HHS Public Access

Author manuscript

Matrix Biol. Author manuscript; available in PMC 2019 November 01.

Published in final edited form as:

Matrix Biol. 2018 November ; 73: 77–104. doi:10.1016/j.matbio.2018.03.005.

Extracellular Matrix in Lung Development, Homeostasis and Disease

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Abstract

The lung's unique extracellular matrix (ECM), while providing structural support for cells, is critical in the regulation of developmental organogenesis, homeostasis and injury-repair responses. The ECM, via biochemical or biomechanical cues, regulates diverse cell functions, fate and phenotype. The composition and function of lung ECM become markedly deranged in pathological tissue remodeling. ECM-based therapeutics and bioengineering approaches represent promising novel strategies for regeneration/repair of the lung and treatment of chronic lung diseases. In this review, we assess the current state of lung ECM biology, including fundamental advances in ECM composition, dynamics, topography, and biomechanics; the role of the ECM in normal and aberrant lung development, adult lung diseases and autoimmunity; and ECM in the regulation of the stem cell niche. We identify opportunities to advance the field of lung ECM biology and provide a set recommendations for research priorities to advance knowledge that would inform novel approaches to the pathogenesis, diagnosis, and treatment of chronic lung diseases.

1. Introduction

Over the last three decades, our understanding of the many, diverse roles of the extracellular matrix (ECM) in mammalian biology has greatly advanced. It is now well established that, in addition to providing a scaffold for cells, the ECM provides essential biochemical and biomechanical cues directing tissue morphogenesis during development, homeostasis and injury-repair responses. The lung is characterized by a unique ECM composition and function that becomes markedly deranged in childhood disorders such as bronchopulmonary dysplasia (BPD), and adult diseases such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) (Figure 1).

In this review, we assess the current state of the field of lung ECM biology, and identify opportunities to advance knowledge that would inform novel approaches to understand, diagnose, and treat lung diseases of childhood and adults. Areas of focus in this review include fundamental advances in ECM composition, dynamics, topography, and biomechanics; the role of the ECM in normal lung development and aberrant development; ECM dynamics and altered deposition in adult lung diseases, namely COPD and IPF; the role of ECM in inflammation/autoimmunity; and maintenance of the stem cell niche. The potential for ECM-based therapeutics for chronic lung diseases is considered. Our goal is to identify specific areas that represent gaps in our understanding of ECM biology, and to provide a set of recommendations for research priorities to advance the field of lung ECM biology.

2. The ECM in Lung Development

The lung begins as a respiratory diverticulum (lung bud) from the foregut at approximately 5 weeks post-conception in the human embryo and develops by stages until full development is complete. Alveologenesis is thought to proceed well into post-natal life in humans, reaching the maximal number of 200–300 million during early adolescence [1]. The stages of lung development consist of a pseudoglandular stage (human: 5–17 weeks of gestation;

mouse: E9.5-E16.6), canalicular stage (human: 16–25 weeks; mouse: E16.6-E17.4), terminal saccular stage (human: 24–32 or 36 weeks; mouse: E17.4-P5), and the alveolar stage (human: 32 or 36 weeks to childhood or early teen years; mouse: P5-P28 or P42) [2, 3]. During these stages, the initial processes of branching morphogenesis, vasculogenesis and angiogenesis transition to alveolar septation and maturation accompanied by marked changes in lung ECM composition. The two main concepts regarding ECM in lung development are: (1) the lung ECM, not only provides vital physical support or a “scaffold” for resident cells of the lung and contributes to its mechanical properties but, is also essential for biophysical and biochemical signaling of lung cells, and (2) reciprocally, lung cells regulate the production and deposition of ECM over the course of development [4]. The processes by which ECM regulates lung cells and lung cells, in turn, produce or break down ECM are critical to normal lung development; alterations in these processes may lead to impaired lung development such as that seen in BPD. Additionally, abnormal recapitulation of developmental processes may contribute to disorders such as IPF, pulmonary arterial hypertension, or lung cancer with corresponding alterations in the ECM [5, 6].

The composition and topography of lung ECM changes over the course of lung development, and is very heterogeneous depending on location (e.g. close to bronchi, in alveolar septum, in pleura etc.) and developmental stage (e.g. saccular stage vs. early alveolar septation vs. mature adult lung). The lung ECM in fetal, neonatal and adult tissues are distinct, and temporally regulates the shape, migration, differentiation of resident cells [7, 8]. For example, during murine embryonic development, all five laminin γ chains are present, whereas adult lungs express primarily laminins γ3, γ4, and γ5 [9–11]. Fetal murine lung tissues contain more total GAGs and proteoglycans, and higher expression of collagen I and III in the pleura and the alveolar septae, in comparison to adult tissues [12]. Collagen comprises 16% of the pulmonary artery in young adults, and decreases to 10% in individuals over 80 years old [13, 14]. The ECM is constantly remodeled, with multiple post-translational modifications of various protein components. Various proteolytic enzymes, such as the matrix metalloproteinases (MMPs), and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), are involved in remodeling the ECM during development and in ECM homeostasis. The fetal human (and mouse) lung is characterized by greater proteolytic profile (higher MMP-2 and less TIMP-3 expression), while the adult lung is more anti-proteolytic (less MMP-2 and greater TIMP-3), with similar constitutive expression of MMP-14, MMP-20, TIMP-1, and TIMP-2 [15].

Airway branching and ECM

Airway branching (branching morphogenesis) that occurs primarily during the pseudoglandular stage of lung development is driven by interactions between the epithelium and the mesenchyme with active participation of ECM components, such as fibronectin, laminin, tenascin, and syndecan [4, 16]. Transplanting mesenchyme from a region wherein branching is occurring can induce branching of epithelial tissue where it does not, otherwise, occur [17]. During early human lung development, the collagens I, III, and VI and PGs (decorin, biglycan, and lumican) are primarily seen at the epithelial-mesenchymal interface, forming a sleeve around the developing airways [18]. The PG component of the ECM may regulate airway branching, in part related to the ability of sulfated PGs to bind FGF10,

which is necessary for branching [19]. Comprehensive gene expression profiling of murine lung development identified patterns of ECM gene expression, and determined possible relationships among groups of these genes that coordinate defined developmental processes [20].

Alveolar septation and ECM

The saccular stage is characterized by further widening of the air spaces and a thinner air-blood interface, accompanied by a reduction in the mesenchymal ECM, and organized deposition of elastin which is maximal along the sites of the future secondary crests (alveolar septa) [4]. Tropoelastin, the precursor of elastin, is produced during alveolar septation and is cross-linked by lysyl oxidase, and this modification facilitates alveolar septation. The deposition of elastin leads to elevation and suspension of the secondary crest that subdivides primary saccules. The secondary crest serves as a ridge that runs along the saccular wall, dividing it into two or more parts [21]. It is thought that the elastin and collagen fibers in the developing secondary crest tether portions of the primary saccular wall, restricting their motion, while the remaining portions of the wall expand further outward, forming the alveolus [21]. The process of alveolar septation requires other ECM proteins such as tenascin-C and growth factors such as PDGF-A [22]. Alveolar septation is followed by maturation of the capillaries that surround the alveolar space [2, 16]. PGs such as chondroitin sulfate and decorin are also localized to the secondary septa, and may also have a role in alveolar septation [16]. Similar to branching morphogenesis, it is likely that the degree of sulfation of the PGs is important for alveolar septation. Mice lacking the enzyme sulfatase-modifying factor 1 (Sumf1) which activates all sulfatases manifest an arrest in alveolarization associated with increased sulfated GAG deposition and increased TGF-β signaling [23]. Membrane-type 1 matrix metalloproteinases (MT1-MMP, also known as MMP-14) is involved in alveolar development, although mice deficient in MMP-3, 7, 9, or 12 develop normal lungs [24]. MMP-2 deficiency leads to delayed alveolarization in association with thickened pulmonary arteries and increased perivascular collagen and elastin [25].

Mechanotransduction from hydraulic pressure and airway peristalsis may also contribute to lung development. For example, marked reduction in amniotic fluid volume (oligohydramnios) is associated with lung hypoplasia in the fetus, presumably due to loss of fluid volume and decreased internal stenting force [26, 27]. Recent studies support synergistic control of alveolar epithelial cell differentiation by the concerted action of mechanical forces and local growth factors [28, 29]. Bronchial myogenesis is regulated by intraluminal pressure, and mechanical stretch modulates alternative splicing of serum response factor (SRF) and, thereby, regulates downstream myogenic genes [30]. Mechanical strain differentially regulates ECM molecules in fetal lung cells [31]. The cross-linker lysyl oxidase modulates tissue stiffness, which regulates low-density lipoprotein receptor-related protein (LRP) 5 and Tie 2 signaling (increased in cells on stiffer matrices), thus, controlling angiogenesis during alveolar septation [32].

Abnormal lung development

BPD that occurs in preterm infants is characterized by an inhibition of alveolar development, with varying degrees of inflammation, fibrosis, and abnormal vascular remodeling [33, 34]. Autopsy studies have documented that parenchymal collagen increases through development, and that preterm infants before 30 weeks have a delicate and intricate interstitial collagen network [35]. In BPD, there is an arrest of septation, with thickened collagenous saccular walls and increased interstitial collagen [35]. The volume of elastic tissue is also tightly regulated during fetal life, and doubles in the lung parenchyma from 22 to 30 weeks, and then doubles again over 20 weeks [36]. The amount of lung elastin in BPD increases with increasing respiratory distress severity, but the lung elastic tissue is disorganized and not in alveolar septa but in aberrant sites such as in the saccular-alveolar duct junction, which may represent sites of highest mechanical stress [36]. There is evidence that alterations in elastin may be secondary to changes in lysyl oxidase expression and activity [37, 38], perhaps combined with increased elastin degradation and uncoupling of its synthesis and assembly [39–41]. Impaired alveolar septation is among the most frequent histopathologic abnormalities found in children with suspected interstitial and diffuse lung diseases, and frequently occurs in association with chromosomal abnormalities and congential heart disease [42]. Abnormal alveolar development is also seen in association with Down syndrome [43].

3. ECM Composition and Dynamics

The matrisome is defined as the ensemble of ~1000 genes encoding ECM and ECM-associated proteins [44, 45]. The nature of many ECM proteins (large size, cross-linking, disulfide bonds, glycosylation, unique post-translational modifications, requirement of chaotropic agents for solubilizing etc.) has hindered their biochemical analysis and, until recently, only surrogate measurements (transcript levels, immunohistochemistry) were available to study the composition of *in vivo* ECMs. Mass spectrometry-based proteomics has emerged as a valuable method to study the global composition of the ECM of tissues and organs [46–54] (Figure 2). The adult murine lung matrisome comprises 143 matrisome proteins that can be divided into core matrisome proteins (glycoproteins, collagens and proteoglycans) and matrisome-associated proteins (including remodeling enzymes and ECM-affiliated proteins) [48, 53, 54] (Table 1). Proteomics has been applied to study the dynamics (turn-over or degradation vs. neo-synthesis) of the ECM compartment in a mouse model of lung fibrosis [53, 55], and more recently to human end stage interstitial lung disease. These studies have identified sets of matrix proteins upregulated during the course of the fibrotic response [56–58]. Such studies will aid in determining how global composition and organization of the lung ECM changes over time and in the context of disease progression.

The development of an atlas of the lung ECM using experimental models and human samples and, using the methods outlined below, has the potential to accelerate knowledge of ECM composition and dynamics. The atlas would compile ECM profiles of normal (different developmental stages, adults) and diseased lung tissues and would, in addition, include regional characterization (pleura, trachea, bronchi, alveolar interstitium, vessels,

upper lobe vs. middle lobe vs. lower lobe). The atlas would integrate global "-omics" data to include: (1) quantitative *proteomics* to identify ECM isoforms and post-translational modifications (such as cross-linking, phosphorylation, etc.) [59]; (2) ECM *degradomics* to identify the release of active cryptic fragments of ECM proteins that play key roles in disease progression, and to identity neo-epitopes within the ECM that could serve as biomarkers of disease progression or response to treatment [60, 61]; and (3) *glycomics* to identify the nature and abundance of polysaccharides and glycosaminoglycans (GAGs) in the lung ECM [62].

Newer -omics technologies will help unravel the complex interactions of genes, proteins, lipids and metabolites at unprecedented levels of detail and resolution. Such methods are comprised of proteomics analysis, including post-translational modifications and activity measurements at ever-lower protein concentrations, single cell fluorescent *in situ* hybridization (FISH) for multiple gene transcripts, and mass spectrometry imaging approaches for proteins, metabolites and lipids [63–70]. For example, a large compendium of peptides identified in a large set of tandem mass spectrometry proteomics experiments from multiple organisms is publicly available (www.peptideatlas.org). Many of these approaches, along with additional imaging platforms (CT, MRI, phase-contrast X-ray, cryomicrotome/optical, confocal, and immunohistochemistry) are currently being utilized by NHLBI's LungMAP consortium to create an open-access reference resource and comprehensive 3D tissue/cellular/molecular atlas of the late-stage developing mouse and human lung (www.LungMAP.net). There are limitations of "omic" type data, specifically due to variations in sample processing, analytical techniques, and normalization of resulting data. These limitations are primarily evident with low abundance proteins that may exhibit marked inter- and intra-sample variation. However, both spatial (location relative to structures, cells, and other ECM proteins) and temporal data can be obtained when techniques such as *in situ* proteomics complement "omic" studies, and when samples are analyzed in series. Together, these data will provide important clues to the identification of structural and regulatory events that occur during lung development, injury, and repair.

In addition to compositional changes of the ECM with disease progression, the architecture of the ECM also changes as indicated by methods that monitor the ECM at the macromolecular scale; one example of such approaches is second harmonic generation microscopy [71]. Finally, technologies are needed to study not just ECM composition and remodeling, but also the post-translational modifications that affect the function of various ECM proteins. Just as cross-linking of collagens can be measured biochemically with lysylpyridinoline, hydroxylysylpyridinoline and pentosidine [72, 73], imaging of such crosslinks using nondestructive methods is likely to be informative. There is ample experimental evidence that sulfation, glycosylation, tyrosine cross-linking, and glycation (among others) are all potentially deranged in human lung diseases [74–77]; visualizing such changes in real-time will further enhance our understanding of the functional significance of such post-translational modifications.

4. ECM Topography and Biomechanics

Matrix topography and stiffness are major physical properties of the ECM. Matrix topography refers to the structural characteristics of the ECM. It includes the architecture, geometry, size and organization of the matrix network, ranging from the nano-scale level to the macro-scale level. The most substantial influence of matrix topography on cells is the impact on cell morphology. Aligned matrix fibers generate anisotropic stress that changes cell shape by a process known as contact guidance [78]. Matrix topography also regulates stem cell differentiation and cancer cell invasion. Human mesenchymal stem cells cultured on titanium dioxide nanotubes with various dimensions differ in cell morphology. Larger nanotubes promote mesenchymal stem cell elongation and differentiation into osteoblasts in the absence of osteogenic media, whereas smaller nanotubes permit stem cell adhesion without significant osteogenic differentiation [79]. In a 3D cell invasion model, highly aligned collagen matrices promote breast cancer cell invasion as compared to low alignment collagen matrices [80]. In normal mammary tissues, collagen fibers are arranged in parallel to epithelium and along the axis of the gland. However, carcinoma-associated fibroblasts remodel tumor ECM and reorient collagen fibers perpendicular to the gland. Such reorganized collagen fibers could act as “highways” to facilitate breast cancer cell invasion into the neighboring tissues [81]. Interestingly, lung (myo)fibroblasts isolated from patients with IPF are characterized by an invasive phenotype [82–84]. Stiffened fibrotic ECM promotes IPF lung myofibroblast invasion into the basement membrane through expression of mechanosensitive α_6 integrin on the cell surface [84]. It has been observed that matrix fibers are arranged with their long axis parallel to the long axis of the alveolar septa at the fibroblastic foci in IPF/UIP [85]. The highly organized, anisotropic matrix fibers and specific integrins may aid IPF (myo)fibroblast invasion to form a continuous fibrotic reticular network (Figure 3).

To date, the mechanotransductive mechanisms involved in the conversion of matrix topographic and stiffness cues into intracellular signals remain poorly understood. Integrins are important signal molecules and membrane receptors that link the cytoskeleton to the ECM. The ECM-integrin-cytoskeleton complex potentially acts as a molecular clutch in the process of contact guidance. Specific topographic features of the ECM may transmit cell signals by spatially biased focal adhesion formation, preferential actin cytoskeletal remodeling, and/or confined protein absorption and patterning [86, 87]. Additionally, matrix topography induces deformation of nuclear architecture, which may alter the profile of gene expression [88].

Many micro/nanofabrication technologies have been developed for tissue engineering applications. These technologies can be utilized to create geometrically defined matrix structures for the study of matrix topography-cell interactions. For example, electrospinning creates fibrous scaffolds with controlled orientation distributions (e.g., random fibers, aligned fibers) [89]. Both natural polymers, such as collagen, and synthetic polymers have been used to create electrospun nanofibers. In contrast, photolithography employs light to produce defined topographic features, such as grooves, pillars and pits [90]. A recently developed topography array incorporates thousands of distinct topographical units on a single chip to enable systematic and high throughput studies of cell-matrix interactions [91].

Biomechanics of lung ECM have been studied using atomic force microscopy (AFM) to analyze micromechanical tissue properties. While this method is invasive and, thereby, imposes substantial alterations from the physiological state of perfused and ventilated lung within the intact thoracic cavity, it has the advantages of high sensitivity and spatial resolution [92]. Using AFM, it has been possible to compare the elastic or Young's modulus of normal and fibrotic lung tissues, with pronounced increases in tissue elastic modulus (stiffening) identified in human IPF-derived lungs both before and after decellularization [93], and similar changes are observed in intact tissue obtained from mouse models of lung fibrosis [92, 94]. AFM methods have also allowed regional differences in lung ECM properties to be measured, including higher modulus values in the pleura and vessels compared to the alveolar walls in decellularized lung matrices [95–97], and higher modulus values in the airways than in the surrounding parenchyma in intact lung tissue [98]. Some systematic differences in measured mechanical properties are observed across these studies, in tandem with variations in the specific AFM methodologies applied (e.g. tip size and shape, indentation depth and velocity) and methods of tissue preparation (e.g. intact versus decellularized tissue, thickness of tissue slice). Thus, while the overall trends consistently demonstrate increased tissue stiffness in fibrotic parenchyma, and increased moduli of pleura and conducting airways and vessels relative to alveolar regions, it will be important to systematically test the influence of tissue preparation and AFM methodology to gain greater confidence in the quantitative values reported for lung tissue mechanical properties. Building on current approaches, AFM should be coupled with other optical techniques to correlate local mechanical properties with the underlying architecture, and perhaps even composition, of the ECM, to provide novel insights into structure-function relationships of the lung ECM. In addition to the study of fibrosis, AFM mechanical characterization should also be applied to the study of other chronic lung remodeling diseases, such as COPD, asthma and pulmonary hypertension; additionally, such approaches could be extremely valuable in characterizing the mechanical microenvironment of the developing lung [32, 38, 99].

Multiple lung cell types, including fibroblasts, macrophages, epithelial and endothelial cells exhibit functional changes that depend on matrix stiffness spanning the range observed in normal and diseased lung tissue [32, 92, 94, 100–106]. Matrix stiffness effects are typically observed in hydrogels, silicon rubbers, and natural biomaterials through changing bulk polymer concentration, crosslink density between polymer chains, or a combination of the two; these materials can be fabricated over a wide range of stiffness from 10^1 to 10^6 Pascal (a unit of stiffness) [107]. Once seeded on these substrates, matrix stiffness-stimulated intracellular signaling appears to occur through conserved growth factor- or transcription factor-mediated pathways. For example, increasing matrix stiffness enhances the capacity of cells to generate tractions, the forces that cells transmit to the ECM [106] and, thereby, activate TGF-β from a latent matrix-bound form [94, 108], linking matrix stiffness to activation of matrix synthesis. Increasing matrix stiffness also engages and activates the mechanoregulatory transcription factors, MRTF [102] and YAP/TAZ [109] in lung fibroblasts (see Section 6 for more details). Pharmacological inhibition of Rho kinase upstream of MRTF-A [110], or MRTF itself [109], attenuates bleomycin-induced fibrosis, while expression of constitutively active YAP or TAZ confers fibrogenic potential to fibroblasts adoptively transferred to the lungs [109]. Interestingly, human fibroblasts isolated

from patients with IPF remain responsive to inactivation of these pathways [109, 110], and to changes in matrix stiffness in general [105], suggesting that targeting the matrix mechanical environment or its downstream signaling pathways may be beneficial in diseases of aberrant matrix remodeling.

5. The ECM in Emphysema

COPD-emphysema (termed COPD here) is a prototypical disease of aberrant lung ECM. The association of excessive elastase activity with alveolar wall breakdown in animal models coupled with the accelerated COPD phenotype in persons with alpha-1 antitrypsin deficiency supports the concept of a protease-antiprotease imbalance [111]. However, several recent observations suggest greater complexity to this paradigm: (a) increased elastin and collagen content in distal compartments of COPD lungs; (b) small airway fibrosis in COPD lungs; (c) emerging subphenotype of combined pulmonary fibrosis and emphysema (CPFE); (d) genetic emphysema syndromes with perturbations in matrix turnover and TGF-β1 pathways; and (e) impaired antioxidant defenses in COPD lungs. These findings invoke a pathogenetic scheme in which altered matrix composition may not only reflect upstream signaling disturbances, but also direct adverse sequelae in the distal lung.

ECM content in COPD lungs

The predominant matrix elements in the distal lung are collagen I, collagen III and elastin [12]. Collagen I likely confers tensile strength; collagen III, flexibility; and elastin, recoil properties. In the COPD lung, the combination of tissue destruction and matrix remodeling leads to dynamic changes in matrix content reflecting primary disturbances and compensatory responses. Although single time-point analyses of matrix composition do not fully describe the sequence of changes throughout the course of the disease, our current understanding of the lung ECM is almost exclusively based on such approaches. Cross-sectional studies albeit with small sample sizes consistently show an increase in collagen content and altered fibril morphology in lungs of patients with moderate and severe COPD [112–114]. Abnormal elastin fibers (fragmented, clumped) with variable changes in elastin content are evident in emphysematous lungs [112, 113, 115, 116]. Animal models of emphysema, typically involving airway elastase instillation or chronic cigarette smoke exposure, also demonstrate increased collagen and elastin synthesis with matrix deposition during the establishment and progression of the airspace lesion [117–121]. Various other ECM components such as proteoglycans, basement membrane components and matrix binding properties are variably altered in human COPD lungs and animal models of emphysema. Lung proteoglycans, known to be increased in COPD, can also inhibit elastic fiber assembly [116, 122]. These data support a more complex process of ECM destruction and defective repair contributing to altered biomechanical forces, COPD development and progression. Whereas much research in patients and animal models has focused on the ECM destruction, the mechanisms of aberrant repair are poorly detailed. A better understanding of the repair axis is crucial as efforts to reconstruct the damaged COPD lung will need to integrate the correction of adverse reparative cascades and the re-initiation of normal matrix synthesis and regenerative programs.

Small airway fibrosis in COPD lungs

Airway obstruction in COPD is primarily caused by architectural and functional changes in small airways attributed to both loss of alveolar attachments (feature of emphysema) and airway wall thickening [123–125]. Several studies demonstrate a significant component of airway wall remodeling with specific matrix alterations in COPD. Increased collagen with reduced elastin in the small airways of COPD lungs has been observed [126–128]. More recently, a loss of distal airways effectively destabilizing the distal airspace has been found to punctuate COPD [129]. How abnormal matrix composition interfaces or contributes to airway loss is unclear. Clearly, the widespread notion that matrix alterations are divergent in the airway and airspace (increased and reduced, respectively) ignores the consistent abnormalities present in both compartments, as described above. However, relative differences in the expression of matrix proteins in alveolar versus airway compartments in a murine COPD model suggest that there may be temporal and compartmental distinctions in the reparative response [130]. By this paradigm, a persistent repair response in the airway culminates in airway fibrosis, while an attenuated response in the alveoli manifests in airspace enlargement.

Combined pulmonary fibrosis and emphysema

A recently recognized subphenotype of COPD is CPFE, an underdiagnosed disorder manifesting in coexisting pulmonary fibrosis and emphysema [131, 132]. Although standardized diagnostic criteria are lacking, several cross-sectional and observational studies suggest that the prognosis for CPFE may be worse than that for emphysema or pulmonary fibrosis alone [133–136]. The demonstration of increased collagen content in the airspace and small airways of COPD lungs may provide a unifying mechanism for this phenotype. In this view, the primary or compensatory increase in collagen deposition in COPD represents an early fibrotic response coincident with developing emphysema. A second hit (cigarette smoke, oxidative stress, inflammation) or simply temporal progression may confer the full CPFE phenotype. Further studies of the CPFE phenotype are required to determine the underlying mechanisms for this mixed phenotype.

Genetic emphysema syndromes

Whether genetic disorders displaying progressive airspace enlargement can inform the understanding of acquired COPD-emphysema is debatable. These syndromes do establish that defects in ECM composition and TGF-β signaling cascades can lead to airspace dysmorphology. Emphysema is a minor phenotype of cutis laxa, Marfan Syndrome and vascular Ehlers Danlos Syndrome, single gene disorders caused by mutations in ECM proteins (fibulin 5, elastin, fibrillin 1, collagen III, latent TGF-β binding proteins, respectively) [137–142]. Whereas several GWAS studies of COPD-emphysema patients did not reveal any matrix proteins as candidate genes, a recent tissue profiling analysis identified fibulin 5 as a candidate COPD gene [143–146]. Additionally, distinct genes in the TGF-β pathway have been implicated in genetic and gene expression studies of COPD [143, 147–149]. This suggests that COPD may result from either primary disturbance in matrix remodeling pathways (e.g. TGF-β1, matrix metalloproteases), secondary mechanisms conferring matrix abnormalities (e.g. exaggerated repair cascades), a combination of these,

or processes altogether distinct from genetic matrix disorders. Additionally, a distinct subset of COPD patients may have low abundance pathogenic alleles in matrix proteins mimicking Mendelian disorders of the matrix. Identification of novel candidate genes in well-characterized subphenotypes of COPD may contribute to our understanding of pathophysiologic patterns of matrix remodeling.

Antioxidant defenses in COPD

Oxidative stress is a known contributor to COPD development and progression, evident in both patient-based studies and animal models [150, 151]. An interesting connection between the abnormal matrix of COPD and oxidative stress is the antioxidant superoxide dismutase 3 (SOD3). This isoform of SOD is secreted and distributes in the extracellular space. Recent studies provide evidence of SOD3 binding to the ECM proteins, fibulin 5 and collagen I [152, 153]. Reduced SOD3 levels have been documented in murine models of BPD and COPD, likely through inhibition of ECM fragmentation and subsequent chronic inflammation [154, 155]. Genetic studies have also implicated SOD3 as a candidate gene for reduced lung function and COPD [156, 157]. Further exploration of the lung matrix as a repository for regulatory proteins that determine lung homeostasis is warranted.

Recent studies of COPD have allowed investigators to move beyond the protease:antiprotease paradigm to incorporate emerging concepts regarding the complex, dynamic aspects of matrix deposition and turnover that define the disease. These mechanisms could determine both COPD severity and progression. Future research efforts should not only characterize the alterations in matrix content and organization in COPD, but also explore strategies to engage reparative and regenerative pathways that restore lung structure and function.

6. The ECM in Fibrosis

Alveolar epithelial injury induces a stereotypic response characterized by disruption of the alveolar basement membrane and the deposition of a provisional matrix rich in fibrin and fibronectin. Reparative fibroblasts are recruited to this milieu where they replace and remodel the provisional matrix into a more organized and cross-linked collagen-rich matrix [158]. In most cases, the repair response resolves with formation of a physiologic scar that does not disrupt tissue architecture or function. Such resolution of wound repair with the restoration of homeostatic function requires the clearance of excessive extracellular matrix and the apoptosis of the fibroblast/myofibroblast population [158]. These processes must be tightly regulated, both temporally and spatially, as the impaired loss of fibroblasts and insufficient clearance of matrix is associated with fibrosis while extensive loss of fibroblasts and matrix might lead to emphysema [159, 160]. The precise mechanisms regulating collagen turnover and fibroblast apoptosis, and the extent to which these biologic processes are linked, remain poorly understood.

The capacity of the injured lung to heal is perhaps best exemplified by the clinical course of patients with acute respiratory distress syndrome (ARDS). Regardless of cause, ARDS manifests as diffuse alveolar damage with a rapid reparative response characterized by the upregulation of collagen detected in the alveolar space [161, 162]. Consistent with other

studies of wound repair, the resolution of ARDS is associated with evidence of fibroblast apoptosis within airspace granulation tissue [163]. Despite the extensive injury and the associated fibrotic response, the majority of ARDS survivors have normal or near-normal restoration of lung mechanics and gas exchange over the course of a year [164].

The ability of the chronically injured lung with established fibrosis (or emphysema) to heal or regenerate is less well established. Evidence in other organs, including kidney, liver, and muscle suggests that fibrosis is not, in and of itself, an irreversible process [165–169]. Furthermore, existing evidence in both human lung disease and animal models supports the concept that fibrosis can resolve [110, 163, 170, 171]. Nevertheless, it is unclear why fibrosis is persistent and progressive in certain disease processes such as IPF. An extensive body of literature demonstrates that “pro-fibrotic” soluble and matrix factors stimulate fibroblast synthesis of collagen and other matrix components. However, the mechanisms regulating matrix degradation, and how these mechanisms are perturbed in chronic lung disease, have received far less attention [160]. An impaired tissue degradative environment has been observed in both pulmonary fibrosis and fibrosis in other organ systems [172, 173]. In IPF patients, for example, there is an imbalance between the production of MMPs and TIMPs with an increase in the ratio of TIMPs:MMPs at the site of scar formation [173]. There is a similar decrease in MMP expression in fibrotic livers [174]. Lysates of tissue biopsies taken from the skin and lungs of scleroderma and IPF patients, respectively, have reduced ability to degrade collagen *in vitro* as compared with control biopsy samples [173, 175].

Matrix turnover involves both extracellular proteolysis and cell-mediated uptake of cleaved matrix fragments [176]. Recent evidence has emerged that genetic mutant mice with impaired cell-mediated collagen uptake develop more severe fibrosis in response to lung injury suggesting that the cell-mediated pathway, in addition to the proteolytic pathways [177, 178], is important in regulating the severity of tissue fibrosis [179, 180]. The mechanisms by which cell-mediated removal of collagen fragments promote resolution of fibrosis is less well understood. One possibility is that collagen internalization negatively regulates the production of new collagen/matrix by either the cells ingesting collagen or cells adjacent to those ingesting collagen.

A recent RNAi-based genomic screen of cell-mediated collagen internalization has identified several other mediators of the intracellular pathway [181]. In addition to identifying the flotillin family of vesicle transport proteins as functioning upstream of uPARAP/endo180 in regulating collagen turnover, the screen also identified two candidate genes, fibroblast activation protein and ATG6/Beclin-1, which have been shown to be important in *in vivo* collagen degradation [182, 183]. The emerging role of autophagy in collagen uptake and degradation coupled with recent studies linking impaired autophagy with lung fibrosis, warrants further investigation of the mechanisms by which autophagy regulates collagen turnover [184–187].

The cells principally responsible for the clearance of collagen have not been elucidated. Macrophages, fibrocytes and fibroblasts have all been shown to ingest extracellular collagen, and fibrocytes may be even more efficient than fibroblasts [188]. A prominent role for

macrophages in collagen resorption is supported by mouse models where genetic deletion or pharmacological depletion of macrophages during the remodeling phase of experimental-fibrosis reduces scar resolution [189–191]. We speculate that fibroblasts may be the predominant cell involved in collagen turnover under homeostatic conditions, while recruitment of macrophages and/or fibrocytes enhances the capacity for collagen clearance in response to lung injury.

The kinetics of collagen turnover in the lungs of patients with IPF remain poorly understood. Specifically, while it is accepted that lung collagen is continuously turned over [179, 192–194], it is unclear whether there exist pools of collagen that are rapidly turned over versus pools that are more stable and long-lived or whether all lung collagens turn over at similar rates [195]. Scar tissue contains a number of other matrix molecules in addition to fibrillar collagens. The relative amounts of each of these matrix molecules are beginning to be understood through proteomic analysis [55]. How these molecules physically interact with each other remains unclear, as does whether the three-dimensional structure of a scar prevents access of proteolytic enzymes to their target sites on collagen, thereby, inhibiting the ability for collagen breakdown.

The fate of the matrix is intricately linked with the fate of the (myo)fibroblasts and, in the context of fibrotic repair, interactions between the ECM and fibroblasts can establish a “feedforward” amplification loop in which myofibroblasts produce matrix and the matrix, in turn, activates signaling pathways that support fibroblast survival [92, 196] (Figure 4). For example, matrix adhesion is essential for myofibroblast differentiation and survival, and these fibroblast phenotypes are further modulated by the biomechanical properties of that extracellular matrix [92, 110, 197–200]. The ability of fibroblasts to sense biomechanical properties of the ECM affects phenotype, survival and resolution of fibrosis [201, 202], but the mechanosensory apparatus of fibroblasts is poorly understood. The pro-fibrotic cytokine TGF-β1 is also a potent stimulus for myofibroblast differentiation and survival [159, 197, 199, 203]. Moreover, the matrix serves as a reservoir for latent TGF-β1 and activation of TGF-β1 from its latent form can be achieved either through proteolytic mechanisms or through a non-proteolytic mechanism mediated by a stiff extracellular matrix [108]. While the ECM and TGF-β1 may each promote myofibroblast differentiation and acquisition of an apoptosis-resistant phenotype, and each interacts with and influences the other, matrix regulation of fibroblast phenotype may occur independent of TGF-β activation [93, 102, 204].

Supporting the interactions between TGF-β1 and matrix-mediated signals in the coordinate regulation of myofibroblast differentiation and survival, TGF-β1 and rigid extracellular matrices utilize common upstream mechanisms, including focal adhesion kinase (FAK) and Rho kinase (ROCK) to regulate transcriptional events dependent on serum response factor (SRF) and myocardin-related transcription factor (MRTF), and/or YAP-TAZ [102, 110, 205–208]. Specifically, TGF-β1 and/or matrix stiffness-mediated activation of FAK, RhoK, SRF/MRTF, and YAP-TAZ have been shown to promote myofibroblast resistance to apoptosis through induction of inhibitor of apoptosis proteins including X-linked inhibitor of apoptosis, survivin, anti-apoptotic BCL-2 family proteins, and through upregulation of plasminogen activator inhibitor-1 (PAI-1), a serpin protease inhibitor that blocks fibroblast

apoptosis induced by plasmin-mediated fibronectin proteolysis [110, 198, 200, 209]. Although the mechanistic hierarchies have not been established and the interactions between matrix and soluble factors in the regulation of each kinase and their transcriptional regulators have yet to be delineated, each of these mediators and proteins has been shown to be increased within the fibroblastic foci of lung tissue from patients with IPF and/or in lung fibroblasts explanted from patients with IPF [110, 209–212]. Moreover, inhibition of each has been shown to enhance fibroblast susceptibility to apoptosis *in vitro* and promote the resolution of lung fibrosis *in vivo*.

In summary, accumulating studies support the concepts that: (1) matrix accumulation is necessary for fibrogenesis and resolution of fibrosis requires matrix degradation; (2) matrix-generated signals maintain an apoptosis-resistant myofibroblast phenotype utilizing mechanisms that coordinately regulated by TGF-β1 signaling; (3) disruption of fibroblast-matrix interactions can induce fibroblast apoptosis while blockade of matrix-mediated signals can enhance fibroblast susceptibility to apoptosis; and (4) inhibition of matrix-derived signals that promote fibroblast survival is associated with resolution of lung fibrosis in murine models. There is relatively limited knowledge about the biological pathways that regulate matrix resorption and myofibroblast apoptosis, the degree to which these processes might be linked, and whether these processes are amenable for therapeutic intervention. Understanding the mechanisms regulating matrix turnover and fibroblast apoptosis, and how these mechanisms are perturbed, is critical for the identification of novel strategies to promote the resolution of lung fibrosis.

7. The ECM in Inflammation and Autoimmunity

The lung microvasculature provides a vast surface area where circulating and activated immune cells mount an appropriate response to eliminate invading pathogens. Although an influx of immune cells into the lungs is designed by nature to protect the host against harmful infectious insults, excessive innate and adaptive immune responses to environmental exposures may promote chronic inflammation that destroys the lung parenchyma [213, 214]. Specifically, chronic exposure to a variety of inhaled noxious stimuli such as environmental pollutants, cigarette smoke, and other sterile toxic fumes could promote recruitment and activation of inflammatory cells in the lungs. Adaptive immune cells such as autoreactive T lymphocytes, directed against the lung's structural molecules or it ECM components, can induce inappropriate immune responses that could trigger lung destruction. Therefore, while activation of innate and acquired immunity are critical in host defense against invading organisms, activated immune cells could evoke untoward responses and promote autoimmune inflammation in the lungs.

Many of the signals that result in aberrant activation of immune cells are embedded within the normal lung stroma that, when altered, are processed and presented by the antigen presenting cells (APCs) to lymphocytes in the context of the MHC complex [215, 216]. While APCs can take in and process many self- and foreign proteins, they require additional signals to become activated and initiate acquired immune responses at the sites of inflammation [217]. Proteolytic degradation or modification of ECMs (e.g., proteoglycans, fibrillar collagens, glycoproteins, elastin, etc.) could generate new antigens that bind and

activate immune cells in the lungs [218–220]. For example, fragments of human lung elastin formed through cleavage by neutrophil elastase have been shown to be chemotactic for monocytes [221], and are strongly immunogenic as demonstrated by the presence of elastin-specific autoreactive T cells in smokers with emphysema [222]. Therefore, in susceptible smokers, in response to specific antigens (e.g., elastin fragments), T lymphocytes proliferate or induce B cells to make autoreactive antibodies to promote chronic inflammation.

Another example of how ECM breakdown can directly affect immune cell activation is the potent bioactive tripeptide, proline-glycine-proline (PGP), proteolytic fragments of type I collagen. PGP is a molecular mimic of several CXC chemokines, such as IL-8, and attract neutrophils through binding to their CXCR1 and CXCR2 receptors [223]. MMPs with strong gelatinolytic activity (e.g. MMP2, MMP9, and MMP13) are released by fibroblasts and innate immune cells; cleavage of collagen by prolyl endopeptidase (PE) further degrades the gelatin fragments to form PGP [224]. Clinical and translational studies in smoking-induced COPD support the concept that PGP-mediated inflammation in the lungs creates a positive feed-back system, which may be independent of acquired immunity [225] (Figure 5).

In addition to affecting immune cell function by generating effector ECM fragments, MMPs can regulate their influx and activation by several other mechanisms [226]. For example, the macrophage secreted MMPs, MMP12 and MMP28, can either promote or restrict macrophage influx into the lungs [227, 228]; MMP28 and TIMP3 moderate pro-inflammatory activation of macrophages [229, 230]. With respect to mechanisms, MMPs quite often affect chemokine availability or activity either directly, by modifying the protein, or indirectly by acting on proteins that modulate their activity [231–233].

Some of the key questions remaining is how newly formed fragments of endogenous proteins and peptides activate immune cells and promote chronic indolent inflammation in the lungs. ECM molecules collectively play an important role in orchestrating the flow of immune cells in and out of the lungs; thus, deciphering how ECM-derived fragments shape immune cell activation represents a major challenge for future investigations. More importantly, it is not clear how ECM-mediated activation of immune cells selectively perpetuate recruitment of inflammatory cells into the lungs. Approaches such as deep sequencing and proteome-wide screening may identify the global effects of different ECM-derived pathways that promote inflammation. Additionally, identification of specific ECM-derived mediators that act upstream of immune cell activation may provide opportunities for therapeutic intervention.

8. The ECM in Regulation of the Stem Cell Niche

Elucidating the regenerative potential of lungs in adult life is critical to the potential reversibility of emphysema and/or fibrosis in humans. However, closing gaps in our knowledge of lung regeneration is limited by the lack of understanding of the composition and maintenance of stem cell niches along the respiratory tract [234–236]. A major challenge is to “decode” the mechanisms by which the normal ECM regulates the stem cell

niche within the lungs. It also remains to be defined how the altered ECM influences niche dysfunction and stem cell behavior in diseased lungs.

Within the alveolar compartment, alveolar epithelial cells (AECs) are closely associated with various cell types such as vascular, mesenchymal, and immune cells. Lipofibroblast cells localized between capillary endothelial cells and AECs have been shown to regulate the proliferation and differentiation of type II AECs which function as facultative stem cells of the alveolar epithelium. Deposition of stem cell-active ECM proteins or proteolytic deployment of encrypted ECM fragments by niche cells could evoke proliferation and modulate differentiation of alveolar stem cells. Thus, uncovering how individual cell types deploy ECM to orchestrate regenerative alveolarization would potentially fill a large gap in our current knowledge of stem cell niches within the lungs; this would also provide an opportunity to mimic these conditions using bioengineering approaches to develop more realistic niches *ex vivo* [236].

There are several potential challenges to decoding the ECM in the lung stem cell niche. First, it is critical to generate a “matrix footprint” by identifying niche-derived ECM factors that modulate alveolar stem cell function, including MMPs, serine proteases, and specific matrix proteins. This will also provide clues to which biochemical and/or biophysical properties of the niche may be capable of guiding stem cell fate. Second, determining how endothelial cells, mesenchymal/stromal, immune and other supporting niche cells coordinately mobilize proper ECM signals to stimulate functional repair and avoid maladaptive repair/fibrosis is essential. It is important to recognize that niche ECM may be critical to the support and maintenance of niche cells, in addition to the stem cells themselves. Third, a long-term goal would be to exploit bioengineering tools to rebuild a faithful niche that recapitulates and facilitates the endogenous stem cell-niche crosstalk. To engineer such synthetic polymer, hybrid, and natural materials, the required tools should include polymer chemistry to create new materials, photolithography to pattern materials, electro-spinning to manufacture these materials, and methods to ensure that the intended properties to be mimicked in the system are indeed present. Such tools will also aid in determining whether the ECM can be manipulated to favor therapeutic lung regeneration.

To this end, there is an urgent need to develop *ex vivo* “macroscale” models to interrogate stem cell niches in the lung. For example, establishing stem cell-niche cell co-culture systems and organotypic models would be helpful to interrogate how the niche-derived ECM imposes the stemness and modulates the balance between regeneration and fibrosis in the injured lung. Generating efficient *ex vivo* co-culture models encompassing both alveolar stem cells and supporting niche cells would permit for mechanistic delineation of cell type-specific contributions in lung stem cell niche. Traditional biochemical means or high-throughput screening systems could be employed in these models to uncover the molecular basis of stem cell-niche cell crosstalk, including responsible ECM molecules deployed by niche cells and corresponding receptors on stem cells. Once the critical ECM components and niche cell types are mapped out, niche re-construction using engineered scaffolds would be an ideal approach to recapitulate the pro-regenerative “matrix footprint” *ex vivo*. Decellularized tissues may be used as a bioreactor that expands different types of lung stem cells and fosters the crosstalk between stem cell and their niches [237]. In addition to these

biochemical considerations, it is also critically important to ensure that cells in this artificial niche experience the appropriate mechanical environment. Lung is a very dynamic tissue, and it is important that, in addition to passive stiffness, the niche is also capable of recapitulating cyclical strain typically placed on it during the breathing cycle. Approaches such as these may inform strategies for more effective cell-based therapies in the future, and provide more fastidious pre-clinical models to assess the efficacy of pro-regenerative therapies.

Establishing animal models of lung regeneration is also pivotal for defining the important characteristics of a lung stem cell niche. Further characterization of animal lung injury models such as hyperoxia, influenza infection, and pneumonectomy would be crucial for revealing the molecular and cellular basis of lung regeneration. By combining “cell-type specific” lineage tracing and “gain and loss function” genetic tools, these animal models would allow us to unravel and verify the functional contribution of key niche ECM molecules during regenerative alveolarization. Of note, one of the most rigorous assays to demonstrate stem cell activity is to test the diverse cell lineage reconstitution in an *in vivo* transplantation system [238]. While limiting dilution transplantation assays have been widely used for the study of hematopoietic stem cells, lung stem cell research has been hindered by the paucity of a faithful cell transplantation system. Developing a lung regeneration/repair model that facilitates functional engraftment of lung stem cells would be invaluable to test, not only the attributes of particular stem cells but, the mechanisms by which host niche cells regulate the homing, adhesion, engraftment and differentiation of transplanted stem cells. As such, mechanistic revelation of the ECM biology in lung stem cell niches requires establishment of both animal models of lung regeneration and cell lineage-specific genetic approaches. These *in vivo* platforms will allow for stringent interrogation of the *in vivo* crosstalk between stem cells and niche cells in lung regeneration that will potentially enable design of pre-clinical models for regenerative therapy.

9. ECM-Based Therapeutics

Recent Phase II/III clinical trials in IPF have targeted ECM composition, crosslinking, and/or matrix-driven signaling [239]. Matrix crosslinking, in particular, seems to be a promising therapeutic target [240, 241]. Targeting the balance of proteases and antiproteases has long been recognized as a potential therapeutic strategy for disorders of excessive matrix accumulation; however, this strategy is complicated by the promiscuity and redundancy of protease-antiprotease pathways, which activate and inactivate a broad array of biochemical signals in addition to, or as a direct result of, their effects on matrix turnover. For example, MMP-8, which is a collagenase, is actually pro-fibrotic via inactivation of particular chemokines [242]. Additionally, matrix fragments may have potent and deleterious inflammatory activity. Nevertheless, altering the balance of collagen production and degradation (collagen turnover) remains an important therapeutic strategy to promote resolution of fibrotic remodeling. An intracellular pathway of collagen turnover may be advantageous, in that it avoids the “friendly fire” problems associated with extracellular proteolysis [179, 180]. Emerging strategies for promoting beneficial matrix turnover include engineering collagenolytic cells for adoptive transfer, or engineering collagen-degrading bacteria.

Another promising approach is targeting or mimicking microRNAs (miRs) which regulate ECM biogenesis. For example, miR-29 mimicry with synthetic RNA duplexes blocks fibroblast collagen synthesis and attenuates bleomycin-induced fibrosis [243]. Matrix signals, particularly those associated with mechanical force, are transmitted through integrins; thus, there are numerous strategies to target particular integrin subunits or integrin-activated signaling cascades [84, 110, 244–246]. Integrin targeting should be undertaken with caution, however, as exemplified by the findings that broad targeting of αv integrin may be beneficial for fibrosis [245], whereas targeting αvβ3 specifically promotes fibrosis progression in the liver [247]. Increased understanding of mechanosensing and mechanotransduction is likely to uncover novel approaches for limiting deleterious responses of cells such as fibroblasts to an altered matrix environment.

10. Emerging Technologies to Study the ECM

The capability to monitor dynamic changes that occur in ECM, in living individuals over time, is a crucial aspect of comprehending the clinical significance of such changes in particular lung diseases. For example, recent technological advances in magnetic resonance imaging (MRI) and ultrasound have allowed us to begin addressing this goal. Magnetic resonance elastography (MRE) is a new tool being used to study hepatic fibrosis in the research setting. Using an ultrasound device compatible with magnetic resonance scanners, mechanical sound waves are generated through the liver while the individual is undergoing magnetic resonance scanning. Using specialized software with a modified phase-contrast gradient-echo sequence, data can be used to generate elastograms and calculate liver Young's modulus [248]. Similarly, ultrasound-based approaches such as transient elastography, in which a controlled vibration produces a mechanical shear wave with consistent amplitude and frequency, clinicians can track the speed and depth of shear wave propagation through the tissue, represented in graphic form and as Young's modulus (stiffness) [249]. Application of ultrasound for the assessment of the lung has been limited to qualitative and semi-quantitative assessments of projections from the lung surface and have not previously allowed a direct assessment of lung physiology or mechanics [250]. However, a recent study used ultrasound combined with speckle tracking software to analyze pleural displacement and showed that this method could be used to estimate lung strain in normal human volunteers and in a murine model of pulmonary fibrosis [250]. This study provides proof-of-concept that non-invasive imaging by ultrasound is a feasible strategy that may be developed as a tool for longitudinal assessments of lung stiffness.

Development and refinement of newer imaging technologies that afford greater spatial resolution than currently available must continue. In the lung, computed tomography (CT) scanning, either alone or in combination with radioactive tracers, positron emission tomography (PET) or single-photon emission computed tomography (SPECT), currently provides the greatest resolution and is the method of choice for 3D imaging. The most significant impediment to performing repeated CT imaging over time is the risk associated with radiation dose, although new CT systems to reduce the radiation dose are being developed. Additionally, improved image sequencing and compressed sensing image reconstruction algorithms are now being developed for MRI that have the potential for creating 3D images of lung tissues, comparable to CT, but without the risk of radiation dose

[251, 252]. These new approaches along with advancements in model-based image reconstruction that compensate for tissue motion cause by breathing or beating of the heart will ultimately facilitate 4D MRI to enhance our ability to evaluate heterogeneities in structural and functional changes in the lung. Many of these new image reconstruction and analysis approaches developed for MRI may also translate to CT or other complementary imaging modalities.

Combining higher-resolution imaging of tissue biomechanics with pulmonary function testing (for example, spirometry) could be transformative in patient care by allowing us to determine whether therapeutic interventions have the intended consequence of affecting matrix dynamics. However, these newer image reconstruction, segmentation, and analysis techniques will need to be automated to user-friendly interfaces to achieve broad acceptance in clinical settings. Similarly, developing probes or tracers for individual ECM components (including second harmonic generation microscopy) that can be imaged in real-time in living individuals will enhance our understanding of disease processes such as COPD, IPF, bronchiectasis, asthma, and even ARDS.

In parallel with improvements in clinical imaging technologies, there are also many developments in tissue engineering, imaging and analysis that will improve our understanding of the role of ECM in development, homeostasis and disease. Many of these new technologies have been developed using animal models and *in vitro* methods [253]. For example, ultra-high-resolution micro-CT approaches have been developed that can image the lungs of mice at 1–2 micron resolution. However, the high radiation doses preclude the ability to use such an approach in live animals [254]. Nevertheless, the ability to image the structures of the lungs from laboratory animals ranging from the trachea to the secondary lobules offers exciting opportunities for measuring changes in 3D structures of the lung, including the ECM at unprecedented resolution. Likewise, multiple laboratories are developing highly detailed 3D images of tissues including the lung using cryomicrotome or vibratome sections of frozen, embedded lungs. By including fluorescent markers for gene expression, proteins, inhalation or intravenously administered microspheres, etc., these techniques offer additional high-resolution 3D images of the lung that will be useful for evaluating the role of the ECM in lung function [255–258].

It is imperative that imaging of ECM degradation/turnover and synthesis be developed with enhanced resolution to better understand human disease processes. By making salient observations of the patient, we will be in a better position to enhance our model systems for studying the human lung. In addition to biochemical surrogates [259, 260], newer imaging tools that allow evaluation of collagen dynamics in patients over time would greatly contribute to our understanding of collagen metabolism during disease progression. Similarly, tools that would allow visualization of collagen ultrastructure within areas of fibrosis should help determine whether the three-dimensional structure of collagen embedded within a scar is stereotypically similar in different scars within the same diseased lung or different for each particular scar. Dynamic synthesis and breakdown of ECM (i.e. “remodeling”) result in the development of novel epitopes of ECM molecules that may be suitable for tracking fibrogenesis and resolution of fibrosis. Similarly, real-time, longitudinal

imaging of lung ECM during development of COPD may help us better understand the nature of alveolar enlargement and septal destruction.

Over the past five years, production of decellularized scaffolds from native lungs has proven feasible. Detergents are commonly used in decellularization to solubilize cell membranes, disengage cytoskeletal proteins from cells, and detach DNA remnants from proteins [261]. As reported by many groups, these scaffolds often retain many of the essential ECM proteins present in the original organ [262–265]. Although decellularized lung tissue can provide a model to study ECM changes during aging or lung disease [93, 266], it is important to recognize that many decellularization protocols render a depleted scaffold that may not be optimal for long-term cell culture or for cell adhesion, survival and proliferation [267]. Currently, next-generation proteomic approaches are under development that will allow quantification of lung matrix composition, and benchmarking of decellularized samples against native tissues.

Successful decellularization should include the removal of cell membrane epitopes, DAMPs, and DNA remnants from the scaffold as these components may induce inflammatory and/or immune reactions [268–271]. Host responses to acellular matrices may include pro-inflammatory or pro-constructive macrophage responses [272, 273]. The threshold level of nuclear material that induces pro-inflammatory responses or adaptive immunity has not yet been established, and hence acceptable levels of decellularization for various organs remain undefined [273, 274]. Despite the lack of clear benchmarks for what constitutes “decellularized”, it has been generally accepted that DNA fragments that are less than 300 bp in length will not elicit negative remodeling responses [274]. In terms of the impact of non-nuclear donor material on adverse immune responses, it remains unclear if proteinaceous cell debris, such as cytoskeletal elements, are problematic. Currently, there are multiple reports of decellularized tissues with detectable cytoskeletal debris, such as actin, [263, 275], although the functional consequences of these remnents have yet to be determined. In the very long term, acellular lung matrices may serve as a bioengineering platform for construction of functional lung tissue. To be functional, a regenerated lung should fulfill specific “design criteria”, including the ability to: (1) maintain lung-specific epithelial, mesenchymal, and vascular cells; (2) provide a barrier to separate blood from air; (3) incorporate a hierarchical branching geometry that provides suitable surface area for gas exchange; (4) contain a perfusable microvasculature that is resistant to thrombosis; and (5) be sufficiently mechanically robust to withstand ventilation and physiological mechanical stresses [276]. Although some progress has been made [262–264, 277–281], these functional criteria have not yet been met. The quality of the underlying matrix scaffold will determine whether these critical design criteria can be met.

11. Critical Questions and Emerging Opportunities in Lung ECM Biology

This review of the role of the ECM in lung development, homeostasis and repair has served to identify several key questions and knowledge gaps in the field. The authors have identified the following critical questions and emerging opportunities in lung ECM biology:

Critical Questions

1. What is the regenerative capacity of the lung in adult life? This question is confounded by the current lack of understanding of the influence of the ECM, and changes to the ECM, on the function of stem cell niches in the lung. There is a critical need to "decode" the mechanisms by which the ECM regulates niche function. Closing these gaps is necessary to answer the question of whether the lung can be stimulated to undergo alveolarization and to, potentially, reverse chronic lung diseases such as emphysema and fibrosis.

2. The extent to which fibrosis is reversible is unknown. In part, this reflects a gap in the tools available to visualize *in vivo* kinetics of collagen turnover or changes in distinct pools of collagen that are responsive to resorptive mechanisms. It is also not understood to what degree biochemical and biomechanical properties of the fibrotic lung alter cellular behavior toward a "point of no return" or, even which cells are the key effectors in collagen turnover. Additional knowledge gaps include the mechanisms mediating collagen uptake and degradation, how intracellular collagen degradation is regulated by ECM interactions, the effects of impaired intracellular collagen processing on cellular behavior, and which cells are the key players in collagen turnover in tissue homeostasis and repair.

3. How does the same injury (e.g. cigarette smoke) give rise to different disease phenotypes, for example, emphysema, fibrosis or combined? It is thought that chronic epithelial stress is relevant to the pathogenesis of both emphysema and fibrosis. It is unknown to what degree alterations in the ECM promote or attenuate epithelial stress and whether such alterations bias an injury response toward one disease pathway or another. Do disease-relevant cellular phenotypes that track toward an emphysematous or fibrotic process emerge mainly from cell autonomous or ECM-regulated effects? If altered ECM is a critical determinant, there remains a lack of understanding of specific alterations in the ECM that promote development of emphysema vs. fibrosis.

4. How do MMPs and ECM-derived proteolytic products contribute to lung repair, regeneration and inflammation? There is evidence that cellular responses to "danger signals" emitted by ECM fragments are an important determinant of inflammation and injury in chronic lung disease. However, to what extent ECM fragments also function as important regenerative signals is unknown. Are adjuvant effects of ECM fragments an important driver of autoimmune adaptive immune responses that then promote progression of chronic lung diseases such as COPD and pulmonary fibrosis? There is limited understanding of the full extent to which ECM fragments contribute to regeneration/recovery or act as perpetrators of disease progression.

5. Is it possible to develop a decellularized scaffold that can serve as a functional bioreactor for lung regeneration? Such a scaffold would need to: (1) reliably maintain lung-specific epithelial, mesenchymal, and vascular cells; (2) provide a barrier to separate blood from air; (3) maintain a hierarchical branching geometry that provides suitable surface area for gas exchange; (4) contain a

perfusable microvasculature that is resistant to thrombosis; and (5) be sufficiently mechanically robust to withstand ventilation and physiological mechanical stresses.

Emerging Opportunities

1. Develop *ex vivo* "macroscale" models to interrogate stem cell niches in the lung; for example, co-culture systems, organotypic models, or stem cell niche re-construction on scaffolds that recapitulate the "matrix footprint". Are biochemical and/or biophysical properties of the niche a determinant of stem cell function and, if so, can the ECM be manipulated to favor regeneration over fibrosis?

2. Establish animal models of lung regeneration as a pivotal avenue for defining the important characteristics of a lung stem cell niche. Further characterization of animal lung repair models such as hyperoxia, influenza infection, and pneumonectomy will be crucial for revealing the molecular and cellular basis of lung regeneration. By combining "cell-type specific" lineage tracing and "gain and loss function" genetic tools, these animal models would allow us to unravel and verify the functional contribution of key niche ECM molecules during regenerative alveolarization, as well as their potential clinical value in improving lung function.

3. Develop novel imaging technologies that allow evaluation of collagen turnover in patients over time. This methodology would greatly contribute to our understanding of the dynamic nature of collagen metabolism at different times during disease progression. Similarly, tools that would allow visualization of collagen ultrastructure within areas of fibrosis should help determine whether the three-dimensional structure of collagen embedded within a scar is stereotypically similar in different scars within the same diseased lung or different for each scar. Establish the goal of functional imaging as a tool to assess drug effects *in vivo* and methods to reach this goal.

4. Define an ECM "map" to include spatial and temporal changes in ECM composition, topography, and biomechanics during injury-repair (animal models), and in human diseases such as emphysema and fibrosis. The atlas should compile ECM profiles of normal (different developmental stages, adults) and diseased lung tissues and, in addition, include regional characterization (pleura, trachea, bronchi, alveolar interstitium, vessels, upper lobe vs. middle lobe vs. lower lobe). The atlas should integrate global -omics data such as: (a) quantitative *proteomics* to identify ECM isoforms and post-translational modifications (such as cross-linking, phosphorylations, etc.); (b) ECM *degradomics* to identify the active cryptic fragments of ECM proteins that play key roles in disease progression, as well as the neo-epitope within ECM proteins that could serve as biomarkers of disease progression or response to treatment; and (c) *glycomics* to identify the nature and abundance of polysaccharides and glycosaminoglycans (GAGs) in the lung ECM.

5. Identify the specific cell types involved in matrix remodeling and how they regulate matrix production and resorption. Specifically, more refined definitions of fibroblasts and macrophages and their precise roles in matrix dynamics are needed. It is currently not known which of these cell types are primarily responsible for collagen/matrix turnover, what the relative contributions of each cell type is, whether there exist fibroblast and/or macrophage subsets that specialize in matrix degradation and, if so, how these cells can be identified. Furthermore, if subsets of cells exist that specialize in matrix degradation, is their differentiation driven through cellular on acellular cues provided by the fibrotic microenvironment surrounding them?

Expansion of our knowledge of the structure, biomechanics and functional properties of the dynamic lung ECM will enrich our understanding of the development, physiology and pathobiology of the lung. This knowledge will advance novel strategies to treat lung diseases across the lifespan, and reduce the incidence of chronic lung disease. This goal can be realized with a collaborative research effort that encompasses matrix biologists, and extends to the larger community of investigators studying lung development, health and disease.

Acknowledgments

We acknowledge the support of the Division of Lung Diseases, National Heart, Lung & Blood Institute which initially organized a workshop on this topic on September 17–18, 2014 in Bethesda, Maryland. All authors of this manuscript participated in this workshop. The authors thank J. Michael Wells, MD, Derek Russell, MD and J. Edwin Blalock, PhD for their contributions to the construction of Figure 5.

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Highlights

- Recent advances in ECM composition, dynamics, topography, and biomechanics
- ECM in normal lung development and aberrant development (BPD)
- ECM dynamics and altered deposition in adult lung diseases, namely COPD and IPF
- ECM in inflammation/autoimmunity; and maintenance of the stem cell niche
- ECM-based therapeutics for chronic lung diseases
- Critical questions and emerging opportunities in lung ECM biology research

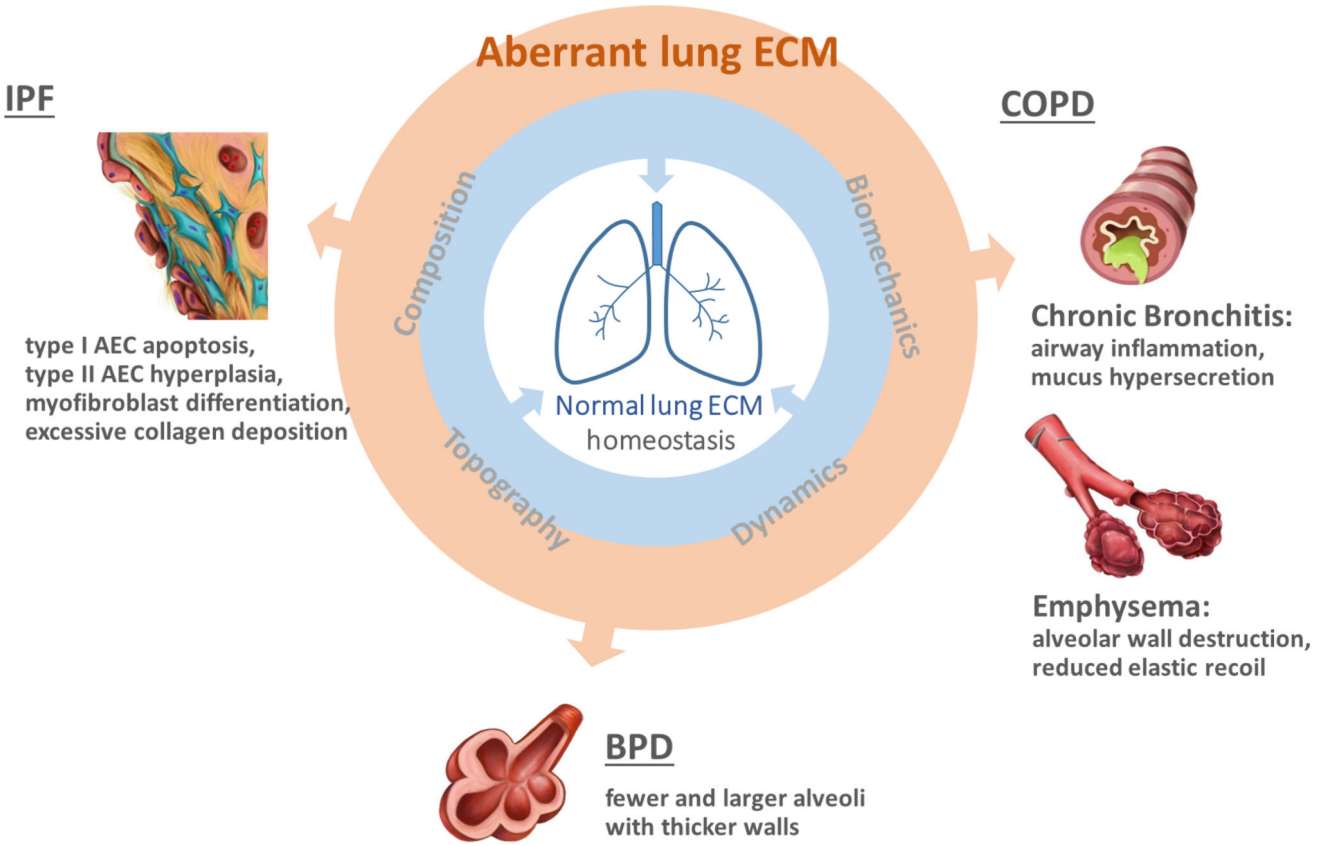

Figure 1. Role of the ECM in lung homeostasis and disease

Normal lung ECM is critical for embryonic lung development and the maintenance of lung homeostasis in adulthood. Aberrant alterations of the properties of lung ECM, including composition, biomechanics, dynamics and topography, are characteristic of a number of adult and child lung diseases, including IPF, COPD and BPD. IPF = idiopathic pulmonary fibrosis; COPD = chronic obstructive pulmonary disease; BPD = bronchopulmonary dysplasia.

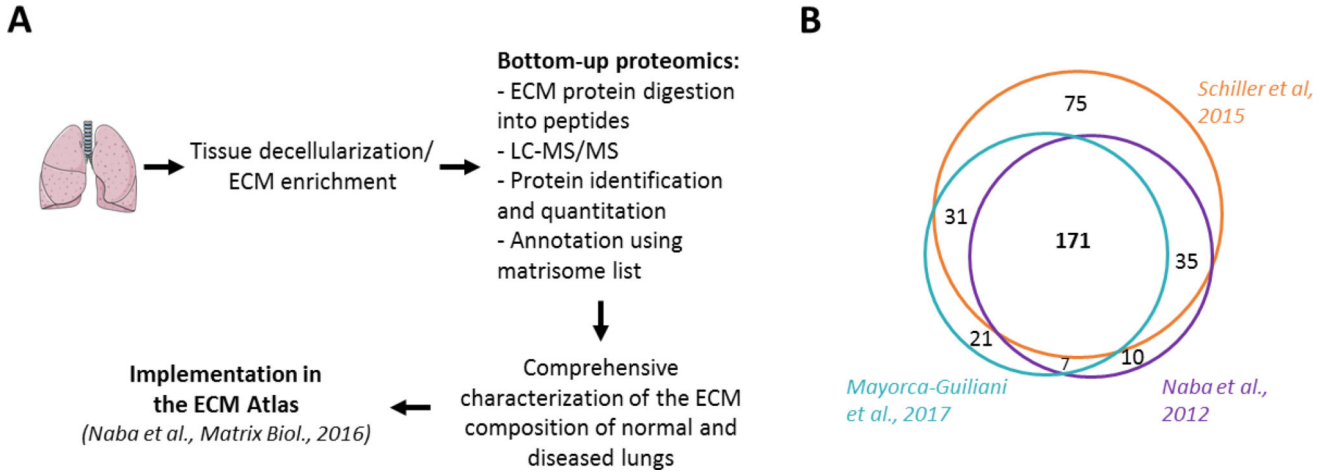

Figure 2.

A, An experimental pipeline to characterize the lung ECM using proteomics. B, Comparison of the lung ECM composition, defined by mass spectrometry-based proteomics from 3 independent studies (as denoted in the figure).

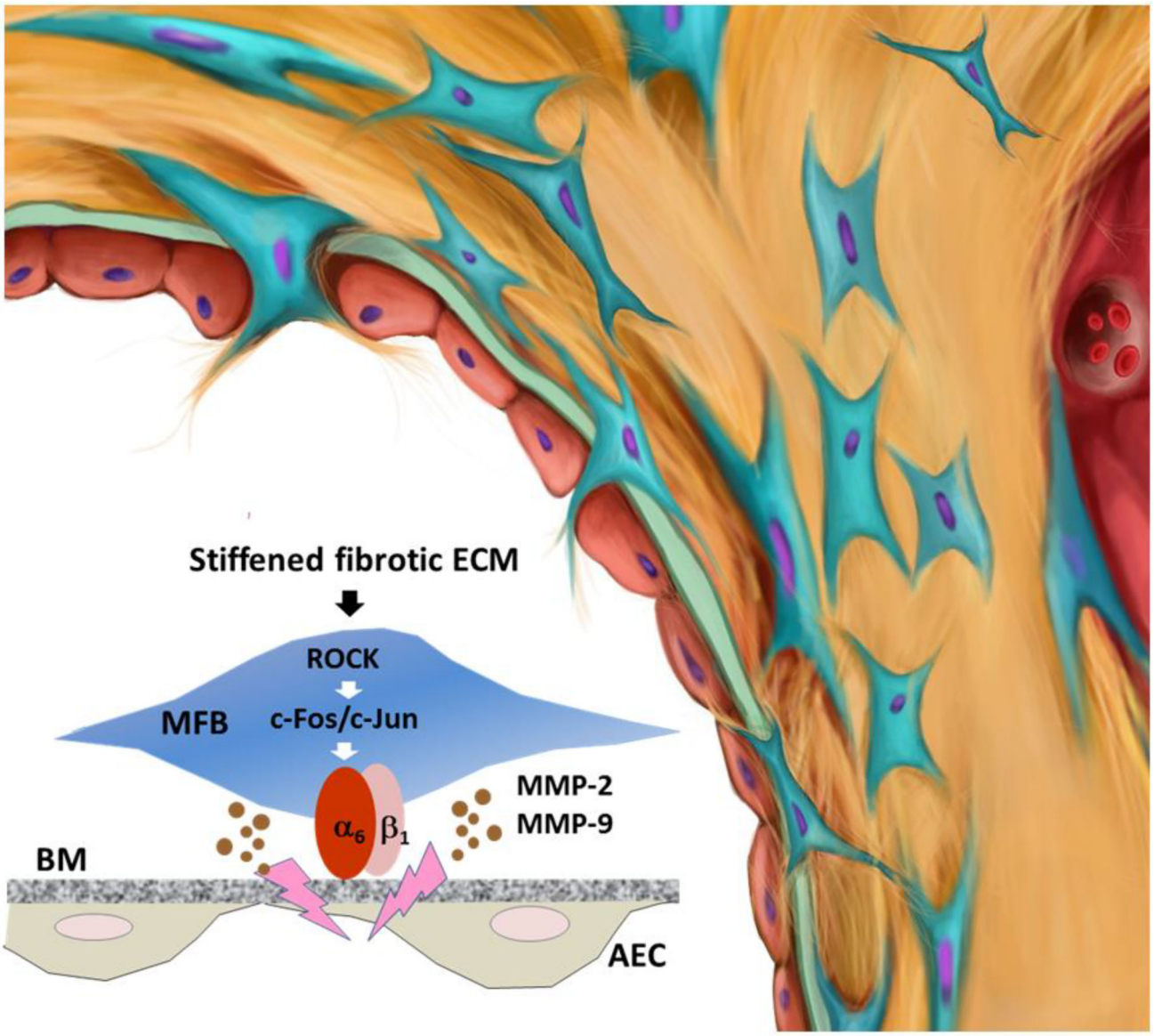

Figure 3. Matrix stiffness and topography guide IPF myofibroblast invasion into the ECM

Stiffened fibrotic matrix upregulates α_6 integrin expression by ROCK-dependent activation of c-Fos/c-Jun transcription factor complex. Interactions between $\alpha_6\beta1$ integrin and the BM bring lung myofibroblasts into the close proximity to the BM. This facilitates MMP-2/9-mediated pericellular proteolysis of BM component collagen IV, leading to lung myofibroblast invasion (see ref. #84 for details). Matrix stiffness sensing by α_6 integrin and the highly organized, anisotropic matrix fibers, which could act as “highways” that aid IPF myofibroblast invasion through the BM and interstitial ECM to form a continuous fibrotic reticular network. MFB = myofibroblast; ROCK = Rho kinase; MMP = matrix metalloproteinase; BM = basement membrane; AEC = alveolar epithelial cell.

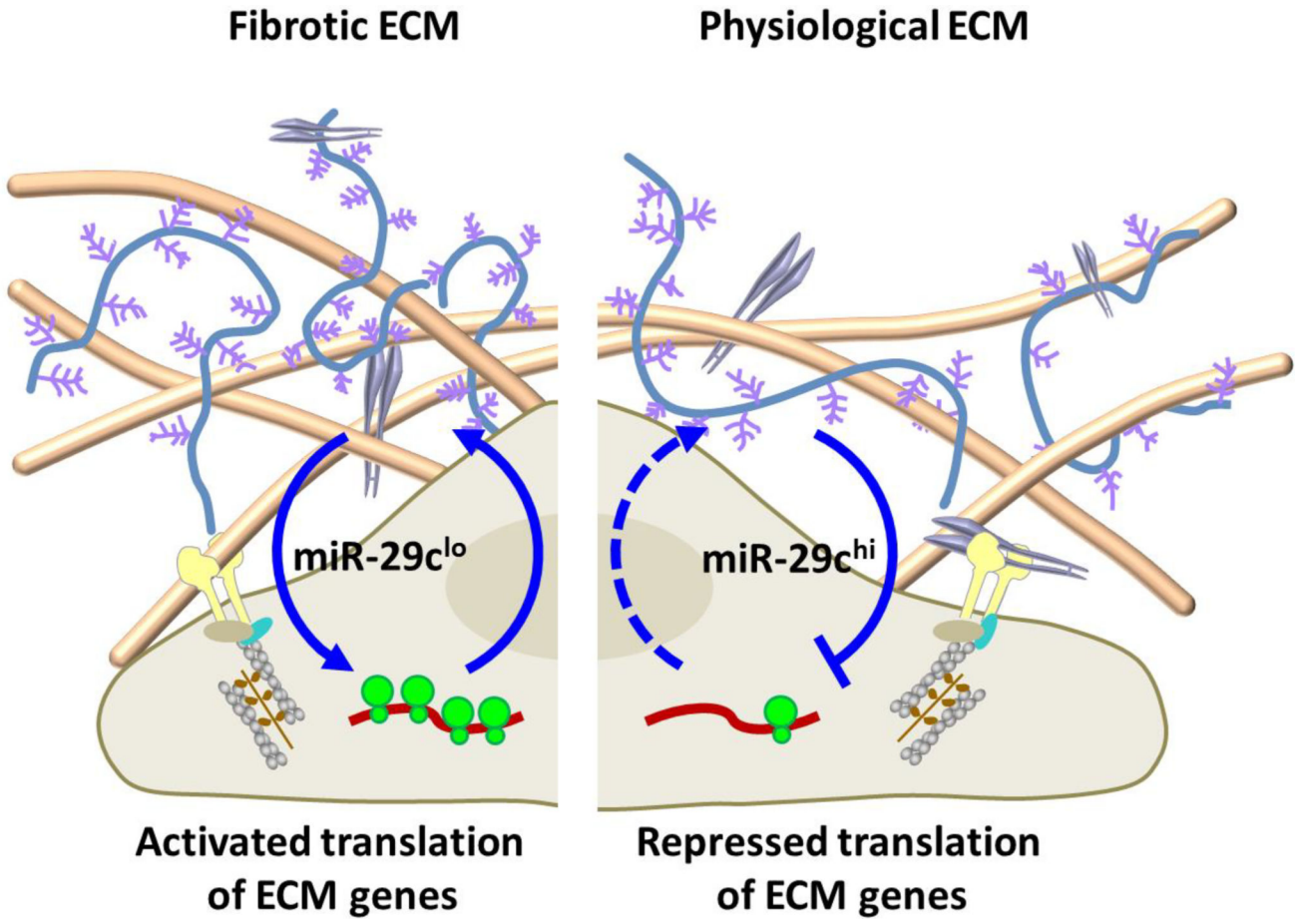

Figure 4. miR-29c-mediated positive feedback between the fibrotic ECM and the fibroblast amplifies the fibrotic phenotype

miR-29c targets ECM genes and limits ECM production in normal lungs. Downregulation of miR-29c activates the synthesis of ECM products by lung fibroblasts and persists in response to the fibrotic ECM (modified from ref. #196).

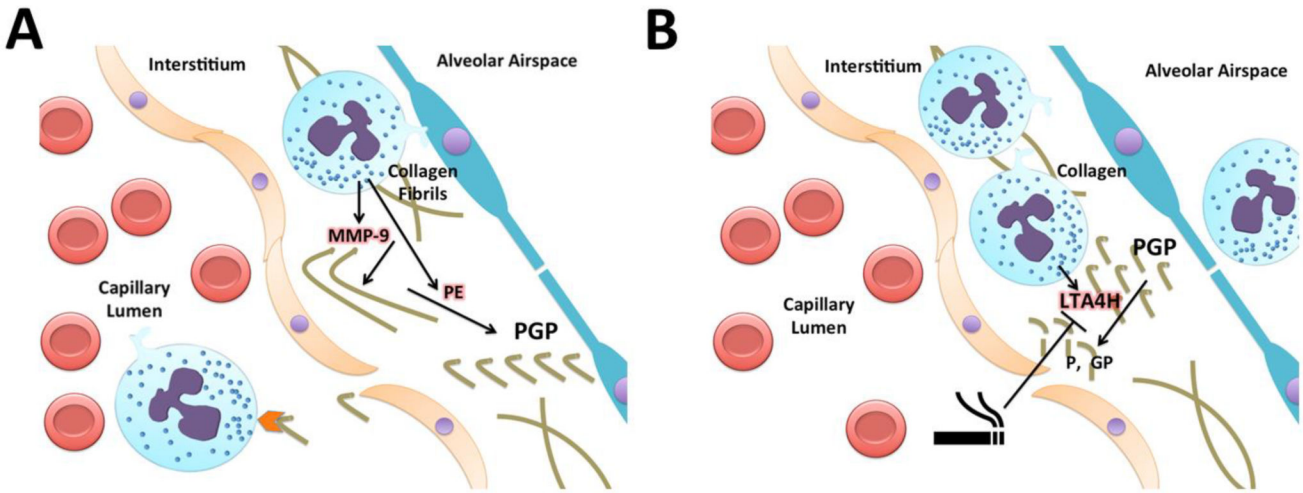

Figure 5. The central role of MMP-derived PGP in smoking-induced pulmonary inflammation

A, Neutrophil-derived MMP9 and prolyl endopeptidase (PE) degrade lung collagens to generate PGP. PGP serves as a chemoattractant to recruit neutrophils to lung interstitium. Cigarette smoke induces increases in MMP-9, PE and PGP production which promotes neutrophil influx. **B**, Leukotriene A4 hydrolase (LTA4H) is a pro-inflammatory enzyme that possesses aminopeptidase activity. LTA4H serves to degrade PGP and stop the PGP-mediated neutrophil chemotaxis in acute inflammation. Cigarette smoke selectively inactivates LTA4H's aminopeptidase function, leading to accumulation of PGP and neutrophils (see ref. #223 for details). This contributes to the chronic inflammation that drives disease progression in COPD.

Table 1

Lung ECM composition

Proteins were identified by mass spectrometry-based proteomics from three independent studies (see text for more details).

CORE MATRISOME			
ECM Glycoproteins		**Collagens**	**Proteoglycans**
5430419D17RIK	LGI3	COL10A1	ACAN
ABI3BP	LTBP1; LTBP2; LTBP3; LTBP4	COL11A1; 11A2	ASPN
ADIPOQ	MATN1; MATN2; MATN4	COL12A1	BGN
AEBP1	MFAP2; MFAP4; MFAP5	COL13A1	CHAD
AGRN	MFGE8	COL14A1	DCN
AW551984	MGP	COL15A1	FMOD
BMPER	MMRN1; MMRN2	COL16A1	HAPLN1
CILP	NDNF	COL17A1	HAPLN3
CILP2	NID1; NID2	COL18A1	HAPLN4
COLQ	NPNT	COL19A1	HSPG2
COMP	NTN1; NTN3; NTN4	COL1A1; 1A2	IMPG1
CRISPLD2	PAPLN	COL22A1	LUM
DPT	PCOLCE; PCOLCE2	COL23A1	OGN
ECM1; ECM2	POSTN	COL24A1	PODN
EFEMP1; EFEMP2	PXDN	COL25A1	PRELP
EGFEM1	RELN	COL27A1	PRG2
ELN	SBSPON	COL28A1	PRG3
EMID1	SLIT3	COL2A1	VCAN
EMILIN1; EMILIN2	SNED1	COL3A1	
FBLN1; FBLN2; FBLN5	SPARC; SPARCL1	COL4A1; 2; 3; 4; 5; 6	
FBN1; FBN2	SPON1	COL5A1; 5A2; 5A3	
FGA; FGB; FGG	SRPX; SPRX2	COL6A1; 2; 3; 4; 5; 6	
FGL2	SVEP1	COL7A1	
FN1	TGFBI	COL8A1; A2	
FRAS1	THBS1; THBS2; THBS3	COL9A1; 9A2; 9A3	
GLDN	THSD4		
HMCN1; HMCN2	TINAG; TINAGL1		
IGFALS	TNC; TNXB		
IGFBP6; IGFBP7	VTN		
IGSF10	VWA1; 3A; 5A; 5B1; A9		
KCP	VWF		
LAMA1; A2; A3; A4; A5; B1; B2; B3; C1; C2; C3	WISP2		

MATRISOME-ASSOCIATED		
ECM-affiliated proteins	ECM Regulators	Secreted Factors
ANXA1; 2; 3; 4; 5; 6; 7; 9; 11	1810010H24RIK	ANGPT1
C1QA; C1QB; C1QC; C1QL2	A2M	ANGPTL2
C1QTNF2; C1QTNF5; C1QTNF7	ADAM10; 17; 19; 9	BMP3; BMP6
CLEC11A; CLEC14A	ADAMTS14; 16; 17; 5; 9	CHRD; CHRDL1
COLEC12	ADAMTSL1; L3; L4; L5	CRLF1
CSPG4; CSPG5	AGT	CXCL15
FCNA	AMBP	EGFL7
FREM1; FREM2	CD109	FAM132A
GPC4	CPN2	FGF1; FGF2
HPX	CSTB	FLG2
ITLN1	CTSB; CTSD; CTSG	HCFC1
LGALS1; LGALS3; LGALS7; LGALS8; LGALS9	ELANE	HGFAC
LMAN1	F13A1; F13B	IL16
MBL1	F2	INHBC
MBL2	HRG	INHBE
PLXDC2	HTRA1	MEGF6
PLXNA1L PLXNB2; PLXNC1	HYAL2	NRG1
SDC3	ITIH1; 2; 3; 4; 5	PDGFB
SEMA3A; 3B; 3C; 3E; 3F; 3BG	KNG1; KNG2	PDGFD
SFTPA1; SFTPB; SFTPC; SFTPD	LOX; LOXL1; L2; L3; L4	PF4
	MMP19; 1B; 2; 20; 28; 9	RPTN
	MUG2	S100A10; A11; A13; A4; A6; A8 A9; B
	PLAT	SCUBE1; SCUBE2
	PLG	TGFB1; TGFB2
	PLOD1; 2; 3	TNFSF10; TNSF12; TNSF13
	PRSS1	VEGFA
	PZP	WNT2; WNT3A; WNT4; WNT5B
	SERPINA1A; 1B; 1D; 1E; 3C; 3G; 3K; 3M; 3N	
	SERPINB12; B1A; B1C; B3A; B3C; B3D; B5; B6A; B6B; B9; B9B; B9C	
	SERPINC1; E1; E2; F1; F2; G1; H1	
	TGM1; 2; 3	
	TIMP3	