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CYTOKINE-INDUCED EPITHELIAL-MESENCHYMAL TRANSITION: MECHANISMS AND IMPLICATIONS FOR FIBROTIC DISEASES OF RESPIRATORY AND INTESTINAL EPITHELIA

by

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Trinity College, Dublin 2, Ireland

Under the direction and supervision of

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and

Dr. Carlos Medina

2010
Declaration

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Stephen Buckley
Summary

Fibrosis is characterised by the accumulation of myofibroblasts, the key effector cell implicated in the fibrogenic reaction. The exact origin of these myofibroblasts remains poorly understood. However, growing evidence suggests that a significant portion of synthetically active myofibroblasts arise from the conversion of epithelial cells through the process of epithelial-mesenchymal transition (EMT). During EMT, the cell-cell adhesion structures of epithelial cells are down-regulated, their cytoskeleton is reorganised and they become more motile, reflecting transition to a mesenchymal phenotype. This PhD project sought to greater characterise EMT and provide additional insights into potential mechanisms underlying this process.

Although widely characterised in the alveolar epithelium, few investigations have explored the extent to which epithelia of the upper airways and large intestine are susceptible to EMT. The first part of this work focused on determining the relative susceptibility of alveolar (A549), bronchial (Calu-3) and intestinal (Caco-2) epithelium to undergo EMT. Data showed that A549 cells readily underwent EMT when exposed to TGF-β1 and/or cytomix, coupled with an enhanced migratory behaviour and diminished proliferative capacity. In contrast, treatment of Calu-3 or Caco-2 cells failed to induce such cellular alterations. Our investigations continued by examining the effect of telmisartan, an angiotensin II receptor antagonist, on alveolar EMT. Concomitant treatment with telmisartan served to reduce cytokine-induced collagen I production and cell migration independently of Smad2/3. However, expression levels of EMT markers remained unaltered.
The extent to which EMT-induced structural changes influence cellular mechanics is unclear. The second part of this work assessed the bio-mechanical properties of A549 cells following exposure to TGF-β1. Atomic force microscopy (AFM) revealed that stimulation with TGF-β1 gave rise to a more than two-fold increase in cell stiffness, which was augmented in the presence of a collagen I matrix. Coupled to this, alterations in topographical features were observed, with stimulated cells exhibiting a rougher surface profile. In agreement, simultaneous quantitative examination of the morphological attributes of stimulated cells using a high-content analysis (HCA) system, revealed dramatic alterations in cell shape, F-actin content and distribution.

Underlying the phenotypical change associated with EMT is a dramatic alteration in cellular structure. The final part of this work explored the hypothesis that RAGE and ERM interact and play a key role in regulating EMT-associated structural changes in alveolar epithelial cells. Exposure of A549 cells to inflammatory cytokines resulted in re-distribution of ERM to the cell periphery and localisation with actin stress fibres. Concurrently, ERM underwent phosphorylation via Rho kinase (ROCK). Inhibition of ROCK attenuated cytokine-induced structural changes. In addition, following cytokine stimulation, expression of RAGE was diminished, with release of its soluble isoform (sRAGE) via a matrix metalloproteinase (MMP)-9-dependent mechanism. Analysis revealed strong association between ERM and RAGE, which was disrupted when challenged with inflammatory cytokines, as ERM complexed with CD44.

Collectively, the results of this work uncover novel functional elements pertinent to the EMT reaction within lung parenchyma, and in this way, contribute to unravelling the complex mechanisms which underlie this process.
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Introduction

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1.1 Epithelial-mesenchymal transition (EMT)

1.1.1 Characteristics of epithelial and mesenchymal cells

Cell phenotype is a comprehensive term describing the general appearance and behaviour of the cell. Epithelial and mesenchymal cells are the two “classic” cell phenotypes, with each exhibiting a number of distinguishing characteristics. Epithelium is typically composed of a sheet of cells. Neighbouring cells are held tightly together by intercellular junctions, which also serve to inhibit the movement of individual cells away from the epithelial monolayer. This internal adhesiveness ensures that they exhibit a defined structure and mechanical rigidity. Epithelial cells are polarised, possessing apical and basal surfaces which may be both visually and functionally different.

In contrast, mesenchymal cells do not possess a regimented structure or tight intercellular adhesion. They are irregular in shape and lack uniformity of composition and density. Owing to weaker adhesion between cells, mesenchymal cells exhibit a greater migratory capacity. In comparison to epithelial cells, they have an elongated, more extended shape with front-to-back leading edge polarity. Importantly, migration of mesenchymal cells is mechanistically different from that of epithelium, with mesenchymal cells moving individually, whilst epithelial cells move as a sheet (Lee et al., 2006).

1.1.2 Key cellular events during EMT

Epithelial mesenchymal transition (EMT) is a complex, extreme manifestation of epithelial plasticity (Thiery, 2002). Consisting of multiple steps, it results in a
reversible phenotypic switch from epithelial to mesenchymal cell type. In order to initiate EMT and facilitate its completion, a series of discrete molecular processes are employed. These include disruption of epithelial cell-cell adhesion contacts, reorganisation and expression of cytoskeletal proteins, and stimulation of mesenchymal gene expression. Morphologically, cells transform from a cuboidal to spindle shape. Moreover, the apical basal polarity of epithelial cells is destabilised as they gain front-to-back leading edge polarity. EMT confers cells with enhanced migratory capacity. This newly developed invasive activity permits them to pass through the underlying basement membrane. Notably, expression of epithelial markers (e.g., E-cadherin) gradually diminishes, whilst in parallel mesenchymal marker expression increases. Such changes in marker expression are characteristic hallmarks of the EMT process (Savagner et al., 2001), and are frequently used as biomarkers to demonstrate passage of a cell through EMT. Although numerous cellular changes are correlated to EMT, the extent to which this collection of changes occurs is variable (Boyer et al., 2001). Although it remains unclear, it is probable that the exact range of changes which occur during EMT is determined by the integration of extracellular signals the cell receives (Thiery et al., 2003) The conversion of an epithelial cell to a mesenchymal cell is a crucial component of embryogenesis and organ development. However, in addition to its importance in biological developmental processes, EMT is also reported to play a significant role in the pathology of a number of diseases.

1.1.3 Inducers of EMT

Numerous growth factors and cytokines have been implicated in the induction of EMT. Epidermal growth factor (EGF), hepatocyte growth factor (HGF) and the
transforming growth factor-beta (TGF-β) family of growth factors are all known to be
directly involved. Among these, TGF-β1 is the most studied mediator. On exposure to
these factors, epithelial cells lose cell-cell adhesion, undergo reorganisation of their
cytoskeletal systems and acquire a fibroblast-like cell shape. In some instances
epithelial cells undergo “reversible scatter” rather than true EMT, following cytokine
stimulation (Kalluri et al., 2003). Reversible scatter looks like EMT with cells
assuming a spindle-like shape and undergoing a brief period of transcription.
However, following removal of the inducing stimulus, the epithelia revert to their
original state (Janda et al., 2002). Significantly, cells undergoing “reversible scatter”
do not express substantial amounts of mesenchymal markers.

Whilst extended exposure to TGF-β1 is a powerful inducer of complete EMT (Willis
et al., 2005), alone, most cytokines are only capable of inducing a reversible scatter
effect. In some instances, cytokines exhibit an additive or synergistic effect when
combined with an inducing factor such as TGF-β1 (Liu 2008; Borthwick et al., 2009).
In general, combinations of cytokines are present in most areas of tissue injury. Each
moiety may contribute a unique inducement to the transition culminating in the
integration of a diverse set of signals. Furthermore, following lung injury, it is thought
that inflammation plays a significant role in the pathogenesis of a number of lung
diseases including pulmonary fibrosis. In particular, interleukin-1β (IL-1β) and
tumour necrosis factor-α (TNF-α) are important cytokines in mediating airway
inflammation (Berger 2002; Lappalainen et al., 2005).

In rat airway epithelial cells, over-expression of IL-1β resulted in an increase in TNF-
α in the broncho-alveolar lavage fluid followed by an increase in TGF-β1, ultimately,
giving rise to airway remodelling and fibrosis (Kolb et al., 2001). Alone, TNF-α has been shown to mediate the transition from inflammation to fibrosis in the lung via recruitment of lymphocytes, which produce TGF-β1 (Oikonomou et al., 2006). In interferon-gamma (IFN-γ) knockout mice, lung inflammation and fibrosis was attenuated following bleomycin exposure (Chen et al. 2001). These studies suggest a possible link between the inflammatory process and fibrosis.

1.1.4 EMT in fibrosis

Fibrosis is defined by the overgrowth, hardening and/or scarring of various tissues (Wynn, 2008). Central to this is the excess deposition of ECM components such as collagen. However, it is the myofibroblast, which is the key cellular mediator of fibrosis. In its activated state, the myofibroblast promotes ECM deposition, acting as the primary source of collagen (Leivonen et al., 2002). Furthermore, it plays a significant role in promoting epithelial injury (Noseda et al., 2006) and release of inflammatory mediators (Zhang et al., 1994). Collectively, these are considered to be key contributors to the fibrotic process.

The origin of these myofibroblasts is controversial, however. Initially it was thought that the sole myofibroblast progenitor after injury of different tissues was locally residing fibroblasts (Hinz, 2007), which transiently differentiate into myofibroblasts. Over the years, it has become evident that myofibroblasts arise from a variety of sources. Recent research suggests that bone marrow-derived fibrocytes may represent an alternative source of myofibroblasts (Schmidt et al. 2003; Forbes et al. 2004). Other studies do not support this view, however. Interestingly, myofibroblasts have also been shown to derive from epithelial cells through a process of EMT (Willis et
Cumulative research has revealed EMT as playing a prominent role in fibrogenesis in a number of tissues, including the kidney and eye (Zeisberg et al., 2004; Saika et al., 2008). In the lung, the possible contribution of EMT to the pathogenesis of fibrosis has only recently received consideration (Willis et al., 2005; Kasai et al., 2005; Ando et al., 2007; Wu et al., 2007). Whilst in the intestine, evidence is emerging which implicates EMT as a mechanism involved in Crohn’s disease, with EMT apparent in areas of fibrosis in the colon (Bataille et al., 2008).

It is known that epithelial cells of the alveolus have the capacity to both produce and respond to TGF-β1 (Kapanci et al., 1994; Khalil et al., 2002; Xu et al., 2003; Kwong et al., 2004), modify cell morphology and gene expression in response to injury (Kasper et al., 1996) and regulate the function and differentiation of fibroblasts. As such, they play an essential role in the pathogenesis of lung fibrosis. Importantly, alveolar epithelial cells (AECs) exhibit considerable plasticity. Following injury, alveolar type II (ATII) cells are capable of giving rise to cells with an ATI-like phenotype through a process of transdifferentiation, in addition to self-renewal (Borok et al., 1998). Furthermore, cells that have acquired ATI-like cell characteristics have been shown to revert to an ATII cell phenotype under certain experimental conditions (Borok et al., 1998). Given this plasticity, it has been suggested that under circumstances in which transition to an ATI cell phenotype is inhibited, ATII cells may have the ability to undergo transition to fibroblasts and myofibroblasts via EMT (Fig. 1). The findings of a number of studies support this hypothesis (Yao et al., 2004; Willis et al., 2005; Kasai et al., 2005; Kim et al., 2006).
Figure 1. Alveolar epithelial transdifferentiation pathways. Under normal physiological conditions, ATII cells transdifferentiate into ATI cells, a process which is reversible in vitro. In reflection of the cellular environment and stimuli, AECs react to injury by travelling down one of a number of potential pathways: (1) apoptosis or necrosis; (2) proliferation, transdifferentiation, and re-epithelialisation; or (3) EMT to a myofibroblast phenotype, resulting in excessive deposition of extracellular matrix components, destruction of lung architecture, and fibrosis (Willis et al., 2006). Reprinted with permission.

Recent in vitro studies indicate that the bronchial epithelium may too undergo EMT in pathological settings (Doerner et al., 2009; Zhang et al., 2009; Câmara et al., 2010), and in this way contribute to tissue remodelling of the airways. However, it remains unclear as to what extent this occurs in vivo, and whether or not it is restricted to specific cell sub-types of the bronchus. In the case of intestinal fibrosis, whilst currently, there is a lack of supportive data, given the abundance of known mediators of EMT in the inflamed intestine, the likelihood of this process occurring may be relatively high. Moreover, cumulative evidence indicates that EMT is a contributor to cancer progression in the intestine, giving rise to a more invasive tumour cell phenotype (Bates et al., 2007), suggesting that epithelial cells of the intestine exhibit sufficient plasticity to undergo EMT.
1.1.5 Inflammation in fibrosis

Manifestation of the fibrotic phenotype is a consequence of complex interactions between numerous cytokines and cell types (Bringardner et al., 2008). Much controversy surrounds the question as to what extent inflammation contributes to its pathogenesis. In the lung, initial work suggested that inflammation initiated by unidentified stimuli gave rise to injury and subsequent fibrosis (Keogh and Crystal, 1982). In particular, evidence indicates an accumulation of pro-inflammatory cytokines (e.g., IL-1β and TNF-α) in the lungs of IPF patients (Piguet et al., 1993; Keane and Strieter, 2002). Additionally, multiple studies have revealed an essential role for pro-inflammatory cytokines in IPF through processes involving the blockade, detection or augmentation of cytokine expression in experimental models of IPF (Sime et al., 1997; Kolb et al., 2001). However, the poor efficacy of anti-inflammatory therapies in resolving the symptoms associated with IPF has led researchers to question the exact function of an inflammatory component (Piguet, 2003). Moreover, the absence of a uniform animal model in which to examine this question has precluded definitive clarification of this issue. In this regard, a number of hypotheses have been proposed. Most notably, Bringardner and colleagues (2008) have suggested that inflammatory cells may function in an atypical manner to facilitate fibrosis. In particular, they speculate that resident inflammatory proteins within the lung matrix may drive the disease, influencing fibrosis and tissue repair.

In the context of fibrosis of the intestinal tract there exists convincing evidence which suggests an important role for inflammation (Rieder and Fiocchi, 2008; Rieder and Fiocchi, 2009). In addition to TGF-β1, the proinflammatory cytokine, TNF-α, appears to exert a critical role in the pathogenesis of fibrotic states such as
inflammatory bowel disease (IBD) (Rieder and Fiocchi, 2009), with recent work indicating that blockade of TNF-α may serve to attenuate intestinal fibrosis (Sorrentino et al., 2008). However, in order to demarcate the precise role of inflammation in organ fibrosis further investigations are required.

1.1.6 Angiotensin II in fibrosis

In addition to TGF-β1 and pro-inflammatory cytokines, angiotensin II - a component of the renin-angiotensin-aldosterone system - has been shown to exhibit profibrotic properties. Studies indicate that angiotensin II plays an important role in the development of fibrosis within cardiac, renal and hepatic tissues (Mezzano et al., 2001; Watanabe et al., 2005). Through stimulation of TGF-β1 production, it facilitates myofibroblast differentiation with resultant accumulation of collagen I (Rosenkranz, 2004). Moreover, it is capable of augmenting TGF-β1 signalling pathways. In particular, exposure to angiotensin II gives rise to increased levels of Smad2 and enhanced nuclear translocation of pSmad3 (Tomasek et al., 2002). Consistent with these observations, recent evidence suggests that exposure to angiotensin II results in EMT (Burns et al., 2010). Thus, collectively, it appears that targeting of angiotensin II may offer a therapeutic approach by which to attenuate or slow the fibrogenic reaction.
1.2 Receptor for advanced glycation end-products (RAGE)

1.2.1 RAGE structure and distribution

RAGE is a member of the immunoglobulin superfamily (Neeper et al., 1992). The receptor itself is composed of an extracellular region containing one “V”-type and two “C”-type immunoglobulin domains. This is followed by a hydrophobic transmembrane-spanning domain which in turn neighbours a highly charged, short cytoplasmic domain that is essential for post-RAGE signalling (Fig. 2). This has been termed “full-length” or membrane RAGE (mRAGE). In addition, a number of isoforms have been identified (see below).

![Diagram of RAGE and isoforms](image)

**Figure 2.** Schematic representation of RAGE and the generation of some of its isoforms commonly found in the lung. In addition to its full-length form (mRAGE), RAGE also exists in a soluble form (sRAGE) which lacks the transmembrane and cytosolic domains found in mRAGE. Production of sRAGE isoforms is via either proteolytic cleavage, which gives rise to cleaved RAGE (cRAGE) or alternative splicing at exon 9 resulting in a C-truncated form termed endogenous secretory RAGE (esRAGE).
RAGE was initially identified and characterised for its ability to bind advanced glycation end products (AGEs), adducts formed by glycoxidation that accumulate in disorders such as diabetes (Schmidt et al., 1992). Subsequently, RAGE has also been shown to be a pattern recognition receptor, recognising families of ligands rather than a single polypeptide. Such ligands include amyloid fibrils, amphoterins, S100/calgranulins, and Mac-1 (Schmidt et al., 2001; Chavakis et al., 2003).

In the majority of healthy adult tissues, RAGE is expressed at a low basal level. The up-regulation of RAGE has been associated with a diverse range of pathological events, from atherosclerosis to Alzheimer’s disease (Schmidt et al., 1999). However, the exact function of RAGE in the lung has yet to be fully characterised. Uniquely, pulmonary tissues express remarkably high basal levels of RAGE suggesting that RAGE may have a number of functions in the lung distinct from that which it holds in other tissues (Fig. 3). Whilst the current body of research indicates important roles in both pulmonary physiology and numerous pathological states, additional work is required to clarify those inconsistencies which currently exist in the literature and further elucidate the important role of RAGE in the lung.
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**Figure 3.** Tissue distribution of RAGE. a) Relative number of expressed-sequence clones per million identified in tissue and species-specific databases (Barrett et al., 2007). The table was updated on 07/20/09. b) Quantitative analysis of RAGE mRNA expression in healthy human tissue. Two sets of RAGE-specific primers, RAqu_2 (black) and RAqu_3 (white), were used to compare RAGE expression in 16 human tissues of healthy donors in quantitative RT-PCR analysis. The transcript numbers of RAGE were compared with the transcript numbers of GAPDH in 2μL cDNA. The mean and SEM of two separate light cycler runs are displayed (adapted from Demling et al., 2006).
1.2.2 RAGE isoforms

In addition to its full-length, membrane-bound form (mRAGE), an increasing number of isoforms of RAGE have been identified (Fig. 2). In particular, RAGE has been shown to exist in a soluble isoform termed soluble RAGE (sRAGE). Whilst the sRAGE isoform contains the same V-type and C-type regions found in mRAGE, it lacks the transmembrane and cytosolic domains. Consequently, sRAGE is found in the extracellular space and is capable of binding RAGE ligands prior to their interaction with mRAGE. sRAGE is produced primarily by two mechanisms: removal of the transmembrane region via alternative splicing and cleavage from the membrane by proteolysis.

Studies have illustrated that alternative splicing at exon 9 gives rise to a C-truncated form termed endogenous secretory RAGE (esRAGE or RAGE_v1) (Yonekura et al., 2003). Proteolytic cleavage at the cell surface results in the production of a further soluble isoform termed cleaved RAGE (cRAGE) (Hanford et al., 2004). Recent findings by Raucci et al. (2008) suggest that this process is mediated by ADAM10, a membrane metallopeptidase. Notably, both soluble isoforms are capable of binding the same RAGE ligands and in this sense their functions are equivalent. In this way, it has been proposed that sRAGE acts as a decoy receptor, preventing the interaction of mRAGE with its ligands.

Furthermore, it has been shown that the expression of many isoforms in tissues or cell lines is dependent on the cell type. Indeed, Gelfter and colleagues (2009) have recently illustrated that certain lung isoforms possess distinct epitopes which were not found elsewhere. Interestingly, non-lung cells and tissues were found to express mRNA
which was more than three times the size of that expressed in the lung. Moreover, the majority of cell lines were revealed to express a cell line isoform whilst lacking the isoform found predominantly in the lung. These findings suggest that those RAGE isoforms unique to the lung may exhibit both structural and functional differences. However, it is currently unclear as to the specific mechanisms which give rise to any lung-restricted isoforms. Undoubtedly, the role of RAGE under both physiological and pathological settings involves elaborate interaction between the numerous isoforms. Thus, further elucidation of the expression of all isoforms of RAGE is essential.

1.2.3 Localisation and physiological role of RAGE in the lung

Initial immunostaining data of bovine tissues showed RAGE to be expressed in pulmonary endothelium, bronchial and vascular smooth muscle, alveolar macrophages, leiomyocytes, and the visceral pleural surface (Brett et al., 1993). Using polyclonal antibodies and specimens from human thoracoscopy, Morbini and co-workers (2006) later observed RAGE in bronchiolar epithelia, ATII cells, macrophages, and some endothelia. However, in both rat and human lungs, RAGE has also been suggested to co-localise with markers specific to ATI cells (Demling et al., 2006, Fehrenbach et al., 1998). However, this contrasts with Katsuoka’s findings (1997) that RAGE mRNA expression was predominantly restricted to ATII pneumocytes in rats. Expression of both RAGE protein and mRNA in A549, a human lung adenocarcinoma cell line with alveolar type II-like properties, has been suggested by some groups (Nakano et al., 2006; Queisser et al., 2008), while others were unable to confirm RAGE to be present in A549 cells at baseline (Nina Demling personal communication).
Further supporting evidence for the localisation of RAGE to alveolar epithelial type I (ATI) cells was provided by Dahlin et al. (2004) who illustrated that RAGE was differentially expressed in rat type I cells. Similarly, the rat type I-like cell line, R3/1, has also been shown to express high levels of RAGE (Koslowski et al., 2004). Collectively, this suggests RAGE being a marker for type I pneumocytes rather than the type II phenotype.

Within the ATI cell, RAGE has been shown to be specifically localised towards the basal cell membrane (Demling et al., 2006, Fehrenbach et al., 1998, Shirasawa et al., 2004). Given its high expression in the lung and specific localisation in ATI cells, an important role for RAGE in maintaining lung homeostasis is likely. However, the exact role of RAGE in lung physiology has yet to be fully elucidated (Fig. 4).

Demling and co-workers (2006) have shown that HEK293 cells overexpressing RAGE adhere much faster to collagen IV, and to a greater extent when compared to mock transfected cells. Furthermore, the degree of adherence was decreased in the presence of an anti-RAGE antibody. Similarly, in A549 cells, blockade of RAGE inhibited the adhesion onto collagen and intact extracellular matrix (Queisser et al., 2008). Pre-incubation with sRAGE was found to reduce this effect, although alone it was found to have no effect on cell adhesion, suggesting that this function is mediated by RAGE in its full-length form. Moreover, expression of RAGE has also been shown to promote spreading of adherent cells on collagen IV. The specificity of this RAGE-collagen IV interaction was underlined by the fact that adherence on fibronectin and laminin was inefficient (Demling et al., 2006). In addition, the findings of Bartling et al. (2005) that RAGE-transfected lung cancer cells exhibited epithelial growth on
collagen layers suggest that this interaction is of functional importance. Most recently, it has been shown that RAGE and collagen IV are co-localised at the basement membrane of normal mouse lungs (Englert et al., 2009). Observations by Hori et al. (1995) that a RAGE-amphoterin interaction is involved in neurite outgrowth during brain development further support a possible role for RAGE in cell-extracellular matrix interactions.

![Diagram](image)

**Figure 4.** RAGE is central to many fundamental biological processes in the lung. Expression of RAGE has been shown to promote spreading of adherent cells on collagen IV and in doing so may ensure effective gas exchange. RAGE-expressing epithelial cells exhibit diminished proliferative capacity compared to non-expressing cells. Cells overexpressing RAGE adhere much faster to collagen IV, and to a greater extent when compared to mock transfected cells suggesting an important role in cell-extracellular matrix interactions.

Using siRNA techniques it was illustrated that knockdown of RAGE in A549 cells and human pulmonary fibroblasts resulted in increased migration as evaluated by chemotaxis migration and scratch wound healing assays (Queisser et al., 2008). Furthermore, both modified cell types exhibited increased proliferation. Of note, the
effect of RAGE knockdown on migration was more pronounced in fibroblasts whilst conversely epithelial cells adopted greater proliferative capacity, suggesting that the specific role of RAGE may vary from one cell type to another.

In a developmental setting, it has been shown in rat lungs that levels of mRAGE and sRAGE at both the mRNA and protein level exhibit a gradual increase from foetal (E19) through to adulthood (Lizotte et al., 2007). Given that the newborn rat lung is not fully alveolarised, this increase in RAGE levels may correspond to alveolarisation and expansion of the ATI cell population. Additionally, since sRAGE has been shown to inhibit RAGE dependent epithelial spreading in vitro (Demling et al., 2006), the deficiency of sRAGE at these early stages of development may serve to promote spreading of ATI cells during development of the alveolus.

Together, these findings indicate that RAGE may assist ATI cells to acquire a spread-out morphology and in doing so ensure effective gas exchange and alveolar stability. Given its apparently important role in modulating adhesion of alveolar epithelial cells to the basement membrane, a role for RAGE in pathologies such as cancer and fibrosis, in which these interactions are altered or impaired, appears likely.

1.2.4 RAGE and pulmonary fibrosis

Fibrotic processes are disorders in which cell attachment and cell communication are critical events. RAGE has been implicated in the fibrotic process in a number of tissues, including the peritoneum, kidney, and liver (De Vriese et al., 2006; Li et al., 2004; Xia et al., 2008), where it has been shown to promote fibrosis. In the lung, evidence continues to accumulate suggesting an important role for RAGE in
pulmonary fibrosis, although data is conflicting if RAGE has a protective function or is indeed a culprit.

In animal models of pulmonary fibrosis, both membrane RAGE and sRAGE protein levels have been shown to be reduced following treatment with bleomycin, asbestos, or silica (Hanford et al., 2003; Englert et al., 2008; Ramsgaard et al., 2008). In the case of bleomycin injury, loss of mRAGE was seen within 24 hours (Hanford et al., 2003) and a reduction in sRAGE observed as early as day 2 (Hanford et al., 2003), with levels of both remaining reduced at day 7 (Hanford et al., 2003; Englert et al., 2008). The deleterious effects of asbestos on expression of both mRAGE and sRAGE were evident within 24 hours and maintained to day 14 following treatment (Englert et al., 2008). In common with asbestos, silica was found to induce a loss of RAGE which was apparent in samples isolated two weeks after treatment (Ramsgaard et al., 2008). A similar decrease in RAGE has also been illustrated in ATI cells isolated from rat lung slices treated with CdCl2 and TGF-β1 (Kasper et al., 2004).

Investigations by Englert et al. (2008) have shown that RAGE-deficient (RAGE −/−) mice spontaneously develop fibrosis-like alterations in lungs, exhibiting enhanced levels of collagen I and increased hydroxyproline content. Furthermore, following treatment with asbestos, these mice develop a fibrosis which is more severe compared to control mice.

Similar findings have been illustrated in lung homogenates and broncho-alveolar lavage fluid (BALF) from patients suffering from idiopathic pulmonary fibrosis (IPF). Both Englert et al. (2008) and Queisser et al. (2008) report that RAGE protein levels in lung homogenates were reduced in comparison to healthy donor samples. A lower
concentration of sRAGE was also found in BALF of IPF patients (Bargagli et al., 2009). Moreover, the RAGE gene has been shown to be significantly down-regulated in IPF lungs (Englert et al., 2008; Selman et al., 2007; Rosas et al., 2008). Together, these findings suggest that loss of RAGE may serve to promote fibrosis in the lung or that RAGE down-regulation is a result of this pathology.

In contrast, He and colleagues (2007) report that RAGE −/− mice were resistant to bleomycin induced lung injury with enhanced survival rates and lower fibrotic scores. They showed that protein levels of the pro-fibrotic cytokines TGF-β1 and PDGF in BALF failed to increase in RAGE −/− mice following bleomycin treatment, in contrast to wildtype mice. Additionally, HMGB1 increased in mice treated with bleomycin. Coupled to this, ATII cells cultured in the presence of HMGB1 were found to undergo epithelial-mesenchymal transition (EMT). In cells isolated from RAGE −/− mice, HMGB1 failed to induce EMT suggesting a potential role for RAGE signalling in HMGB1-induced EMT. These findings are in accordance with the traditionally held view of RAGE as being pro-fibrotic (De Vriese et al., 2006; Li et al., 2004; Xia et al., 2008) and that the RAGE expression is controlled by cytokines (Tanaka et al., 2000).

Findings by Morbini et al. (2006) lend further evidence to a role for RAGE in promoting fibrosis. In immunohistochemical studies of lung samples from IPF patients, over-expression of RAGE was found in reactive pneumocytes, bronchiolar metaplastic epithelium, and endothelium. Interestingly, over-expression was most apparent in fibroblastic foci. Additionally, in a recent study by Chen et al. (2009) it was shown that AGE levels in rat lungs were increased significantly following
bleomycin instillation. Interestingly, when formation of AGEs was blocked through treatment with aminoguanidine, bleomycin-induced fibrosis was attenuated. It is unclear, however, as to what extent this involves its receptor, RAGE.

Currently, it is unclear as to the exact role of RAGE in fibrosis of the lung. A number of studies undertaken using animal models of fibrosis have produced conflicting results. These findings highlight the acknowledged limitations of such models (Moore et al., 2008) and consequently limit the extent to which inference can be drawn from them. Furthermore, whilst a number of studies report a loss of RAGE under experimental conditions of fibrosis, it is unclear as to whether this is due to a down-regulation of RAGE itself or simply a loss of alveolar type I epithelial cells, the primary expresser of RAGE.
1.3 In vitro models of the epithelium

1.3.1 Primary cell cultures

Primary cells are thought to offer the most accurate in vitro representation of the epithelium in its native form. In particular, they typically exhibit characteristics which more closely resemble those observed in vivo. Utilisation of epithelial cells for primary culture entails their fresh isolation from tissues. As a consequence, primary cultures are associated with a number of limitations. For example, availability of tissues is often restricted, in particular those derived from humans. Moreover, cell yields may be poor whilst donor-to-donor variation can reduce the usefulness of these cultures. In contrast to cell lines, primary cultures are also limited by rather short lifespans. As a result, the use of primary cell cultures is associated with high costs and can be time-consuming.

1.3.3.1 Primary cell cultures of tracheo-bronchial epithelia

Over the last 30 years, protocols for the isolation and culture of primary tracheo-bronchial epithelial cells obtained from lungs of many species haven been developed. They include primary cultures of airway epithelial cells of the mouse (Oreffo et al., 1990), hamster (Kaufman, 1976), guinea pig (Robison et al., 1993), rat (Suda et al., 1995), ferret (Chung et al., 1991), rabbit (Liedtke, 1988; Mathias et al., 1995), dog (Welsh, 1985), pig (Black et al., 1989), cow (Sisson et al., 1991), horse (Sime et al., 1997) and human (Masui et al., 1986; de Jong et al., 1993; Galietta et al., 1998). Most of the protocols result in well differentiated epithelial cells with mixed phenotypes. However, following the first couple of subcultures they tend to lose their ability to form tight junctions and their capacity to generate high transepithelial electrical
resistance (TEER) is lost. Indeed, successive passages of human tracheal epithelial cells have been shown to exhibit a linear drop in short-circuit current ($I_{sc}$) (Zabner et al., 2003). Using Ussing chamber techniques, these subcultures were shown to exhibit minimal rates of active $\text{Na}^+$ and $\text{Cl}^-$ transport by passage 3 and beyond passage 4 the cells failed to generate any active ion transport. These data suggest that it is important to develop airway epithelial cell lines that retain the ability to differentiate, form tight junctions and maintain ion channel/pump activities when grown in vitro (Zabner et al., 2003).

Ready-to-use culture systems of human tracheo-bronchial cell layers exhibiting well-differentiated ciliated and goblet cell phenotypes are commercially available (Epiairway™ system, Mattek Corporation, Ashland, MA, USA) (Chemuturi et al., 2005). Despite Epiairway™ being marketed for use in drug delivery studies, little data have been reported to date. This may be reflective of low uptake and/or due to cost or usage in industry that is subject to confidentiality. Overall, primary culture is less convenient and economical than the use of cell lines.

1.3.3.2 Primary cell cultures of alveolar epithelia

Given the scarcity of appropriate alveolar epithelial cell lines, an increasing number of in vitro studies use primary cultures of AEC. Primary mammalian AEC culture techniques involve isolation, purification and cultivation of ATII cells from tissues obtained either after lung resection or isolated perfused lungs. When plated on permeable supports or plastics under appropriate culture conditions, these ATII cells acquire features of type I cell-like phenotype and morphology (Demling et al., 2006; Fuchs et al., 2003; Danto et al., 1995). Isolation of ATI pneumocytes from rat lungs
has been recently described (Borok et al., 2002; Johnson et al., 2002; Chen et al., 2004). However, development of confluent ATI cell monolayers exhibiting electrically tight characteristics has yet to be reported. Of note, unlike many other cells in primary culture, AEC typically exhibit a very limited proliferative capacity and therefore are not suitable for passaging. Consequently, each data set requires a fresh preparation of cells resulting in a marked increase in costs. Moreover, it necessitates a reliable normalisation scheme of data from each set of cell preparations.

Owing to the lack of availability of human lung tissues and ethical issues pertaining to the use of human tissues, the majority of investigations utilising primary cultures of alveolar epithelia have been based on isolation and culture of cells from animals, including mouse (Corti et al., 1996), rat (Goodman et al., 1982), rabbit (Shen et al., 1999) and pig (Steimer et al., 2007). Since evidence for species difference between human and rodents might be more significant than once assumed (King et al., 2001), the importance of confirmation studies using primary human pneumocyte cultures cannot be underestimated.

1.3.3.3 Primary cell cultures of intestinal epithelia

The establishment of simple methods of primary culture for gut epithelial cells is highly desirable. In light of this, comprehensive examination of the primary culture of intestinal cells has been undertaken to enhance isolation procedures for live epithelial cells (Whitehead et al., 1993; Quaroni and Beaulieu, 1997; Panja, 2000; Marian, 2002; Moue et al., 2008). Specifically, the objective is to preserve the phenotypic properties of these cells as progenitors and committed proliferative, differentiated or senescent cells, and to devise controlled culture conditions. However, despite progress
in isolation methodologies and manipulation of the cell microenvironment, long-term primary culture of intestinal cells remains difficult. Although the generation of primary cultures of rodent enterocytes has been optimised, the viability of these culture remains limited (Evans et al., 1994). Moreover, enterocytic differentiation is limited, due to poor survival of cells and insufficient knowledge regarding the factors necessary to facilitate differentiation.

1.3.2 Continuous cell cultures

The use of continuous cell cultures in cell biology is widespread. They include those derived from tumours and transformed cell lines. Importantly, in contrast to primary cell cultures, they are immortal. The principal benefit of cell lines is their convenience of use. Culturing and maintenance of epithelial cell lines can be performed using standard reagents and protocols. Moreover, the yield associated with each passage of a particular cell line is typically high. Furthermore, they offer greater flexibility, with cells capable of being frozen and stored. In terms of cost, they are considerably more economical to use.

However, the use of cell lines is also associated with a number of disadvantages. In the case of transformants, they often lose differentiated properties with increasing passage number. In the case of tumour-derived cells, they exhibit excessive proliferative capacity, in addition to other abnormalities (e.g., mucus and surfactant content) in comparison to normal healthy cells (Ehrhardt et al., 2002).
1.3.2.1 Tracheo-bronchial epithelial cell lines

In contrast to gastrointestinal in vitro testing where Caco-2 cells have emerged as the gold standard, there is no such consensus to date on the preferred cell line(s) for modelling the bronchial epithelium in vitro. Several detailed protocols for culture, maintenance, growth and permeability assessment of tracheo-bronchial epithelial cell lines have been published in recent years (Forbes and Ehrhardt, 2005), with the most commonly used systems being the Calu-3, BEAS-2B and 16HBE14o- cell lines.

Calu-3 (American Type Culture Collection; ATCC HTB-55) is a human bronchial epithelial cell line derived from an adenocarcinoma of the lung (Fogh and Trempe, 1975). This cell line has been shown to exhibit serous cell properties and form confluent monolayers of mixed cell phenotypes, including ciliated and secretory cell types (Shen et al., 1994), but the cilia are formed very irregularly and seem to disappear with increasing passage number (Carsten Ehrhardt personal communication).

The BEAS-2B cell line was derived from normal human epithelial cells that were immortalised using the hybrid virus of adenovirus 12 and Simian virus 40 (Reddel et al., 1988). BEAS-2B is available from the ATCC (CRL-9609) and has been popular in studies of airway epithelial cell structure and function, including phenotyping and mechanistic investigation of cytokine regulation (Atsuta et al., 1997). BEAS-2B cells have also been used to evaluate responses to challenges such as tobacco smoke (Sun et al., 1995), environmental particles (Steerenberg et al., 1998; Veranth et al., 2007) and hyperoxia (Odoms et al., 2004).
Another continuous bronchial epithelial cell line, 16HBE14o-, was generated by transformation of normal bronchial epithelial cells obtained from a one year old male heart-lung transplant patient. Transformation was accomplished with SV40 large T antigen using the replication defective pSVori- plasmid (Cozens et al., 1994).

16HBE14o- cells can be obtained from Dieter C. Gruenert, Ph.D., at the California Pacific Medical Center. The non-commercial availability of the cell line might be one of the reasons why they have been less widely used than Calu-3 or BEAS-2B. 16HBE14o- cells have a non-serous, non-ciliated phenotype and are generally rounder in shape and smaller in size than Calu-3. When grown under liquid-covered culture (LCC) conditions, 16HBE14o- form confluent, polarised cell layers with functional tight junctions (Ehrhardt et al., 2002, 2003). In contrast, air-interfaced culture (AIC) conditions lead to cell layers of less desirable phenotypic and morphological traits. Since most epithelial cells that are normally located at an air interface in vivo function optimally under AIC conditions for cultivation, the superior performance of the 16HBE14o- cell line under LCC conditions is an exception for which the exact reasons or mechanism are currently unknown.

1.3.2.2 Alveolar epithelial cell lines

While a number of immortalised cell lines emanating from different cell types of the airway (i.e., tracheo-bronchial) epithelium of lungs from various mammalian species are available, reliable and continuously growing cell lines that possess alveolar epithelial cell morphology and phenotype are not reported to date.

Probably the most frequently used alveolar epithelial model is the A549 cell line (American Type Culture Collection, ATCC CL-185); continuously growing cells
derived from a human pulmonary adenocarcinoma that have some morphologic and biochemical features of the human pulmonary alveolar type II cell in situ (Lieber et al., 1976). A549 cells contain multilamellar cytoplasmic inclusion bodies, like those typically found in human lung ATII cells, although these hallmarks disappear as culture time increases. At early and late passage levels, the cells synthesise lecithin with a high percentage of disaturated fatty acids utilising the cytidine diphosphocholine pathway (Lieber et al., 1976). The cell line has been utilised for many biological studies, albeit A549 cells lack the ability to form tight monolayers of polarised cells, due to the inability to form functional tight junctions (Foster et al., 1998; Elbert et al., 1999; Kim et al., 2001; Forbes and Ehrhardt, 2002). Notwithstanding, A549 cells remain a widely used model in studies pertaining to the alveolar epithelium.

Other cell lines of an alveolar epithelial origin that are reported to date include R3/1 and L-2 (rat), MLE-12 and 15 (mouse), and H441 and TT1 (human). Of these, NCI-H441 cell line (ATCC HTB-174), emanated from a human lung adenocarcinoma, has been described to have characteristics of both ATII (Duncan et al., 1997; Rehan et al., 2002) and bronchiolar (i.e., Clara) epithelial cells (Newton et al., 2006; Zhang et al., 1997).

Wikenheiser and co-workers generated a series of continuous alveolar epithelial cell lines (MLE-7, -12, and -15) from transgenic mice harbouring the SV40 large T antigen under the control of the human SP-C promoter region. These MLE cell lines maintained morphological and functional characteristics of distal respiratory epithelial cells normally lost after isolation and primary culture (Wikenheiser et al., 1993),
which are consistent with those seen in non-ciliated bronchiolar and ATII epithelial cells. However, morphological and functional characteristics associated with an individual cell type do not always appear to co-exist in a clonal cell line. For example, MLE-12 cells express SP-C mRNA (i.e., indicative of ATII cells in the adult mouse), but other characteristics (e.g., SP-A mRNA expression or presence of lamellar bodies) are missing in MLE-12 cells. The heterogeneity of cellular markers in MLE cells may be related to the immortalisation of ATII cells at various stages of lung development. Alternatively, MLE cell lines might represent distinct subtypes of distal respiratory epithelial cells. Culture conditions may also be an important determinant of expression of various cellular markers in MLE cells. When grown under AIC on hollow fibres, MLE-15 cells contain numerous lamellar bodies and secrete both SP-A and SP-B (Grek et al., 2009). Currently, only MLE-12 can be obtained from the American Type Culture Collection (ATCC CRL-2110).

L-2 cells (ATCC HTB-149) have been isolated by clonal culture techniques from the adult rat lung. These cells appear to retain differentiated functions that are present in ATII cells of intact rat lungs. L-2 cells are diploid, epithelial cells. They contain osmiophilic lamellar bodies in their cytoplasm and synthesise lecithin by the same de novo pathways as in intact lungs (Douglas and Kaighn, 1974). L-2 cells are capable of forming confluent monolayers, although they do not exhibit as high a TEER as ATII cells in primary culture do (Helms et al., 2006). Moreover, they possess ion transport and single channel characteristics indistinguishable from primary cultures of ATII cells, together with comparable amiloride sensitive transepithelial current (Helms et al., 2006).
The rat cell line R3/1 was established from cells obtained from broncho-alveolar tissues of foetal Wistar rats at 20 days of gestation. This cell line displays a phenotype with several characteristic features of ATI cells. R3/1 cells were analysed to show a positive expression for both mRNA and protein for markers related to the ATI cell type (T1a, ICAM-1, connexin-43 and caveolin-1 and -2) (Koslowski et al., 2004). However, it has been shown very recently that R3/1 cells are unable to form confluent monolayers due to a lack of expression of several tight junction proteins, such as occludin (Horálková et al., 2009).

Most recently, a new alveolar type I-like cell line, transformed type 1 (TT1), has been established (Kemp et al., 2008). They are an immortalised cell line produced by retroviral transduction of primary human ATII cells with the catalytic subunit of human telomerase (hTERT) and a temperature sensitive mutant of Simian virus 40 large antigen (U19tsA58 LT). They exhibit a flattened and thin cell morphology which is typical of type I cells. It has also been shown that TT1 cell contain endosomal vesicles within the cytoplasm and invaginations at the cell membrane. Importantly, they express type I cell markers such as caveolin-1, whilst lacking type II cell markers such as TTF-1, pro-SPC and alkaline phosphatase (Kemp et al., 2008). They show positive staining for pan-cytokeratin, confirming their epithelial phenotype. However, they exhibit poor barrier properties. Staining for ZO-1 in TT1 cells is weak and discontinuous consistent with a TEER of ~55 ohm·cm² and flu-Na P_{app} of ~6 x 10^{-6} cm/s (van den Bogaard et al., 2009).
1.3.2.3 Intestinal epithelial cell lines

Owing to the lack of appropriate cell lines from normal human intestinal epithelium, the majority of studies investigating the regulation of human intestinal cell functions have utilised continuous cell cultures derived from experimental animals and human colon cancers. In the majority of in vitro studies of the gut, human colon tumourigenic cell lines such as Caco-2, T84 and HT-29 have been used. Undoubtedly, the most widely used of these is Caco-2. Isolated from a 72-year old Caucasian male, Caco-2 is a colorectal adenocarcinoma cell line (Fogh et al., 1977). In culture, they differentiate spontaneously into polarised intestinal cells possessing an apical brush border and tight junctions between adjacent cells, and they express hydrolases and typical microvillar transporters (Pinto et al., 1983). Although derived from adult human colon, Caco-2 cells express enzymes that are typical of normal small-intestinal villus cells; they also transport ions and water toward the basolateral membrane, forming domes in culture (Pinto et al., 1983). In spite of their apparent usefulness for biological investigations relating to the intestine, it must be emphasised these highly enterocyte-like differentiated cells are not normal cells but malignant cells carrying mutations in several genes such as p53, APC, β-catenin and Smad4 (Gayet et al., 2001). Moreover, it has been shown that the properties of Caco-2 monolayers can vary with time in culture, passage number and culture medium composition (Le Ferrec et al., 2001).

The human HT-29 adenocarcinoma cell line was isolated in 1964 from the colon tumour of a 44-year old Caucasian female, and is considered to be a pluripotent intestinal cell line (Simon-Assmann et al., 2007). Interestingly, these cells have been found to have a notably high rate of glucose consumption. In the presence of both
glucose and serum, HT-29 cells are undifferentiated, growing as a multilayer of non-polarised cells and devoid of any markers of functional epithelial cells (Neutra et al., 1989; Lesuffleur et al., 1991). However, under the influence of culture medium changes or of differentiation inducers, these cells are capable of expressing various differentiation characteristics. Under such conditions, they exhibit ultrastructure features such as microvilli, microfilaments, large vacuolated mitochondria with dark granules, smooth and rough endoplasmic reticulum with free ribosomes, lipid droplets, few primary and many secondary lysosomes (Chen et al., 1987; Didier et al., 1996; Takahashi et al., 1996). In addition, the express brush-border enzymes, although their activities are much lower than those observed in the normal small intestine or in the Caco-2 cells (Simon-Assmann et al., 2007).

Derived from the rat small intestine, the IEC-6 and IEC-18 cell lines are the most widely used rodent lines (Quaroni et al., 1978; Quaroni and Isselbacher, 1981). These cell lines exhibit morphological and functional characteristics suggestive of crypt cells (Quaroni and Beaulieu, 1997). In particular, they have been employed to examine the role of growth factors in epithelial cell physiology, in addition to studies on the specific functions of intestinal cells (Kedinger et al., 1987). More recently, the normal human crypt small intestinal cells HIEC-6 were isolated by Beaulieu et al. at the University of Sherbrooke, Canada (Beaulieu, 1997; Beaulieu, 1999). Although not yet fully characterised, they have been extensively used in integrin and extracellular matrix research (Benoit et al., 2009; Dydensborg et al., 2009). H4, a human foetal small intestinal cell line, was established by Walker and colleagues (Nanthakumar et al., 2000), and is representative of normal enterocytes of immature human intestinal epithelium (Claud et al., 2003; Lu et al., 2008, 2009). Subsequently, it has been sub-
cloned, giving rise to pure epithelial cells with the capacity to establish transepithelial resistance (Cencić et al., 2010). The IPEC-J2 cell line is a non-transformed intestinal cell line originally derived from jejunal epithelia isolated from a neonatal, unsuckled piglet, maintained as a continuous culture and characterised (Schierack et al., 2006). Similarly, it has been sub-cloned to produce cultures of enterocyte-like and mucin producing cell types (Cencić et al., 2010).
1.4 Aims of this work

Growing evidence suggests an important role for EMT in fibrotic disorders. A need for greater characterisation and insight into the mechanisms and events underlying this key biological process led us to the studies discussed in this thesis. The specific objectives of this PhD project were:

1. To investigate the susceptibility of epithelial cells of the alveolus, bronchus and colon \textit{in vitro} to EMT and the effect of the angiotensin II receptor (AT2R) antagonist, telmisartan, on attenuation of this process.

2. To study the bio-mechanical changes in alveolar epithelial cells associated with EMT-induced cytoskeletal rearrangement using atomic force microscopy (AFM), and the influence of extracellular matrix (ECM) components on this process. Furthermore, to comprehensively evaluate the dynamic changes in cytoskeletal structure during EMT using high content analysis (HCA) techniques.

3. To characterise the role of the receptor for advanced glycation end-products (RAGE) in EMT of alveolar epithelial cells and their relationship with focal adhesion molecules.
1.5 References


Boyer B, Vallés AM, Edme N (2000) Induction and regulation of epithelial-mesenchymal transitions. Biochem Pharmacol 60(8); 1091-9


Câmara J, Jarai G (2010) Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-alpha. Fibrogenesis Tissue Repair 3(1):2


Doerner AM, Zuraw BL (2009) TGF-beta1 induced epithelial to mesenchymal transition (EMT) in human bronchial epithelial cells is enhanced by IL-1beta but not abrogated by corticosteroids. Respir Res 10:100


cell line as an *in vitro* model for bronchial drug absorption studies. Cell Tissue Res 308(3):391-400


Morphological characterisation and expression of caveolin-1 and surfactant protein-C.
Cell Tissue Res 311(1):31-45


through the RAGE-ERK1/2 MAP kinase signaling pathway. Am J Pathol 164(4):1389–1397


induced, metabolic and stress pathways marks neutrophil entry into cystic fibrosis
lungs. Proc Natl Acad Sci U S A 106(14):5779–5783

Marian B (2002) In vitro models for the identification and characterization of tumor-
promoting and protective factors for colon carcinogenesis. Food Chem Toxicol
40(8):1099-104

Type beta transforming growth factor is the primary differentiation-inducing serum
factor for normal human bronchial epithelial cells. Proc Natl Acad Sci U S A
83(8):2438-2442

characterization of rabbit tracheal epithelial cell monolayer models for drug transport

Hypertension 38(3, Pt 2):635–638

Physiol Lung Cell Mol Physiol 294(2):L152–L160

for advanced glycation end products and its ligands: a new inflammatory pathway in


endproducts (RAGE) is produced by proteolytic cleavage of the membrane-bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). FASEB J 22(10):3716–3727


Wikenheiser KA, Vorbroker DK, Rice WR, Clark JC, Bachurski CJ, Oie HK, Whitsett JA (1993) Production of immortalized distal respiratory epithelial cell lines
from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. Proc Natl Acad Sci U S A 90(23):11029-11033


Chapter 2

Differential susceptibility to epithelial-mesenchymal transition (EMT) of alveolar, bronchial and intestinal epithelial cells in vitro and the effect of angiotensin II receptor inhibition

Parts of this chapter have been published in:

2.1 Abstract

The generation of myofibroblasts via epithelial-mesenchymal transition (EMT), a process through which epithelial cells lose their polarity and become motile mesenchymal cells, is a proposed contributory factor in fibrosis of a number of organs. Currently, it remains unclear as to what extent epithelia of the upper airways and large intestine are susceptible to this process. Herein, we investigated the ability of model cell lines of alveolar (A549), bronchial (Calu-3) and colonic (Caco-2) epithelial cells to undergo EMT when challenged with TGF-β1 and other pro-inflammatory cytokines. Western blot and immunofluorescence microscopy demonstrated that A549 cells readily underwent EMT, as evidenced by a spindle-like morphology, increase in the mesenchymal marker, vimentin and down-regulation of E-cadherin, an epithelial marker. In contrast, neither Calu-3 nor Caco-2 cells exhibited morphological changes or alterations in marker expression associated with EMT. Moreover, whilst stimulation of A549 cells enhanced migration and reduced their proliferative capacity, no such effect was observed in epithelial cell lines of the bronchus or colon. In addition, concomitant treatment of A549 cells with telmisartan, an angiotensin II receptor antagonist with antifibrotic properties, was found to reduce cytokine-induced collagen I production and cell migration, although expression levels of vimentin and E-cadherin remained unaltered. Mechanistically, telmisartan failed to inhibit phosphorylation of Smad2/3. Together, these results, using representative in vitro models of the alveolus, bronchus and colon, tentatively suggest that epithelial cell plasticity and susceptibility to EMT may differ depending on its tissue origin. Furthermore, our investigations point to the beneficial effect of telmisartan in partial abrogation of alveolar EMT.
2.2 Introduction

Fibrosis is characterised by the accumulation of myofibroblasts, the key effector cell implicated in the fibrogenic reaction. The exact origin of these myofibroblasts remains poorly understood (Hinz et al., 2007). However, growing evidence suggests that a significant portion of synthetically active myofibroblasts arise from the conversion of epithelial cells through the process of epithelial-mesenchymal transition (EMT) (Willis et al., 2006; Willis et al., 2007; Guarino et al., 2009). During EMT, the cell-cell adhesion structures of epithelial cells are down-regulated, their cytoskeleton is reorganised and they become more motile, reflecting transition to a mesenchymal phenotype. In essence, EMT is a striking manifestation of epithelial plasticity (Grüntert et al., 2003). However, epithelial cells from different organs exhibit a variability of receptors and kinases, which in turn determine their preference for signalling pathways. As a consequence, their plasticity may differ and therefore, the extent to which they can undergo EMT.

A collection of soluble growth factors have been implicated in the induction of EMT (Kalluri et al., 2003; Savagner et al., 2001). Chief amongst these is TGF-β1, which is expressed at sites of epithelial injury (Xu et al., 2003). Additionally, TGF-β1 is acknowledged as being an important mediator of fibrosis in a number of organs, including lung and gut (Willis et al., 2007; Vallance et al., 2005). It serves to increase transcription of collagens, and in parallel, prevents their degradation via inhibition of collagenase activity through increased production of matrix metalloproteinase (MMP) inhibitors such as tissue inhibitor of matrix metalloproteinase (TIMP) (Gharaee-Kermani et al., 2009). Typically, fibrotic disease is precipitated by an inciting
injurious event, giving rise to the production and release of pro-inflammatory cytokines (e.g., TNF-α and IL-1β) (Razzaque et al., 2003) which, in turn, modulate TGF-β1 activity. In this way, the inflammatory milieu may serve to augment the EMT-inducing effects of TGF-β1, or conceivably, induce EMT of its own accord.

In the lung, (myo-)fibroblast accumulation and resultant excess deposition of extracellular matrix (ECM) components (e.g., collagen I and fibronectin) is a common characteristic feature of idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD) and asthma (Selman et al., 2002; Holgate et al., 2000). Cumulative data, both in vitro and in vivo, suggests that alveolar epithelial cells contribute to the interstitial myofibroblast pool in IPF, via EMT (Willis et al., 2005; Kim et al., 2006). More recently, in vitro studies indicate that the bronchial epithelium may too undergo EMT in pathological settings (Doerner et al., 2009; Zhang et al., 2009; Câmara et al., 2010), and in this way contribute to tissue remodelling of the airways. To what extent this occurs in vivo, however, remains unclear. Furthermore, evidence is emerging which implicates EMT as a mechanism involved in Crohn’s disease, with EMT apparent in areas of fibrosis in the colon (Bataille et al., 2008).

Although EMT has been widely characterised as playing an important role in fibrosis of the kidney (Burns et al., 2007), liver (Choi et al., 2009) and lung (Willis et al., 2006), an understanding of the relative susceptibility of residing epithelial cells of the bronchus and intestine to EMT is in its infancy. In this study, we examined the ability of representative epithelial cell lines of the human alveolus (A549), bronchus (Calu-3)
and intestine (Caco-2) to undergo EMT \textit{in vitro}, when exposed to TGF-β1 and pro-inflammatory cytokines.

In addition, we focused our investigation on potential amelioration of EMT by way of pharmacological inhibition. Previously, angiotensin II receptor (AT2R) antagonists have been shown to effectively attenuate bleomycin-induced pulmonary fibrosis in several animal models (Molina-Molina \textit{et al.}, 2006; Yao \textit{et al.}, 2006; Waseda \textit{et al.}, 2008). Hence, we hypothesised that an AT2R antagonist, telmisartan, may, at least in part, serve to inhibit EMT \textit{in vitro}.
2.3 Materials and Methods

2.3.1 Materials

Telmisartan, mouse monoclonal anti-vimentin antibody (V5255) and TRITC-phalloidin were purchased from Sigma-Aldrich (Dublin, Ireland). Goat polyclonal anti-phospho-Smad2/3 antibody (SC-11769) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Mouse monoclonal E-cadherin (MCA-1482) and collagen I antibodies (2150-0001) were purchased from AbD Serotec (Oxford, UK). Recombinant human TGF-β1, IL-1β, TNF-α and IFN-γ were purchased from PeproTech (London, UK). Cell culture media, foetal bovine serum (FBS) and all other chemicals were purchased from Sigma-Aldrich.

2.3.2 Cell culture conditions

A549 cells (American Type Culture Collection, ATCC CL-185) were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK) and used between passage numbers 63 and 76. Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 medium (DMEM/F-12) supplemented with 5% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Calu-3 cells (ATCC HTB-55) were obtained from ECACC and were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 μg/ml streptomycin and 100 U/ml penicillin. Passage numbers 31 to 42 were used in experiments.
Caco-2 cells (ATCC HTB-37) were obtained from ECACC and were grown in DMEM supplemented with 20% FBS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin. Passage numbers 24 to 40 were used in experiments.

All cells were cultured on either tissue cultured-treated plastic or glass at 37°C and 5% CO₂ atmosphere; all culture media were exchanged every 48 h. For studies described below, cell cultures at approximately 70% confluence (i.e., after one day in culture (A549); three days in culture (Calu-3) or two days in culture (Caco-2)) were serum-starved overnight and then treated with cytokines in culture medium containing 1% FBS for 72 h, with or without addition of telmisartan (1 µM final concentration).

2.3.3 Western blot analysis

Cell cultures were lysed with cell extraction buffer (Biosciences, Dun Laoghaire, Ireland) on ice and briefly sonicated. Protein sample concentrations were determined using a standard protein concentration assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer’s instructions. Samples were separated by SDS-PAGE and transferred to immunoblot polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (pH 7.4) for 1 h at room temperature. Incubation with the respective primary antibodies was carried out overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody at room temperature for 1 h. Peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland). Relative levels of protein expression were quantified by densitometric analysis of the immunoblot using a ChemiDoc documentation system.
(Bio-Rad). To ensure equal loading, protein was normalised to β-actin using an anti-
β-actin monoclonal antibody (Sigma-Aldrich).

2.3.4 Immunofluorescence microscopy

Lab-Tek chamber slides (Thermo Fisher, Dublin, Ireland) were used to grow
epithelial cells under conditions as described above. Cells were fixed for 10 min with
2% (w/v) paraformaldehyde and blocked for 10 min in 50 mM NH₄Cl, followed by
permeabilisation for 8 min with 0.1% (w/v) Triton X-100 in PBS. After a 60 min
incubation with 150 μl dilution (E-cadherin 1:100; vimentin 1:1000; pSmad 2/3
1:100) of the respective primary antibody, the cell layers were washed three times
with PBS, before incubation with 100 μl of a 1:200 dilution of relevant Alexa Fluor-
488-labelled F(ab')₂ fragment (Invitrogen, Karlsruhe, Germany) in PBS containing
1% (w/v) BSA. Propidium iodide (1 μg/ml in PBS) was used to counterstain cell
nuclei. In the case of F-actin staining, TRITC-phalloidin was used at a concentration
of 500 ng/ml in PBS. After 30 min of incubation, the specimens were again washed
three times with PBS and embedded in FluorSave anti-fade medium (Merck,
Nottingham, UK). Images were obtained using a confocal laser scanning microscope
(CLSM, Zeiss LSM 510, Göttingen, Germany).

2.3.5 Wound healing assay

Cells were grown in six-well plates until a confluent monolayer had developed.
Confluent cultures were serum starved for 24 h to minimise the effect of growth
factors in serum, before being wounded with the use of a 200 μl pipette tip with three
parallel scrapes that extended the full diameter of the well. Cell debris was removed
from the cultures by washing twice with PBS, followed by incubation in the relevant
cell culture medium (1% FBS) supplemented with or without cytokines and/or telmisartan (1 μM). Images of the wounds were captured on an inverted light microscope (Leica DM IL, Wetzlar, Germany) immediately following wounding (0 h) and 24 h later. In between imaging, cells were returned to 37°C, 5% CO₂ humidified incubation. The wound area in each image was measured using the NIH ImageJ freeware program and quantified by following the change in wound area over time compared with the original wound area. These values are representative of between four and nine experiments and are expressed in graphs as percentage of original wound area ± SD.

2.3.6 Cell proliferation assay

The proliferation of A549, Calu-3 and Caco-2 cells was measured using the Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich). Briefly, cells were plated at a density of 1 x 10⁴ in 96-well plates. After 24 h, culture medium was removed and replaced with cell culture medium plus 1% FBS supplemented with or without cytokines. After 72 h, ten microlitres of CCK-8 reagent were added to the medium (100 μl) for 2 h at 37°C. Absorbance was read at 450 nm using a FLUOstar OPTIMA microplate reader. The values expressed are representative of between three and six experiments.

2.3.7 Statistical analysis

Results were expressed as mean ± S.D., compared using one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test. P < 0.05 was considered as significant.
2.4 Results

2.4.1 Morphologic alterations induced by TGF-β1 and pro-inflammatory cytokine treatments

In the absence of TGF-β1 or pro-inflammatory cytokines, A549, Calu-3 and Caco-2 cells maintained a cobblestone appearance characteristic of epithelial cells, together with a normal growth pattern (Fig. 1). Following exposure to TGF-β1 (5 ng/ml) for 72 h, A549 cells adopted a spindle-shaped phenotype and exhibited reduced cell-cell contacts. Similarly, treatment with cytomix [IL-1β, IFN-γ and TNF-α] (10 ng/ml) gave rise to a more elongated morphology in A549 cells. These morphologic alterations were most marked in A549 cells when challenged with a combination of both TGF-β1 (5 ng/ml) and cytomix (Fig. 1, top panel). In contrast, following treatments, monolayers of both Calu-3 and Caco-2 cells maintained an unchanged appearance (Fig. 1, middle and bottom panel).

The arrangement of the actin filaments was visualised by TRITC-phalloidin labelling. Under control conditions, all cell types exhibited highly organised filamentous actin (F-actin) immediately underneath the cell membrane and extending to the cellular junctions (Fig. 1). Exposure to either TGF-β1 or cytomix resulted in a re-distribution of F-actin in A549 cells (Fig. 1, top panel). Evidence of formation of F-actin stress fibres was most pronounced in A549 cells stimulated with both TGF-β1 and cytomix. Neither Calu-3 nor Caco-2 cells exhibited such rearrangements of their cytoskeletons, with both retaining well organised actin fibres beneath the plasma membrane (Fig. 1, middle and bottom panel).
Figure 1. Morphological changes induced by TGF-β1 and pro-inflammatory cytokines in A549 (top panel), Calu-3 (middle panel) and Caco-2 (bottom panel) cells. All cells were grown to ~70% confluence and stimulated with TGF-β1 (5 ng/ml) [b, f], cytomix (10 ng/ml) [c, g] or TGF-β1 and cytomix [d, h] for 72 h. For visualisation of F-actin, cells were fixed and labelled using TRITC-phalloidin (red). Under control conditions, all cells exhibited a cobblestone appearance typical of epithelia. Following treatment, A549 cells showed evidence of loss of cell-cell contacts, acquisition of a more fibroblast-like morphology and formation of F-actin stress fibres. Calu-3 and Caco-2 cells retained their epithelial morphology. Bars 20 μm.
2.4.2 Effects of TGF-β1 and pro-inflammatory cytokine exposure on E-cadherin and vimentin expression levels

Down-regulation of E-cadherin, a transmembrane protein of the adherens junctions contributing to the maintenance of the epithelial barrier, is characteristic for early EMT (Thiery et al., 2006). Using CLSM, the pattern of E-cadherin expression prior to, and following cytokine treatment, was evaluated. Under control conditions, all cell types stained positively for E-cadherin along their intercellular interfaces (Fig. 2a, 3a, and 4a). Treatment of A549 cells with TGF-β1, cytomix or TGF-β1 and cytomix resulted in loss of membrane-bound E-cadherin (Fig. 2b-d). Coupled to this, Western blot analysis showed that E-cadherin protein levels were reduced in treated A549 cells (Fig. 2i). Unlike A549 cells, both Calu-3 and Caco-2 cells showed no disruption of E-cadherin staining, maintaining their epithelial phenotype (Fig. 3a-d and 4a-d).

Moreover, protein levels remained unchanged, as illustrated by Western blot (Fig. 3i and 4i). In parallel, stimulation of A549 cells gave rise to a fibrillar rearrangement of vimentin filaments, as evidenced by CLSM (Fig. 2f-h). This was coupled with a significant increase in expression of vimentin (Fig. 2j). Treatment of Calu-3 and Caco-2 cells, however, failed to modify their vimentin structures, with protein levels remaining unaltered (Fig. 3j and 4j).
Figure 2. Effect of TGF-β1 and pro-inflammatory cytokines on expression of E-cadherin and vimentin in A549 cells. a-h) Immunofluorescence staining for E-cadherin and vimentin (green). Cells were grown to ~70% confluence on chamber slides, serum deprived for 24 h and then treated with TGF-β1 (5 ng/ml) [b, f], cytomix (10 ng/ml) [c, g] or TGF-β1 and cytomix [d, h]. Nuclei were counterstained with propidium iodide (red) Bars 20 μm. i, j) Western blot analysis of cell lysates for E-cadherin and vimentin expression. Expression levels were evaluated by densitometric analysis and standardised by comparison to the β-actin loading control. Treatment of A549 cells gave rise to loss of E-cadherin together with marked increase in vimentin expression. All images and blots are representative of three independent experiments. Means ± SD; * P < 0.05, ** P < 0.01.
Figure 3. Effect of TGF-β1 and pro-inflammatory cytokines on expression of E-cadherin and vimentin in Calu-3 cells. a-h) Immunofluorescence staining for E-cadherin and vimentin (green). Cells were grown to ~70% confluence on chamber slides, serum deprived for 24 h and then treated with TGF-β1 (5 ng/ml) [b, f], cytomix (10 ng/ml) [c, g] or TGF-β1 and cytomix [d, h]. Nuclei were counterstained with propidium iodide (red). Bars 20 μm. i, j) Western blot analysis of cell lysates for E-cadherin and vimentin expression. Expression levels were evaluated by densitometric analysis and standardised by comparison to the β-actin loading control. Expression levels remained unchanged in Calu-3 cells. All images and blots are representative of three independent experiments. Means ± SD.
Figure 4. Effect of TGF-β1 and pro-inflammatory cytokines on expression of E-cadherin and vimentin in Caco-2 cells. a-h) Immunofluorescence staining for E-cadherin and vimentin (green). Cells were grown to ~70% confluence on chamber slides, serum deprived for 24 h and then treated with TGF-β1 (5 ng/ml) [b, f], cytokin (10 ng/ml) [c, g] or TGF-β1 and cytokin [d, h]. Nuclei were counterstained with propidium iodide (red). Bars 20 μm. i,j) Western blot analysis of cell lysates for E-cadherin and vimentin expression. Expression levels were evaluated by densitometric analysis and standardised by comparison to the β-actin loading control. Expression levels remained unchanged in Caco-2 cells. All images and blots are representative of three independent experiments. Means ± SD.

2.4.3 Influence of TGF-β1 and pro-inflammatory cytokines on cell migration and proliferation

EMT is characterised by marked actin rearrangements with the dismantling of cell-cell junctions. As a consequence, cells become independently motile during EMT.

Given this, the effect of TGF-β1 and cytokin on the migration of A549, Calu-3 and Caco-2 cells was evaluated using a scratch wound assay. Incubation of A549 cells with TGF-β1 following injury to the monolayers, had little effect on wound closure
compared to control (Fig. 5a-i). In contrast, stimulation with cytomix had a significant 
($P < 0.05$) effect on wound healing, with over 90% of the wound recovered after 24 h 
(Fig. 5a-i). Similarly, co-treatment with TGF-β1 and cytomix was found to 
significantly ($P < 0.05$) enhance the rate of wound closure (Fig. 5a-i). Notably, in 
Calu-3 cells, no differences were seen between treatment with TGF-β1, cytomix or 
co-treatment with both for 24 h, compared to controls (Fig. 5i). Similarly, incubation 
with TGF-β1, cytomix or TGF-β1 and cytomix failed to alter the migration pattern of 
Caco-2 cells when compared to control conditions (Fig. 5i).

Proliferation of stimulated A549 cells decreased significantly ($P < 0.05$), when 
compared to untreated cells (Fig. 5j). TGF-β1, cytomix and co-treatment with both, 
produced similar anti-proliferative effects (Fig 5j). The proliferative capacity of both 
Calu-3 and Caco-2 cells were unaffected by treatment with TGF-β1, cytomix or TGF-
β1 and cytomix (Fig. 5j).
Figure 5. a-h) Representative images of wound healing assay in A549 cells at 0 h (top panel) and 24 h (bottom panel) in the presence of medium only (a, e), TGF-β1 (b, f), cytomix (c, g) or TGF-β1 and cytomix (d, h). Mechanical wounds were produced by scratching, followed by incubation with TGF-β1 (5 ng/ml), cytomix (10 ng/ml) or TGF-β1 and cytomix. Images were captured at 0 h and 24 h. The area of the wound was measured using the NIH ImageJ freeware program and the % wound closure was calculated. Bars 300 μm. i) Evaluation of migratory capacity of A549, Calu-3 and Caco-2 cells following stimulation with TGF-β1 and/or pro-inflammatory cytokines. Both cytomix and TGF-β1 and cytomix were found to significantly increase migration of A549 cells. In the case of Calu-3 and Caco-2 cells, the different cytokine treatments failed to alter migration. Means ± SD from between 3 and 9 independent experiments. * P < 0.05, ** P < 0.01 versus medium alone. j) Proliferation of A549, Calu-3 and Caco-2 cells following stimulation with TGF-β1 and/or pro-inflammatory cytokines. Following serum starvation for 24 h, cells grown in 96-well plates were treated with TGF-β1, cytomix or TGF-β1 and cytomix for 72 h, at which point 10 μl of CCK-8 reagent was added to each well. After 2 h incubation at 37°C, absorbance was read at 450 nm. Cytokine treatments significantly reduced the proliferative capacity of A549 cells, however, no effect was observed in both Calu-3 and Caco-2 cells. Means ± SD from between 3 and 6 independent experiments. * P < 0.05.

2.4.4 Telmisartan inhibits pro-inflammatory cytokine-induced cell migration and collagen I production in A549 cells but fails to abrogate EMT

A549 cells were treated with the angiotensin II receptor antagonist, telmisartan in the presence of TGF-β1, cytomix or TGF-β1 and cytomix. Analysis of the EMT markers
by Western blot revealed no effect on E-cadherin or vimentin expression levels (Fig. 6a and b). Telmisartan, however, significantly ($P < 0.05$) abolished cytokine-induced collagen I expression (Fig. 6c). Moreover, this effect was found to be dose-dependent (Fig. 6d). Additionally, it reduced the migratory capacity of A549 cells incubated with TGF-β1, cytomix or a combination of both, as evidenced by impaired wound closure (Fig. 6e). It had no effect on migration of cells in their resting state, however.
Figure 6. Effect of telmisartan on EMT markers, collagen I production and migration in A549 cells. Western blot analysis revealed that telmisartan (1 μM) had no observable effect on expression of (a) E-cadherin and (b) vimentin following stimulation of A549 cells with TGF-β1, cytomix or TGF-β1 and cytomix. c) Collagen I levels were significantly attenuated following co-treatment with telmisartan. d) Telmisartan’s inhibitory effect on collagen I production was shown to be dose-dependent. e) Migratory capacity of A549 cells was diminished when treated concomitantly with telmisartan, as evidenced by reduced wound closure. Untreated (black bars); telmisartan-treated (grey bars) In all cases, means ± SD from 3 independent experiments. * P < 0.05.
2.4.5 Smad 2/3 is not involved in telmisartan inhibitory effect on cell migration and collagen I production

Next, the potential pathway by which telmisartan exerted its inhibitory effects on collagen I production and cell migration was investigated. Phosphorylation of Smad 2/3 was assessed by immunofluorescence microscopy. TGF-β1, TGF-β1 and cytomix, and to a lesser extent, cytomix, induced Smad2/3 phosphorylation (Fig. 7a-d). However, concomitant treatment with telmisartan did not prevent this increase in levels of phosphorylated Smad2/3 (Fig. 7e-h).

Figure 7. Telmisartan’s actions are independent of Smad 2/3. Immunofluorescent staining for pSmad 2/3 (green) following treatment with TGF-β1 (b, f), cytomix (c, g) or TGF-β1 and cytomix (d, h) for 1 h. Nuclei were counterstained with propidium iodide (red). A549 cells exhibited marked phosphorylation of Smad 2/3 following cytokine stimulation (top panel). Concomitant treatment with telmisartan (1 μM) failed to attenuate Smad 2/3 phosphorylation (bottom panel). Bars 20 μm.
2.5 Discussion

Numerous studies have illustrated that epithelial cells have the ability to acquire mesenchymal features, affording proof of principle for the process of EMT (Willis et al., 2005; Kim et al., 2006). In doing so, EMT may contribute to the deterioration of epithelial structures and generation of fibroblasts associated with the accumulation of extracellular matrix in chronic fibrotic disorders (Gharae-Kermani et al., 2009). In this study, we addressed whether model cells of human alveolar (A549), bronchial (Calu-3) and intestinal (Caco-2) epithelia undergo EMT in vitro, when exposed to TGF-β1 and pro-inflammatory cytokines.

Our investigations confirmed that A549 cells underwent both morphologic and biochemical changes consistent with that seen in EMT. Interestingly, in addition to the classical EMT-inducer, TGF-β1, treatment with cytomix alone also gave rise to a mesenchymal phenotype. Moreover, in some instances it was found to augment the pro-EMT effects of TGF-β1, although this was not uniformly the case. This supports the suggestion that inflammation of the airways may contribute to abnormal tissue repair (e.g., fibrogenesis) (Lappalainen et al. 2005; Liu et al., 2008). In contrast, Calu-3 and Caco-2 cells failed to undergo such phenotypic changes, as the expression and localisation of E-cadherin and vimentin was unaltered by treatments in both cell types. Consistent with these results, morphologic alterations from an epithelial to fibroblastic appearance were not observed.

Since acquisition of an elongated morphology and rearrangement of the cytoskeleton facilitates cell migration, we also evaluated each cell type’s migratory capacity using
a scratch wound assay. In line with their apparent differential susceptibility to EMT, only A549 cells exhibited enhanced wound closure following stimulation with pro-inflammatory cytokines. Our observations of alveolar EMT are in accordance with previous findings (Yao et al., 2004; Kasai et al., 2005; Willis et al., 2005) and support the suggestion that EMT may contribute to the accumulation of myofibroblasts in IPF (Willis et al., 2006). However, our investigations using the model systems Calu-3 and Caco-2 suggest that both bronchial and intestinal epithelial cells are not amenable to this process. This finding substantiates the assertion that the phenotype which undergoes EMT is limited to just a few cell populations (Brown et al., 2004).

In contrast to our findings, a number of studies have shown that bronchial epithelial cells are capable of undergoing EMT (Borthwick et al., 2010; Hejink et al., 2010). However, it is important to note that several morphologically distinct cell types comprise the human tracheo-bronchial epithelium. Research by Kuroishi et al. (2009) using TGF-β1-stimulated mouse tracheal epithelial cells in primary culture revealed that α-SMA positive cells co-expressed cytokeratin-5 and -7, which are primarily expressed in basal epithelial cells. Moreover, α-SMA was not co-localised with either β-tubulin IV or mucin 5AC, which are expressed by ciliated and mucous cells, respectively. The bronchial epithelial cell line used in these investigations, Calu-3, exhibits characteristics of submucosal gland epithelium (Shen et al., 1994; Lee et al., 1998). This suggests that EMT of bronchial epithelial cells may be restricted to those cells exhibiting a basal phenotype. Additionally, both cell lines which have been previously shown to undergo EMT, 16HBE14o- (Zhang et al., 2009; Hejink et al., 2010) and BEAS-2B (Doerner et al., 2009), possess properties most characteristic of basal-like cells (Zhu et al., 1999). Similarly, this has also been shown in epithelia of
the breast, whereby basal-like cells appear to have an intrinsic phenotypic plasticity for mesenchymal transition (Sarrió et al., 2008).

That EMT may contribute to the accumulation of myofibroblasts in intestinal fibrosis has only recently been suggested (Bataille et al., 2008; Rieder et al., 2009). Currently, there is a lack of supportive data. However, known mediators of EMT (e.g., TGF-β1 and other inflammatory cytokines) are abundant in the inflamed intestine, supporting the hypothesis that the likelihood of this process occurring may be relatively high. Moreover, cumulative evidence indicates that EMT is a contributor to cancer progression in the intestine, giving rise to a more invasive tumour cell phenotype (Bates et al., 2007), suggesting that epithelial cells of the intestine exhibit sufficient plasticity to undergo EMT. Our data, on the other hand, using the Caco-2 cell line, suggest that colonic epithelial cells fail to undergo EMT in the presence of TGF-β1, cytomix or a combination of both. This finding may indicate that intestinal EMT is restricted to only certain pathological settings or cell types.

Notably, these studies have utilised continuous cell cultures which, in the main, are representative of alveolar, bronchial and colonic epithelia. However, it must be highlighted that these in vitro models do not retain all of the characteristics of such cells in vivo. In particular, they are free from the regulatory mechanisms of surrounding tissues (e.g., basement membrane, interstitium). Consequently, we can only speculate that these observations are replicated in vivo. Nevertheless, they do provide preliminary evidence to suggest susceptibility to EMT differs depending on epithelial cell origin.
Given the apparent importance of alveolar EMT, as evidenced by both our study and those of others (Yao et al., 2004; Kasai et al., 2005; Willis et al., 2005), the second part of our investigations focused on potential amelioration of EMT by way of pharmacological inhibition. Previously, angiotensin II receptor (AT2R) antagonists have been shown to effectively attenuate bleomycin-induced pulmonary fibrosis in several animal models (Molina-Molina et al., 2006; Yao et al., 2006; Waseda et al., 2008). Although it remains unclear as to the exact mechanism by which they exert their anti-fibrotic effect, treatment with AT2R antagonists has been shown to effectively reduce serum TGF-β1 levels (Yao et al., 2006). In addition, it has been shown in the lung that stimulation with TGF-β1 gives rise to enhanced production of angiotensinogen, a precursor of angiotensin II, initiating an autocrine signalling loop (Uhal et al., 2007). Moreover, angiotensin has been identified as a profibrotic agent in IPF and an inducer of EMT in other tissues (Rodrigues-Diez et al., 2008). In light of this, we hypothesised that AT2R antagonists may, at least in part, serve to inhibit EMT. However, treatment using telmisartan, an AT2 type 1 receptor antagonist which also exhibits PPAR-γ agonist properties (Benson et al., 2004), failed to influence the expression level of EMT markers, E-cadherin and vimentin.

An integral component in the fibrogenic reaction is the excess deposition of ECM, in particular collagen I (Selman et al., 2002). Thus, we investigated telmisartan’s effects on collagen I synthesis in A549 cells. Under basal conditions, A549 cells synthesise minimal amounts of collagen I, however, following stimulation with TGF-β1, cytomix and most markedly TGF-β1 and cytomix, levels were dramatically increased as the cells acquired myofibroblastic characteristics. Interestingly, concomitant treatment with telmisartan was able to significantly reduce collagen I levels in treated
A549 cells. These findings are in agreement with previous studies performed in breast cancer cells and mesangial cells (Yao et al., 2008; Kociecka et al., 2010). Notably, telmisartan’s inhibitory action on collagen biosynthesis was mediated via PPAR-γ activation in both cases. Furthermore, we studied the effect of telmisartan on the migration of A549 cells in the presence of TGF-β1, cytomix and TGF-β1 and cytomix. Significantly, the enhanced migratory capacity of treated cells was attenuated in the presence of telmisartan. Although AT2R inhibition may not prevent the acquisition of a mesenchymal phenotype, conceivably, its effect on collagen biosynthesis and cell migration may offer protection from the progression of fibrosis.

In addition to examination of telmisartan’s effects on EMT marker expression, collagen synthesis and cell migration, we undertook further investigations to evaluate potential mechanisms through which it exerts its action. Smad signalling pathways, in particular that of Smad2 and Smad3, are known to be activated in alveolar epithelial cells by TGF-β1 (Kasai et al., 2005), and indirectly by cytomix (Liu, 2008), and are thought to be integral to the EMT signalling cascade (Xu et al., 2009). Moreover, both AT2R antagonists and PPAR agonists have been previously shown to modulate the TGF-β1/Smad signalling pathway in a number of cell types (Yao et al., 2007; Mulay et al., 2010). In contrast, our microscopy studies qualitatively revealed that telmisartan failed to inhibit the phosphorylation of Smad 2/3, suggesting that its effects may be independent of this pathway.

Alternative mechanisms for the inhibitory effects observed for collagen I production and cell migration may include blockade of mitogen activated kinases (MAPK). For example, p38 MAPK and ERK 1/2 have been implicated in some models of EMT.
(Ellenrieder et al., 2001; Rodrigues-Díez et al., 2008). Furthermore, telmisartan has been shown to inhibit collagen biosynthesis in breast cancer epithelial cells via a p38 MAPK-dependent pathway (Kociecka et al., 2010), whilst candesartan, another AT2R antagonist, effectively inhibits activation of both p38 and ERK 1/2 signalling pathways in vascular-derived fibroblasts (Liu et al., 2010). However, given telmisartan’s pleiotropic nature (Rizos et al., 2009), inhibition of components of alveolar EMT could be mediated by numerous potential pathways.

In summary, our investigations revealed that exposure of bronchial and intestinal epithelial cells to TGF-β1 and pro-inflammatory cytokines failed to induce phenotypic alterations associated with EMT. This was in contrast to epithelial cells of the alveolus which readily underwent EMT following stimulation. These findings suggest that epithelia of the alveolus, bronchus and gut exhibit differential cell plasticity and susceptibility to EMT and raise the question as to what extent EMT may contribute to fibrogenesis in the proximal airways and large intestine. Unlike serous-like epithelial cells of the bronchus and enterocytes, alveolar type II cells exhibit progenitor-like properties whereby they can transdifferentiate to alveolar type I cells following injury to the epithelium (Willis et al., 2006). Conceivably, this may afford them enhanced capacity to undergo EMT. However, further investigations are required to elucidate the exact properties which provide Calu-3 and Caco-2 cells greater protection against this biochemical process. Given that our studies utilised human cell lines and, as such, are associated with some limitations, additional studies utilising primary cultures are also necessary. Moreover, in the context of alveolar EMT, we revealed that the AT2R antagonist and partial PPAR-γ agonist, telmisartan, is capable of ameliorating collagen I synthesis in A549 cells undergoing EMT, and at
the same time inhibits development of the migratory phenotype typically associated with transition to a mesenchymal phenotype. These findings point to a potential use as adjunct therapy in the treatment of IPF. Crucially, further investigations are required to elucidate the exact signalling pathways involved, in addition to characterisation of its effect *in vivo*.
2.6 References


Câmara J, Jarai G (2010) Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-alpha. Fibrogenesis Tissue Repair 3(1):2


Doerner AM, Zuraw BL (2009) TGF-beta1 induced epithelial to mesenchymal transition (EMT) in human bronchial epithelial cells is enhanced by IL-1beta but not abrogated by corticosteroids. Respir Res 27;10:100


vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci U S A 103: 13180-13185


fibroblasts via RAGE, MAPK and NF-kappaB pathways. Atherosclerosis. 208(1):34-42


Pharmacological modulation of epithelial mesenchymal transition caused by angiotensin II. Role of ROCK and MAPK pathways. Pharm Res 25(10):2447-61


Chapter 3

Cytoskeletal re-arrangement in TGF-β1-induced alveolar epithelial-mesenchymal transition studied by atomic force microscopy and high-content analysis

Parts of this chapter have been submitted as:


and

Stephen T. Buckley, Anthony M. Davies, Carsten Ehrhardt (2010) Atomic force microscopy (AFM) and high-content analysis (HCA) - two innovative technologies for dissecting the relationship between epithelial-mesenchymal transition (EMT)-related morphological and structural alterations and cell mechanical properties. Methods in Molecular Biology.
3.1 Abstract

Epithelial-mesenchymal transition (EMT) is closely implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF), serving as a purported source of fibrogenic myofibroblasts. Associated with this phenotypic transition is acquisition of an elongated cell morphology and establishment of stress fibres. However, the extent to which these EMT-associated changes influences cellular mechanics is unclear. Herein, we assessed the bio-mechanical properties of alveolar epithelial cells (A549) following exposure to TGF-β1 in the presence or absence of a collagen I substrate. Using atomic force microscopy (AFM), changes in cell stiffness and surface membrane features were determined. Stimulation with TGF-β1 gave rise to a more than two-fold increase in stiffness. Moreover, this effect was found to be augmented in the presence of a collagen I matrix. Coupled to this, alterations in topographical features were observed, with TGF-β1-treated cells exhibiting a rougher surface profile with notable protrusions. In line with these findings, simultaneous quantitative examination of the morphological attributes of stimulated cells using an image-based high-content analysis (HCA) system, revealed dramatic alterations in cell shape, F-actin content and distribution. Together, these investigations point to a strong correlation between the cytoskeletal-associated cellular architecture and the mechanical dynamics of alveolar epithelial cells undergoing EMT.
3.2 Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic pulmonary disease of largely unknown aetiology, characterised by progressive fibrotic changes which ultimately leads to irreversible distortion of the lung architecture (Thannickal et al., 2004). Central to this pathogenesis is the formation of fibrotic foci, consisting predominantly of activated myofibroblasts (Selman et al., 2003). Whilst the precise origin of these fibrogenic myofibroblasts has yet to be fully elucidated, cumulative evidence suggests that epithelial-mesenchymal transition (EMT) of resident cells of the alveolar epithelium, a process whereby these cells lose their characteristic structural and biochemical attributes and adopt features typical of a mesenchymal phenotype, may be one such possible source (Willis et al., 2006; Willis et al., 2007). Moreover, in support of this, a collection of cytokines are known to be activated in IPF, and a number of these have been shown to promote EMT (e.g., TGF-β1).

Implicit in this conversion from epithelial to mesenchymal phenotype are distinct alterations in cellular morphology, architecture, adhesion and migratory capacity (Kalluri et al., 2009). In particular, a hallmark of EMT and loss of epithelial function is the formation of actin stress fibres (Radisky et al., 2007). This results in dynamic changes in the structure of the cytoskeleton, endowing affected cells with a spindle-shaped morphology. Notably, the impact of these structural modifications on cell mechanical properties remains poorly understood. The stiffness of the cytoskeleton is determined to a great extent by the actin network (Stricker et al., 2010). Thus, conceivably, given the dramatic acquisition of actin stress fibres during alveolar EMT, such changes should also be reflected in alterations in the mechano-elastic
properties of the cells. In particular, EMT may represent a potential contributor to the abnormal tissue hardening observed in IPF. In this regard, measuring the viscoelastic properties of transitioning living cells should provide novel insights into the influence of EMT-associated cytoskeletal restructuring on the stiffening of lung parenchyma and reveal to what extent the elastic properties are caused by cellular components, particularly parts of the cytoskeleton.

The extracellular matrix (ECM) provides a dynamic support structure on which epithelial cells can grow. Moreover, it also serves to influence cellular behaviour (e.g., migration, proliferation and morphology) (Daley et al., 2008). Indeed, evidence indicates that ECM possesses the capacity to bring about transformation of epithelium to mesenchyme (Hay, 1993). Although the primary inducer of EMT is TGF-β1, the importance of the ECM is becoming increasingly apparent, with recent studies suggesting that these components influence and augment the pro-fibrotic effects of TGF-β1 (Zeisberg et al., 2001; Kim et al., 2006; Shintani et al., 2008; DeMaio et al., 2010). Additionally, from a bio-mechanical perspective, it is known that through its interactions with alveolar epithelial cells, the ECM exerts important centrifugal tethering forces, which serve to balance those centripetal forces exerted by the cytoskeleton (Dudek et al., 2001). Specifically, a number of studies in different cell types have shown that collagen I, a major component of the basement membrane in fibrotic tissues, may serve to induce EMT (Zeisberg et al., 2001; Shintani et al., 2008).

In this study, we used atomic force microscopy (AFM) to examine the mechanical stiffness of cells in response to TGF-β1, and the influence of the ECM component,
collagen I, on this process. Moreover, using high-content analysis (HCA) immunofluorescence imaging techniques, we were able to comprehensively quantify the morphological changes induced by TGF-β1 stimulation, and correlate it with those observations made using AFM imaging and force measurement analysis. Together, this enabled us to reveal the crucial importance of the actin network in determination of the structural and mechanical properties of alveolar epithelial cells during EMT. To our knowledge, this is the first such study which utilises both AFM and HCA as novel tools to assess EMT *in vitro.*
3.3 Materials and Methods

3.3.1 Materials
Hoechst 33258 was purchased from Invitrogen (Karlsruhe, Germany). Recombinant human TGF-β1 was purchased from PeproTech (London, UK). Cell culture medium, foetal bovine serum, TRITC-phalloidin and all other reagents were purchased from Sigma-Aldrich (Dublin, Ireland).

3.3.2 Cell culture conditions
A549 human alveolar epithelial cells (American Type Culture Collection, ATCC CL-185) were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK) and used between passage numbers 65 and 80. Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 medium (DMEM/F-12) supplemented with 5% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO₂ atmosphere and culture media was exchanged every 48 h. For live cell AFM studies, A549 monolayers were cultured in 6-well plates containing 24 mm glass coverslips. In AFM studies using fixed cells, A549 monolayers were grown in chamber slides (Nunc, Roskilde, Denmark). For HCA analysis, cells were cultured in 96-well plates (Nunc). Where cells were cultured on a collagen I substrate, a solution of rat tail collagen I was diluted using sterile water and each well was coated at a concentration of 20 μg/ml. The protein was allowed to bind for several hours at room temperature after which the excess fluid was removed and each well washed twice with PBS. In all studies, following one day in culture, cell medium was replaced with that containing 1% FBS and cells were treated with TGF-β1 (5 ng/ml) for 48 h.
3.3.3 High-content analysis (HCA)

Following culturing as detailed above, A549 monolayers were subsequently fixed by gently adding an equal volume of pre-warmed (37°C) 8% paraformaldehyde to culture medium for 15 min at 37°C. The cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 min, before being washed three times with 1% bovine serum albumin (BSA)/PBS. The cells were then incubated with TRITC-phalloidin to visualise filamentous (F-)actin and Hoechst 33258 to counter-stain the cell nuclei, for 30 min at 37°C. Following three washes using 1% (w/v) BSA/PBS, the plates were resuspended in PBS and stored at 4°C in the dark until further analysis. The 96-well plates were imaged using an InCell 1000™ Analyser Cellular Imaging and Analysis platform (GE Healthcare, Piscataway, NJ). A total of 15 fields per well were imaged under 20x magnification using 2 separate filters to capture the nucleus (blue) and F-actin (red), respectively. Image analysis was performed using the InCell Morphology 1 analysis software (GE Healthcare). This software detects cells for morphology analysis by nuclear dye uptake, with quantification of cellular morphologies and fluorescent intensities determined from 1 or more intracellular stains (e.g., F-actin). Morphological and fluorescence intensity staining parameters were automatically recorded for every cell in the field, and these parameters were also automatically recorded numerically as average values per field and average values per well. Morphological and fluorescence intensity/distribution parameters that were recorded included 1/(form factor), cell area, cell gyration radius, cell/nuclear area, nuclear displacement, intensity (nucleus and cytoplasm, N + C), IxA (nucleus and cytoplasm, N + C), intensity coefficient of variation (CV) and cell count. The definitions of these parameters are described in Table 1. Background fluorescence was subtracted for fluorescence intensity measurements.
Table 1. Description of InCell Analyzer 1000™ morphological and fluorescence intensity parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>l/(form factor)</td>
<td>Measure of cell roundness</td>
</tr>
<tr>
<td>Cell area</td>
<td>Area of the identified cell body</td>
</tr>
<tr>
<td>Cell gyration radius</td>
<td>Measure of the spread of the cell. Defined as the square root of the mean squared distance between the cell's pixels and its centre of gravity</td>
</tr>
<tr>
<td>Cell/nuclear area</td>
<td>Cell to nucleus area ratio</td>
</tr>
<tr>
<td>Nuclear displacement</td>
<td>Distance between the nucleus’s centre of gravity and the cell’s centre of gravity, divided by the gyration radius of the nucleus</td>
</tr>
<tr>
<td>IxA (N+C)</td>
<td>The amount of light emitted by the whole cell. It is equal to cytoplasm average intensity multiplied by cell area</td>
</tr>
<tr>
<td>Intensity CV</td>
<td>Coefficient of variation of the fluorescence intensity of pixels within the cytoplasm</td>
</tr>
<tr>
<td>Intensity spreading</td>
<td>Intensity-based descriptor allowing estimation of the extent of intensity concentration near the boundary of the object</td>
</tr>
</tbody>
</table>

3.3.4 Atomic force microscopy (AFM)

AFM experiments were performed using a NanoWizard II (JPK Instruments, Berlin, Germany) combined with a Nikon Eclipse Ti-E inverted microscope (Nikon Instruments, Surrey, UK). Force measurements were carried out on live A549 cells in a temperature-controlled liquid cell at 37°C. SiN cantilevers with a nominal spring constant of 0.35 N/m (DNP; Veeco Instruments, Santa Barbara, CA, USA) were used.
In contact mode, at least five $4 \times 4$ force-distance curves were recorded per cell. The maximum load was kept constant at 0.3 nN for all measurements and the amplitude was maintained at 5 μm. The depth of indentation did not exceed more than 10% of the cell thickness. Each force curve was fitted according to the Hertz model to obtain the elasticity at each point. Imaging was performed on fixed cells in PBS at room temperature using PPP-FM tips (NanoSensors, Neuchatel, Switzerland) with a nominal spring constant of 2.8 N/m. Samples were scanned in intermittent contact mode at a scan rate of 0.3 Hz at $512 \times 512$ pixels resolution. During imaging the AFM tip indents the cell membrane, producing deflection images in which the submembrane structures appear elevated, and in this way facilitates the acquisition of high resolution images, providing structural resolution on the nanoscale. Feedback gains were manually adjusted to obtain the best resolution in both height and deflection channels. Images were processed and analysed using JPK Image Processing Software v3 (JPK Instruments). In the case of cell surface roughness measurements, at least fifteen $4 \times 4$ μm$^2$ areas of the topographical images were randomly selected for each condition and the root mean square (RMS) roughness ($R_q$) and peak-to-valley roughness ($R_t$) were calculated using JPK Image Processing Software v3.

3.3.5 Statistical analysis

Results are expressed as means ± S.D., compared using one-way analysis of variance (ANOVA) followed by the Student Newman–Keuls post-hoc test. $P < 0.05$ was considered as significant.
3.4 Results

3.4.1 Alveolar epithelial cells undergo EMT-associated morphological changes following exposure to TGF-β1 which are enhanced by collagen I

Using a HCA immunofluorescence imaging platform the morphological alterations induced following treatment with TGF-β1 were quantitatively evaluated. Analysis of cellular morphology was based on fluorescent staining of F-actin components of the cytoskeleton (Fig. 1a-d). Of primary interest was the morphology parameter, \( \frac{1}{\text{form factor}} \), which serves as a measure of cell roundness. Values range from 1 to infinity, whereby 1 reflects a perfect circle. A significant increase in \( \frac{1}{\text{form factor}} \) was observed following stimulation of cells with TGF-β1 in comparison to those untreated (Fig. 1e). This finding is in accordance with cells undergoing EMT and adopting a more spindle-shaped morphology. Moreover, culturing on collagen I served to significantly augment TGF-β1-induced increase in \( \frac{1}{\text{form factor}} \) (Fig. 1e).

Additional morphological parameters were also evaluated, namely, cell area, cell gyration radius, ratio of cell/nucleus area, and nuclear displacement. Although cell area was not significantly altered (data not shown), the extent to which cells spread, as determined by cell gyration radius, was found to be significantly \( (P<0.05) \) enhanced following TGF-β1 exposure (Fig. 1f). In line with increasing morphological alterations, stimulation of cells grown on collagen I was also shown to markedly enhance cell gyration radius in comparison to both untreated cells, and those exposed to TGF-β1 alone, in the absence of a collagen I matrix support (Fig.1f). In contrast, neither cell/nuclear area nor nuclear displacement was found to be influenced by TGF-β1 or collagen I.
Figure 1. Morphological analysis of the cytoskeletal architecture of alveolar epithelial cells. A549 cells were grown in 96-well plates and treated with TGF-β1 (5 ng/ml) for 48 h. Cells were then fixed using paraformaldehyde. For visualisation of F-actin, cell were labelled using TRITC-phalloidin (red) and their nuclei counter-stained using Hoechst 33258 (blue). Imaging and analysis was performed using InCell 1000™ Analyzer Cellular Imaging and Analysis platform. a-d) Immunofluorescence staining for F-actin. Untreated cells showed a cobblestone appearance characteristic of epithelia. Following treatment, A549 cells exhibited loss of cell-cell contacts, acquisition of a more fibroblast-like morphology and formation of F-actin stress fibres. e, f) Quantitative morphological analysis. TGF-β1 stimulation resulted in significant increases in both 1/(form factor), a measure of cell roundness, and cell gyration radius, a measure of cell spreading, and these effects were significantly augmented by culturing on a collagen I substrate. In all cases, means ± SD from 3 independent experiments. * P < 0.05.
3.4.2 F-actin content and distribution is altered following TGF-β1 stimulation

Formation of F-actin stress fibres is a characteristic development in cells undergoing EMT. Hence, we also investigated the F-actin content and its distribution in cells upon stimulation with TGF-β1. The IxA (N+C) parameter effectively detects changes in cytoskeletal fluorescence intensity and correlates with alterations in F-actin content. Here, we observed an increase in fluorescence intensity of ~45% in those cells treated with TGF-β1 (Fig. 2a). In addition to F-actin content, the distribution of F-actin was assessed. Two appropriate parameters were utilised to evaluate such changes; intensity spreading, which estimates the extent of fluorescence intensity near the boundary of the cell, and intensity CV, which describes the coefficient of variation of the fluorescence intensity of pixels within the cytoplasm (i.e., an even distribution of F-actin staining would give a low intensity CV, whereas formation of discrete F-actin structures would increase the value of this measurement). TGF-β1 stimulation evoked a significant increase in the F-actin content of cells, as illustrated by a higher IxA (N+C) value (Fig. 2a). Although no significant change in intensity spreading was observed (data not shown), a marked increase in intensity CV was noted following treatment (Fig. 2b). These findings were supported by visual inspection of immunofluorescence images, which revealed formation of discrete stress fibres within treated cells. Similarly, TGF-β1 exposure to cells grown on collagen I evoked increases in both IxA (N+C) value and intensity CV (Fig. 2a and b). Interestingly, these effects showed apparent independence of growth support.
Figure 2. TGF-β1 exposure affects F-actin content and distribution. Using InCell 1000™ Analyzer Analysis software the cytoskeletal fluorescence intensity and distribution was evaluated using relevant parameters. Treatment with TGF-β1 (5 ng/ml) resulted in an increase in F-actin content as determined by the IxA (N+C) parameter (a). Significant alterations in F-actin distribution upon TGF-β1 exposure was confirmed by a marked increase in the intensity CV parameter (b). These effects were found to be independent of growth support. In all cases, means ± SD from 3 independent experiments. * P < 0.05 versus uncoated; # P < 0.05 versus collagen I.

3.4.3 AFM identifies EMT-associated structural alterations in alveolar epithelial cells

Using AFM the sub-membranous components of the cytoarchitecture, in particular the stiffer filamentous structures were visualised. The AFM deflection images in Fig. 3 show the morphology at the cell surface, indicating the sub-membrane structural organisation. In untreated samples, the cells are composed of poorly defined filamentous structures that appear as disordered ridges (Fig. 3a and c). In contrast, TGF-β1 stimulated cells exhibit well-aligned filamentous structures directly beneath its membrane (Fig. 3b and d). Given that actin is the predominant constituent of the cytoskeleton which localises under the cellular membrane, these observed structures are likely to be actin stress fibres.
Figure 3. Deflection images of alveolar epithelial cells measured by atomic force microscopy. A549 cells were grown on chamber slides and treated with TGF-β1 (5 ng/ml) for 48 h. Cells were then fixed using a mixture of paraformaldehyde and glutaraldehyde. Images were acquired using a cantilever with 2.8 N/m spring constant in PBS buffer at room temperature. Visualisation of the cell ultrastructure revealed numerous distinct filamentous structures (arrows) in those cells treated with TGF-β1 on glass (b) or on collagen I (d), representative of F-actin stress fibres. In contrast, untreated cells (a, c) exhibited indistinct filament arrangements. Images shown are representative data of 3 independent experiments. Bars 10 μm.

Further insight into their topographical features was provided by three dimensional reconstructions of the corresponding height images. Cross-section analysis revealed distinct differences in surface height patterns between untreated cells and those stimulated with TGF-β1 (either on glass or collagen I). Untreated cells exhibited a relatively smooth curved surface, typical of an epithelial cell type (Fig. 4a and c). In contrast, cells exposed to TGF-β1 showed a remarkably rougher surface profile.
interspersed with notable protrusions (Fig. 4b and d) suggestive of EMT-related fibrous filamental structures running parallel to the cell’s long axis.

Figure 4. Height images of alveolar epithelial cells obtained by atomic force microscopy. A549 cells were grown on chamber slides and treated with TGF-β1 (5 ng/ml) for 48 h. Cells were then fixed using a mixture of paraformaldehyde and glutaraldehyde. Images were acquired using a cantilever with 2.8 N/m spring constant in PBS buffer at room temperature. Cross-section analysis of three dimensional height projections of cells illustrated marked modifications in surface height patterns following stimulation with TGF-β1 (either on glass or collagen I). Untreated cells exhibited a relatively smooth curved surface, typical of an epithelial cell type (a, c). In contrast, cells exposed to TGF-β1 showed a remarkably rougher surface profile interspersed with notable protrusions, indicative of actin stress fibres (b, d). Images shown are representative data of 3 independent experiments.
An established quantitative method for demonstrating differences between surfaces is surface roughness. Using the JPK Image Processing Software v3 quantitative surface analysis was performed. Two parameters, root mean square (RMS) roughness ($R_q$), which represents the average of the measured height deviations taken within the evaluation length and measured from the mean line, and peak-to-valley roughness ($R_t$), a measurement of the absolute value between the highest and lowest peaks, were assessed. The data is summarised in Table 2. In support of analyses of image cross sections, these data demonstrate that TGF-$\beta$1-stimulation gave rise to significantly ($P<0.05$) rougher cells ($R_q = 44.32$ nm and $R_t = 203.43$) compared to untreated cells ($R_q = 44.32$ nm $R_t = 203.43$). Moreover, this effect appeared to be significantly ($P<0.05$) augmented when cultured on a collagen I substrate ($R_q = 63.31$ nm $R_t = 284.14$).

**Table 2.** Summary of calculated RMS roughness ($R_q$) and peak-to-valley roughness ($R_t$) values. In all cases, values are expressed as means ± SD of at least 15 values from three independent experiments.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$R_q$ (nm)</th>
<th>$R_t$ (nm)</th>
</tr>
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<tbody>
<tr>
<td>Uncoated</td>
<td>12.76 ± 1.76</td>
<td>73.03 ± 9.04</td>
</tr>
<tr>
<td>Collagen I-coated</td>
<td>15.38 ± 1.36</td>
<td>79.92 ± 5.98</td>
</tr>
<tr>
<td>Uncoated + TGF-β1</td>
<td>44.32 ± 4.91</td>
<td>203.43 ± 18.08</td>
</tr>
<tr>
<td>Collagen I-coated + TGF-β1</td>
<td>63.31 ± 6.55</td>
<td>284.14 ± 32.91</td>
</tr>
</tbody>
</table>
3.4.4 TGF-β1 stimulation results in increased cell stiffness and is augmented by collagen I

Given the enhanced F-actin content at the surface of stimulated cells, it was anticipated that the local mechanical properties of these cells would change in response to these structural rearrangements. In contact mode, living cells were gently indented, and in doing so, a force was applied to induce cell membrane deformation. The level of deformation reflected the stiffness of the cell, and was illustrated in the resultant indentation force curves produced. Using JPK Image Processing software, Young’s moduli were extracted from the force curves according to the classical Hertzian model. The results are summarised in Figure 5. Histograms of the apparent Young’s modulus showed a log-normal distribution (Fig. 5a-d). Stimulation of cells with TGF-β1 gave rise to a notable shift in population distribution towards higher elasticity values (Fig. 5b and d.). Prior to treatment, cells exhibited a stiffness of 8.3±1.1 kPa when cultured on glass. Similarly, those grown on a matrix of collagen I displayed a Young’s modulus of 9.1±2.9 kPa. Following treatment, cell stiffness significantly increased, with a value of 21.5±5.2 kPa for those grown on glass. This represents more than a two-fold increase following TGF-β1 stimulation (Fig.5e). Interestingly, increases in cell stiffness were most apparent in cells cultured on a collagen I substrate. Our data indicates that collagen I significantly (P<0.05) augments the pro-stiffening effects of TGF-β1 (Fig.5e). Together, these results confirm that actin filament organisation is a determinant factor in the modulation of cell stiffness, and suggest that ECM interactions may also play a contributory role.
Figure 5. Mechanical alterations in alveolar epithelial cells following TGF-β1 stimulation measured by atomic force microscopy. A549 cells were grown on glass coverslips (uncoated or collagen I-coated) and treated with TGF-β1 (5 ng/ml) for 48 h. The elastic modulus of cells was measured in contact mode using a DNP tip. At least five 4×4 force-distance curves were recorded per cell. a-d) Histogram showing the overall distribution of elastic modulus. Under all conditions cells exhibited a log-normal distribution. Following TGF-β1 treatment (either on glass or collagen I), a notable population shift to higher elasticity values was observed. e) Bar graph illustrating the average cell stiffness representing > 750 single force–distance curves (means ± SD). On glass, TGF-β1 stimulation resulted in an increased stiffness of more than a two-fold compared to untreated cells. Exposure to TGF-β1 of cells cultured on collagen I enhanced stiffness four-fold and significantly augmented the effect of TGF-β1 alone.
3.4.5 Increase in cell stiffness is strongly correlated with alterations in cell shape

Cell shape is thought to be an important determinant in cellular biomechanics. Using data obtained from both AFM and HCA analysis, we plotted values for 1/(form factor) against relative elasticity (Fig. 6). A strong linear correlation between these two parameters was obtained ($R^2=0.98$). This correlative analysis indicates that the greater cells deviate from their cobblestone-like epithelial state towards a spindle-shaped mesenchymal phenotype, the more pronounced cell stiffness becomes.

Figure 6. Correlation between cell stiffness and shape. Values for 1/(form factor) were plotted against relative elasticity. Analysis revealed a strong correlation between both parameters ($R^2=0.98$). Elasticity values represent measurements from > 750 single force-distance curves. 1/(form factor) values represent data from 15 fields of >30 wells from three independent experiments. All values are expressed as means ± SD.
3.5 Discussion

Alterations in the mechanical properties of tissues and living cells are associated with a number of pathological processes (Krouskop et al., 1998; Kilpatrick et al., 2002; Tajaddini et al., 2003; Samani et al., 2004). Fibrosis, characterised by a thickening and scarring of tissue, is a notable example. In the lung, an organ which exhibits unique mechanical properties crucial for breathing, fibrosis results in a marked increase in tissue stiffness (Thannickal et al., 2004). The myofibroblast is the primary effector cell in IPF, and is responsible for synthesis, deposition and remodelling of ECM (Selman et al., 2003). Although their exact origin remains unresolved, evidence continues to suggest that EMT may be a contributory source (Willis et al., 2005; Kim et al., 2006). Here, in this study, application of AFM and HCA technologies has facilitated an entirely novel assessment of key mechano-cellular features of alveolar EMT, offering highly valuable insight into how the mechanical attributes of these transitioning cells are related to their underlying structure.

The cell cytoskeleton is a profoundly dynamic structure which is responsible for the determination of both cell shape and mechanical integrity (Mofrad et al., 2006). Typically, every cell type has a specific size and shape, with each holding a specialised function. Under circumstances whereby cells are unable to maintain their inherent shape, such functions are compromised. In this way, modifications of cytoskeletal structure can potentially give rise to changes in cell behaviour and phenotype. In line with this, and in agreement with previous studies (Kasai et al., 2005; Willis et al., 2005; Buckley et al., 2010), our investigations revealed that TGF-β1 stimulation of alveolar epithelial cells induced dramatic changes in cytoskeleton
The importance of the cytoskeleton in governing cell shape was emphasised by our quantitative analysis of cellular morphology, which highlighted a striking deviation away from the cobblestone appearance associated with epithelial cells, to a more elongated fibroblast-like form, following TGF-β1 exposure. Moreover, stimulated cells were shown to exhibit an enhanced capacity to spread, suggestive of a greater migratory potential, and characteristic of the mesenchymal phenotype (Crosby et al., 2010).

Immunofluorescence analysis of the cytoskeletal component, F-actin, revealed rearrangements in its organisation in accordance with transition to a (myo)fibroblast state. Before treatment, the majority of F-actin was localised to the periphery near intercellular junctions. TGF-β1 induced disruption of this arrangement, giving rise to increased numbers of F-actin stress fibres, assembled parallel to the long axis of cell bodies. Multiparametric analysis using a HCA platform permitted comprehensive quantitative assessment of these changes. Both F-actin intensity and distribution were shown to be significantly altered following TGF-β1 exposure. Additionally, culturing cells on collagen I appeared to enhance TGF-β1’s EMT-inducing effect. Of note, use of the image-based HCA technology facilitated a rapid, accurate and quantitative evaluation of the EMT-related changes in cytoskeletal architecture. To our knowledge, this represents the first such wide-ranging assessment of a series of morphological and fluorescence intensity parameters in the context of alveolar EMT.

Recent studies suggest that cellular mechanical properties may serve as novel biological markers of cell phenotypes, reflecting changes in differentiation or cellular transformation (Darling et al., 2007; Tilghman et al., 2008). In this regard, AFM has
emerged as a powerful technique capable of providing valuable insights into the nanomechanical properties of cells. Using AFM indentation we quantitatively assessed cellular elasticity in response to TGF-β1. Previously, it has been shown that spindle-shaped cells tend to be stiffer than round cells (Kidoaki et al., 2007). In agreement with this, we observed a significant increase in Young’s modulus in those cells stimulated with TGF-β1. These findings corroborate the suggestion that EMT gives rise to stiffer cells, and supports recent observations in epithelium of the kidney (Thoekling et al., 2010). Such changes in the mechanical properties brought about by the phenotypical transformation process may be attributed to the increase in the amount of organised actin filaments or stress fibres, which appear as stiff cables in a soft matrix. Indeed, a number of reports indicate that cytoskeletal structures, and in particular F-actin, are intimately involved in determination of cellular mechanics. Notably, Hertz’s model was utilised to analyse data from the indentation of cells by AFM. Whilst assumptions of homogeneity, isotropicity, and material elasticity cannot be wholly satisfied, it nevertheless offers a good estimate of the Young’s modulus (Kuznetsova et al., 2007).

Previous studies indicate that basement membrane architecture is critical in maintaining an epithelial phenotype, and that alterations in its composition may promote phenotypic change (Zeisberg et al., 2001). Collagen I is the most common component of the ECM and its production is markedly up-regulated in fibrotic lungs. Our findings reveal that cultivation of alveolar epithelial cells on a collagen I substrate promote a significantly greater increase in cell stiffness in response to stimulation with TGF-β1, than when grown on glass. In addition to cell shape and motility, it is acknowledged that the mechanical properties of living cells may be influenced by
biochemical and physical cues in their surroundings (McPhee et al., 2010). In this regard, the ECM appears to be an important determinant. Indeed, McPhee and colleagues (2010) have recently shown that fibroblasts cultured on fibronectin show notably higher stiffness in comparison to those grown on glass. Our findings lend further support to an important role for the ECM in determining cell biomechanics. In addition, in agreement with previous findings (Kim et al., 2006; Shintani et al., 2008; DeMaio et al., 2010) it appears that such cell-matrix interactions may facilitate TGF-β1-induced EMT. Although not yet established, contact to a collagen I matrix may serve to activate EMT-related signalling pathways, and in this way promote a greater observable effect upon TGF-β1 exposure.

In conclusion, our results illustrate that treatment of alveolar epithelial cells with TGF-β1 significantly augmented the Young’s modulus of living cells. Moreover, our analysis reveals that the ECM component, collagen I, significantly amplifies the effects of TGF-β1. In light of the fact that cellular elasticity is strongly influenced by cell shape and cytoskeletal structure, we examined the morphology and organisation of these structures upon exposure to TGF-β1. Quantitative analysis revealed significant increases in F-actin content and distribution together with changes in cell shape. Importantly use of both AFM and HCA permitted kinetic monitoring in vitro of live cells in real time and fixed cells, and in this way facilitated the measurement of novel and distinct events associated with EMT of alveolar epithelial cells. Moreover, this complementary approach showed high precision, accuracy and reproducibility, allowing measurements to be made directly at the individual cell level, thus minimising artefact and ensuring they are reflective of cell effects. To our knowledge, it represents one of the first such studies of this kind in the context of alveolar EMT.
Collectively, these analyses indicate that the mechanical dynamics of transitioning alveolar epithelial cells are greatly controlled by the cytoarchitecture, as evidenced by the strong correlation between cellular elasticity and cytoskeletal arrangement.
3.6 References


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(2001) Renal fibrosis: collagen composition and assembly regulates epithelial-
mesenchymal transdifferentiation. Am J Pathol 159(4):1313-21
Chapter 4

Interaction of the receptor for advanced glycation end-products with focal adhesion molecules in epithelial-mesenchymal transition of alveolar epithelial cells

Parts of this chapter have been submitted (under revision) as:

4.1 Abstract

Fibrosis of the lung is characterised by the accumulation of myofibroblasts, a key mediator in the fibrogenic reaction. Cumulative evidence indicates that epithelial-mesenchymal transition (EMT), a process whereby epithelial cells become mesenchyme-like, is an important contributing source to the myofibroblast population. Underlying this phenotypical change is a dramatic alteration in cellular structure. RAGE has been suggested to maintain lung homoeostasis by mediating cell adhesion, whilst the family of ezrin/radixin/moesin (ERM) proteins, on the other hand, serve as an important cross-linker between the plasma membrane and cytoskeleton. In the current investigation, we test the hypothesis that RAGE and ERM interact and play a key role in regulating EMT-associated structural changes in alveolar epithelial cells. Exposure of A549 cells to inflammatory cytokines resulted in re-distribution of ERM to the cell periphery and localisation with EMT-related actin stress fibres. Simultaneously, ERM underwent phosphorylation via ROCK, whilst blockade of this pathway attenuated cytokine-induced structural changes. Additionally, RAGE expression was diminished following cytokine stimulation, with release of its soluble isoform (sRAGE) via a matrix metalloproteinase (MMP)-9-dependent mechanism. Immunofluorescence microscopy and co-immunoprecipitation revealed strong association between ERM and RAGE under basal conditions, which was disrupted when challenged with inflammatory cytokines, as ERM in its activated state complexed with membrane-linked CD44. These data suggest that dysregulation of this newly characterised ERM-RAGE complex might be an important step in re-arrangement of the actin cytoskeleton during pro-inflammatory cytokine-induced EMT of human alveolar epithelial cells.
4.2 Introduction

Idiopathic pulmonary fibrosis (IPF) is characterised by a marked disruption in the integrity and structure of the alveolar epithelium. Specifically, injury to the alveolar epithelium gives rise to delayed re-epithelialisation leading to a denuded, disrupted basement membrane (Thannickal et al., 2004). In light of their compromised capacity to re-establish a physiological epithelial lining, increasing evidence suggests that affected populations of alveolar epithelial cells may undergo epithelial-mesenchymal transition (EMT), and in this way serve as a source of pathogenic mesenchymal cell types (Willis et al., 2006, 2007). Core to this process is the cytoskeletal re-arrangement of their actin structures. Following induction of EMT by pro-inflammatory cytokines (e.g., TGF-β1, TNF-α, IL-1β), actin filament architecture changes dynamically from a cortical actin network to stress fibres (Kalluri and Neilson, 2003). Although, currently, very little is known about the mechanisms which underlie these structural re-arrangements, conceivably, a better insight into these mechanisms may provide novel targets or approaches to ameliorate or prevent EMT-related structural alterations of the alveolus in IPF.

The ezrin/radixin/moesin (ERM) family of proteins provide a regulated linkage from the filamentous (F-)actin to membrane proteins on the surface of cells (Louvet-Vallée, 2000). In their native state they remain in a folded conformation due to an intramolecular interaction between their N- and C-terminal domains, which masks the membrane and F-actin binding sites. Upon phosphorylation, ERM proteins become activated enabling interaction with integral membrane proteins (e.g., CD44) and F-actin. Cumulative research suggests that proteins of the ERM family play a crucial
functional role in regulating cell shape (Fehon et al., 2010). Notably, in a drosophila-based model of epithelia, altered expression of ERM proteins resulted in disruption of epithelial morphology and integrity, with cells exhibiting a more invasive migratory pattern (Speck et al., 2003). Moreover, whilst their exact function in pulmonary physiology remains poorly characterised, a recent study suggests that ERM proteins may play a key role in the regulation of alveolar structure and lung homeostasis (Hashimoto et al., 2008).

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily (Neeper et al., 1992). Uniquely, it is expressed at extremely high levels in the lung under physiological conditions, whereas in most other organs low baseline RAGE levels can be observed, which increase during various pathologies such as diabetes, inflammation and cancer (Buckley et al., 2010). Previously, our laboratory has shown that RAGE plays an important role in alveolar epithelial cell function, facilitating cell spreading and adherence (Demling et al., 2006). Its importance is further emphasised by the fact that impairment of RAGE signalling gives rise to enhanced cell migration and proliferation (Queisser et al., 2008). Additionally, RAGE appears to be linked to cytoskeletal components within pulmonary epithelial cells (Demling et al., 2006; Queisser et al., 2008), and in this way, may mediate its regulatory adhesion function. From these observations, RAGE has been concluded to be involved in the maintenance of lung homoeostasis. In particular, this has been highlighted in pathological settings, whereby loss of RAGE has been associated with a fibrotic response (Englert et al., 2008; Queisser et al., 2008).
In this study, we tested the hypothesis that both ERM and RAGE serve as important regulators in the maintenance of normal alveolar epithelial structure and function, and that disruption of their basal expression patterns may be an important feature of alveolar EMT. Our findings revealed a strong co-association between ERM and RAGE in the A549 human alveolar epithelial cell line. Cytokine stimulation elicited a dramatic re-organisation of ERM towards the cell periphery together with proteolytic cleavage of RAGE, in a Rho kinase (ROCK) and matrix metalloproteinase (MMP)-9-dependent manner, respectively. In addition, we observed strong interactions between newly-formed actin stress fibres and ERM proteins. Taken together, these data suggest that dysregulation of the ERM-RAGE complex might be a key event in actin microfilament re-arrangement during cytokine-induced alveolar EMT.
4.3 Materials and Methods

4.3.1 Materials

Goat polyclonal anti-RAGE antibody (AB5484) and rabbit monoclonal anti-MMP-9 were purchased from Millipore (Carrigtwohill, Ireland). Rabbit polyclonal anti-ERM antibody (3142) and anti-pERM antibody (3141), and mouse monoclonal anti-CD44 antibody (3570) were purchased from Cell Signaling (Danvers, MA). TRITC-phalloidin was purchased from Sigma-Aldrich (Dublin, Ireland). Recombinant human TGF-β1, IL-1β, TNF-α and IFN-γ were purchased from PeproTech (London, UK). Cell culture medium, foetal bovine serum and all other reagents were purchased from Sigma-Aldrich.

4.3.2 Cell culture conditions

A549 human alveolar epithelial cells (American Type Culture Collection, ATCC CL-185) were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK) and used between passage numbers 65 and 89. Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 medium (DMEM/F-12) supplemented with 5% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured at 37°C in 5% CO₂ atmosphere and culture media was exchanged every 48 h. For studies described below, following one day in culture, cells were serum-starved overnight and then treated with cytokines in culture medium containing 1% FBS for 72 h as previously described (Buckley et al., 2010). In those experiments using either Y-27632 (Sigma-Aldrich) or MMP-9 inhibitor I (Calbiochem, San Diego, CA), cells were pre-incubated for 2 h prior to cytokine stimulation.
**4.3.3 Western blot analysis**

Cell cultures were lysed with cell extraction buffer (Invitrogen Karlsruhe, Germany) on ice and briefly sonicated. In the case of cell supernatants, culture medium was removed following treatments as detailed above. Protein sample concentrations were determined using a standard protein concentration assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer’s instructions. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to immunoblot polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (pH 7.4) for 1 h at room temperature. Incubation with the respective primary antibody was carried out overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody at room temperature for 1 h. Peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore). Relative levels of protein expression were quantified by densitometric analysis of the immunoblot using a ChemiDoc documentation system (Bio-Rad). When appropriate, blots were stripped and analysed for β-actin (Sigma-Aldrich) as internal control and total ERM.

**4.3.4 Immunofluorescence microscopy**

Lab-Tek chamber slides (Nunc, Roskilde, Denmark) were used to grow A549 cells under conditions as described above. Cells were fixed for 10 min with 2% (w/v) paraformaldehyde and incubated for 10 min in 50 mM NH₄Cl, followed by permeabilisation for 8 min with 0.1% (w/v) Triton X-100 in PBS. After a 60 min incubation with 150 μl dilution (CD44 1:100; ERM 1:100; pERM 1:100; RAGE 1:300; MMP-9 1:100) of the respective primary antibody, the cell layers were washed three times with PBS, before incubation with 100 μl of a 1:200 dilution of relevant
Alexa Fluor-labelled F(ab')$_2$ fragment (Invitrogen) in PBS containing 1% (w/v) BSA. Propidium iodide (1 µg/ml in PBS) was used to counterstain cell nuclei. In the case of F-actin staining, TRITC-phalloidin was used at a concentration of 500 ng/ml in PBS. After 30 min of incubation, the specimens were again washed three times with PBS and embedded in FluorSave anti-fade medium (Merck, Nottingham, UK). Images were obtained using a confocal laser scanning microscope (CLSM, Zeiss LSM 510, Göttingen, Germany) with the instrument’s settings adjusted so that no positive signal was observed in the channel corresponding to the fluorescence of the isotypic controls.

4.3.5 Gelatin zymography

Gelatinase activity (MMP-2 and -9) was measured in A549 cells-conditioned media as previously described (Medina et al., 2003). Samples were subjected to 10% SDS-PAGE with co-polymerised gelatin 0.2% (w/v). After electrophoresis, the gels were washed twice for 20 min each with 2% (w/v) Triton X-100, and then incubated in development buffer (50 mmol Tris-HCl, 200 mmol NaCl, 10 mmol CaCl$_2$ and 1 mmol ZnCl$_2$; pH 7.5) at 37°C overnight for the development of enzyme activity bands. Conditioned medium of HT-1080 human fibrosarcoma cells that contains high amounts of both MMP-2 and -9 was used as standard. After incubation, gels were fixed and stained in 40% methanol, 10% acetic acid and 0.1% (w/v) Coomassie Blue for 1 h and then de-stained. The gelatinolytic activities were detected as transparent bands against the background of Coomassie brilliant blue-stained gelatine. Images were analysed using a ChemiDoc documentation system (Bio-Rad).
4.3.6 Co-immunoprecipitation

Two hundred microlitres of lysate from A549 cells grown as described above was added to pre-washed protein A/G beads (20 μl of 50% bead slurry; Sigma-Aldrich) and incubated at 4°C for 60 min. The mixture was then centrifuged for 10 min at 4°C. Next, the supernatant was incubated with goat antibody against RAGE or CD44 at 4°C overnight. Pre-washed protein A/G beads (20 μl at 50%) were then added to the mixture and incubated for 3 h at 4°C. Following centrifugation, beads were washed five times with solubilisation buffer (20 mM Tris-HCl, pH 7.4; 5 mM EDTA; 150 mM NaCl; 10% glycerol; 1% Triton X-100). Isolated protein complexes were denatured for 5 min at 95°C, analysed by gel electrophoresis, and transferred to PVDF membranes, followed by immunoblotting with anti-ERM or anti-pERM antibodies and detection with Immobilon Western Chemiluminescent HRP substrate.

4.3.7 Statistical analysis

Results are expressed as mean ± S.D., compared using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test. $P < 0.05$ was considered as significant.
4.4 Results

4.4.1 ERM proteins undergo re-distribution and activation following exposure to TGF-β1 and/or cytomix and co-localise with actin stress fibres

Under basal conditions, ERM proteins exhibited a predominantly cytoplasmic distribution in A549 cells (Fig. 1a). Following treatment with TGF-β1 and/or cytomix, a marked re-distribution of ERM towards the plasma lemma was observed (Fig. 1b-d). This was coupled with alterations in cell morphology, with cells adopting a fibroblast-like shape. In tandem, immunoblot analysis revealed that cytokine treatment induced activation of ERM proteins as evidenced by their phosphorylation (Fig. 1e). To explore their involvement in EMT-associated cytoskeletal re-arrangement, co-localisation studies were performed by CLSM. In untreated cells, actin was strongly associated with the adherens junctions (Fig. 1f), whereas stimulation with TGF-β1 and/or cytomix resulted in re-organisation of cortical filaments, with cells exhibiting elongated stress fibres similar to those found in fibroblasts. Notably, ERM proteins were found to strongly co-localise with these newly-formed F-actin fibres (Fig. 1g-i).
Figure 1. a-d) Re-distribution and activation of ezrin/moesin/radixin (ERM) proteins following stimulation with TGF-β1 and/or cytomix and co-localisation with actin stress fibres. Immunofluorescence staining for ERM (green). A549 cells were grown on chamber slides and stimulated with TGF-β1 (5 ng/ml) [b], cytomix (10 ng/ml) [c] or TGF-β1 and cytomix [d] for 72 h. Nuclei were counterstained with propidium iodide (red). Treatment of A549 cells gave rise to marked re-distribution of ERM from the cytoplasm to the cell periphery. e) Western blot analysis of cell lysates for pERM. Treatment of A549 cells with TGF-β1 and/or cytomix induced phosphorylation of ERM proteins. Expression levels were evaluated by densitometric analysis and standardised by comparison to total ERM. f-i) For visualisation of F-actin, cells were fixed and labelled using TRITC-phalloidin (red). Following treatment with TGF-β1 (5 ng/ml) [g], cytomix (10 ng/ml) [h] or TGF-β1 and cytomix [i], A549 cells acquired a more fibroblast-like morphology together with formation of F-actin stress fibres. ERM proteins (green) exhibited strong co-localisation with these newly-formed stress fibres. Means ± S.D. from >3 independent experiments; * P<0.05. Bars 20 μm.

4.4.2 ROCK-dependence of ERM phosphorylation and actin stress fibre formation

In order to determine the signalling pathway through which pro-inflammatory cytokines activate ERM, A549 cells were incubated with Y-27632, a specific inhibitor of ROCK, followed by treatment with TGF-β1, cytomix or both. As observed by Western blot, inhibition of ROCK signalling prevented ERM phosphorylation (Fig.
2a). Its effect was most apparent in cells treated with TGF-β1 in the presence or absence of cytomix, whilst less so in those stimulated by cytomix alone. This suggests that phosphorylation of ERM mediated by pro-inflammatory cytokines is primarily mediated via a ROCK-dependent pathway. Of note, morphological analysis using phalloidin staining revealed that exposure to the ROCK inhibitor, Y-27632, prevented formation of mesenchymal-associated actin stress fibres in those cells stimulated with TGF-β1, whilst reducing stress fibre numbers in cytomix and TGF-β1 plus cytomix treated cells (Fig. 2b-i).

![Western blot analysis and F-actin structures](image)

**Figure 2.** ROCK-dependent phosphorylation of ERM proteins and actin stress fibre formation. A549 cells were incubated with Y-27632 (5 μM), a specific inhibitor of ROCK, followed by treatment with TGF-β1, cytomix or TGF-β1 and cytomix for 72 h. a) Western blot analysis revealed that cytokine-induced ERM phosphorylation was blocked following inhibition of ROCK signalling. b-i) F-actin structures were visualised using TRITC-phalloidin and examined by CLSM. Pre-incubation with Y-27632 ensured maintenance of cortical actin architectures in cells stimulated with TGF-β1, whilst in those treated with either cytomix or TGF-β1 and cytomix, the incidence of stress fibre formation was notably diminished. Means ± S.D. from >3 independent experiments; * P<0.05. Bars 25 μm.
4.4.3 RAGE is down-regulated and released in its soluble form when challenged by pro-inflammatory cytokines

We have previously shown that RAGE associates with intermediate filaments and microfilaments of the cytoskeleton (Demling et al., 2006). Given this, we investigated its expression following cytokine-induced remodelling of the cytoskeleton. Western blot analysis revealed abundant expression of RAGE in alveolar epithelial (A549) cells (Fig. 3a), with immunofluorescence microscopy indicating that it was predominantly localised to the membrane (Fig. 3b-e). Exposure to TGF-β1 and to a greater extent, cytomix and TGF-β1 and cytomix alone, resulted in a marked diminution of RAGE expression (Fig. 3a-e).

RAGE exists in a number of different isoforms (Gefter et al., 2009; Buckley et al., 2010). We hypothesised that RAGE may be cleaved, giving rise to release of its soluble isoform, sRAGE. Using Western blot, the level of RAGE in cell medium was assessed. Investigations revealed that RAGE levels in cytokine-stimulated medium were significantly higher than that in medium from control cells (Fig. 3f).
Figure 3. Down-regulation of RAGE and release of sRAGE when treated with TGF-β1 and/or cytomix. a) Western blot analysis demonstrated that expression of RAGE in A549 cells was significantly (P<0.05) diminished following treatment with TGF-β1 and/or cytomix for 72 h. b-e) Immunofluorescence staining for RAGE (green) illustrated that under control conditions, cells exhibited diffuse cytoplasmic staining [b] which was decreased on cytokine stimulation [c-e]. Nuclei were counterstained with propidium iodide (red). Bars 20 μm. 

f) Analysis by Western blot of corresponding cell supernatants revealed significantly (P<0.05) enhanced expression of sRAGE in those samples stimulated with TGF-β1 and/or cytomix. Expression levels were evaluated by densitometric analysis and standardised to controls. Means ± S.D. from >3 independent experiments; * P<0.05; ** P<0.01.
Release of sRAGE is mediated by MMP-9

Next, we determined the mechanism for release of sRAGE. MMPs are known to be up-regulated in fibrotic disorders of the lung, actively contributing to tissue remodelling (Atkinson et al., 2003). Specifically, MMP-9 is a known mediator of alveolar basement membrane disruption (Pardo et al., 2006), and has been previously implicated in protein ectodomain shedding (McCawley et al., 2001). In light of this, we investigated if proteolytic cleavage of RAGE was mediated by MMP-9.

In A549 cells pre-treated with MMP-9 Inhibitor I, a selective inhibitor of MMP-9, prior to cytokine stimulation, there was a marked decrease of RAGE in cell supernatants (Fig. 4a). Using gelatin zymography, we verified that treatment with TGF-β1 and/or cytomix enhanced MMP-9 activity. MMP-2, however, remained unchanged (Fig. 4b). In agreement with these findings, we observed enhanced MMP-9 expression in treated cells, as evidenced by CLSM (Fig. 4c-f). Together, these results suggest that MMP-9 contributed to the release of sRAGE into the cell medium.
Figure 4. MMP-9 mediates release of sRAGE. a) When cultures were pre-treated with MMP-9 Inhibitor I, cytokine-mediated release of sRAGE into the supernatant was blocked as indicated by Western blot analysis of relevant supernatants. b) Conditioned medium from untreated A549 cells and from TGF-β1, cytomix or TGF-β1 plus cytomix treated cells was subjected to analysis of gelatinolytic activities by zymography. HT1080 media (lane 1) was used as a marker of pro-MMP-2 and -9. Gelatinases of molecular weights of 72 and 92 kDa corresponding to pro-matrix metalloproteinases (MMP)-2 and pro-MMP-9, respectively, were detected. TGF-β1 and/or cytomix caused significant strong increases in pro-MMP-9 band activities which was regressed by MMP-9 Inhibitor I (5 nM). c-f) Immunofluorescence staining for MMP-9 (green) revealed enhanced expression levels in stimulated cells. Means ± S.D. from >3 independent experiments; * P<0.05. Bars 20 μm.

4.4.5 Uncoupling of the RAGE-ERM complex following cytokine stimulation

In untreated A549 cells, RAGE and ERM proteins exhibited marked co-localisation, with both showing diffuse cytoplasmic distribution as shown in Fig. 5a. Upon treatment with TGF-β1 and/or cytomix, this interaction was uncoupled, with ERM proteins re-distributing to the cell membrane and RAGE expression diminishing markedly (Fig. 5b-d). Co-immunoprecipitation analysis confirmed the association of RAGE and ERM proteins in A549 cells (Fig. 5e).
Figure 5. Cytokine stimulation uncouples ERM-RAGE complex. a-d) A549 cells cultured with or without TGF-β1, cytomix or TGF-β1 plus cytomix for 72 h were subjected to immunofluorescence staining with antibodies to ERM (green) and to RAGE (red). Untreated cells exhibited strong co-localisation of ERM and RAGE, with both showing diffuse cytoplasmic distribution. Following treatment, this association was disrupted as ERM redistributed towards the cell periphery and RAGE underwent proteolytic cleavage. Bars 20 μm. e) Cell lysate from A549 cells was immunoprecipitated using anti-RAGE antibody or IgG. ERM associated with RAGE was detected by anti-ERM antibody as shown in this representative Western blot. Cell lysate from A549 cells is used as positive control for ERM. All images and blots are representative of at least three independent experiments.

4.4.6 TGF-β1 and pro-inflammatory cytokines enhance CD44 expression and promote CD44-ERM interaction through phosphorylation of ERM proteins

CD44 is a cellular adhesion receptor that is up-regulated following tissue injury, purportedly playing a role in remodelling of pulmonary tissues (Kasper and Haroske., 1996). Moreover, ERM proteins in their active state are known to bind CD44, facilitating their cross-linking with actin filaments (Louvet-Vallée, 2000). Thus, we next examined the changes in expression and localisation of CD44 in A549 cells using CLSM. In their resting state, A549 cells exhibited weak and diffuse localisation of CD44 throughout the cytoplasm (Fig. 6a). When challenged with TGF-β1 and/or cytomix, enhanced expression of CD44 directly below the plasma membrane was observed (Fig. 6b-d). In agreement with immunofluorescence findings, analysis by
Western blot revealed an increase in protein expression of CD44 following treatment, with the effects of both cytomix, and TGF-β1 and cytomix being most marked (Fig. 6b).

To examine the relationship between CD44 and ERM, co-localisation studies using CLSM were performed, revealing that cytokine treatment induced association of pERM with CD44 (Fig. 6f-i). In particular, strong signals were observed towards the peri-nuclear region of cells, with more diffuse co-localisation noted in cytoplasmic areas. Consistent with CLSM analysis, CD44 was identified in the protein complex immunoprecipitated by anti-pERM antibody, but not by IgG control (Fig. 6j).
Figure 6. TGF-β1 and/or cytomix enhance CD44 expression and promote CD44-ERM interaction via phosphorylation of ERM proteins. a-d) A549 cells cultured with or without TGF-β1 [b], cytomix [c] or TGF-β1 plus cytomix [d] for 72 h were subjected to immunofluorescence staining with antibodies to CD44 (green) alone. CLSM analysis revealed increased expression of CD44 directly under the plasma membrane following cytokine treatment. e) Western blot analysis demonstrated that expression of CD44 in A549 cells was significantly (P<0.05) enhanced following treatment with TGF-β1 and/or cytomix for 72 h. f-i) Using CLSM co-localisation studies were performed for pERM (red) and CD44 (green). Representative images reveal a strong association between pERM and CD44, following cytokine treatment as detailed above. j) A549 cells cultured as mentioned above were lysed and subjected to immunoprecipitation with antibodies against CD44 or with control IgG. The resulting precipitates as well as the cell lysates were subjected to immunoblot analysis with antibodies against ERM. The representative blot illustrates a strong association between CD44 and pERM in A549 cells following cytokine stimulation. Means ± S.D. from >3 independent experiments; * P<0.05. Bars 20 μm.
4.5 Discussion

There is an ever-growing body of evidence supporting a role for EMT in IPF (Willis et al., 2005; Kim et al., 2006; Jayachandran et al., 2009; Tanjore et al., 2009). However, whilst studies continue to elucidate the underlying pathways of this biological process, the nature of the mechanisms which give rise to the major rearrangements of the sub-membrane cytoskeleton remain poorly understood. In this study, we assessed the relationship between ERM proteins and RAGE and their functional role in cytokine-induced EMT of an alveolar epithelial cell line, A549. Our observations revealed a close association between ERM and RAGE, which was disrupted following cytokine stimulation, as evidenced by proteolytic cleavage of RAGE and re-distribution and activation of ERM. Collectively, our findings suggest that this newly identified ERM-RAGE complex is important in the maintenance of normal alveolar structure and function, and that uncoupling of this complex is a potentially important step in EMT-associated acquisition of a mesenchymal phenotype (Fig. 7).
Figure 7. Proposed schematic of disruption of ERM-RAGE complex in EMT of alveolar epithelial cells. Pro-inflammatory cytokines induce the expression of CD44, a cellular adhesion receptor involved in pulmonary tissue re-modelling, and the phosphorylation of ERM, in a manner dependent on ROCK activation. Simultaneously, MMP-9 activity is increased following cytokine stimulation and mediates proteolytic cleavage of RAGE. Collectively, these events serve to promote deterioration of the ERM-RAGE complex. Disruption of this stabilising complex gives rise to re-modelling of the actin cytoskeleton and formation of the ERM-CD44 complex, and ultimately lead to EMT induction.

Members of the ERM family of proteins have previously been shown to be important in regulation of cytoskeletal structure and determination of cellular shape and motility (Takenouchi et al., 2004; Zeidan et al., 2008; Rollason et al., 2009; Rebillard et al., 2010). Recently, investigations by Takahashi et al. (2010) illustrated that
phosphorylation of ERM and subsequent complexation with CD44 was an important step in EMT of retinal pigment epithelial cells, mediating the loss of cell-cell contacts and associated morphological changes. Moreover, in carcinoma-derived renal epithelial cells and pulmonic vascular endothelial cells, cytoskeleton re-arrangement was associated with redistribution of members of the ERM family of proteins, and their phosphorylation (Takenouchi et al., 2004; Koss et al., 2006). Extending this to the alveolar epithelial setting, our current investigations illustrated that ERM proteins localise towards the cell periphery following cytokine stimulation, entering their activated state and complexing with membrane-associated CD44. Additionally, our studies revealed a striking interaction between the redistributed ERM proteins and the F-actin stress fibres formed as cells underwent transition to a mesenchymal phenotype, further emphasising the importance of ERM in determination of cell shape. Collectively, this co-localisation is suggestive of a close relationship between cytokine-induced ERM re-distribution and phosphorylation, and EMT-associated actin remodelling in alveolar epithelial cells.

Several kinases have been implicated in the regulation of ERM protein function. In particular, the Rho-activated kinase, ROCK, appears to be key in this context (Koss et al., 2006). Furthermore, the ROCK family are important regulators of actin cytoskeleton organisation (Pellegrin and Mellor, 2007). Notably, in human colonic epithelial cells, inhibition of ROCK signalling has been shown to attenuate actin remodelling and prevent ERM phosphorylation, whilst in migrating intestinal epithelial cells, enhanced co-association of ezrin with CD44 was found to be mediated, in part, by ROCK (Hopkins et al., 2007; Rebillard et al., 2010). In light of these findings, we investigated the effect of ROCK inhibition on ERM...
phosphorylation state. Immunoblot analyses revealed that activation of ERM was blocked by the inhibitor, Y-27632, suggesting that ROCK is responsible for the cytokine-induced phosphorylation of ERM proteins. In addition, our investigations revealed that inhibition of ROCK and thus ERM phosphorylation, contributed towards attenuation of EMT-associated actin stress fibre generation. This implicates ROCK and its downstream effects in the regulation of cytoskeletal structure, and is in agreement with previous findings in epithelia of the lens and kidney (Das et al., 2009; Cho and Yoo, 2007). We hypothesised that inflammatory cytokines, in particular TGF-β1, induce F-actin stress fibres as a consequence of ROCK activating ERM proteins.

Preservation of an epithelial phenotype and its associated morphological and functional characteristics is crucial to homoeostatic maintenance of the alveolar unit. In this regard, RAGE, which is intimately involved in the regulation of adhesion, migration and proliferation of alveolar epithelial cells (Demling et al., 2006; Queisser et al., 2008), exerts an important role. Moreover, pathologically, loss of RAGE has been implicated in fibrosis of the pulmonary epithelium (Hanford et al., 2003; Englert et al., 2008; Queisser et al., 2008). In accordance with these observations, we detected a marked decrease in RAGE expression in alveolar epithelial cells following stimulation with TGF-β1 and/or cytomix. Given that RAGE associates with components of the extracellular matrix (Demling et al., 2006), conceivably, loss of RAGE may lead to diminished affinity for the ECM and in this way increase the susceptibility of epithelial cells of the alveolus to injurious events which propagate the fibrogenic reaction. Given our findings, and that of Queisser and colleagues (2008), it appears likely that cytokine-induced RAGE deficiency may be an important
component of the EMT process - an acknowledged key contributor to the expanded fibroblast population in IPF.

Next, we investigated the potential mechanism by which RAGE expression levels are diminished. Previous studies have shown that membrane RAGE may be cleaved by proteases to form soluble RAGE (sRAGE) (Raucci \textit{et al.}, 2008; Zhang \textit{et al.}, 2008), and in this way, give rise to lower expression of full-length, membrane-bound RAGE. Indeed, in models of acute lung injury (ALI), sRAGE has been found to accumulate in the broncho-alveolar lavage fluid (BALF) (Uchida \textit{et al.}, 2006). Clinical studies have also highlighted the value of sRAGE as a pathogenic and prognostic marker. A strong correlation has been shown to exist between poorer clinical outcomes in patients with ALI and higher baseline plasma sRAGE levels (Calfee \textit{et al.}, 2007), whilst both airspace and perfusate sRAGE levels appear to be negatively correlated with alveolar fluid clearance (AFC) (Frank \textit{et al.}, 2007; Briot \textit{et al.}, 2009). Similarly, under hyperoxic conditions, levels of sRAGE in BALF and lung homogenates have been shown to increase (Su \textit{et al.}, 2009; Reynolds \textit{et al.}, 2010). Interestingly, in a bleomycin model of pulmonary fibrosis, sRAGE BALF levels were found to decrease following injury (Englert \textit{et al.}, 2008), whilst in a silicosis model, very little was detected, both before and after injury (Ramsgaard \textit{et al.}, 2010). However, it has been suggested that these conflicting results may be due to rapid clearance of sRAGE following the injurious event. Here, in A549 human alveolar epithelial cells, we observed increased levels of sRAGE in the cell supernatant following stimulation with pro-inflammatory cytokines. This finding is consistent with the suggestion that increased sRAGE levels are a biological indicator of alveolar epithelial cell injury. Matrix metalloproteinases are one of the main proteolytic enzymes involved in tissue
remodelling during the fibrotic process. In particular, MMP-9 (gelatinase B) has been shown to mediate the disruption of the alveolar basement membrane (Pardo et al., 2006). Hence, we investigated the effect of inhibition of MMP-9 activity on sRAGE release. Activated MMPs are rarely detected, on account of their fast degradation, and, therefore, changes in activity of pro-forms were investigated (Medina et al., 2003). Using gelatin zymography we verified that stimulation with TGF-β1 and/or cytomix gave rise to enhanced MMP-9 activity. Further, use of an inhibitor of MMP-9 revealed marked abrogation of RAGE release into the cell media of our in vitro model. Together, these results lend support to the hypothesis that proteolysis caused by MMP-9 contributed to sRAGE release into the media; albeit it is noted that inhibition of RAGE cleavage did not alter the morphological changes observed in vitro.

Using immunofluorescence confocal microscopy and co-immunoprecipitation, we explored the potential interaction between both ERM proteins and RAGE. Interestingly, for the first time, we showed a strong association between both proteins in alveolar epithelial cells under control conditions. Moreover, we found that this complex was disrupted following stimulation with TGF-β1 and/or cytomix, as cells lost their epithelial phenotype and gained characteristics typical of a mesenchymal phenotype, forming stress fibres and adopting a more fibroblast-like appearance. It is important to note that EMT is not a single event, rather, it consists of multiple steps, including - epithelial de-aggregation, detachment from basement membrane and cytoskeletal re-arrangement (Kim and Chapman, 2007). Our investigations suggest that disruption of this ERM-RAGE complex is an important component in this process. Conceivably, this event gives rise to a de-stabilised epithelial state and in this
way, serves to "prime" epithelial cells of the alveolus for transition to a mesenchymal phenotype. In particular, it appears that in this vulnerable state such cells are especially receptive to the downstream effects of ROCK signalling, resulting in marked cytoskeleton re-configuration.

In conclusion, in the present study we have identified a novel ERM-RAGE complex and shown that disturbance of this complex in a MMP-9 and ROCK-dependent manner is an important step in alveolar EMT. Our results highlight the importance of both ERM proteins and RAGE in alveolar function and dysfunction, and offer insight into a potential therapeutic target for amelioration of EMT-related fibrosis of the lung. Whilst our investigations have utilised a human alveolar epithelial cell line (A549) and as such are associated with some limitations, previous studies indicate that they are an appropriate model in which to study the biology of both EMT and RAGE (Kasai et al., 2005; Queisser et al., 2008). However, importantly, further experiments are required to confirm these observations in both primary cell culture models and in vivo, and to elucidate additional underlying mechanistic details, in particular, the exact identity of those member(s) of the ERM family of proteins implicated in the effects observed in our studies.
4.6 References


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Chapter 5

Conclusions
Although much remains to be explored regarding EMT and its association with fibrotic pathologies, the presented work provides novel insights into this complex biological process, allowing the following conclusions to be drawn, thus far:

- In discordance with alveolar epithelial cells, which exhibit a high degree of plasticity and are readily amenable to undergoing EMT, resident epithelial cells of the bronchus and colon fail to exhibit unisonous phenotypical alterations associated with EMT in response to EMT-inducing stimulants. This draws into question the precise contribution of EMT in the development of fibrotic foci in the proximal airways and large intestine.

- Telmisartan effectively attenuates key EMT-associated events, namely collagen I synthesis and development of a migratory phenotype. In this regard it may represent a useful therapeutic moiety in the treatment of IPF.

- Transition of alveolar epithelial cells to a mesenchymal state is associated with an increase in cellular stiffness. This change in mechanical properties is strongly correlated with EMT-related alterations in cytoskeletal structure. Collagen I, a principal component of the ECM in fibrotic tissues, effectively augments the effects of TGF-β1, both structural and mechanical. Collectively, AFM and HCA are pertinent research tools in the elucidation of the relationship between cellular architecture and their mechano-elastic attributes.

- A newly identified ERM-RAGE complex is a key determinant in preservation of normal alveolar epithelial cell structure. Ablation of this functionally important
complex is an important step in the acquisition of a mesenchymal phenotype and is mediated via MMP-9- and ROCK-dependent pathways. Together, this uncovers a novel mechanism which may serve as a potential therapeutic target for amelioration of EMT-related fibrosis of the lung.

Undoubtedly, future work will shed more light on the molecular mechanisms which underlie alveolar EMT. On account of the findings of these studies, this should include evaluation of the effects of telmisartan in vivo, further characterisation of the ERM-RAGE complex in both primary cultures and in situ, and investigation of prospective therapeutic opportunities derived thereof.
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Finally, I wish to thank Eva - for her support, encouragement, love and understanding.

Tusind tak ;-)
List of abbreviations

16HBE140- human bronchial epithelial cell line
A549 human alveolar epithelial adenocarcinoma cell line
AEC alveolar epithelial cell
ADAM a disintegrin and metalloproteinase
AFC alveolar fluid clearance
AFM atomic force microscopy
AGE advanced glycation end-products
ALI acute lung injury
ANOVA analysis of variance
ATI alveolar epithelial type-I cell
ATII alveolar epithelial type-II cell
AT2R angiotensin II receptor
BALF broncho alveolar lavage fluid
BEAS-2B human bronchial epithelial cell line
BSA bovine serum albumin
Calu-3 human bronchial epithelial adenocarcinoma cell line
Caco-2 human colorectal epithelial adenocarcinoma cell line
CCK-8 cell counting kit 8
CF cystic fibrosis
CLSM confocal laser scanning microscope
COPD chronic obstructive pulmonary disease
cRAGE cleaved RAGE
DMEM Dulbecco's modified Eagle's medium
ECM extracellular matrix
EGF epidermal growth factor
ERK extracellular signal-regulated kinases
ERM ezrin/radixin/moesin
EMEM Eagle's minimum essential medium
esRAGE endogenous secretory RAGE
FBS foetal bovine serum
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<th>Abbreviation</th>
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<tr>
<td>H4</td>
<td>human foetal small intestinal cell line</td>
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<td>H441</td>
<td>human respiratory epithelial adenocarcinoma cell line</td>
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<td>HCA</td>
<td>high content analysis</td>
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<td>HEK-293</td>
<td>human embryonic kidney cell line</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<td>HIEC-6</td>
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<td>HMGB1</td>
<td>high-mobility group protein B1</td>
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<td>HRP</td>
<td>horseradish peroxidise</td>
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<td>HT-29</td>
<td>human intestinal epithelial adenocarcinoma cell line</td>
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<td>hTERT</td>
<td>human telomerase</td>
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<td>IBD</td>
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<td>IFM</td>
<td>immunofluorescence microscopy</td>
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<td>IFNγ</td>
<td>interferon gamma</td>
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<td>immunoglobulin G</td>
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<td>interleukin 1beta</td>
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<td>idiopathic pulmonary fibrosis</td>
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<td>LCC</td>
<td>liquid-covered culture</td>
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<td>MMP</td>
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<td>mRAGE</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>P&lt;sub&gt;app&lt;/sub&gt;</td>
<td>apparent permeability</td>
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<td>PBS</td>
<td>phosphate buffer saline</td>
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<td>PPAR</td>
<td>peroxisome proliferator agonist receptor</td>
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<td>R3/1</td>
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<td>RAGE</td>
<td>receptor for advanced glycation end-products</td>
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<td>ROCK</td>
<td>rho kinase</td>
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<td>Rq</td>
<td>root mean square surface roughness</td>
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<tr>
<td>Rt</td>
<td>peak-to-valley surface roughness</td>
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<td>SD</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>siRNA</td>
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<td>transepithelial electrical resistance</td>
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<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
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<tr>
<td>TRITC</td>
<td>tetramethyl rhodamine iso-thiocyanate</td>
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