The extracellular matrix to bind them all: interactions between ECM and cells in lung fibrosis and influences thereof
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CHAPTER 2

The multi-faceted extracellular matrix: unlocking its secrets for understanding the perpetuation of lung fibrosis

Mehmet Nizamoglu & Janette K. Burgess

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ABSTRACT

Purpose of review: Lung fibrosis is currently thought to stem from an aberrant wound healing response after recurring (micro) injuries in the lung epithelium, together with disrupted crosstalk between epithelial and stromal cells. An important factor in lung fibrosis is the abnormal deposition of extracellular matrix (ECM). In this review, we extend the view of ECM to summarize how aberrant structural organization and degradation of ECM contributes to (perpetuation of) lung fibrosis.

Recent findings: Fibrotic changes in ECM including altered composition, such as increased collagens, coupled with mechanical properties, such as increased stiffness or abnormal fiber crosslinking, promote pro-fibrotic responses in cells in this microenvironment. Similarly, changes in matrix degrading enzymes and release of degradation products from ECM proteins also perpetuate cellular fibrotic responses.

Summary: In lung fibrosis, irreversible ECM structure, organization and architectural alterations drive a perpetuating fibrotic response. Targeting strategies abrogating the abnormal ECM or ECM-degrading enzymes accompanied by prognostic and/or diagnostic approaches based on ECM fragments may provide novel alternatives to current therapeutic approaches for lung fibrosis.
INTRODUCTION

Lung fibrosis is a common characteristic of the heterogeneous group of interstitial lung diseases (ILDs). Of these, the most common is idiopathic pulmonary fibrosis (IPF), which is a chronic, progressive lung disease, with a very poor survival rate (median: 3–5 years) [1]. Currently, there is no cure for IPF, other than lung transplantation, and while there are two therapeutic agents, pirfenidone and nintedanib, that can slow the disease progression, these therapies are not effective in all patients and have adverse, sometimes severe side effects [2]. Lung fibrosis is currently thought to result from an aberrant wound healing response following recurrent microinjuries to the alveolar epithelium, augmented by aberrant cross-talk between the fibroblasts and epithelial cells resulting in an excessive and abnormal deposition of extracellular matrix (ECM) proteins [3–5].

Under normal physiological conditions, ECM is composed of a multitude of different proteins, glycosaminoglycans (GAGs), and glycoproteins (collagen types I, III, IV and VI, fibronectin, laminin, peristin, and hyaluronic acid are a few examples), forming a dynamic network that provides support to the cells embedded within it [6, 7]. In addition to its structural support function, ECM is a bioactive component of the tissue and it provides cues to all cells to influence/instruct their behavior. In fibrosis, deposition of several different ECM proteins such as collagens and fibronectin is increased, while others are decreased, changing the biochemical composition of the tissue [8]. As a natural consequence of the changes in the protein composition and organization, the biomechanical properties of fibrotic lung tissues are also altered: fibrotic lungs are stiffer, have a greater degree of collagen crosslinking and altered topography [9–11]. This catalogue of changes was previously thought to only be the result of the fibrotic process within the tissue; however, a plethora of recent studies have illustrated the changes in the ECM are an emerging contributor to the disease progression process itself, influencing different cell types and cellular mechanisms [12–17]. Moreover, with the advances in the single-cell RNA sequencing methods, lung resident cell populations are shown to have great heterogeneity in lung fibrosis, compared to healthy lungs, which would also impact the diversity of ECM changes in fibrosis [18–21]. Interestingly, it has recently been suggested (in the context of embryonic development) that each cell type expresses its own unique ECM gene profile (indicative of the production of an individual ECM protein profile) that becomes more refined as the cells differentiate towards end-stage cells such as fibroblasts [22]. This finding implies the importance of the ECM microenvironment, which is disrupted in fibrosis, for the maintenance of a homeostatic status in tissue. However, the detailed mechanisms regarding how altered properties of ECM affect cellular responses or contribute to the cellular heterogeneity present in fibrosis and the consequent influence upon the disease outcome are yet to be investigated completely.
In this review, we summarize the varied aspects of the contribution of ECM in lung fibrosis and how ECM influences cellular responses. First, we focus on updates for understanding how changes in ECM composition, coupled with altered mechanical properties, impact cellular responses. Then, we look beyond the ECM scaffold to illustrate how ECM degradation and the released bioactive ECM fragments play a role in lung fibrosis. Finally, we reflect on how targeting (changes in) ECM can be leveraged to provide new avenues for managing lung fibrosis.

**ECM CHANGES IN FIBROSIS AND THEIR FUNCTIONAL CONSEQUENCES**

**Composition and Crosslinking**

In pulmonary fibrosis, changes in the quantities of ECM proteins have been extensively described [6, 9, 23]: including, but not limited to, increased collagen types I and III, fibronectin, periostin, and hyaluronic acid. One of the most important pieces of evidence illustrating how fibrotic ECM induces fibrotic responses in fibroblasts, as a result of the feedback in two dimensional (2D) cell culture models, was described by Parker et al [14]. In concert, primary lung fibroblasts cultured on scaffolds made with stacked sections of decellularized IPF lung were shown to produce a protein output that mirrored the fibrotic matrix composition compared to the fibroblasts cultured on scaffolds made with control lung tissue [24]. By comparing the decellularized fibrotic and alveolar tissue-derived sections of mouse ex vivo lung tissue scaffolds, the fibrotic microenvironment was found to decrease the spontaneous movement speed of immortalized mouse fibroblasts, compared to healthy mouse tissue [13]. The effect of the microenvironment was shown to also influence responses in other cells: Monocyte-derived macrophages in the fibroblastic-foci were found to perpetuate the fibrotic response, suggesting the fibrotic microenvironmental cues were guiding these cellular responses [25]. Similarly, pericytes were also shown to have higher gene and protein expression of α smooth muscle actin (α-SMA) when cultured on decellularized IPF lung samples compared to decellularized control lung samples [26]. Interestingly, culturing alveolar epithelial cells on IPF lung derived decellularized matrices was found to protect alveolar epithelial cells from transforming growth factor β (TGF-β) induced apoptosis, while additionally strengthening the profibrotic response of IPF lung-derived decellularized matrix-seeded fibroblasts to TGF-β via engagement of integrin α2β1, compared with cells seeded on non-disease control lung derived decellularized matrices [27]. These studies collectively show the influence of the fibrotic ECM on different cells, illustrating the different responses of the cells to the changing microenvironment in lung fibrosis.
Along with the changes in the biochemical distribution of the ECM proteins in fibrosis, post-translational modifications of these proteins are also altered. Collagen protein synthesis starts within the rough endoplasmic reticulum, with post-translational modifications adding hydroxyl groups to proline and lysine residues (Figure 1) [28]. Individual collagen molecules come together within the Golgi to form the triple helical structure, forming the procollagen molecule. This trimer then is secreted from the Golgi into the extracellular space, where its pro-collagen ends at both the C- and N-terminals are cleaved to generate the mature collagen molecule. The collagen molecules self-assemble to begin forming fibrils before lysyl oxidases (LOX), LOX–like enzymes (LOXLs) and transglutaminases (TGs) actively crosslink the triple helices to each other, forming the collagen fibers [28]. Increased expression and amount of LOXL1 and LOXL2 was reported in IPF lung tissue compared with non–disease control lung tissue [11]. In concert, fibrotic fibroblasts were found to have higher expression of TG2 compared with healthy fibroblasts in vitro [29]. Crosslinking of the collagen fibers by LOX/LOXL has also been shown to promote the TGF–β induced stiffening of the microenvironment [11]. ECM deposited by from IPF–lung derived fibroblasts increased the expression of LOXL3 and TG2; and in turn, the increased crosslinking of this ECM was demonstrated to boost fibroblast proliferation and adhesion [29]. These data together suggest that the increased collagen crosslinking, and dysregulation of the crosslinking enzyme amounts in pulmonary fibrosis could contribute to the positive feedback loop which Parker et al. first described [14].

Figure 1: Schematic illustration of synthesis, secretion and crosslinking of collagen fibrils. RER: Rough endoplasmic reticulum; LOX: lysyl oxidase; LOXL: LOX–like; TG: transglutaminase.
**Stiffness and Viscoelasticity**

Changes in the biomechanics of fibrotic lung tissue result directly from the altered and abnormal distribution and modification of the ECM proteins in lung fibrosis. Lung ECM has a viscoelastic nature that can dissipate the stress applied to it via various sources, such as mechanical forces changing with breathing in and out [10]. Among many biomechanical parameters, stiffness of the tissue is strongly associated with lung fibrosis and has been well-documented: native IPF lung samples were shown to have higher stiffness than control lung samples (1.96 ± 0.13 kPa vs 16.52 ± 2.25 kPa) and this difference remained similar also in decellularized lung samples (IPF lung sample: 7.34 ± 0.6 kPa, control lung sample: 1.6 ± 0.08 kPa) [9, 10]. Stiffness, like many other mechanotransducers, induces the Hippo pathway through yes-association protein (YAP) – PDZ-binding motif (TAZ) signaling, resulting in the perpetuation of fibrosis [30]. Several in vitro models have been developed to assess the effect of stiffness on lung cells: fibroblasts cultured on 2D hydrogels with higher stiffness were shown to migrate faster, along with a greater cell spread area, compared to the fibroblasts cultured on hydrogels with lower (more physiological-like) stiffness [31]. Similarly, a stiffer 2D culture environment was shown to increase fibroblast activation via chromatin remodeling compared to softer surfaces, accompanied by increased nuclear volume in these fibroblasts [32]. Higher stiffness of fibronectin coated polyacrylamide hydrogels was shown to decrease fibroblast activating protein expression while increasing the cell spreading area and αSMA expression in murine lung fibroblasts compared with softer hydrogels; on the other hand, changes in the stiffness of collagen type I coated polyacrylamide hydrogels did not change the cellular response [33]. Likewise, comparing the effect of different stiffness values of polyacrylamide hydrogels and incorporation of solubilized matrix from healthy or IPF lungs on pericytes seeded on these hydrogels showed that increased cell area and higher expression of αSMA resulted from the increase in the stiffness of the hydrogel rather than the ECM composition [26]. Interestingly, a study by Matera et al. suggested opposing effects of stiffness on lung fibroblasts in 2D and 3D cultures: higher stiffness of 2D cultures promoted myofibroblast differentiation while stiffer 3D cultures limited the differentiation of the lung fibroblasts [12]. Lastly, blocking the YAP – TAZ pathway of mechanotransduction in IPF lung-derived fibroblasts resulted in decreased expression of ECM proteins while ECM-degradation enzyme gene expression levels increased compared to the untreated IPF fibroblasts [34]. All of these studies together indicate different effects of stiffness on cells. It is highly possible that the combination of altered composition and increased stiffness induces different cellular responses in different cells. More investigation on separating the contribution of altered composition and stiffness of the fibrotic microenvironment...
could improve our understanding and help identify novel therapeutic approaches for targeting the progression of lung fibrosis.

Another emerging parameter among the biomechanical properties of ECM is the viscoelasticity, which is the ability to dissipate an applied stress through time [35]. The importance of the viscoelasticity of ECM both in healthy and diseased conditions has been recently reviewed elsewhere [36]. Similar to many other tissues and organs, lung ECM has viscoelastic properties and the loss of viscoelastic relaxation in fibrotic tissues has recently been established by our group [10]. The implications of (the loss of) viscoelasticity on cellular function \textit{in vivo} is yet to be clarified; however, it is known that changes in the viscoelasticity of the microenvironment can affect cell migration, proliferation and ECM deposition by the cells [36]. All of these cellular functions are recognized as being altered in lung fibrosis, so now the challenge lies in separating the individual contributions of these different mechanical stimuli to the perpetuation of the fibrotic response. Promisingly, a recent study revealed the possibility of modifying stiffness and viscoelasticity independently of each other [37]. Developing advanced \textit{in vitro} culture systems will further our understanding of viscoelasticity and its contribution to the progression of the lung fibrosis.

**Topography**

Topography of the ECM influences many cellular responses including migration and proliferation, as recently reviewed by Ouellette et al. [38]. While the altered composition alone could influence the topography of the ECM in lung fibrosis, the increased crosslinking and abnormal alignment of the fibers in the ECM are two other important factors changing the topography. In lung fibrosis, the topography of the ECM is drastically altered (Figure 2), due to the increased mature and organized collagen content, compared to the healthy lungs [11].
One of the mechanisms by which abnormal topography plays a role in lung fibrosis is the altered microstructures in the protein organization. In a study by Seo et al., comparison of adipose-derived stromal cells seeded on collagen type I networks with thin fibers and low pore size to cells seeded on networks with thick fibers and bigger pore size revealed that changing the microstructure increased differentiation of these stromal cells to myofibroblasts ~1.5X [39]. Along with fiber thickness and pore size, fiber alignment is an important parameter in ECM topography. Increased migration speed of primary lung fibroblasts seeded on collagen type I-methacrylated gelatin hydrogels was observed in highly aligned network samples, compared to hydrogels with less aligned networks [40]. In another study, increasing fiber density independent of the stiffness resulted in higher surface area of seeded dermal fibroblasts in 3D in vitro culture [41]. While collagen type I hydrogels with different stiffness values were used to test the effect of microstructure in the study by Seo et al., it is difficult to conclude the stiffness-independent contribution of the microstructure. As the abovementioned changes (stiffness, viscoelasticity and topography) in the fibrotic ECM occur simultaneously during fibrosis, more studies using advanced biomaterials are required to examine the individual contributions of such properties to the perpetuation of the fibrotic response.
Storage of growth factors in the ECM

As the non-cellular part of the tissue microenvironment, the ECM serves as a storage depot for many different growth factors and other soluble proteins, many of which are important for regulating the fibrotic response. Within the ECM, it is predominantly the glycosaminoglycans (GAGs) that serve as a reservoir for growth factors in the extracellular space. The negatively charged residues within the GAGs provide multiple binding sites for the positively charged amino acids within many growth factors. Through these binding interactions the growth factors are bound to the ECM, protecting them from degradation and conserving them until they are required for local signaling [42, 43]. The changing protein content and organization in fibrotic lung diseases is likely to alter the presence, amount, and the availability of the factors stored within ECM. Among these ECM proteins, fibronectin can bind many growth factors and soluble proteins, including latent TGF-β-binding protein-1 (LTBP-1) [44]. Increased amounts of fibronectin and LTBP-1 in fibrosis could lead to a greater storage capacity of ECM for TGF-β. Activation of the stored TGF-β can be directed via mechanical stimuli due to prestress on the ECM, applied by cells or decreased viscoelastic relaxation of the ECM itself [45]. TGF-β activation is also regulated by mechanisms driven by other proteins including fibulin-1, an ECM glycoprotein, which is also found in greater amounts in the lung tissues of IPF patients [46, 47].

The binding of growth factors important for lung development and repair, including TGF-β, fibroblast growth factor (FGF)1 and FGF2 and hepatocyte growth factor (HGF), and their interaction with their relevant receptors are dependent on the sulfation state of GAGs such as heparan sulfate, dermatan sulfate and chondroitin sulfate [42, 48]. Using hydrogels established using decellularized lung ECM (which is devoid of most GAGs) combined with heparan sulfate, dermatan sulfate or chondroitin sulfate with either TGF-β, FGF2 or HGF, Uhl and colleagues recently illustrated that matrix associated growth factor-dependent and –independent GAG effects in parallel with GAG-dependent and –independent matrix-associated growth factor effects are important for regulating cellular responses in lung in vitro models [48]. There is an increase in heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronan in IPF lungs compared to controls [49], suggesting a greater capacity for anchoring important growth factors for regulating reparative or fibrotic processes in these tissues. Analyses of the sulfation state of the GAGs in the IPF tissues found that the highly sulfated GAGs were located predominantly in the regions of interaction between the fibrotic and less fibrotic tissues, potentially indicating a central role for the GAGs in providing growth factors for promoting the high fibrotic activity within these regions.
### Table 1: Summary of recent studies illustrating changes related to ECM in fibrosis and their impacts on cell responses.

<table>
<thead>
<tr>
<th>Category</th>
<th>Changes in ECM</th>
<th>Cellular Response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM Crosslinking</td>
<td>Pan-inhibition of LO activity</td>
<td>Lower rates of TGF-β induced collagen remodeling and ECM stiffness in human primary lung fibroblasts seeded on decellularized cell-derived ECM</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td>Increased ECM crosslinking by TG2</td>
<td>Higher rates of proliferation in human primary lung fibroblasts seeded on decellularized IPF cell-derived ECM</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td>Knock out of LOXL3 by siRNA</td>
<td>Lower rates of fibroblast-to-myofibroblast differentiation in normal human lung fibroblasts</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td>Deficiency of LOXL1</td>
<td>Lower expressions of TGF-β, collagen type I and αSMA in TGF-β overexpression mice model of fibrosis at day 35 compared to wild type mice of the same model</td>
<td>[51]</td>
</tr>
<tr>
<td>Stiffness</td>
<td>Increased stiffness in 2D polyacrylamide hydrogels</td>
<td>Faster migration and higher spread area normal human lung fibroblasts</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>Increased stiffness in 2D collagen type I–coated culture</td>
<td>Higher rate of activation and higher nuclear volume in murine primary lung fibroblasts</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Increased stiffness of fibronectin coated polyacrylamide hydrogels</td>
<td>Higher αSMA expression in murine lung fibroblasts</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>Increased stiffness of polyacrylamide hydrogels functionalized with solubilized matrix from lungs</td>
<td>Higher cell area and higher αSMA expression in human primary microvascular pericytes</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>Increased stiffness in 2D dextran-based hydrogels functionalized with MMP-cut sites</td>
<td>Higher myofibroblast differentiation in normal human lung fibroblasts</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>Increased stiffness in 3D dextran-based hydrogels functionalized with MMP-cut sites</td>
<td>Lower myofibroblast differentiation in normal human lung fibroblasts</td>
<td>[12]</td>
</tr>
<tr>
<td>Topography</td>
<td>Thicker collagen fibers in 3D collagen type I hydrogels</td>
<td>Higher myofibroblast differentiation in human adipose derived stromal cells</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Increased alignment of collagen type I–methacrylated gelatin hydrogel networks</td>
<td>Increased migration speed in human primary lung fibroblasts in 2D</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Increasing fiber density in 3D dextran–based hydrogels functionalized with MMP–cut sites</td>
<td>Higher myofibroblast differentiation, cell spread area, YAP translocation and proliferation rate in normal human lung fibroblasts</td>
<td>[12]</td>
</tr>
</tbody>
</table>

It is not unlikely that the ECM in fibrotic lung disease would have enhanced storage capacity for bioactive factors as a result of the increased amount of the abovementioned ECM proteins, among others, and the activation and release of these factors from the ECM would be boosted by the biomechanical changes in the tissues in lung fibrosis. Further investigations regarding the ECM–stored bioactive factors are necessary for improving our understanding of the contribution of the repository function of the ECM to the progression of lung fibrosis.

Collective impact of the altered ECM scaffold in fibrotic lung disease

All in all, the altered ECM in fibrosis generates diverse influences which impact cellular phenotypes; as summarized in Table 1. While the biochemical changes in the fibrotic microenvironment have been demonstrated with proteomics analyses via mass spectrometry [6], the accompanying biomechanical changes, such as increase in stiffness or loss of viscoelastic relaxation [10], require further investigation. While these changes could be simply the result of the altered biochemical composition, there are other emerging contributing factors such as collagen crosslinking that require further clarification. With the new developments in the field of biomaterials, advanced in vitro culture systems will be generated to mimic the specific biomechanical properties of the fibrotic microenvironment. Such systems will further improve our understanding of how the biomechanical properties of the ECM, either individually or collectively, contribute to the perpetuation of fibrotic disease in the lung. Eventually, such knowledge should illuminate how such properties could be targeted via therapeutic intervention for treatment of lung fibrosis.

BEYOND THE ECM SCAFFOLD

The role of ECM degrading enzymes and their regulators

The ECM is a dynamic microenvironment that is constantly being remodeled as elements are degraded and newly deposited during normal tissue maintenance and particularly under conditions of disease pathogenesis. While all cell types synthesize, secrete and orchestrate deposition of ECM (including epithelial cells, mesenchymal cells, endothelial cells and immune cells), the fibroblasts are recognized as the major ECM producing cell type in fibrotic tissues. Leukocytes and macrophages, but also mesenchymal and epithelial cells produce enzymes that regulate the degradation of the ECM. The most well recognized group are the matrix metalloproteinases (MMPS), but also serine or cysteine proteases have a role in maintaining a healthy homeostasis within the ECM [52, 53]. Activity of the enzymes that degrade the ECM is tightly balanced by endogenous inhibitors (tissue inhibitors of MMPs (TIMPs)), serpins or cystatins [54–56].
In fibrotic lung disease, an easy assumption would be that there would be reduced levels of matrix degradative enzymes, in particular MMPs, as this is where the greatest amount of research has focused, as an explanation as to why there is increased ECM deposition. However, multiple studies report increased levels of several MMPs associated with fibrotic lung disease, reviewed in [53, 57, 58]. While this appears paradoxical, it is important to realize that in addition to degrading ECM proteins, the range of substrates MMPs can process and activate includes cell receptors, chemokines and growth factors [59]. Through the generation of chemotactic gradients or activation of specific proinflammatory or profibrotic factors, in cooperation with disruption of basement membranes and other physical barriers within the tissue, MMPs can influence the influx of inflammatory cells into the site of tissue injury, which in turn then contribute to the development / perpetuation of fibrosis [60, 61]. A number of MMPs have been specifically linked to fibrosis in the lungs, summarized in Table 2. A selection of MMPs associated with fibrotic lung disease, particularly IPF, are highlighted herein.

**MMP1**

MMP1, considered a “classic” collagenase, cleaves interstitial collagens including collagen type I and III. MMP1 protein levels are increased in bronchial alveolar lavage and gene and protein expression levels are increased lung tissue from IPF, compared to non–fibrotic, patients [63, 69, 79, 96]. A single nucleotide polymorphism, within the AP–1 binding domain of the MMP1 promoter (which increases transcription of MMP1) is observed more frequently in patients with IPF who smoke, than those who do not [97]. In a murine system, MMP1 enhanced cellular migration, increased wound closure rate, and protected cells from apoptosis. Increased MMP1 in alveolar epithelial cells repressed mitochondrial respiration, reduced the production of reactive oxygen species (both total and mitochondrial) and also, under normoxic conditions, increased expression of hypoxia–inducible factor-1α (HIF-1α) [98]. The fact that MMP1 was upregulated via increased HIF-1α induction under hypoxic conditions in the alveolar epithelial cells suggests a role for MMP1 in bidirectional cross talk regulating alveolar epithelial cell functions in fibrosis.

Intriguingly, MMP1 has recently been reported to be part of a set of signature genes illustrating the link between IPF and lung cancer. MMP1 was suggested to be a promising candidate gene driving significant expression changes through the transition from healthy tissue to IPF and non-small cell carcinoma [99]. MMP1 is primarily located in reactive bronchial epithelial cells, hyperplastic type 2 pneumocytes in honeycomb cysts and in alveolar macrophages, with little to no expression being observed in interstitial mesenchymal cells, suggesting that the localization of the increased MMP1 in fibrotic lung tissue does not facilitate the degradation of the fibrotic deposits [69].
Table 2: Different types of MMPs, their origins, involvement in lung fibrosis and possible mechanisms of action. Substrates for cleavage sourced primarily from [62].

<table>
<thead>
<tr>
<th>MMP</th>
<th>Source in vivo</th>
<th>Localization in lung tissue</th>
<th>Profile in lung fibrosis</th>
<th>Substrates for Cleavage</th>
<th>Possible mechanism of action</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>MMP1 Collagenase 1</td>
<td>Bronchial and AECs, Macrophages</td>
<td>Reactive bronchial epithelial cells, hyperplastic type 2 pneumocytes and in alveolar macrophages</td>
<td>↑ in plasma, serum &amp; BAL from IPF, ↑ gene and protein in IPF lung tissue</td>
<td>ECM substrates: Collagen types I, II, III, VII and X; gelatin; aggrecan; link protein; entactin; tenasin; perlecans Non-ECM substrates α2-M; α1-PI; α1-antichymotrypsin; IGFBP-2, 3, 5; proIL-1β; CTGF</td>
<td>Regulates AEC migration, wound closure and resistance to apoptosis. Possible bidirectional cross talk (via HIF1α) regulating AEC functions in fibrosis</td>
<td>[63–67]</td>
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<tr>
<td>MMP2 Gelatinase A</td>
<td>Bronchial and AECs, Fibroblasts and fibrocytes</td>
<td>Subepithelial myofibroblasts foci, close to basement membrane disruption. In ECM surrounding fibroblast foci, AECs and basal bronchiolar epithelial cells</td>
<td>↑ in plasma and BAL from IPF, ↑ protein in IPF lung tissue</td>
<td>ECM substrates Gelatin; collagen types IV, V, VII, X and XI; Ln; Fn; elastin; aggrecan; link protein Non-ECM substrates ProTG-β; FGF receptor 1; MCP-3; IGFBP-5; proIL-1β; galectin-3; plasminogen</td>
<td>Postulated disrupted integrity of sub-epithelial/endothelial basement membrane resulting in infiltration of factors and interstitial cells to the alveolar space promoting fibro-proliferative response</td>
<td>[67–71]</td>
</tr>
<tr>
<td>MMP3 Stromelysin 1</td>
<td>Bronchial and AECs, Alveolar Macrophages, and fibroblasts</td>
<td>Regions of bronchiolization close to aberrant ECM deposits. Weaker expression in lymphoid aggregates</td>
<td>↑ in plasma and BAL from IPF, ↑ gene and protein in IPF lung tissue</td>
<td>ECM substrates Aggrecan; decorin; gelatin; Fn; Ln; collagen types III, IV, IX and X; tenascin; link protein; perlecans Non-ECM substrates lamin, casein, IGFBP-3; proIL-1β; HB-EGF; CTGF; E-cadherin; α1-antichymotrypsin; α1-PI; α2-M; plasminogen; uPA; proMMP-1, 7, 8, 9, 13</td>
<td>Regulating activation of TGF-β through release from latency associated peptide and latent TGF-β binding protein 1 Induction of CTGF</td>
<td>[67, 72–77]</td>
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<td>MMP</td>
<td>Source in vivo</td>
<td>Localization in lung tissue</td>
<td>Profile in lung fibrosis</td>
<td>Substrates for Cleavage</td>
<td>Possible mechanism of action</td>
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<td>MMP7</td>
<td>Bronchial epithelial cells, aberrantly activated AECs, mononuclear phagocytes and fibrocytes</td>
<td>Aberrant activated AECs and in bronchiolar epithelial cells</td>
<td>↑ in plasma and serum from IPF ↑ gene and protein in IPF lung tissue</td>
<td>ECM substrates Aggrecan; gelatins; Fn; Ln; elastin; entactin; collagen type IV; tenascin; decorin; link protein, Non-ECM substrates osteopontin, β4 integrin, E-cadherin, syndecan, FasL, plasminogen, Proα-defensin; proTNFα; CTGF; HB-EGF; RANKL; IGFBP-3; plasminogen</td>
<td>Regulating neutrophil transepithelial influx, via the shedding of syndecan-i-CXCL1 complexes, Protecting the fibroblast from undergoing apoptosis via removal of FasL from their surfaces</td>
<td>[63, 65-68, 72, 78-82]</td>
</tr>
<tr>
<td>MMP8</td>
<td>Bronchial epithelial cells, neutrophils, macrophages</td>
<td>Bronchial epithelial cells in regions of moderately severe and severe fibrosis, macrophages</td>
<td>↑ in plasma and BAL from IPF ↑ gene and protein in lung homogenates and tissue from IPF</td>
<td>ECM substrates Collagen types I, II and III; gelatin; aggrecan; link protein Non-ECM substrates αl-PI</td>
<td>Promotes fibrocyte migration</td>
<td>[58, 66-68, 72, 83, 84]</td>
</tr>
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<td>MMP9</td>
<td>AECs, neutrophils, macrophages, fibrocytes and fibroblasts in fibrotic foci</td>
<td>Metaplastic AECs, alveolar and interstitial macrophages and fibrocytes in fibrotic foci</td>
<td>↑ in plasma from IPF ↑ gene and protein in lung tissue from IPF</td>
<td>ECM substrates Gelatins; collagen types III, IV and V; aggrecan; elastin; entactin; link protein, vitronecin; N-telopeptide of collagen type I Non-ECM substrates ProTGFB-β1; IL-2 receptor α; Kit-L; IGFBP-3; proIL-1β; ICAM-1; αl-PI; galectin-3; plasminogen</td>
<td>Fibroblasts in healthy lung tissue express Thy-1 (a glycosphosphatidylinositol-linked glycoprotein) which suppresses MMP9 expression. IPF fibroblasts lack Thy-1 leading to expression of MMP9, which correlates with regions of active fibrogenesis, suggesting a role for MMP9 in induction of fibrosis. Activation of TGF-β1 in the extracellular space</td>
<td>[67-69, 72, 79, 85]</td>
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<tbody>
<tr>
<td>MMP10</td>
<td>Stromelysin 2</td>
<td>Bronchial and AECs, alveolar macrophages and peripheral bronchiolar epithelial cells</td>
<td>↑ in serum and BAL from IPF; ↑ protein in lung tissue from IPF</td>
<td>ECM substrates Aggrecan; Fn; Ln; collagen types III, IV and V; link protein, gelatin, Non–ECM substrates lamin, casein, Pro-1, 8, 10</td>
<td>Possible role in regulation of macrophage migration and polarization driving fibrotic response</td>
<td>[58, 86–88]</td>
</tr>
<tr>
<td>MMP12</td>
<td>Macrophage elastase</td>
<td>Macrophages, and lung stromal cells</td>
<td>↑ in serum and BAL from IPF; ↑ fragment from collagen type IV released after MMP12 degradation in IPF</td>
<td>ECM substrates Elastin; aggrecan; Fn; collagen type IV; osteonectin; Ln; nidogen Non–ECM substrates Plasminogen; apolipoprotein(a)</td>
<td>Postulated disrupted integrity of sub–epithelial/endothelial basement membrane resulting in infiltration of factors and interstitial cells to the alveolar space promoting fibro–proliferative response</td>
<td>[67, 89, 90]</td>
</tr>
<tr>
<td>MMP13</td>
<td>Collagenase 3</td>
<td>Bronchial and AECs, alveolar macrophages and fibroblasts</td>
<td>↑ in serum and BAL from IPF; ↑ protein in lung homogenates from IPF</td>
<td>ECM substrates Collagen types I, II, III, IV, IX, X and XIV; aggrecan; Fn; tenascin; osteonectin; Ln; Perlecain Non–ECM substrates CTGF; ProTGF–β; MCP–3; α1–antichymotrypsin</td>
<td>Not known as yet</td>
<td>[67, 91]</td>
</tr>
<tr>
<td>MMP14</td>
<td>MT1–MMP</td>
<td>AECs, alveolar macrophages and endothelial cells.</td>
<td>↑ gene and protein in lung tissue from IPF</td>
<td>ECM substrates Collagen types I, II and III; gelatin; aggrecan; Fn; Ln; fibrin; Ln–5 Non–ECM substrates ProMMP–2; proMMP–13; CD44; MCP–3; tissue, transglutaminase</td>
<td>Facilitates fibroblast migration through disruption of ECM barriers</td>
<td>[69, 70, 92]</td>
</tr>
</tbody>
</table>
Table 2: Different types of MMPs, their origins, involvement in lung fibrosis and possible mechanisms of action. Substrates for cleavage sourced primarily from [62]. (continued)

<table>
<thead>
<tr>
<th>MMP</th>
<th>Source in vivo</th>
<th>Localization in lung tissue</th>
<th>Profile in lung fibrosis</th>
<th>Substrates for Cleavage</th>
<th>Possible mechanism of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP15 MT2-MMP</td>
<td>AECs and endothelial cells</td>
<td>AECs</td>
<td>↑ gene and protein in lung tissue from IPF</td>
<td>ECM substrates Fn; tenasin; nidogen; aggrecan; perlecan; Ln ProMMP-2; tissue transglutaminase</td>
<td>Not known as yet</td>
<td>[70]</td>
</tr>
<tr>
<td>MMP16 MT3-MMP</td>
<td>AECs and fibroblasts</td>
<td>AECs and fibroblasts in fibroblastic foci</td>
<td>↑ gene and protein in lung tissue from IPF</td>
<td>ECM substrates Collagen type III; Fn; gelatin Non-ECM substrates ProMMP-2; tissue transglutaminase</td>
<td>Not known as yet</td>
<td>[70]</td>
</tr>
<tr>
<td>MMP19</td>
<td>Monocytes, macrophages, fibroblasts, and endothelial cells</td>
<td>Hyperplastic AECs overlying fibrotic areas</td>
<td>↑ gene in lung tissue from IPF</td>
<td>ECM substrates Collagen type IV; gelatin; Fn; tenasin; aggrecan; COMP; Ln; nidogen Non-ECM substrates IGFBP-3</td>
<td>Stimulates epithelial cell wound healing and migration Promotes fibroblast migration, proliferation and ECM component synthesis</td>
<td>[93]</td>
</tr>
<tr>
<td>MMP28 Epilysin</td>
<td>Bronchial and AECs</td>
<td>Bronchial and AECs</td>
<td>↑ in serum from IPF ↑ gene and protein in lung tissue from IPF</td>
<td>ECM substrates Unknown Non-ECM substrates Casein</td>
<td>Promoting M2 macrophage programming</td>
<td>[64, 94, 95]</td>
</tr>
</tbody>
</table>

AEC – alveolar epithelial cells; TGF – transforming growth factor; α2-M, α2-macroglobulin; α1-PI, α1–proteinase inhibitor; COMP, cartilage oligomeric matrix protein; CTGF, connective tissue growth factor; Fas-L, Fas ligand; FGF, fibroblast growth factor; Fn, fibronectin; HB-EGF, heparin–binding epidermal growth factor like growth factor; IGFBP, insulin–like growth factor binding protein; ICAM-1, inter–cellular adhesion molecule 1; Kit-L, kit ligand; Ln, laminin; MCP-3, monocyte chemotactic protein–3; MMP, matrix metalloproteinases; MT–MMP, membrane–type MMP; PG, proteoglycan; proIL–1β, pro interleukin–1β; Pro, proteinase type; proTNF–α, pro tumor necrosis factor–α; proTGF–β, pro transforming growth factor β; ProMMP, latent MMP; RASI-1, rheumatoid arthritis synovium inflamed–1; RANKL, receptor activator for nuclear factor κB ligand; uPA, urokinase plasminogen activator
MMP3
MMP3, also known as stromelysin-1, can degrade a variety of ECM proteins including collagen types II, III, IV, IX, and X, proteoglycans, fibronectin, elastin, and laminin. MMP3 levels are increased in IPF patients with progressive disease who died within three years of follow-up, compared to those who survived [72]. Both MMP3 gene and protein expression are increased in IPF patients' lung tissue and serum compared to controls [73, 74]. MMP3 is predominantly expressed in regions of bronchiolization close to aberrant ECM deposits within the IPF lung tissue, with some evidence of weaker expression in lymphoid aggregates [74]. MMP3 is important for regulating the activation of the profibrotic growth factor TGF-β through facilitating the release of TGF-β homodimer from the latency associated peptide and latent TGF-β binding protein 1 [75]. Also of importance for the development of fibrosis in the lungs, MMP3 can induce gene and protein expression of connective tissue growth factor (CTGF/CCN2), independent of its proteolytic activity [76, 77].

MMP7
Increased levels of MMP7, referred to as matrilysin, have been recognized as a biomarker for IPF [100, 101]. Increases in serum and plasma protein levels and in lung tissue gene and protein expression are well documented in IPF patients compared to healthy controls or other forms of fibrotic lung disease [63, 65, 67, 78, 79]. Two MMP7 promoter polymorphisms (rs11568818 and rs11568819), which result in higher levels of MMP7 in plasma, have been associated with IPF [102]. MMP7 is synthesized and released from lung bronchial epithelial cells and aberrantly activated alveolar epithelial cells, mononuclear phagocytes and circulating fibrocytes [65, 68]. It has proteolytic activity against a wide range of ECM proteins including collagen type IV, laminin, elastin and fibronectin. In addition, it cleaves gelatin, osteopontin (a multifunctional cytokine which controls cell adhesion and migration), transmembrane tumor necrosis factor α (pro-TNF-α), β4 integrin, E-cadherin, syndecan, FAS ligand (FasL), plasminogen and insulin-like growth factor binding protein–3 (IGFBP–3), among others [57, 103]. MMP7 colocalizes with osteopontin in alveolar epithelial cells in IPF tissues and has an important role in regulating neutrophil transepithelial influx via the shedding of syndecan-1–CXCL1 complexes, thereby facilitating epithelial cell damage which then promotes fibrosis [80, 81]. The cleavage of FasL from the IPF fibroblast surface (releasing sFasL into the circulatory system), and thus protecting the fibroblast from undergoing apoptosis induced by T cells, has also recently been suggested as a mechanism by which MMP7 contributes to the development of fibrosis [82].
MMP28
MMP28 (epilysin), the newest member of the MMP family, has recently been identified as a possible biomarker for IPF [94]. Unlike other MMPs MMP28 is constitutively expressed in healthy tissue, including lung, leading to the suggestion that it has a role in maintenance of tissue homeostasis [104–106]. It is localized in bronchial and alveolar epithelial cells in IPF lung tissues [95] with the gene and protein levels being increased in IPF lung tissues, compared to other fibrotic lung diseases or normal controls, and the protein levels are increased in serum of IPF patients [66, 94, 95]. MMP28 is also expressed in macrophages and has been shown to reduce proinflammatory (M1) macrophage functions while promoting anti-inflammatory / profibrotic (M2) programming, thereby supporting development of lung fibrosis [107].

Interestingly, MMPs diffuse along ECM protein fibers, with different MMPs having affinity for different collagen fiber structures (for example MMP1, 8 or 13 unwind and cleave collagen fibers at specific internal sites within the fibers, whereas MMP2 and 9 will move along the fibers and digest predominantly at the termini) [108]. Through these patterns of behavior, the MMPs orchestrate a programmed functional outcome within a tissue environment. In a fibrotic environment, where the topography and arrangement of the ECM fibers is disrupted, the regulated function of the MMPs is predicted to be adversely affected.

ECM FRAGMENTS IN LUNG FIBROSIS

The resultant products from endogenous enzyme activity in the fibrotic lung environment, while often overlooked, are potentially key players in the disease process. These released fragments, called matricryptins or matrikines or ECM fragments (the term by which they will be referred to in this review), are bioactive and have been reported to regulate processes as diverse as cell signaling, gene expression, angiogenesis, adipogenesis, tumor growth and metastasis, wound healing and fibrosis. The ECM fragments can interact with growth factor receptors, toll–like receptors, integrins and other diverse cell surface receptors through which they actively induce cellular responses that often differ from events induced by their parent molecule. ECM fragments can also act as proteolytic enzymes or inhibitors of enzyme activity themselves or can be involved in the process of proenzyme activation. In all these functional capacities, ECM fragments may contribute to the disrupted ECM remodeling that is characteristic of the fibrotic lung, as summarized in Table 3.
<table>
<thead>
<tr>
<th>Parent ECM molecule</th>
<th>ECM fragment name</th>
<th>Molecular mass (kDa)</th>
<th>Evidence for alterations in pulmonary fibrosis</th>
<th>Mechanism of action</th>
<th>Other comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>Proline–glycine–proline (PGP) (can also be found in Collagen III &amp; IV)</td>
<td>0.269</td>
<td>Not detectable in BAL of IPF patients Administration of PGP to bleomycin challenged mice is protective from development of fibrosis</td>
<td>Enhances MMP9 and neutrophil elastase secretion Chemoattractant for leukocytes Promotes repair of epithelial cells, and neo-angiogenesis</td>
<td>Engages CXCR1/2</td>
<td>[109–111]</td>
</tr>
<tr>
<td>Collagen type Iα1</td>
<td>Product from cleavage between amino acid positions 1158/1159</td>
<td></td>
<td></td>
<td>Promotes ECM deposition, fibroblast migration</td>
<td></td>
<td>[112]</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>Arresten (α1 chain)</td>
<td>26</td>
<td>Increased in serum of IPF patients. Increased fragment from collagen IV released after MMP12 degradation in IPF Higher levels related to mortality in ILD. Tumstatin and Lamstatin absent in lung tissues of patients with UIP but present in controls</td>
<td>Inhibits angiogenesis Promotes fibrosis, MMP2 expression Inhibits lung fibroblast migration, myofibroblast contraction Turnover is predictive of mortality in COPD</td>
<td></td>
<td>[90, 113–118]</td>
</tr>
<tr>
<td></td>
<td>Canstatin (α2 chain)</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumstatin (α3 chain)</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tetrastatin (α4 chain)</td>
<td>25 20 AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lamstatin / Pentastatin (α5 chain)</td>
<td>25 2.45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexastatin (α6 chain)</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent ECM molecule</td>
<td>ECM fragment name</td>
<td>Molecular mass (kDa)</td>
<td>Evidence for alterations in pulmonary fibrosis</td>
<td>Mechanism of action</td>
<td>Other comments</td>
<td>References</td>
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</tr>
<tr>
<td>Collagen type VI</td>
<td>Endotrophin (α3 chain)</td>
<td>5.8</td>
<td>Elevated in serum of IPF patients</td>
<td>Increases expression of collagen and LOX family genes</td>
<td>Regulates TGF-β, Promotes fibrosis</td>
<td>High levels predict mortality in COPD</td>
</tr>
<tr>
<td>Collagen XVIII</td>
<td>Endostatin (α1 chain)</td>
<td>21</td>
<td>Increased in serum, plasma and BAL of IPF patients</td>
<td>Anti-fibrotic activity in in vitro and in vivo models of fibrosis</td>
<td>Regulates angiogenesis, adipogenesis, autophagy. Inhibits activation and activity of MMPs</td>
<td>Interacts with integrins and VEGFR2</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Fibstatin (C-terminal heparin binding domain)</td>
<td>29</td>
<td>Increased in plasma of ILD patients</td>
<td>Domains 12–14 (C-terminus) abolishes agonist activity</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>Fragment containing FN III EDA Anastellin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[128]</td>
</tr>
<tr>
<td>Perlecan</td>
<td>Endorepellin</td>
<td>85</td>
<td>Parent molecule increased in lung tissue from IPF patients</td>
<td>Counteracts endostatin's regulation of angiogenesis.</td>
<td>Interacts with integrins and VEGFR2</td>
<td>[49, 129, 130]</td>
</tr>
<tr>
<td>Fibulin-1</td>
<td>Fibulin-1C1</td>
<td></td>
<td>Increased in serum and lung tissue of IPF patients</td>
<td>Regulates fibrosis and activation of TGFβ</td>
<td>Interacts with LTBP-1, collagen and fibronectin</td>
<td>[46, 47, 131, 132]</td>
</tr>
</tbody>
</table>

ROLE OF ECM FRAGMENTS IN THE FIBROTIC PROCESS

Active ECM fragments are generated throughout all phases of tissue repair and may contribute to the ongoing fibrotic process [117]. These fragments have roles in the regulation of the inflammatory responses (which have recently been reviewed in [111, 133], but will not be the subject of this review), angiogenic and fibrogenic responses. The altered ECM landscape in the fibrotic lung contains an increased proportion of many ECM molecules that yield active fragments that regulate angiogenesis (see Table 3). Given that angiogenesis generally precedes fibrosis in an area of tissue undergoing repair, these regulatory ECM fragments may impact the tissue repair as a result of the altered structure of the fibrotic ECM. In turn, the consequential sprouting of fresh vessels from pre-existing vasculature within the damaged tissues may also impact the dysregulated and aberrant ECM composition and the continuing production of active ECM fragments.

Fibroblasts are recognized as the key active cell during the fibrogenic phase of the repair process. Aberrant fibroblast responses to the altered ECM environment that they encounter in the fibrotic lung may lead to overabundant ECM fragment production, which would further compound the matrix remodeling driving progressive fibrosis. The role of the ECM scaffold in IPF lung tissues has been elegantly illustrated [9, 24] as a driver of cellular responses; however, how this environment impacts the release of active ECM fragments has yet to be explored.

Collagen type I
The smallest identified ECM active fragment (PGP) comes predominantly from collagen type I but is also present in collagens type III and IV. The role of this fragment has been well characterized in inflammatory processes, as reviewed in [111], but less is known about its potential roles in regulating other processes in the angiogenic or fibrogenic responses.

The collagen type I α1 fragment released following MMP 2 and 9 cleavage between amino acids 1158/1159 induces fibroblast migration and enhances deposition of a variety of ECM proteins, contributing to the fibrotic response [112].

Collagen type IV
The six α chains that generate the heterotrimers of collagen type IV have all been characterized to release active ECM fragments, which are predominantly active in the regulation of neo-angiogenesis and -lymphangiogenesis. In addition to these functions these ECM active fragments also regulate the activity of mesenchymal
cells. While most of the information about these functions have been elucidated in the cancer field, emerging evidence points to key roles for these fragments in pulmonary diseases.

**Arresten (α1 non-collagenous region 1 (NC1))** binds to integrin α1β1 to inhibit angiogenesis through impacting endothelial cell migration, proliferation and the ability to form tubes. These actions occur in part by blocking its parent molecule binding to the same integrin [134]. Arresten may also interact with heparan sulfate proteoglycans to further enhance its effects. Arresten is increased in lung tissue of patients with usual interstitial pneumonia (UIP) [118].

**Canstatin (α2 NC1)** binds to integrins αvβ1, αvβ3 and αvβ5 on endothelial cells to inhibit tumor associated angiogenesis through disrupting cell–matrix interactions. Some studies suggest that interactions with αvβ3 and αvβ5 induces apoptosis, while inhibiting migration and proliferation in the endothelial cells [135]. Canstatin is also increased in lung tissue of patients with usual interstitial pneumonia (UIP) [118].

**Tumstatin (α3 NC1)** binds to the CD47/αvβ3 integrin complex to inhibit proliferation of melanoma and epithelial cells. It inhibits neo-angiogenesis but also has antitumorigenic activities that are associated with distinct regions within this active ECM fragment [136]. Tumstatin binds to airway smooth muscle cells to influence the ECM they deposit, which in turns impacts the migration of endothelial or inflammatory cells within this matrix environment [137, 138]. The levels of tumstatin are reduced in airway tissues from individuals with asthma and lymphangioleiomyomatosis [114, 139], and recently were reported to undetectable in lung tissues from patients with UIP [118].

Similarly, tetrastatin (α4 NC1) and hexastatin (α6 NC1) also bind to integrins (αvβ3 and αvβ1, αvβ3 and αvβ5 respectively). While the direct integrin that lamstatin/pentastatin (α5 NC1) interacts with has not been reported, it is reasonable to assume this active ECM fragment will also interact in a similar manner to its family members. Similar to tumstatin, lamstatin/pentastatin has been reported to be absent in lung tissues from patients with lymphangioleiomyomatosis and UIP [118, 139].

**Arresten, canstatin and tumstatin** all interact directly with fibroblasts to exert organ-specific effects on migration and proliferation [140], with induction of migration being noted in cardiac fibroblasts but inhibition in lung fibroblasts [118, 141]. In the lung fibroblasts, TGF-β induced conversion of fibroblasts to myofibroblasts was linked with canstatin release.
The multi-faceted extracellular matrix: unlocking its secrets for understanding the perpetuation of lung fibrosis

Fibronectin
Fibronectin is recognized to have central roles in regulating fibroblast migration when incorporated in the ECM, while the released active fragments of fibronectin also regulate fibroblast functions, including their phenotype differentiation [117]. These fragments also regulate endothelial cell responses, particularly during wound healing and possibly fibrosis. Anastellin, a peptide derived from the first type III module in fibronectin, helps orchestrate fibronectin fibriilogenesis and is anti-angiogenic but promotes fibroblast survival [142].

Fibulin-1
Fibulin-1 usually acts as a bridging molecule in the ECM facilitating the assembly of the larger structural proteins to which it binds, including collagen type I, elastin and fibronectin. In vitro studies have identified a peptide from fibulin-1 that activates lung-derived fibroblasts, inducing attachment, enhanced viability, proliferation and mitochondrial activity [131]. Fibulin-1 levels are increased in serum and lung tissues of IPF patients, with high levels being related to disease progression [47]. Mice that lack the fibulin-1C isoform are protected from the development of pulmonary fibrosis, through regulation of TGF-β activation via interactions with latent TGF-β binding protein [46, 132], but the levels of circulating fragments from fibulin-1 have not been measured in these animals.

Perlecan
When incorporated in the basement membrane, perlecan is recognized to have a pro-angiogenic function. However, when the active fragment, endorepellin, is released from its parent molecule, it has the opposite effect. Through binding to integrin α2β1 on endothelial cells, endorepellin mediates interruption of cell migration, via disruption of cytoskeletal arrangement and focal adhesions [143]. Endorepellin cooperates with endostatin, a polypeptide derived from the carboxy-terminus of collagen type XVIII, to enhance the effectiveness of each ECM fragment [130]. It also interacts with fibroblasts, protecting them from apoptosis, hence possibly mediating a role in fibrosis through this promotion of fibroblast survival [129].

ECM fragments as (bio)markers of an active fibrotic process
In addition to being active contributors to the pathological processes occurring during lung fibrosis, ECM fragments can also serve as sentinel indicators of these processes. During the cleavage processes that result in the release of ECM fragments from the deposited ECM in lung tissues, or indeed during the processes that enable the ECM fibers to be incorporated into the ECM bed, neo-epitopes are exposed on these fragments. Monitoring of the exposure of these neo-epitopes, or the levels of
the recognized ECM fragments, including those discussed above, released from lung tissues has the potential to inform us about the fibrotic processes that are active in a patient. Such fragments can be considered as possible biomarkers for fibrotic lung disease.

In a population-based multi-ethnic study, Madahar and colleagues reported that levels of two collagen fragments, carboxyl-terminal telopeptide of collagen type I (ICTP) and amino-terminal propeptide of procollagen type III (PIIINP), were associated with sub-clinical interstitial lung disease (interstitial lung abnormalities) detected through computed tomography screening [144]. These associations were not influenced by sex, race or smoking status. This report suggests that these ECM fragments represent a sensitive indication of fibrotic activity that can be detected well before lung function parameters can register disease activity.

In the PROFILE study, ECM degradation markers were found to have significantly different levels between controls and IPF patients [145]. When assessed longitudinally, changes in the levels of six neoepitopes (MMP−degraded−collagen type I (C1M), −collagen type III (C3M), −collagen type VI (C6M) and −C-reactive protein (CRPM), collagen type III degraded by ADAMTS-1/4/8 (C3A) and citrullinated vimentin degraded by MMP−2/8 (VICM)) were indicative of IPF patients with a greater likelihood of disease progression compared to those with stable disease. The baseline levels of C1M and C3A were associated with increased mortality. The levels of markers of ECM synthesis, neoepitope of collagen type III (PRO-C3) and collagen type VI (PRO-C6), were also elevated in IPF patients compared to healthy controls and again were increased in progressive disease compared to stable [146]. In addition, during exacerbations, patients with idiopathic interstitial pneumonia, including IPF, had increased levels of MMP−degraded collagen type IV (C4M) and C6M but decreased levels of MMP7−degraded elastin (ELM7) and MMP−degraded versican (VCANM) compared to patients with stable disease. Lower VCANM levels during exacerbation were associated with increased mortality [147]. Serum levels of laminin, collagen type IV, PIIINP and hyaluronic acid were also higher in a cohort of IPF and connective tissue disease patients, compared to controls, and were associated with mortality [116].

The serial measurement of ECM fragment or neoepitope markers in serum has the potential to inform about parameters important for clinical management of disease in these patients. These markers may bring manifestation of precision medicine in pulmonary fibrosis one step closer.
CONCLUSION

There is an urgent need for establishing effective processes for diagnosing patients with fibrotic lung diseases, and an even greater need for being able to accurately identify the underlying pathological cause to then be able to effectively manage these patients, without causing further harm. The ultimate goal is to enable the development of therapeutic approaches that are able to reverse the destructive changes in the lung tissue to regenerate effective gas exchange units and to return the longevity and quality of life for these patients. Thinking about the ECM as an active contributory element within the disease process has the potential to provide far reaching opportunities for novel advances in identifying disease-modifying mechanisms for pulmonary fibrosis. A consideration of the diversity of “hidden” changes within the ECM milieu that go far beyond the well-recognized changes in the composition and amount of ECM in a fibrotic lesion within lung tissues augments the novel directions that can be pursued when searching for future therapeutic targets (Figure 3). Future studies using emerging novel *in vitro* models that incorporate dimensionality and mechanical elements that exist in the lung, coupled with state-of-the-art transcriptomic and spatial proteomic profiling of fibrotic lung tissues, have the potential to ensure exciting developments in our understanding and management of pulmonary fibrosis in the near future.

Figure 3: ECM changes and potential therapeutic targeting sites/points. ECM: Extracellular matrix; LOX: Lysyl oxidase; LOXL: Lysyl oxidase–like; TG: Transglutaminase.
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