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Review

Emerging Concepts in the Pathogenesis of Lung Fibrosis

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Fibrogenesis is an often-deadly process with increasing world-wide incidence and limited therapeutic options. Pulmonary fibrogenesis involves remodeling of the distal airspace and parenchyma of the lung, and is characterized by excessive extracellular matrix deposition and accumulation of apoptosis-resistant myofibroblasts. Recent studies have added significantly to our understanding of the complex mechanisms involved in lung fibrogenesis. Emerging concepts in this field include the critical role of the epithelium, particularly type II pneumocytes, in the initiation and perpetuation of fibrosis in response to either endogenous or exogenous stress; a growing awareness of alternative activation of macrophages in tissue remodeling; growing appreciation of the alternative origins and phenotypic plasticity of fibroblasts; the roles of epigenetic reprogramming and context-dependent signaling in profibrotic phenotype alterations; and recognition of the importance of cross talk and convergence of intracellular signaling pathways. *In vitro*, *in vivo*, and *in silico* approaches support a paradigm of "disordered re-development" of the lung. Designing effective antifibrotic interventions will require accurate understanding of the complex interactions among the genetic, environmental, epigenetic, biochemical, cellular, and contextual abnormalities that promote pulmonary fibrogenesis. *(Am J Pathol 2009, 175:3–16; DOI: 10.2353/ajpath.2009.081170)*

Lung fibrosis occurs in interstitial lung diseases (ILDs) and idiopathic interstitial pneumonias (IIPs), as part of several systemic connective tissue diseases and childhood interstitial lung disease syndrome, and in response to many types of lung injury, including radiation and some chemotherapeutic drugs. Idiopathic pulmonary fibrosis (IPF) is perhaps the most pernicious and enigmatic form of the greater problem of lung fibrogenesis. IPF claims more lives annually in the United States than many types of cancer[1]; however, effective therapy is lacking. Recent evidence indicates that both IPF prevalence and mortality are growing in the United States and elsewhere.[2] The increasing burden of IPF is not simply reflective of an aging population, as age-adjusted mortality for IPF is increasing as well.[2]

In much of the latter half of the 20th century, perhaps as the result of successful use of anti-inflammatory therapies such as corticosteroids for some of the IIPs, fibrosis was believed to be initiated and propagated by persistent lung inflammation.[3,4] However, corticosteroids and other anti-inflammatory therapies have been uniformly unhelpful for IPF, and may in fact be harmful.[5] This fact, combined with absence of classic inflammatory biomarkers in IPF, and evidence from animal models in which fibrosis proceeds in the absence of inflammation, led to reassessment of the inflammatory paradigm.

The view that fibrotic remodeling, both of the lung parenchyma (eg, in IPF) and airways (eg, in asthma) instead represents a form of disordered wound healing, in which epithelial-mesenchymal interactions are predominant, has become canonical in the literature of the past two decades.[6–8] Selman et al accordingly proposed reconsideration of IPF as a disorder of epithelial-fibroblast interaction.[8] Interactions between the epithelium and mesenchyme have been shown to be critical for developmental morphogenesis of the lung,[9] and are also prominent in lung fibrogenesis. Recent microarray data from IPF and experimental models of fibrosis demonstrated recapitulation of expression patterns and signaling pathways critical during lung development.[10,11] Thus

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it appears that many of the cellular and molecular events critical to "modeling" of the lung during development are recapitulated during the "re-modeling" that occurs during fibrogenesis. Lung fibrosis may thus not only be conceptualized as a form of aberrant repair,[6,12–14] but also as a "disordered re-development" of the lung.

Because nearly 45% of all deaths in the developed world are attributed to some type of chronic fibroproliferative disease,[15] it is important periodically to review new developments in the study of fibrogenesis. This review focuses on recent experimental findings with regard to the pulmonary epithelium and fibroblasts, which include novel observations regarding the role of the epithelium in the initiation and maintenance of pulmonary fibrogenesis, as well as support newer concepts regarding the origins and differentiation of fibroblasts in fibrotic processes. In addition, it will review the latest data on intracellular signaling pathways and cross talk within and between the fibroblasts and epithelial cells that mediate pulmonary fibrosis. Understanding the emerging concepts discussed in this review and their relative roles in lung fibrosis will facilitate development of novel therapeutic approaches to ameliorate the global burden of fibroproliferative disease.

Role of Epithelium in Genesis and Perpetuation of Fibrosis

Epithelium as a Mediator of ILD

The pulmonary alveolar epithelium is the final barrier interface to inhaled substances. Together with sentinel macrophages, the epithelium controls pulmonary homeostasis by responding to environmental challenges through continuous reciprocal interactions with mesenchymal and vascular cells. Chronic epithelial cell stress, due to either intrinsic cellular defects or extrinsic insults such as infection, can promote epithelial cell death, impair normal re-epithelialization and alter epithelial–mesenchymal interactions, leading to fibroproliferation.[16]

Defined etiologies for pulmonary fibrosis secondary to intrinsic cellular defects include genetic deficiency of the pulmonary surfactant protein C (SP-C) and ATP binding cassette protein (ABC)A3. Persistent infection by viruses (specifically Herpesviridae) targeting the respiratory epithelium have also been associated with a fibrotic response. These distinct origins of human fibrosis have related mouse models that share many features of human disease, thus better delineating epithelial-based mechanisms of lung fibrosis.

Abnormalities of Surfactant Protein C in Idiopathic and Familial Pulmonary Fibrosis

Pulmonary SP-C is a highly hydrophobic protein that enhances surface activity and contributes to innate immune defense of the lung. SP-C is synthesized and secreted only by alveolar type II cells, which are the site of initial injury in SP-C dysfunction disease. A variety of mutations in the *SFTPC* gene have since been identified that strongly associate this gene with disease. *SFTPC* mutations include point mutations that alter single amino acid residues, frame shift mutations that change or terminate downstream translation, and splice site mutations that delete entire exons to produce a truncated proSP-C protein.[17,18] The natural history and severity of SP-C-associated lung disease is highly variable and may reflect the cellular response to the distinct mutations in the *SFTPC* gene coupled with the action of undefined modifier genes.

Alterations of a proprotein structure due to mutations can potentially inhibit proper folding to achieve correct conformation and function (loss of function). Likewise, impaired processing can lead to accumulation of nonfunctional protein that the cell must now eliminate (toxic gain of function). Maturation of newly translated proteins occurs as a series of folding events as proteins transit the endoplasmic reticulum and Golgi for release into cellular compartments for secretion. Cells have adapted a series of endoplasmic reticulum-based responses to restore proper folding or guide elimination of terminally misfolded proteins to relieve further stress. The cascade of responses is termed the unfolded protein response (UPR). If the UPR does not ultimately relieve cellular stress, then caspase-dependent cellular apoptosis pathways can be activated to eliminate cells entirely. The presence of aberrant forms of the SP-C precursor protein is a particular threat to homeostasis due to the high levels of SP-C normally synthesized, processed, and stored for secretion by the type II epithelial cells.

UPR marker immunostaining is increased in the lungs of individuals with both SP-C mutations and IPF.[19] These observations have been extended to non-SP-C-related sporadic IPF. Increased expression of UPR mediators and caspase 3 were demonstrated by immunoblotting in IPF but not in chronic obstructive pulmonary disease. Immunostaining co-localized markers of UPR and apoptosis to type II cells in regions of dense fibrosis.[20] Collectively, these findings are consistent with a generalized endoplasmic reticulum stress response of epithelial cells in fibrotic tissue.

In vitro expression of SP-C constructs encoding an *SFTPC* mutation found in patients resulted in SP-C aggregation, impaired epithelial cell growth, increased expression of an endoplasmic reticulum stress response and epithelial cell apoptosis.[21,22] Co-chaperones precipitated with the truncated SP-C, indicating association of UPR pathway proteins with the truncated and potentially misfolded SP-C. Expression of the *SFTPC* exon-four deletion mutation in the lungs of transgenic mice was lethal at birth, and analysis of the lungs demonstrated severe hypoplasia, reduced branching morphogenesis, and epithelial cell death.[23] These and additional studies are consistent with the overproduction of non-native proSP-C eliciting a misfolded protein stress response and epithelial cytotoxicity that contributes to the progressive lung disease in affected individuals.

Usual interstitial pneumonia, the hallmark histopathological lesion in IPF, has also been described in two reports of SP-C deficiency without identified mutations in the *SFTPC* gene, in which no mature SP-C and greatly

diminished proSP-C were detected.[24,25] Fibrotic disease in these affected individuals implies that the SP-C null condition may also produce altered type II cell or alveolar function that results in disease. When SP-C deficient mice were generated by gene targeting techniques, the mice developed a strain-specific ILD that progressed with age to irregular fibrosis.[26] The severity of bleomycin-induced lung fibrosis was increased in the lungs of SP-C deficient mice, suggesting that the absence of SP-C predisposes the lung to fibrosis.[27] The SP-C deficient mice do not produce any proSP-C that could stimulate an UPR. Taken together, the origins of SP-C-related lung disease may be multifactorial, resulting from cumulative epithelial cell injury initiated by the lack of SP-C in the airspace, abnormality of cellular SP-C processing events, or the presence of aberrant forms of proSP-C and an UPR.

ATP Binding Cassette Family Member ABCA3 and Interstitial Lung Disease

The ABC transporter family is a diverse group of large transmembrane proteins that use ATP to translocate substrates. The ABCA subfamily facilitates the movement of cholesterol or specific phospholipids. ABCA3 is highly expressed in the lung relative to other organs. The localization of ABCA3 to the limiting membrane of lamellar bodies in type II cells implicated ABCA3 in the assembly of the intracellular storage form of pulmonary surfactant.[28,29]

ABCA3 mutations are recessive and associated with a highly variable disease phenotype. Infants homozygous for mutations in the *ABCA3* gene generally develop severe and usually fatal neonatal respiratory distress; however, a subset has been described with *ABCA3* mutations presenting as ILD in later childhood, some of whom had transient neonatal symptoms.[30–32] Histopathology indicates alveolar proteinosis and interstitial thickening, with ultrastructural evidence of small lamellar bodies with dense eccentrically positioned membranes, consistent with a surfactant lipid transport function for ABCA3.[30]

Data are limited regarding disease progression in older individuals and the potential to develop clinical fibrosis. However, an adolescent heterozygous for three novel variants of *ABCA3* was the first well-documented case of a child with usual interstitial pneumonia, which was not previously thought to occur in children.[32] A recent study identifies a modifier effect of *ABCA3* mutation in individuals with a single specific *SFTPC* point mutation. The presence of the *ABCA3* mutation with the *SFTPC* mutation increased the severity of clinical disease in comparison to individuals with only the *SFTPC* point mutation.[33] This study highlights the possibility that idiopathic lung disease may result either from single mutations altering epithelial cell integrity or multiple gene mutations that disrupt different components of a common process.

Distinct mutations have been identified that alter ABCA3 structure, including nonsense mutations that prevent any ABCA3 production.[34] *Abca3*$^{-/-}$ mice die of neonatal respiratory failure. Analysis of lungs of *Abca3*$^{-/-}$ mice demonstrates no detectable surfactant with impaired lamellar body formation, similar to lamellar body defects of patients.[35] *ABCA3* missense mutations have also been identified that likely generate a UPR.[34] Thus the mechanisms of ABCA3-related ILD may also be multiple and complex, involving decreased surfactant production and cellular stress from UPR responses, similar to SFTPC-related ILD.

Relationship of Pulmonary Fibrosis to Herpesvirus Infection

Viral infection of the pulmonary epithelium is traditionally viewed as a transient injury. However, the capacity of some viruses to establish latent infection has been hypothesized to mediate a state of chronic or repetitive damage to the infected epithelium that eventually results in fibrosis. Herpesvirus family members can establish latency after acute infection, and various herpesviruses have been detected in tissue of patients with IPF. In one study, Epstein-Barr virus was found in lung tissue of almost half of patients with IPF, and a more recent study identified either Epstein-Barr virus, cytomegalovirus, human herpesvirus 7, human herpesvirus 8 (also known as Kaposi's sarcoma herpesvirus) or combinations of these herpesviruses in the lungs of IPF patients.[36,37,38] Herpesviruses were detected in the lungs of non-IPF patients at a lower prevalence. Herpesvirus infection (Epstein-Barr virus, cytomegalovirus, or human herpesvirus 8/Kaposi's sarcoma herpesvirus) was detected by immunohistochemistry in the lungs of 15/23 individuals with IPF, with similar prevalence in sporadic, non-*SFTPC*-associated familial, and *SFTPC*-associated cases, but none was detected in controls. Notably, herpesvirus colocalized with cells expressing increased UPR markers. These results support the hypothesis that herpesvirus infections may be a modifier that exacerbates the severity of SP-C dysfunction disease, as well as an independent cause of disease.

The concept of persistent herpesvirus infection-induced IPF is supported by reports of gamma herpesvirus in equine pulmonary fibrosis and murine gamma herpesvirus (MHV-68)-induced lung fibrosis in interferon γ-deficient mice.[39,40] Gamma herpesvirus infection also accentuates the severity of chemically-induced lung fibrosis in mice.[41] Fibrosis following chronic viral exposure may also result in part from altered function of alveolar macrophages.

In murine MHV-68 infection models and human IPF lung tissue, macrophages found in fibrotic regions expressed markers of alternatively activated macrophages (AAMs).[42,43] AAMs have reduced phagocytic and bactericidal activity and express genes consistent with a repair and remodeling phenotype. Markers associated with the AAM phenotype are better defined in mice, but both mouse and human AAMs express increased arginase 1 activity compared with classically activated macrophages.[42,43] Arginine turnover by arginase 1 activity limits nitric oxide production and enhances production of collagen precursors used in wound healing processes. Arginase 1 activity is similarly induced in interstitial fibroblasts from the lungs of bleomycin-treated mice.[42,44] Both tissue arginase 1 and AAM arginase 1 activity are

increased by chemically induced and herpesvirus-associated fibrosis and may contribute to the aberrant accumulation of matrix.

The pathology of infants with SFPTC and ABCA3-related disease is frequently classified as desquamative interstitial pneumonia (DIP), indicative of a macrophage-dominated histopathology.[34,45–46] *Sftpc*$^{-/-}$ mice have been reported to have an abnormal macrophage phenotype and impaired microbial killing and express markers consistent with an AAM phenotype.[47] The relationship between macrophage activation patterns and epithelial dysfunction, and their role in the generation of disease is still unclear.

Section Summary

Disease arising from mutations and deficiencies of SP-C or ABCA3 is uniquely alveolar type II cell in origin, emphasizing the role of the epithelium in ILD/IPF. Type II cell injury from either SP-C or ABCA3 dysfunction may be sufficient to drive pathogenesis; alternatively, either of these deficiencies may render the alveolar epithelium more vulnerable to additional insults such as infection. Chronic herpesvirus infection linked to familial and SFTPC-related IPF supports the concept of an infectious process promoting ILD/IPF progression in the setting of alveolar epithelial compromise. Activation of macrophages in a manner identified with tissue repair rather than microbial protection suggests that epithelial cell stress may signal to the innate macrophage population in an effort to restore alveolar integrity. Thus signaling by impaired epithelial cells may be multidirectional, altering function of both fibroblasts and macrophages (Figure 1).

Origins and Phenotypic Regulation of Fibroblasts

Central Role of the Fibroblast in Fibrosis

Although evidence is mounting that the epithelium in many cases initiates and perpetuates fibrogenic signaling, the fibroblast is by definition the principal effector cell in fibrosis; its very name implies fibrogenesis. Persistence of fibroblasts in the altered extracellular matrix within damaged airspaces, and their excessive matrix deposition, exemplified in IPF by the histopathological lesions termed "fibroblastic foci," are the features that correlate most clearly with poor outcomes.[48,49]

Fibroblasts are somewhat enigmatic cells in that they lack a universal marker. They are present in most tissues, particularly those with prominent epithelial and microvascular components (eg, skin, lung, liver, kidney). Often they are defined by their location in interstitial/mesenchymal compartments, and their elongated morphology. In tissue culture, they are often defined morphologically and by the absence of other specific markers.

Fibroblasts have significant roles in three principal areas in normal biology: development, tissue homeostasis, and wound healing. In organs and tissues which have a prominent epithelial derivation, such as the lung, kidney, liver and skin, fibroblasts are critical to normal development and function. Injury and repair in such tissues follows a fairly defined pathway. Epithelial and/or endothelial damage results in exudation of platelet-rich plasma into procoagulant-rich tissue spaces, where it forms a fibrinous clot (provisional matrix).[50] Fibroblasts migrate into the provisional matrix, proliferate, and produce additional extracellular matrix components, such as fibronectin and collagen, resulting in fibroblast-populated granulation tissue. Simultaneously, fibroblasts acquire a myofibroblastic phenotype. The role of myofibroblastic differentiation, the acquisition by fibroblasts of smooth muscle-like phenotypic features such as expression of α-smooth muscle actin, in wound healing and fibrosis has been extensively reviewed recently.[51,52] Normal repair depends on re-epithelialization, remodeling of provisional matrix, and eventual removal of fibroblasts, probably through apoptosis.[53] Recent findings regarding the origins of fibroblasts in fibrotic tissues, and regulation of their differentiation will be considered in greater detail here.

Circulating Fibroblast Precursors

Multiple studies in the past decade have considered alternative origins of lesional fibroblasts in fibrosis in the

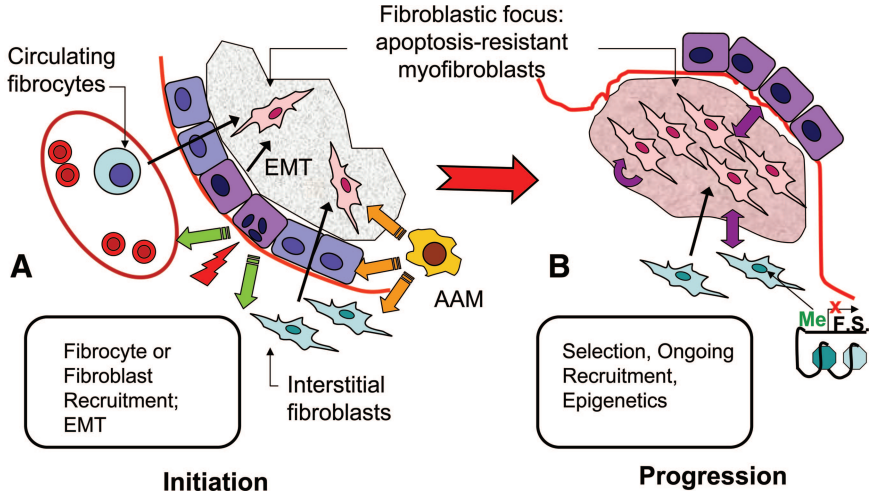

Figure 1. Schematic representation of cellular and contextual abnormalities in pulmonary fibrogenesis (IPF fibroblastic focus). **A:** Fibrogenesis is initiated by injury to, infection of, or intrinsic abnormalities (eg, surfactant dysfunction mutations) within alveolar epihtelial cells. Capillary disruption results in deposition of fibrin-rich provisional matrix, into which fibroblasts (either resident interstitial fibroblasts, fibrocytes, or EMT-derived) migrate. Signals for perpetuation of fibrogenesis (eg, fibroblast proliferation, myofibroblastic differentiation, extracellular matrix remodeling) may arise from the altered epithelium, alternatively activated macrophages (AAM), composition and/or biomechanical properties of ECM, or the fibroblasts themselves, recapitulating developmental pathways and resulting in progression of fibrogenesis (**B**). Epigenetic alterations, such as DNA methylation or histone modifications, lead to silencing of fibrosis supressor (FS) genes, further promoting an apoptosis-resistant myofibroblast phenotype.

lung and other tissues besides differentiation, proliferation, or migration of resident tissue fibroblasts. Bucala popularized the term fibrocyte to refer to circulating fibroblast precursors.[54] The idea of circulating fibroblast precursors participating in wound healing dates to the 19th century,[55] but the characteristics of this population of cells, their differentiation, and mechanisms of their recruitment have only recently been defined in numerous elegant studies.[56–59]

The contribution of fibrocytes to hypertrophic scars and keloids,[60] scleroderma, renal fibrosis,[61] airway remodeling in asthma,[62,63] and experimental models of lung fibrosis[59] have been well-described. A recent study demonstrated fibrocytes, defined predominantly by co-expression of CXCR4, a fibrocyte-associated chemokine receptor, with myofibroblast markers such as procollagen I, α-smooth muscle actin, and prolyl-4-hydroxylase, in lung tissue of 8/9 patients with lung fibrosis, but none in normal lungs.[64] There was a positive correlation between the abundance of fibroblastic foci and the number of lung fibrocytes ($r = 0.79$; $P < 0.02$). However, the exact role of bone marrow-derived fibroblasts in IPF has not been definitively established.

Although cultured fibrocytes can be induced to differentiate into myofibroblasts *in vitro*, it is not clear *in vivo* that the bone marrow-derived cells recruited to the lung contribute to pathological fibrosis.[59,65,66] In the setting of fibrogenic injury, bone-marrow derived mesenchymal stem cells may promote repair and thus ameliorate fibrosis.[67,68] Previous studies had indicated that bone marrow-derived cells can become type II pneumocytes after hematopoietic stem cell transplantation.[69] A large number of studies have followed, using different stem cell pools and different animal models, with varying results (reviewed in[70]). This phenomenon has been suggested to occur in humans undergoing sex-mismatched lung transplants as well, by demonstration of epithelial cells of recipient origin in donor lungs.[71] Different populations of fibroblast precursors may be recruited to the lung after injury (eg, fibrogenic fibrocytes versus repair-promoting mesenchymal stem cells). Factors present in the local environment are likely to influence the differentiation of fibroblastic cells from multiple sources, as discussed further below. Additional studies are needed to clarify some of these controversies.

Epithelial-Mesenchymal Transition

During development, cells show remarkable phenotypic plasticity. In gastrulation, endodermal epithelial cells differentiate into mesenchyme, which subsequently contributes to the formation of germ layers.[72] After this primary epithelial–mesenchymal transition (EMT), there are additional instances of EMT and also of mesenchymal–epithelial transition during development.[72,73] The molecular changes that occur in EMT—loss of cell–cell adhesion, increased motility, cytoskeletal and morphological changes, and resistance to apoptosis—are also changes that occur during wound healing. Because there is similarity among phenotypic characteristics of epithelial and mesenchymal cells in development, wound healing, and fibrosis, it is not surprising that the molecular pathways which regulate EMT overlap significantly with those associated with fibrogenesis.

Numerous studies have characterized EMT *in vitro*. A549 cells, primary human and rat alveolar epithelial cells, and human bronchial epithelial cell lines can all be induced to undergo EMT *in vitro*.[74–75] There is *in vivo* evidence of EMT in fibrogenesis as well. In both bleomycin-induced fibrosis and in the transforming growth factor (TGF)-β-overexpressing model, up-regulation of α-smooth muscle actin and vimentin in E-cadherin and surfactant-protein C-expressing cells, respectively, has been observed, suggesting EMT.[75,76] In obliterative bronchiolitis following lung transplantation, localization of S100A4/fibroblast-specific protein, a mesenchymal marker, has been observed in bronchial epithelium; explanted bronchial epithelial cells have increased expression of matrix metalloproteinases 2 and 9 and are collagen-invasive, all consistent with EMT.[77] Two studies have demonstrated colocalization of epithelial markers, such as thyroid transcription factor-1 or pro-surfactant protein B or C, with α-smooth muscle actin or N-cadherin in cells overlying fibroblastic foci in IPF.[78,76] However, others have failed to detect dual expression of epithelial and mesenchymal markers *in vivo* in either clinical samples or the bleomycin model.[79]

An intriguing recent study of 30 cases of IPF and multiple disease controls demonstrates a unique and novel finding of cells with a bronchiolar basal cell phenotype in a layer between myofibroblasts and overlying alveolar or bronchial epithelium, perhaps introducing a novel cellular player into the field.[80] These cells co-express markers associated with increased cellular motility (laminin-5 γ-2 and facsin), as well as markers of activation of the Wnt-β-catenin pathway, suggesting they may be undergoing EMT. However, the relative contribution of EMT to the pathogenesis of IPF is by no means clear. As fibrogenesis is considered to be a displaced or dysregulated repair process and many of the cellular and molecular events necessary for lung modeling during development are recapitulated during remodeling, the weight of evidence suggests that EMT is an important contributor to pulmonary fibrogenesis. Data from microarray analyses bear this out further, with enrichment of genes representative of multiple developmental pathways in IPF, including Wnt-β catenin, the TGFβ-bone morphogenetic protein family, and multiple developmental transcription factors.[10]

Fibroblast Heterogeneity

In addition to (or perhaps as a result of) the different possible origins of fibroblasts, it is clear that they exist in a remarkable range of phenotypes. Fibroblast heterogeneity has been the subject of extensive review in the past.[81] Differences among subsets of normal lung fibroblasts have been characterized on the basis of size and shape, surface markers, cytoskeletal composition, lipid content, cytokine profile, and expression of cyclooxygenase 2 and telomerase.[81–82] Fibroblasts from lungs with active fibrosis have increased proliferation, display an-

chorage-independent growth, and are morphologically distinct.[83–84] Fibroblasts from fibrotic tissue also demonstrate enhanced migration and invasion of matrices, processes critical to their entering damaged airspaces.[85,86]

One of the most extensively studied *in vitro* models of fibroblast heterogeneity is based on the surface expression of the lipid raft glycoprotein Thy-1. Thy-1(−) and Thy-1(+) mouse lung fibroblasts are morphologically distinct, with Thy-1(−) being more rhomboid and compact, and Thy-1(+) having the characteristic spindle shape of normal fibroblasts with more abundant intracellular lipid and rough endoplasmic reticulum. The two subpopulations differ in cytokine and growth factor production, cytokine receptor expression, and expression of major histocompatibility complex (MCH) class II.[87–92] Recent studies demonstrate that Thy-1 is an important modulator of latent TGF-β activation, myofibroblast differentiation, and survival.[93,94] Thy-1 thus appears to function as a "fibrosis suppressor," analogous to tumor suppressors. Another candidate fibrosis suppressor, which like Thy-1 affects lipid raft-associated signaling, is caveolin-1, which modulates multiple aspects of fibrogenic signaling. Its role in pulmonary fibrosis and scleroderma has been recently reviewed.[95]

Emerging Mechanisms of Regulation of Fibroblast Phenotype

Recently, clinical and laboratory data have generated renewed interest in the role of telomerase in fibroblasts.[96] Telomeres are guanine-rich repeat sequences on the ends of chromosomes and are regulated by telomerase, a ribonucleoprotein complex with an RNA component (Terc, hTR) that serves as a template for addition of repeat sequences, and a reverse transcriptase catalytic subunit (Tert).[97] The absence of telomerase leads to progressive telomere shortening with each round of cellular replication, resulting in a eventual loss of cellular viability characteristic of replicative senescence.

Telomerase is up-regulated in many malignancies. Terc null mice are initially phenotypically normal, but subsequent generations develop loss of fertility, a number of premature aging phenotypes, and decreased longevity.[97] Induction of telomerase activity has been noted in rat lung fibroblasts in bleomycin-induced fibrosis.[98] Dyskeratosis congenita is a rare disorder associated with mutations in telomerase genes, with skin manifestations, bone marrow failure, and some cases, usual interstitial pneumonia, often at an early age.[99,100] Two recent studies demonstrated telomerase mutations (hTERT, hTR) in families with IPF and in some sporadic cases, associated with abnormal telomere length.[101,102] Furthermore, telomerase (TERT)-deficient mice are protected from bleomycin-induced fibrosis; transplantation of wild-type bone marrow into TERT-null mice restores sensitivity to bleomycin, and transplantation of TERT-null bone marrow into wild-type mice is protective.[103] This set of studies indicates that telomerase expression is required for the profibrotic fibroblast phenotype, and that bone marrow-derived cells have a critical role in bleomycin-induced fibrosis. Interestingly, patients with IIPs, including IPF, have shorter telomeres than age-matched controls even in the absence of telomerase mutations.[104]

Epigenetic alterations result in heritable changes in gene function without changes in the DNA sequence, thus offering an extra layer of transcriptional control regarding how, when, and where genes are expressed.[105] Epigenetic regulation is important for the diversity of cell types arising during development, and is critical to maintaining the stability and integrity of expressed gene profiles. DNA methylation and chromatin modifications have been extensively studied in cancer research. Other epigenetic mechanisms, such as microRNAs and chromatin structural alterations, are increasingly recognized as critical to defining and maintaining cell phenotype.

There is growing evidence for epigenetic alterations in fibrotic diseases. Methylation of *FLI1* is associated with increased collagen expression in scleroderma fibroblasts[106]; histone deacetylase 4 is required for TGF-β-induced myofibroblastic differentiation of skin fibroblasts.[107] However, the extent to which epigenetic reprogramming is responsible for the multiple cellular phenotypic alterations in fibroblasts (or for that matter in other cell types) associated with pulmonary fibrosis has yet to be determined. A recent study demonstrated epigenetic silencing of Thy-1 by DNA hypermethylation specifically within fibroblastic foci in IPF, suggesting that this may be an important mechanism for pathogenic fibroblast alterations.[108]

MicroRNAs are single-stranded RNA molecules of 21 to 23 nucleotides in length that can be complementary to multiple mRNAs and induce silencing of multiple transcripts. They have been found to regulate reprogramming of gene expression in several types of cancer and in fibrosis in other organs, such as the heart.[109] Specific microRNA genes are selectively expressed in the lung, implicating them as candidate regulatory factors in development or disease. Lung epithelial specific overexpression of the miR-17-92 microRNA cluster in transgenic mice stimulated epithelial cell proliferation, while impairing distal lung alveolarization.[110] When the miR-223 gene was deleted *in vivo*, the mice developed a progressive pulmonary inflammation consisting of neutrophils that have an increased oxidative response to challenge.[111] These findings indicate that microRNAs are an important component of pulmonary gene regulation; however, the role of microRNA in pulmonary fibrogenesis has yet to be determined.

Mechanisms controlling cellular phenotype such as epigenetic programming, in addition to being regulated temporally (as in development or aging) or environmentally (such as by nutrition or exposure to toxicants) may also be regulated contextually (such as by local biochemical or mechanical signals). A recent study supporting contextual programming applied microarrays and hierarchical clustering to characterize fibroblasts derived from 43 different anatomical sites, demonstrating marked heterogeneity of gene expression.[112] Although there were general groupings indicating compartmentalization among anteroposterior, proximal–distal, and dermal–nondermal locations, there were also strong and specific location-restricted profiles such that, for example, fore-

arm dermal fibroblasts had expression profiles more similar to adult lung fibroblasts than to lower leg dermal fibroblasts. Within organs there are also likely to be significant positional differences based on structural localization.[113] Epigenetic mechanisms are believed to drive this context-dependent programming.[112] Local biochemical signals are critical in contextual programming; for example, regional differences in oxygen tension are important in airway branching.[114] Significant influence may also come from the composition or biophysical properties of the extracellular matrix, both of which in turn are altered by the cells themselves.

A large and growing literature, largely from the cancer field, demonstrates the effects of matrix composition and stiffness on cell behavior (reviewed in[115,116]). A dramatic example of the role of cell–matrix biophysical interactions in regulating phenotype demonstrated that constraining individual mesenchymal stem cells on either a large (10,000 μm^2) or small (1024 μm^2) surface area of fibronectin promoted differentiation into osteoblast or adipocyte lineage, respectively, even in the absence of classic differentiation-promoting factors.[117] Prostaglandin E2 was recently shown to inhibit TGF-β1-induced myofibroblast differentiation through cell shape and adhesion-dependent signaling.[118] In addition, fibroblasts from IPF patients elude the normal antiproliferative effects of polymerized collagen through alterations in integrin-dependent signaling.[119] Normal myofibroblasts undergo apoptosis within contracting collagen gels; fibroblasts lacking Thy-1 escape this mechanism, which is restored on transfection of Thy-1.[93]

Myofibroblast differentiation has been demonstrated to be maximized in the presence of both active TGFβ and mechanical tension.[51] A related study showed that myofibroblast contraction mediates latent TGF-β1 activation through a direct biophysical alteration of the matrix where it is sequestered.[120] Focal adhesion kinase and focal adhesion kinase-related non-kinase, which are critical regulators of cytoskeletal organization downstream of matrix binding, have opposing effects on TGFβ-induced myofibroblastic differentiation.[121] A recent intriguing finding is that of genome-wide alterations in translational control in IPF fibroblasts, downstream of aberrant integrin signaling.[122]

Section Summary

The origin and regulation of fibrogenic myofibroblasts in IPF and other fibrotic disorders are complex and variable. Local structural fibroblasts, those arising through transdifferentiation of other cells, and those recruited from bone marrow or circulating progenitors, are all subject to reprogramming by a number of mechanisms, resulting in developmental or wound-healing expression repertoires that may self-perpetuate, resulting in aberrant fibrogenesis (Figure 1). The immediate biochemical/biomechanical context within fibrotic lesions, and the cell's ability both to respond to and alter that context, significantly affect fibroblast differentiation, persistence, and survival.

Signaling Pathways and Programming Paradigms

Fibrogenic Signaling: Lessons from Animal Models

Multiple rodent models have been established that recapitulate mechanisms leading to pulmonary fibrosis. Most involve the administration of drugs, chemical compounds, irradiation, or infections.[123] Administration of bleomycin through a number of routes to rodents is among the earliest and most widely published methods, causing a transient, inhomogeneous fibrotic response associated with early inflammatory infiltration of macrophages and lymphocytes.[124] The use of this model to test antifibrotic interventions has been recently reviewed, demonstrating its very limited ability to predict clinical responses in humans, and underscoring the important differences between bleomycin-induced fibrosis and human IIPs.[125] Instillation of other chemicals, including fluorescein isothiocyanate, asbestos fibers, or silica particles, into rodent lungs also results in chronic and progressive inflammation with fibrosis.[126,127] In addition, radiation-induced pulmonary fibrosis results in a more uniform cellular injury compared to drug- or chemical-induced lung injury. Within 1 month following irradiation, inflammatory cells are recruited into the air spaces and are closely associated with fibrotic lesions.[128] Also, mice defective in interferon-gamma receptor signaling chronically infected with the murine γ-herpesvirus 68, a virus that is closely related to Epstein-Barr virus and human herpesvirus 8, develop progressive interstitial lung fibrosis associated with a robust inflammatory response, as well as enhanced fibrotic responses to bleomycin or fluorescein isothiocyanate.[123,129]

Limited Role of Inflammation per se and Pre-Eminence of TGF-β

Injury models use agents known to cause pathological disease in humans, and have identified many cells, pathways, and a vast number of chemokines, cytokines, and growth factors that mediate pulmonary fibrosis. The disadvantage of these models is their reliance on acute injury leads to a broad inflammatory response, which is not characteristic of IPF or many other IIPs.

Replication-deficient adenoviral vectors containing cDNAs of specific genes have been transferred to the lung epithelium of rodents to mimic these diseases. Expression of the chemokines macrophage inflammatory protein-2, RANTES, IP-10, monokine induced by gamma interferon and lymphotactin all resulted in marked increases in bronchoalveolar lavage inflammatory cells and tissue pneumonitis, but did not induce lung fibrosis or residual remodeling.[130–133] The cytokines tumor necrosis factor-α, granulocyte macrophage colony-stimulating factor, and interleukin-1β also induced an acute inflammatory response with variable degrees of alveolar destruction,[134–136] along with a graded fibrotic response ranging from marginal (tumor necrosis factor α) to severe (interleukin-1β). The degree of fibrosis in these models was found to correlate directly to both the amount and

duration of expression of active TGFβ1.[137] Confirmation of the critical role of TGFβ1 in fibrogenesis was demonstrated by overexpression of active TGFβ1 in rat lung, resulting in prolonged and severe interstitial and pleural fibrosis.[138] TGFβ1-induced fibrosis developed and progressed without extensive inflammation, and was not induced by expression of the latent form.[138] Together these studies demonstrate that induction of lung fibrosis is not directly dependent on the degree or characterization of the inflammatory response, but rather, the amount and length of TGFβ1 induced downstream of cytokine signaling.

Further evidence supporting the important role of TGFβ1 in modulating the fibrotic response has been demonstrated by other transgenic models. Overexpression of the cytokine IL-13 using the Clara cell secretory protein regulatable airway epithelial promoter led to a marked inflammatory response with airway and parenchymal fibrosis.[139] IL-13-induced fibrosis was significantly ameliorated by treatment with a TGFβ antagonist, demonstrating that the fibrotic effects of IL-13 are mediated to a great extent through the TGFβ1 pathway.[140] In addition, there is a balance between TGFβ and bone morphogenetic proteins, which appears to be disrupted in some animal models and in human IPF by expression of Gremlin, a bone morphogenetic protein antagonist that is up-regulated by TGFβ in a MAPK-dependent manner. Restoration of bone morphogenetic protein-7 signaling inhibits asbestos-induced fibrosis in mice.[141]

TGF-β Downstream Signaling

Collectively, studies in both viral-delivered and pulmonary-specific transgenic models demonstrate that TGFβ is a key modulator of lung fibrosis and support targeting TGFβ pathways to prevent or reverse fibrosis. Responses elicited by TGFβ are dependent on and specific for the target cell lineage and are classically mediated by intracellular signaling via Smad proteins. In mesenchymal cells, the fibrotic effects of TGFβ depend on active TGFβ release from the matrix-bound latent complex. Factors which activate latent TGFβ include thrombospondin and the integrin αvβ6.[142,143] Once activated, TGFβ family members initiate signaling by interacting with and complexing the TGFβ type II receptors and the TGFβ Type I receptor (ALK5). Smad2 and Smad3 proteins then associate with the activated receptor and become phosphorylated, allowing the formation of an oligomeric complex with Smad4. This complex translocates into the nucleus and binds to specific nucleotide motifs to regulate transcription of target genes. Multiple studies in fibroblasts have established that the Smad pathways modulate TGFβ-induced cellular processes associated with lung fibrosis including enhanced collagen synthesis, proliferation, migration, adhesion, and transdifferentiation into myofibroblasts.[144,145] The role of Smad signaling in TGFβ-driven fibrosis has been demonstrated *in vivo* using Smad3 null mice, which are resistant to TGFβ1 mediated pulmonary fibrosis.[146] Furthermore, administration of a selective kinase inhibitor of Alk5 prevents the induction of fibrosis from adenovirus-mediated gene transfer of active TGFβ1, and also blocks progressive fibrosis when administered transiently to rats with established fibrosis.[147]

Although the Smad pathway is believed to be the primary conduit for signals from the TGFβ1 receptors, emerging evidence highlights the importance of non-Smad pathways. Fibroblasts stimulated with TGFβ1 demonstrate Smad-independent proliferation and matrix protein production which are regulated by mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) pathways.[145,148,149] c-Albelson is a proto-oncogene regulated by PI3K; TGFβ stimulates c-Albelson kinase activity in fibroblasts independent of Smad2 and Smad3 phosphorylation.[150] Loss of c-Albelson kinase prevents TGFβ-mediated stimulation of extracellular matrix gene expression and cell proliferation. *In vivo*, transgenic mice that conditionally over express TGFβ1 in the lung epithelium develop epithelial apoptosis and extensive inflammation followed by progressive lung fibrosis.[151] Mice that are deficient in the semaphorin 7A, a protein believed to function in angiogenesis, apoptosis, and immune responses, when crossed with TGFβ1 overexpressing mice, develop markedly less fibrosis and alveolar remodeling, despite TGFβ1 activation of Smad proteins.[152] Together, these findings indicate that TGFβ1-initiated cellular responses are regulated by relative contributions and cross talk between both Smad-dependent and Smad-independent signaling pathways.

Non-TGF-β Pathways to Fibrogenesis

Recent studies have shown that fibrosis and lung remodeling may also develop independently of TGFβ1 signaling. Mice exposed to house dust mite antigen and concurrently treated with a pan-neutralizing anti-TGFβ antibody developed airway remodeling comparable with mice exposed to house dust mite and treated with irrelevant antibody control.[153] Similarly, house dust mite-exposed Smad 3 knockout mice developed remodeling to the same extent as house dust mite-exposed wild-type littermate control mice.

TGFα binds to the epidermal growth factor receptor. Overexpression of TGFα using the regulatable Clara cell secretory protein promoter causes progressive and extensive pulmonary fibrosis characterized by pleural, perivascular, and peribronchial matrix deposition. Fibrosis in this transgenic model develops and progresses in the absence of detectable inflammation or activation of TGFβ.[154] Oncostatin-M, an inflammatory cytokine that is elevated in IPF BALF, causes exuberant inflammation and fibrosis in an animal model. Interestingly, the oncostatin-M-mediated fibrosis appears independent of both inflammation and TGF-β signaling.[155]

Potential Downstream Confluence of Signaling Pathways

As fibrosis is likely heterogeneous in etiology and molecular pathophysiology, attempts to block or counteract single pathways may not be sufficient to inhibit cellular processes associated with fibrosis. Ongoing studies

have identified potential points of confluence, where multiple inputs eventually converge to elicit the cellular response of mesenchymal proliferation and matrix deposition.

PI3K is a signal transduction enzyme that catalyzes the phosphorylation of phosphatidylinositol (4,5)-biphosphate to form phosphatidylinositol (3,4,5)-triphosphate in response to activation of receptor tyrosine kinase, G-protein coupled receptors or cytokine receptors. Activation of PI3K is essential to a number of cellular processes associated with fibrogenesis including cell growth, proliferation, migration, survival, and collagen gene expression.[156] The tumor suppressor phosphatase and tensin homolog is a negative growth regulator of the PI3K-Akt pathway, for which baseline activity is believed to be constitutively high. Both cell proliferation and collagen production are up-regulated in lung fibroblasts deficient in phosphatase and tensin homolog.[157] Moreover, in phosphatase and tensin homolog-haploinsufficient mice, using both cutaneous wound healing and bleomycin-induced lung injury models, deficiency in phosphatase and tensin homolog results in a more durable fibroproliferative response.[119] Further evidence supporting the PI3K pathway mediating lung fibrosis is demonstrated in transgenic models. Pulmonary fibrosis in the regulatable TGFβ1 transgenic model was significantly attenuated when mice were treated with an Akt inhibitor.[152] The PI3K-Akt pathway is also activated in the regulatable TGFα transgenic model, and lung fibrosis in this model is prevented when mice are treated with a PI3K-specific inhibitor (WH, unpublished observations). The platelet derived growth factor family, another profibrotic cytokine group implicated in inflammatory models of lung fibrosis,[158,159] also activates PI3K.[160] These data further support the PI3K as a common pathway where multiple cytokines synergistically function or become confluent.

While emerging data supports targeting common signaling pathways such as PI3K-Akt, the plethora of cellar processes mediated and potentially affected by broad signaling inhibitors poses significant challenges.[161] Therefore, more specific downstream effectors involved in matrix synthesis and proliferation may be better therapeutic targets. The mammalian target of rapamycin (mTOR) is a highly conserved intracellular serine/threonine kinase and a major downstream component of the PI3K pathway.[162] Inhibitors of mTOR, such as rapamycin, bind to an intracellular cytoplasmic receptor, the FK506-binding protein-12.[162] The complex formed then interacts and disrupts mTOR function and leads to cell cycle arrest in the G1 phase. In addition to blocking cell proliferation, mTOR inhibitors have been identified with anti-inflammatory, anti-tumor, and anti-fibrotic properties.

The role of mTOR in fibrosis has been demonstrated *in vivo* in rodent models of renal fibrosis and cirrhosis where rapamycin treatment has provided effective in either reversing or preventing fibrosis.[149,163] In the lung, the rapamycin analog SDZ RAD reduced bleomycin-induced pulmonary fibrosis by 75% compared to vehicle control.[164] In the TGFα transgenic model, rapamycin prevented development of epidermal growth factor receptor-induced fibrosis as assessed by lung histology, lung collagen content, and changes in lung mechanics.[165] Activation of mTOR leads to interaction with downstream effectors such as p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor-4E-binding protein-1. Through these pathways, mTOR controls cellular growth, proliferation and translation. Both S6K and 4E-binding protein-1 possess several phosphorylation sites that are points of convergence of several pathways including not only mTOR, but also PI3K and MAPK.[162,166] Future research targeting S6K and 4E-binding protein-1 phosphorylation in fibrosis models will be needed to determine whether these proteins represent effective and specific therapeutic targets.

Recent studies have underscored the role of transcription factors in the regulation of fibrogenesis. The transcription factors Fra-2/AP-1 have been shown to be profibrogenic in lung, in part by regulating coordinated expression of non-TGF-β fibrogenic mediators.[167] Tumor necrosis factor-α induces expression and DNA binding of AP-1 in fibroblasts, resulting in increased transcription of the TGF-beta 1 gene.[168] Additional studies are needed to further define the roles and hierachies of transcription factors in regulating the "fibrogenic transcriptome" IPF and other IIPs.

Section Summary

Animal models of pulmonary fibrosis have revealed multiple cytokines and growth factors leading to fibrosis through a number of different pathways including TGFβ (Smad dependent and non-Smad dependent) and non-TGFβ pathways. However, to date there is no single model that duplicates the correct temporal, spatial, and dynamic aspects of human fibrotic disease.[169] Currently, methods to identify specific pathways causing fibrosis in human disease are limited to "footprints" of molecular activation, such as immunostaining for phosphorylated signaling intermediates. Development of biomarkers for assessment of activation of cellular and molecular pathways associated with fibrogenesis is an active area of investigation. Identification of common downstream "funneling" factors where signals converge is likely to provide optimal therapeutic targets that will allow treatment of fibrosis regardless of the upstream initiating events.

Summary and Future Directions

Recent studies have added significantly to our understanding of pulmonary fibrogenesis. Emerging concepts include the critical role of the epithelium, particularly type II pneumocytes, in the initiation and perpetuation of fibrosis, in response to either endogenous or exogenous stress; a growing awareness of alternative activation of macrophages in tissue remodeling; growing appreciation of the alternative origins and phenotypic plasticity of fibroblasts; the roles of epigenetic reprogramming and context-dependent signaling in profibrotic phenotype alterations; and recognition of the importance of cross talk and convergence of intracellular signaling pathways in designing antifibrotic interventions. Remarkable overlap exists in molecular and cellular programming among nor-

mal development, wound healing, fibrogenesis, and cancer,[170] such that careful attention to novel findings in related fields is warranted. Overall, the paradigm that emerges is one of "disordered re-development" of the lung. Only by careful analysis of the developmental context, understanding of the key molecular "players" and mechanisms of context disruption, and by using a coordinated, multidisciplinary approach, can we hope to discover the critical points of convergence and begin to restore order and function.

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