies a distinct variability in structural and cellular composition across the lung that may have implications in various lung pathologies. For instance, thickening of reticular basement membrane – one of the two structural layers of the bronchial epithelial basement membrane – is usually observed in multiple pathological conditions such as chronic obstructive pulmonary

disease (COPD) and asthma (Figure 2), as discussed in detail later in this review. Also, in COPD, the basement membrane becomes fragmented as a result of EMT.

EMT in lung development

The first instance of EMT during embryonic development occurs during gastrulation when initially the mesenchyme is formed. Thereafter, multiple rounds of EMT and MET are often invoked in formation of various organs (Nieto et al., 2016). Particularly, during branching morphogenesis – a hallmark of organ development such as lung, kidney and mammary gland, where a tubular epithelial structure splits repeatedly to generate a ductal tree, and the tip cells located at the front – also referred to as terminal end buds (TEBs) – display a partial EMT phenotype (Revenu and Gilmour, 2009). This process requires bidirectional communication between the epithelium and mesenchyme, mediated by multiple signaling pathways such as TGF-β /bone morphogenetic protein (BMP), Sonic hedhehog (Shh), retinoic acid (RA), FGF or Wnt (Bartis et al., 2014). During branching morphogenesis, TEB cells maintain cell-cell adhesion with their neighbours, and transiently acquire mesenchymal traits such as altered cell polarity and enhanced migration in response to extracellular signals. Consequently, these TEB cells state migrate collectively forming finger-like projections and are prevented from undergoing a full EMT by the action of 'molecular brakes on EMT' such as the transcription factors GRHL2 and its downstream target OVOL2 (Watanabe et al., 2014; Aue et al., 2015; Walentin et al., 2015).

GRHL2 is a transcription factor that exclusively mediates the development of both the airways and alveolar epithelium at all stages, but is absent in lung mesenchyme (Varma et al., 2012). GRHL2-knockout phenotype is lethal at the beginning of lung development, underlining its key role in lung epithelium morphogenesis (Rifat et al., 2010). In lung epithelium, GRHL2 activates the transcription of a key lung transcription factor *Nkx2-1*, and that of E-cadherin (*Cdh1*) (Varma et al., 2012). Nkx2-1 can also reciprocally activate GRHL2, and both these transcription factors are among the top 25 activators of E-cadherin (Shimamura et al., 2011). Thus, this GRHL2/NKX2-1 feedback loop forms a central axis that contributes to preserving an epithelial phenotype (Varma et al., 2012). Besides, GRHL2 and its transcriptional target OVOL2 can regulate epithelium lumen formation via activating *Rab25*, *Cldn4* (claudin 4) and *Cldn3* (claudin 3) (Senga et al., 2012). Similar roles for GRHL2 have been mentioned in renal epithelium development (Aue *et al.*, 2015). Such feed-forward loop formed by GRHL2, OVOL2, and their targets typically stabilize the target gene

expression, thus emphasizing the role of GRHL2 in inducing and maintaining an epithelial identity (Mangan and Alon, 2003). Furthermore, GRHL2 is required for establishing human mucociliary airway epithelium (Gao et al., 2013). GRHL2 point mutations lead to many developmental defects such as reduction of lung size, and hindered lung inflation at birth, potentially due to reduced and/or mislocalized E-cadherin (Pyrgaki et al., 2011). In a nutshell, GRHL2 regulates a large repertoire of genes driving epithelial morphogenesis, intercellular adhesion, and cell motility in lung development, and may play similar roles in development of many other epithelial tissues (Werth et al., 2010).

Besides its central role in orchestrating lung epithelium development, GRHL2 also regulates the expression of hTERT (human Telomerase Reverse Transcriptase) (Chen et al., 2010). Given that genetic mutations in hTERT may predispose for idiopathic pulmonary fibrosis (IPF) (Tsakiri et al., 2007; Alder et al., 2008), GRHL2 may be dysregulated in IPF progression. Consistent with its role in lung development, GRHL2 can (a) inhibit EMT-inducing transcription factor ZEB1 directly, and is inhibited during TGFβ-mediated EMT (Cieply et al., 2012; Cieply et al., 2013; Xiang et al., 2017), (b) induce epithelial gene expression signature during cancer progression (Xiang et al., 2012), and (c) maintain collective migration in non-small-cell lung cancer (NSCLC) H1975 cells that display a stable hybrid E/M phenotype (Jolly et al., 2016).

EMT in lung injury repair

Lung homeostasis after injury depends on efficient recovery of an intact lung epithelium. In the airways, acute injury response on a timescale of 12-24 hours is cell spreading and migration, while proliferation of progenitor cells picks up later and may continue for weeks (Crosby and Waters, 2010). TGF-β, a potent EMT inducer, has been suggested to stimulate airway epithelial repair by inducing cell migration (Yu et al., 2008). These migratory cells that spread over large surfaces to provide an intact lining transiently gain mesenchymal features atleast partially (Vaughan and Chapman, 2013). Consistently, in a recent study of repair after napthalene-induced airway epithelium injury, SNAIL1 expression was induced, leading to a transient increase in Vimentin and αSMA (alpha-smooth muscle actin) that contributes to regeneration of intact epithelial barriers (Volckaert et al., 2011).

EMT in chronic lung disease

EMT is well described in lung embryogenesis (Lee et al., 2006), metastatic malignant disease (Bjornland et al., 1999), but it has only recently been recognised in the chronic human lung and airway diseases (Ward et al., 2005a; Willis et al., 2006; Hodge et al., 2009; Sohal et al., 2010a; Sohal et al., 2011; Sohal and Walters, 2013b; Sohal and Walters, 2013a; Ojo et al., 2014; Jonsdottir et al., 2015; Mahmood et al., 2015; Mahmood et al., 2017a). In the sections below, we will review the current literature on EMT in chronic lung disease.

Chronic obstructive pulmonary disease (COPD)

COPD is a diseased condition of inflamed lung and airways that leads to shortness of breath; this condition is progressive and irreversible, and mainly caused by smoking, (Sohal et al., 2013b). however, other factors such as biomass fuel consumption has also been reported as major cause of COPD in countries like China and India (Kurmi et al., 2012). The prime pathology in COPD pertains to small airway fibrosis that leads to disrupted airway function (Sohal et al., 2013a). Small airway destruction occurs quite early in the disease and also often correlates with impaired lung tissue involved in gaseous exchange (a process called emphysema). The other closely associated pathology with COPD is the development of airway epithelial cancer, predominantly in large airways (i.e. squamous cell carcinomas), although adenocarcinomas are also seen (Sohal, 2015). One potential mechanism which might be central to these pathologies is the process of EMT (Sohal et al., 2014a; Sohal, 2015).

EMT has been recently reported to be an active process in the airways of smokers and COPD patients. Several other groups have since confirmed these findings (Gohy et al., 2015; Milara et al., 2013). The reticular basement membrane (Rbm) in both small and large airways is often highly fragmented with many clefts (fissures or vertical indentations in the tissue) evident, which itself is a structural hallmark of active EMT (Sohal et al., 2010a; Soltani et al., 2010; Sohal et al., 2011; Sohal and Walters, 2013a; Sohal and Walters, 2013b). Airway epithelium and cells within these clefts stained positive for markers of EMT and were found to be related to decrease in lung function and the smoking history. Co-expression of an epithelial marker cytokeratin and EMT marker S100A4 during COPD (Sohal et al., 2011) was indicative of cells undergoing partial EMT. A recent study furthered this notion and showed an increase in EMT-TFs and mesenchymal markers in human bronchial epithelial cells (HBEs) derived from COPD patients. Cultured media from human COPD lung fibroblasts induced a partial EMT in HBEs, suggesting that interactions between resident

fibroblasts and epithelial cells may be critical in driving EMT in COPD (Nishioka et al., 2015).

To further contextualize EMT in smokers and COPD patients, we stained endobronchial biopsies and resected small airway tissue for vessel markers. We found large airway Rbm to be hyper-vascular whereas small airway to be hypo-vascular (Sohal and Walters, 2013d; Sohal and Walters, 2013c; Sohal et al., 2014a; Mahmood et al., 2015). Further, vessels were also observed penetrating into the epithelium in large airways. Active EMT associated with increased angiogenesis has been regarded as a hallmark of early growth of primary epithelial cancers (Kalluri and Weinberg, 2009). These observations fit well with the underlying pathology of COPD, i.e. cancer formation, especially squamous cell carcinoma, which is common in large airways (Sohal et al., 2014a; Sohal, 2015; Eapen et al., 2016).

As mentioned earlier, we have also found active EMT in adenocarcinomas in smokers but the orgin of adenocarcinomas is debatable. Since small airway Rbm is devoid of active angeiogensis it is quite possible that adenocarcimomas might be orginating from type-II pneumocyte, but this warrants further work. We also recently reported transcriptional control of EMT in COPD, describing active Smad signalling in the airway. However, no significant correlation was observed for TGF-β1 expression and pSmad or EMT markers such as S100A4, suggesting that factors other than or in addition to TGF-β1 are also involved in modulating EMT during COPD (Mahmood et al., 2017a).

Lung cancer and COPD share a common etiology i.e. tobacco smoking. Further, presence of COPD can increase the risk of developing of lung cancer by 4-5 folds, even when the smoking history is controlled for (Parimon et al., 2007a), implying that mechanisms specific to COPD may be involved in development of lung cancer (Petty, 2005; Kiri et al., 2009; Kiri, 2010). Pathologically, COPD and lung cancer share many biological mechanisms, including chronic inflammation, ECM disruption, cell proliferation, abnormal wound repair and angiogenesis, and EMT (Yang et al., 2011; Sohal, 2017). EMT can play crucial roles in the pathogenesis of epithelial cancers (Tarin et al., 2005; Thompson et al., 2005); several key pathways driving EMT during embryogenesis get aberrantly activated in cancer. Very recently, we reported active EMT in the leading edge of invasive non-small cell lung cancer (NSCLC), both squamous cell and adenocarcinoma cell subtypes; the extent of EMT was strongly correlated with the aggressive behaviour of the tumor (Mahmood et al., 2017b).

Furthermore, EMT activity within the tumours closely correlated with EMT activity in non-tumour–affected airway wall epithelium, suggesting that the level of EMT activity in the airway wall, even in large airways that are amenable to bronchoscopic biopsy, can potentially be used as a marker for smokers most likely to develop both COPD and lung cancer. These observations are clearly suggestive of EMT as a potential link between COPD and lung cancer (Sohal, 2015; Mahmood et al., 2017b; Sohal, 2017).

The other prime pathology associated with COPD is small airway fibrosis and obliteration, and this could potentially be related to active Type-II EMT, as it does not involve angiogenesis usually (Mahmood et al., 2015). It is of interest and relevance in this context that over 90% of human cancer arises in epithelia, and the involvement of EMT in all of these may be central, specially in Type-III EMT that typically associates with angiogenesis (Garber, 2008; Barnes and Adcock, 2011; de Torres et al., 2011). COPD-related cancer may well be just another example of this core principle of unstable epithelium in the context of tissue inflammation and/or chronic stimulation (Nowrin et al., 2014; Eapen et al., 2016). Due to increased vascularity of the Rbm, it is also possible that the process of endothelial to mesenchymal transition (EndMT) – an analogous process to EMT – is also active in smokers and COPD (Sohal, 2016). EndMT has been reported to be crucial during fibrosis (Zeisberg et al., 2008), and similar to EMT (Jia et al., 2015; Jolly et al., 2015; Grigore et al., 2016; Nieto et al., 2016), EndMT need not be a binary process (Welch-Reardon et al., 2015), therefore fibrosis progression might involve multiple stages of EMT and EndMT.

Lung cancer

In cancer, induction of a partial or full EMT has been associated with enhanced tumor-initiation potential (Mani et al., 2008; Morel et al., 2008; Jolly et al., 2014), resistance against multiple therapies (Singh and Settleman, 2010), immune-evasion (Tripathi et al., 2016), altered metabolism (Dong et al., 2013), and genomic instability (Comaills et al., 2016), thus suggesting implications of EMT in multiple hallmarks of cancer. EMT leading to single-cell dissemination has been long considered to be a key driver of the metastasis-invasion cascade. However, recent studies have questioned the indispensability of EMT in metastasis, but strengthened its proposed role of EMT in chemoresistance (Fischer et al., 2015; Zheng et al., 2015).

Particularly in NSCLC, EMT phenotype correlates with drug resistance, mutations of EGFR (epidermal growth factor receptor), and formation of Cancer Stem Cells (CSCs) characterized

by an enriched stem cell signature and a heightened tumorigenic potential (Bartis et al., 2014). EMT can drive resistance to multi-targeted anti-folate (MTA) chemotherapy (Liang et al., 2015) as well as to EGFR inhibition (Thomson et al., 2005); NSCLC lines in a mesenchymal state were insensitive to growth arrest effect of EGFR inhibition both *in vitro* and in xenografts (Thomson et al., 2005). Further, the gefitinib-resistant subline of A549 – A549/GR – exhibited a spindle-shape morphology and higher levels of mesenchymal marker vimentin with concomitant decrease in CDH1, suggesting an EMT (Rho et al., 2009). Also, activation of TGF-β signaling in NSCLC cells can induce EMT and render cells insensitive to erlonitib; and erlotinib-resistant 'mesenchymal-like' cells are already present in cell lines and tumors prior to erlotinib treatment, indicating intratumoral heterogeneity (Yao et al., 2010). Finally, EMT can drive evasion against T-cell mediated immunotherapy; mesenchymal NSCLC cells display low levels of immunoproteasome and inhibit the production of relevant antigens for CD8+ T-cell presentation (Tripathi et al., 2016). Not surprisingly, immunoproteasome deficiency is associated with poor prognosis in NSCLC (Tripathi et al., 2016). Thus, EMT lies at the nexus of resistance against multiple therapies.

Also, induction of EMT via Dickkopf-1 (DKK-1) in NSCLC – a proposed biomarker for lung cancer (Yamabuki et al., 2007) – can drive vasculogenic mimicry (Yao et al., 2016) that may accelerate tumor aggressiveness by enabling direct access of blood vessels to cancer cells. Consistently, miR-206 that can inhibit HGF-induced EMT in NSCLC cells and reduce the migration and tube formation of human endothelial vascular cells (HUVECs) compromised metastasis and angiogenesis of lung cancer *in vivo* (Chen et al., 2016). Furthermore, TGF-β induced EMT in A549 cells can shift the metabolism towards more oxidative phosphorylation that can generate sufficient ATP needed for metastasis (Jiang et al., 2015). Therefore, EMT activation can modulate aggressiveness of NSCLC cells via multiple routes.

Proteomic and morphological analysis of a panel of NSCLC cell lines reveal that similar to developmental EMT, EMT in lung cancer need not be an 'all-or-none' response; instead, many cells lines can display a hybrid E/M status (Schliekelman et al., 2015). These hybrid cell lines can contain both individual cells co-expressing epithelial and mesenchymal markers (H1975), and sub-populations of epithelial and mesenchymal cells (H2291) (Jolly et al., 2016). Similarly, co-existence of subpopulations displaying epithelial, mesenchymal and hybrid E/M phenotypes was observed in multiple cell lines (i.e. isogenic populations) – A549, LT473, and H460 – in different ratios (Andriani et al., 2016), thereby indicating a role

of non-genetic heterogeneity in EMT decision-making (Lu et al., 2013; Mooney et al., 2016). Furthermore, in a cohort of 60 NSCLC patients, those displaying a balanced co-expression of CDH1 and SLUG (an EMT-TF) showed a significantly reduced survival, but neither of these markers alone was predictive of outcome (Andriani et al., 2016). These results strongly suggest the enhanced aggressive nature of a hybrid E/M phenotype as compared to the cells locked in a fully mesenchymal state. Consistently, the expression of GRHL2 – a 'phenotypic stability factor' that can maintain cells in a hybrid E/M phenotype – correlates with poor survival, independent of the breast cancer subtype (Mooney et al., 2017).

A detailed comparison of multiple traits of hybrid E/M cells vs. fully mesenchymal cells still remains to be accomplished, but initial evidence in breast cancer indicates that cells co-expressing an epithelial marker (CD24) and a mesenchymal marker (CD44), instead of those expressing a mesenchymal marker only, may manifest an adaptive drug-tolerant phenotype (Goldman et al., 2015; Boareto et al., 2016). These cells can possess enhanced tumor-initiation potential both *in vitro* and *in vivo* as compared to canonically regarded CSCs in breast cancer – CD44+/CD24- (Goldman et al., 2015; Grosse-Wilde et al., 2015). Thus, further analysis of EMT in lung cancer both in the context of metastasis and drug resistance needs a careful and more nuanced classification regarding EMT status.

Asthma

Airway remodeling and thickening of basement membrane via collagen deposition has been consistently reported in asthma (Bergeron et al., 2010), but the exact source of this extra collagen deposition remains unclear. EMT has been proposed to be the source of fibroblasts that can deposit collagen and thus contribute to airway remodeling in asthma (Hackett, 2012). However, other major criteria for recognising EMT *in vivo* – Rbm fragmentation, accompanied by cell migration and expression of mesenchymal markers (Zeisberg and Neilson, 2009) – are typically not observed in asthma (Sohal et al., 2010b; Soltani et al., 2012). In asthma, Rbm is thickened, but shows no sign of fragmentation, hypercellularity or hypervascularity**.** Hence, unlike in COPD, lung airways in adult asthma give no suggestion of an active EMT. However, published histopathology micrographs from asthmatic children are suggestive of active EMT (Jenkins et al., 2003), and these neglected observations deserve follow up.

Biochemical changes typically accompanying EMT can be induced in epithelial cell cultures, but such changes can be 'metastable', i.e. present only for a short period of time. Those changes do not necessarily reflect EMT rather a process called "reversible scatter", where cells assume a spindle-like shape following cytokine stimulation, but the transcriptional changes are not sustainable; thus, upon withdrawal of the inducer, the epithelium returns to its original state (Kalluri and Neilson, 2003). Such response has been observed for alveolar epithelial cells (AECs) in culture upon TGF-β stimulation, but the asthmatic epithelium *in vivo* showed no empirical sign of EMT (Hackett et al., 2009). We believe that the current suggestions about EMT in the context of asthma are controversial and mainly based on molecular and cell culture techniques findings (Bartis et al., 2014), and the asthmatic tissue does not show the core structural hallmarks of EMT (Sohal et al., 2010b).

Idiopathic pulmonary fibrosis (IPF)

IPF is a devastating disease in which lung tissue becomes scarred (the condition of fibrosis) over time, thus obstructing the flow of oxygen from lungs into the bloodstream for distribution to other organs, and difficulty in breathing. With very limited treatment options and poorly understood underlying causes, IPF has a median survival of 3 years after diagnosis (Rudd et al., 2007). It is histopathologically characterized by heterogeneously distributed areas with progressive scarring in basal and lateral parts of the lung (King et al., 2011). A hallmark for these scarred areas is the presence of distinct structures called fibroblast foci (FF) – collections of fibroblasts/myofibroblasts that produce ECM actively (King et al., 2011). Present at the boundary of normal and fibrotic tissue, these FFs denote the leading edge for the propagation of the tissue remodelling or scarring (Bagnato and Harari, 2015).

The source of myofibroblasts in FFs has been debated over the years, and they have been proposed to from resident tissue fibroblasts, bone marrow-derived progenitor cells (so-called fibrocytes), or from AECs that have undergone EMT (King et al., 2011). The proposition that AEC may be a source for fibroblasts/myofibroblasts in FFs has been supported by studies showing that AECs that circumscribe FFs express both mesenchymal and epithelial markers (Willis et al., 2005; Yamaguchi et al., 2016). Consistent with this notion, AECs isolated by laser microdissection from IPF patients expressed mesenchymal markers, such as collagen-1 (Marmai et al., 2011). Furthermore, *in vivo* lineage tracing experiments in mouse models of pulmonary fibrosis have demonstrated that cells expressing mesenchymal markers had

epithelial origin, including AECs (Kim et al., 2006; Kim et al., 2009; Rock et al., 2011; DeMaio et al., 2012).

However, the presence of these cells co-expressing epithelial and mesenchymal markers denotes an incomplete transition, and not necessarily a complete transition of an epithelial cell into a ECM-producing myofibroblast. Similar to these reports of incomplete transition, cells in alveolar epithelium of IPF lungs co-expressed epithelial marker GRHL2 with EMT markers VIM or ZEB1 (Varma et al., 2014), indicating a hybrid E/M phenotype. Similarly, bronchiolar basal metaplastic cells in IPF patients lose E-cadherin expression only partially (Morbini et al., 2011). Therefore, in most IPF studies, an EMT process was not observed to be fully completed. These observations concur with partial EMT observed in fibrosis of tubular epithelial cells of the mice. These cells do not completely convert to interstitial fibroblasts, but stay within the tubule and contribute to interstitial fibrosis, inflammation, and recruitment of immune cells (Lovisa et al., 2016).

Several potential triggers can activate EMT in IPF. In 1980s, epithelial injury alone was shown to be able to trigger fibrogenic processes (Adamson et al., 1988). Many subsequent studies that have identified markers for epithelial injury and remodelling in IPF (Kuwano et al., 1996; Uhal et al., 1998) have commonly observed increased number of apoptotic/necrotic cells in alveolar epithelium, and stress markers – such as those for ER stress and for unfolded protein response (UPR) – in the surviving cells (Tanjore et al., 2012). AECs from a subset of individuals with familial pulmonary fibrosis (a scenario in which IPF occurs in many members of the same family) that are predisposed to develop the disease have been reported to be positive for ER stress and UPR markers (Mulugeta et al., 2005). These individuals also carry mutation in proteins that are exclusively expressed by alveolar type cells, such as surfactant protein C, surfactant protein A2 (Nogee et al., 2001; Wang et al., 2009), suggesting that ER stress caused by the mutations might contribute to disease development, by potentially inducing EMT in AECs, as shown *in vitro* (Tanjore et al., 2011; Zhong et al., 2011).

Besides ER stress, TGF-β_1 is an important player in progression of fibrosis in many organs including the lung. TGF-β_1 can induce both EMT with concomitant increase in α-SMA expression and collagen-1 production in isolated AECs (Kalluri and Neilson, 2003), and has

the ability to differentiate fibroblasts into α-SMA-positive myofibroblasts that produce ECM in an exaggerated manner (Hu et al., 2003). Thus, overexpression of TGF-β_1 may induce a prominent fibrotic response. Consistently, TGF-β_1 blocking antibodies attenuate the fibrotic response in bleomycin-treated mice (Sime et al., 1997; Bonniaud et al., 2005). Increased staining of TGF-β_1 has been reported in the alveolar epithelium in patients with IPF. Alveolar epithelium may both produced and activate TGF-β_1 due to increased levels of integrin αvβ6 (Munger et al., 1999) that can release and activate sequestered TGF-β_1 in the ECM (Annes et al., 2003). Increased active TGF-β_1 in the alveolar epithelium may stimulate repair responses such as EMT or differentiation of sub-epithelial fibroblasts.

In summary, evidence for active EMT in IPF suggests it may play a role in the pathogenesis. To further understand the impact of EMT in IPF, it remains to be elucidated if EMT-derived cells can undergo a full transition into ECM-producing fibroblasts/ myofibroblasts *in vivo*.

Bronchiolitis obliterans syndrome (BOS)

Lung transplantation is an effective therapeutic option for carefully selected patients with advanced lung disease. Long-term survival after transplantation is, however, limited by chronic lung allograft dysfunction (CLAD) that prevents transplanted lung from functioning properly. CLAD most commonly manifests itself as Bronchiolotis Obliterans Syndrome (BOS) - defined by a sustained reduction in the forced expiratory volume in first second of expiration (FEV_1). BOS may be observed in 50% of recipients within five years post-transplant, and is associated with morbidity and mortality (Royer et al., 2016). Early identification of BOS, thus, represents a key goal for all transplant centres and new therapies are required.

The pathophysiology of BOS involves inflammation, fibroblast proliferation and ECM deposition in the small airways. We have previously shown that BOS is accompanied by airway neutrophilia (an increase in neutrophilic lymphocytes) in both airway biopsies and via bronchoalveolar lavage (BAL) – a medical technique by which cells and fluid from the broncho-alveolar airspace are isolated for disease diagnosis (Snell et al., 1997; Ward et al., 1997; Zheng et al., 1997; Ward et al., 1998; Zheng et al., 2000; Whitford et al., 2001). Airway neutrophilia has been consistently reported in BOS by other transplant groups, despite the variation in patient management and sampling from the different centres (Royer et al., 2016). Neutrophils are a key producer of MMP-9, a type IV collagenase associated with

cell invasion and basement membrane damage. Thus, EMT may be exhibited in human airways in BOS (Ward et al., 2005b), as recently substantiated by an observed increase in three EMT markers - αSMA, S100A4, and EDA-FN (a splice variant of fibronectin) - by flow cytometry of brochial epithelial cells in BOS patients (Hodge et al., 2009). Intriguingly, these epithelial cells did not lose entirely the expression of epithelial cell antigen, thereby indicating a possibility of a hybrid E/M phenotype in BOS (Hodge et al., 2009). These observations are reminiscent of the first description of EMT in the human airway in a study of lung allograft patients that suggested EMT as an important process in BOS and pathophysiology of other common airway diseases (Ward et al., 2005a) , where 15% of cells in the airway epithelium in these biopsy samples co-stained for S100A4.

Signalling pathways usually associated with EMT have also been observed to be active in BOS. We have shown that epithelial released alarmins (molecules released from damaged tissue to induce an immune response), TGF-β and SMAD signalling and infection can drive EMT in the lung allograft airway (Parker et al., 2008; Borthwick et al., 2009; Borthwick et al., 2010; Borthwick et al., 2011; Gardner et al., 2012; Suwara et al., 2014). Furthermore, we observed that members of miRNA200 family – a crucial brake on EMT (Park et al., 2008; Schliekelman et al., 2011; Lu et al., 2014; Huang et al., 2015; Sundararajan et al., 2015; Somarelli et al., 2016) – are involved in a TGF-β driven EMT in BEAS2-B cells (normal primary epithelial cells) and primary airway cultures established from allograft brushings (Ladak et al., 2014; Ladak et al., 2016a; Ladak et al., 2016b). Consistent observations have been made by other groups. For instance, miR-21, a potential activator of EMT and CSC-like state (Han et al., 2012), was found to be overexpressed specifically in fibroblasts and in activated epithelial cells in all human BOS cases and in rat grafts, whereas it was absent in normal human and rat lungs (Di Carlo et al., 2016). Similarly, (a) miR-323a-3p that can attenuate TGF-β signaling by directly targeting SMAD2, was downregulated in epithelium of lungs with BOS after lung transplant (Ge et al., 2016); and (b) miR-144, a potential amplifier of TGF-β signaling, was overexpressed in lungs with BOS (Xu et al., 2015). With clinical trials showing promising results for miRNA leading to successful treatment of hepatitis, the abovementioned findings indicate that a greater understanding of the molecular pathophysiology of EMT may have therapeutic potential for both CLAD and BOS, and potentially other airway and lung diseases too.

EMT as a therapeutic target

The current pharmaceuticals available to treat COPD does not really alter disease progression, at least when given late in the disease's natural history as per the current convention. However, emerging evidence points that some of these drugs may affect the underlying process of EMT and therefore they may be more beneficial if given earlier. For example, Roflumilast™ (an inhibitor of phosphodiesterase-4 (PDE4) that elevates secondary signalling molecule cyclic adenosine monophosphate (cAMP)) has been shown to block cigarette smoke-induced EMT in bronchial epithelial cells (Milara et al., 2014). Inhaled corticosteroids (ICS) have been reported to protect COPD patients against lung cancer (Parimon et al., 2007) and we have reported that inhaling the corticosteroid fluticasone propionate suppresses EMT in COPD patients on six months treatment (Sohal et al., 2014b). This first study reporting anti-EMT effects of ICS in COPD demonstrated marked reduction in EGFR, Rbm fragmentation, S100A4 and MMP-9 expression in the active arm compared to placebo (Sohal et al., 2014b), but Rbm hyper-vascularity remained unchanged (Soltani et al., 2016). It is quite possible that a longer treatment by ICS may also affect Rbm hyper-vascularity. This anti-EMT effect of ICS may contribute, at least in part, to the effect that patients on ICS are associated with a significant (50%) reduction in the risk of lung cancer (Parimon et al., 2007b; Kiri et al., 2009; Kiri, 2010).

Epigenetic inhibition of EMT has also been suggested, for instance, by sorafenib in A549 lung adenocarcinoma cell line via an increase in histone acetyltransferase (HAT) expression and consequent decrease in histone deacetylase (HDAC) (Steiling et al., 2013). Similar effects of sorafenib are observed in liver (Chen et al., 2011) and urothelial carcinoma *in situ* (Steinestel et al., 2013) by targeting STAT3 and urokinase plasminogen activator (uPA). Targeted silencing of uPAR (uPA-receptor) using small hairpin RNA (shRNA) inhibited EMT in cultured human small airway epithelial cells (Wang et al., 2015). Camara *et al* suggested another potential therapeutic target for EMT - TGF-β1/Smad2/3 (Camara and Jarai, 2010), which we found to be active and associated with EMT in smokers and COPD patients (Mahmood et al., 2017a). Yang *et al.* further demonstrated that crosstalk between muscarinic acetylcholine receptor (mAChR) and TGF-β1 can induce EMT in lung epithelial cells (A549) suggesting a role of non-neuronal cholinergic system in EMT and a potential novel therapeutic target for EMT (Yang et al., 2014).

Similar to COPD, two drugs have recently been introduced in IPF which significantly decrease the rate of decline in lung function due to fibrosis (Brusselle and Bracke, 2014;

Richeldi et al., 2014). Nintedanib is a tyrosine-kinase inhibitor and pirfenidone mainly inhibits TGF-β1 agent. These drugs affect the key pathways implicated in EMT - Smad and β-catenin (Hostettler et al., 2014; Wollin et al., 2015; Cholankeril et al., 2016; Knuppel et al., 2017). Nintedanib also inhibits angiogenesis, thus it may have implications for Type III EMT (pro-fibrosis and pro-cancer), while Pirfenidone is likely to be more specific to Type II EMT due to its anti-fibrotic action.

Another putative anti-EMT agent is azithromycin (Banerjee et al., 2012), but part of its anti-inflammatory properties can also be attributed to its action on macrophages (Banjanac et al., 2012). Effects of azithromycin on epithelial cells seems to include modulation of transcription factors, ER stress, lysosomal accumulation, and excessive autophagy (Parnham et al., 2014). Similarly, other commonly used drugs for cardiovascular risk management that are not usually considered as respiratory treatment, but may yield long term fortuitous beneficial effects in COPD through mechanisms discussed earlier are statins (Yang et al., 2013) and angiotensin-converting enzyme (ACE)-inhibitors (Qian et al., 2013). Indeed, atorvastatin can partially inhibit TGF-β1-driven EMT in small cell lung cancer cells (Fan et al., 2016), and ACE has also been implicated in EMT-driven metastasis in lung cancer (Qian et al., 2013).

Conclusions

We reviewed the implications of EMT in lung development, homeostasis, and many chronic lung diseases. From *in vitro* and *in vivo* studies in development, and pathological investigations of human samples, two key notions seem to emerge: a) functional role of EMT in driving the pathophysiology underlying COPD, BOS and IPF, and b) recognition of EMT not being a binary process with only two end-points. Very few studies have reported anti-EMT effects of drugs. Data are especially sparse from *in vivo* human clinical investigations; most of the conclusions are drawn from *in vitro* studies. Thus, therapeutic approaches to block this profound epithelial plasticity are still in their infancy. Given the fact that disease-associated EMT can be of two types – fibrosis and cancer, each with some unique phenotypic and signalling manifestations – it becomes more complicated to tease out what proteins/pathways are contributing independently to fibrosis and cancer. Further *in vivo* studies and pathological investigations of human samples may have important translational insights regarding the progression of both these diseases, suggesting novel treatment strategies which are urgently required.

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Competing Interests

The authors declare no competing interests.

Figure legends

Figure 1: EMT in lung development and disease. EMT and MET can be multi-step processes with potential intermediate or hybrid state(s) that can be stable phenotypes, i.e. may reflect the end of a transition unless otherwise perturbed (highlighted by STOP signs at a hybrid E/M phenotype). Key references implicating a partial or full EMT in lung development and other chronic lung diseases such as fibrosis, COPD, and lung cancer.

Figure 2: Pictorial illustrations of the central compartment and their sub-divisions of human lung. Schematic adapted by permission from Macmillan Publishers Ltd. Nat Rev Cancer (Sun et al., 'Lung cancer in never smokers: a different disease'), copyright (2007) shows the central compartment (top left), and structure of large bronchus (top right) and respiratory bronchioles (bottom left). Histological representation of thin-walled small airways (SA) (A and C) from normal non-smoker, thickened in COPD (B and D) (bottom right). A, B at 50X, and C, D. are at 200X magnification.

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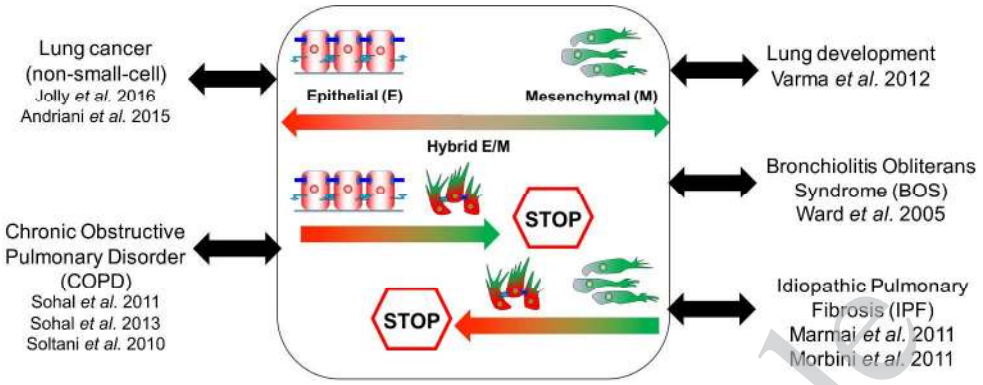

Figure 1.

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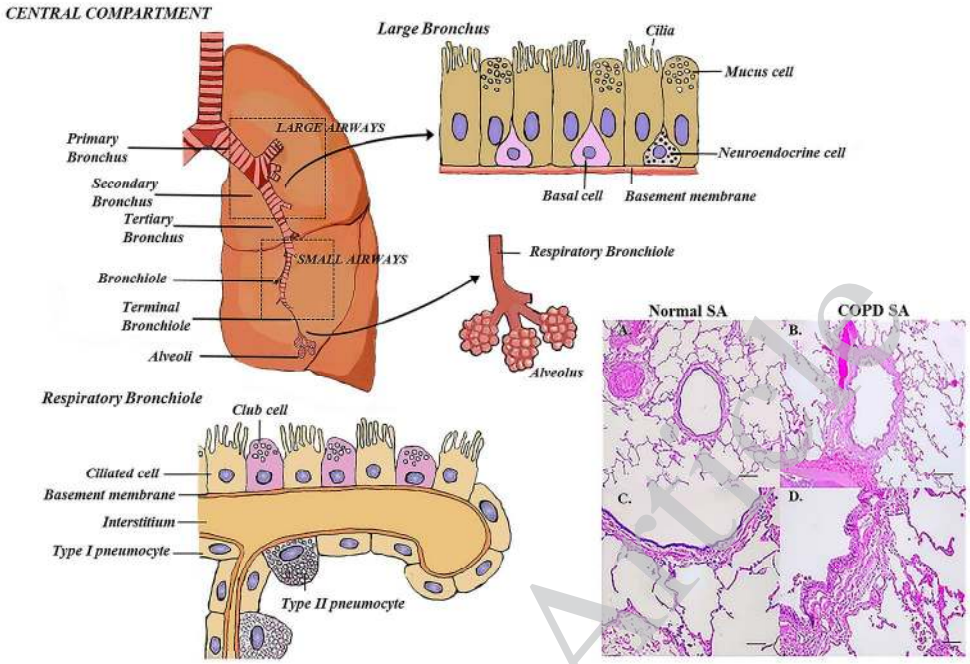

Figure 2.

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