Chapter 7
Mechanisms of Fibrosis in IPF

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Abbreviations

AEC	Alveolar epithelial cell
BAL	Bronchoalveolar lavage
BMPs	Bone morphogenetic proteins
cav-1	Caveolin-1
Col I	Collagen I
CTGF	Connective tissue growth factor
DAMPs	Danger-associated molecular patterns
ECM	Extracellular matrix
ED-A	Extra type III domain A
ELMOD2	ELMO domain containing 2
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
IL-1β	Interleukin-1β
IL-13	Interleukin-13
IL-17A	Interleukin-17A
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-8/CXCL8	Interleukin-8
IPF	Idiopathic pulmonary fibrosis
LAP	Latency-associated peptide

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LOXL2	Lysyl oxidase 2
LPA	Lysophosphatidic acid
LTBP-1	Latent binding protein-1
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1α
MMPs	Matrix metalloproteinases
MUC5B	Mucin 5B gene
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PAMPs	Pathogen-associated molecular patterns
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PINK1	Putative kinase 1
PRA	Proteinase-activated receptors
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTGER2	Prostaglandin E2 receptor
RAGE	Receptor for advanced glycation end products
SASP	Senescence-associated secretory phenotype
SNP	Single nucleotide polymorphism
SP-C	Surfactant protein C
TERC	RNA component of telomerase
TGF-β	Transforming growth factor-β
TGF-βR1	TGF-β type I receptor
TGF-βR2	TGF-β type II receptor
TIMPs	Tissue inhibitors of matrix metalloproteinases
TNF-α	Tumor necrosis factor-α
TOLLIP	Toll-interacting protein
UIP	Usual interstitial pneumonia
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
WISP	WNT-induced signaling protein
α-SMA	α-Isoform of smooth muscle actin

Introduction

The normal reparative response to tissue injury involves the orchestrated involvement of multiple cell types under the influence of a myriad of autocrine, paracrine, and inflammatory mediators, with a goal of reestablishing tissue integrity and barrier function. Wound-healing in the adult human does not fully recapitulate embryologic developmental patterning, resulting in the formation of a scar at the site of injury [1, 2]. Resolution of the reparative response is important to preserve existing

normal tissue architecture and involves the tight spatiotemporal regulation of signaling involved in wound healing [3]. Fibrosis, characterized by excessive extracellular matrix accumulation and disruption of normal tissue architecture, can occur as a result of chronic injury, chronic inflammation, or dysregulation of the normal reparative process within a tissue bed.

In the lung the alveolar walls are formed by delicately apposed monolayers of alveolar epithelial cells and endothelial cells that are separated only by their respective basement membranes [4]. This delicate architecture forms the primary gas exchanging interface of the lung, allowing rapid diffusion of oxygen and carbon dioxide between the alveolar airspace and the alveolar capillary blood. The surrounding supporting interstitial spaces of the lung are comprised of a fine network of fibrillar proteins (collagens, fibronectin, elastin) in composite with hydrated glycosaminoglycans [5]. In pulmonary fibrosis, there is a dramatic disruption of this intricate structural organization with expansion of the connective tissue compartment of the lung due to accumulation of matrix components, associated alveolar obliteration and collapse, and progressive distortion of normal lung architecture [6]. These changes result in disturbances in gas exchange and, when progressive, respiratory failure and death.

IPF is one of several disorders of the lung characterized by aberrant tissue fibrosis. In contrast to several other forms of pulmonary fibrosis, such as the fibroproliferative phase of acute respiratory distress syndrome (diffuse alveolar epithelial cell injury), or fibrotic sarcoidosis (exuberant granulomatous inflammation), the underlying etiology of the fibrotic response has historically not been immediately clinically apparent in IPF, hence its name. However, aging is one of the single most important demographic risk factors for the development of IPF, making it a prototypical aging-related disorder. More recently, several genetic alterations have been identified that increase the risk for development of the disease. Understanding how age-related changes in cell function intersects with underlying genetic susceptibilities and exogenous insults to drive recurrent injury and the non-resolving wound-healing response may be getting us closer to understanding the etiology of this disorder.

The histopathology of IPF is defined by the usual interstitial pneumonia (UIP) pattern [7], which is characterized by spatial variegation of the fibrotic process [6, 8, 9] with normal-appearing areas of the lung adjacent to areas characterized by severe scarring and architectural distortion and the presence of microscopic honeycombing [6, 7].

Staining for early collagen forms that are indicative of collagen synthesis reveals that active, synthetic fibroblasts are present in clusters near the air-tissue interface, termed fibroblastic foci [6]. The presence of these spatially discreet foci of “activated” fibroblasts in juxtaposition to areas of “old” scar containing fewer fibroblasts and more mature collagen along with normal-appearing alveoli suggests an indolent but progressive process. The presence of these various stages of fibrosis within the same pathologic specimen is termed “temporal heterogeneity” and is a required diagnostic element of the UIP pattern [7]. While areas of scarring may contain a

mild, mixed inflammatory infiltrate as part of the UIP pattern, it does not predominate when compared to the fibrotic reaction.

The distinctive lesions of IPF, which are the fibroblastic foci, lend some insight into the underlying biology mediating this disorder. The fibroblastic foci are the site of "new fibrosis" with fibroblasts most proximal to the airspace demonstrating the greatest amount of collagen synthesis. Basal lamina remnants appear on the interstitial side of the fibroblastic focus, suggesting that this structure has developed within the previously intact airspace [6]. Supporting this concept, foci are often associated with a poorly adherent, hyperplastic epithelial cell layer on their luminal (airspace) side along with areas of epithelial sloughing. These structures form a reticulated network of fibrosis throughout the lung and are thought to represent the "leading edge" of new fibrosis [10]. The numbers of these structures present on surgical lung biopsy correlate with survival [11, 12], consistent with this role in disease progression.

An Overview of the Current and Evolving Model of IPF Pathogenesis

Given that several forms of pulmonary fibrosis are the result of a robust inflammatory response [13], it is not surprising that historically, IPF was originally viewed as a disorder primarily characterized by an early, macrophage-mediated alveolitis, with resultant progressive tissue fibrosis [14, 15]. The development of more precise classification schemes for the idiopathic interstitial pneumonias resulted in an improved appreciation of the lack of extensive inflammation in the histopathology of IPF [7] and called into question the role of inflammation in the disease process. It is now clear that broad inhibition of immune function using corticosteroids and azathioprine does not positively affect disease progression and patient outcomes [16–18]. Thus, the concept of IPF as a product of a robust, disordered inflammatory response has been supplanted by the current concept of IPF as a disorder characterized by repetitive alveolar epithelial cell injury and an aberrant, non-resolving wound-healing response [19, 20].

The temporal relationships of the key pathogenic events of IPF are largely inferred from the knowledge gained from several decades of investigations into the mechanisms of epithelial cell injury and the reparative response of cells and tissues [2, 21, 22]. This work from animal models of pulmonary fibrosis and correlative studies in IPF lung specimens has now been enriched by recent systems biology (genomic, transcriptomic, proteomic, etc.) studies that had significantly increased our understanding of IPF, leading to the current model of disease pathogenesis.

IPF pathogenesis is characterized by repetitive injury to AEC leading to apoptosis and disruption of the AEC layer. The risk for repetitive AEC injury in any one individual may be modified by genetic factors, age-related changes in AEC biology, and exogenous exposures. Dysregulated telomere biology, aging-related changes,

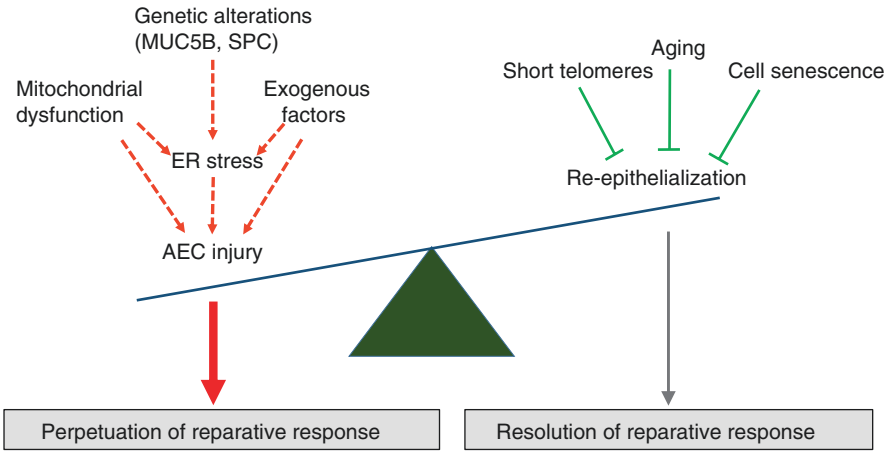

Fig. 7.1 Factors contributing to AEC injury and failure of reepithelialization in IPF. Reepithelialization is essential for resolution of the wound-healing response. Genetic susceptibility, aging-related changes in mitochondrial function, and repetitive exposure to exogenous factors all predispose to the development of UPR, ER stress, and ultimately AEC injury (apoptosis and dropout). In IPF, normal reepithelialization is hindered by telomere dysfunction, aging-related factors, and AEC senescence. Lack of reepithelialization (and loss of homeostatic AEC-derived signals) results in perpetuation of the wound-healing response

and the development of cell senescence interact to inhibit the reestablishment of a normal AEC barrier (reepithelialization), thereby perpetuating the aberrant wound-healing response (Fig. 7.1). AEC-derived signals play an important role in the potentiation of the wound-healing response via secretion of profibrotic cytokines (especially transforming growth factor-β [TGF-β]), chemokines, and proteases that trigger the recruitment and activation of inflammatory cells and fibroblasts. Local elaboration of matrix metalloproteinases results in disruption of the basal lamina of the alveolus. AEC injury is accompanied by the formation of a serum-derived, fibrinous exudate, which serves as a provisional matrix analogous to that of dermal wounds [23]. Chemokines and serum-derived factors present in the provisional matrix lead to the influx and expansion of local mesenchymal cell progenitor populations from the local interstitium, and there is also recruitment of circulating cell populations and acquisition of aberrant epithelial cell phenotypes that may play a role in the perpetuation of the fibrotic response. Activation of fibroblasts by TGF-β results in a highly contractile and synthetic phenotype, termed the myofibroblast, which serves as the primary effector cell for matrix production and tissue remodeling. Myofibroblast activation and tissue remodeling persist in IPF, possibly due to failure to reestablish normal epithelialization plus aberrant behavior of the myofibroblasts, which may be related to age-related cellular senescence, epigenetic reprogramming, or matrix-driven propagation of the fibrotic response. Progressive matrix deposition and remodeling ensue, resulting in a severely disordered tissue architecture (honeycombing) and organ dysfunction (Fig. 7.2).

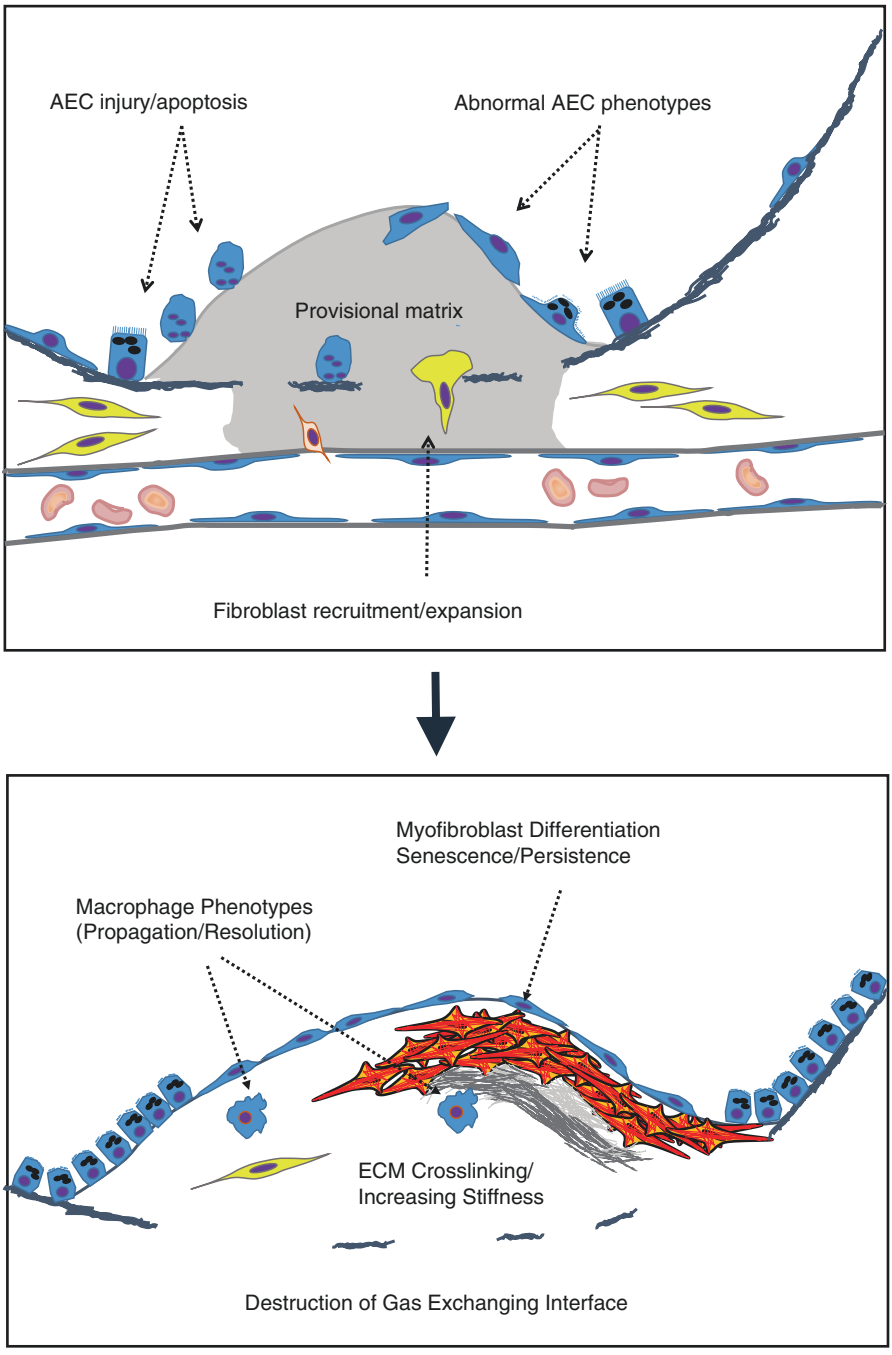
AEC injury/apoptosis
Abnormal AEC phenotypes
Provisional matrix
Fibroblast recruitment/expansion
Myofibroblast Differentiation
Senescence/Persistence
Macrophage Phenotypes
(Propagation/Resolution)
ECM Crosslinking/
Increasing Stiffness
Destruction of Gas Exchanging Interface

Fig. 7.2 Model of fibroblast focus formation. *Upper panel:* Epithelial cell injury leads to apoptosis and AEC dropout, resulting in a denuded basement membrane. There is an attempt to reepithelialize the airspace, but multiple aberrant AEC phenotypes emerge that lack proper homeostatic function and elaborate profibrotic mediators. Disruption of the normal basement membrane (broken lines) and alterations in alveolar capillary permeability result in the elaboration of a serum-derived exudate (provisional matrix) within the alveolar airspace. Localized progenitor populations expand the fibroblast population, while circulating bone marrow-derived cells also populate the wound. *Lower panel:* In response to TGF-β, matrix cues, and other soluble mediators, fibroblasts differentiate into (myo)fibroblasts and elaborate and incorporate abundant ECM. ECM deposition and cross-linking stiffen the matrix. Increases in tissue stiffness promote further TGF-β signaling, myofibroblast differentiation, and apoptosis resistance, perpetuating the fibrotic response. Cell-intrinsic or acquired metabolic derangements and senescence factors also perpetuate the fibrotic response. Progressive fibrosis emanating from this lesion results in obliteration of the adjacent capillaries, prevents gas exchange, and ultimately results in macroscopic architectural distortion and honeycombing of the lung

Several novel concepts that build upon this conceptual framework have emerged in recent years and are discussed in greater detail later in the chapter. Repetitive epithelial cell injury may be triggered by a combination of genetic and age-related factors that lead to increased susceptibility to alveolar epithelial cell stress, coupled with a "second hit" of exogenous "triggers" such as tobacco smoke, gastroesophageal reflux, changes in the local bacterial microbiome, or indolent viral replication that result in epithelial injury [24–26]. Global analysis of gene expression and non-coding microRNA in human subjects with IPF have demonstrated that signals associated with embryologic development and TGF-β-associated signals comprise a significant portion of the reparative gene expression response in humans with IPF [27]. These pathways are linked to the epithelial cell responses to injury, repair of the disrupted alveolar cell layer, and myofibroblast activation in IPF. How differences between the developmental and reparative response in these signaling pathways lead to the propagation of fibrosis remains an area of investigation [28], but given the strong link between aging and the development of IPF, it is likely that cell-intrinsic changes with age modify these responses.

Finally, remodeled, fibrotic matrix is not merely the end result of the fibrotic response. Biomechanical features of the matrix environment, such as its stiffness, are an independent determinant of fibroblast response and fibrotic progression, suggesting a new mechanism of aberrant cell behavior/function in IPF. In total, these mechanisms result in a mutually reinforcing cycle of fibrotic signaling, leading to non-resolving tissue fibrosis. The subsequent sections explore these concepts in detail.

Alveolar Epithelial Cell Injury and Failure of Normal Reepithelialization

Alveolar epithelial cell injury The normal alveolar epithelial lining of the lung is comprised of two types of epithelial cells, type I and type II AEC, forming a single-

cell thick layer. Type I cells are flat, highly specialized cells whose membrane comprises the bulk of the alveolar-capillary interface in normal lung tissue [29]. Type II cells have a cuboidal morphology, with intracellular lamellar bodies. Type II cells secrete surfactant proteins, retain proliferative capacity, and are responsible for the regeneration of epithelium after injury [30], including trans-differentiation to type I cells [31]. In IPF, alveolar epithelial cell morphology is severely deranged, with overt epithelial cell necrosis and denudation of the capillary basement membrane [32], as well as extensive type II pneumocyte apoptosis [33]. Alveolar spaces that have been disrupted by extensive fibrotic changes in IPF lungs are lined with numerous, hyperplastic type II pneumocytes, cuboidal epithelial cells that may be derived from the adjacent bronchiolar lining cells [34], and abnormal-appearing, elongated epithelial cells [35]. The presence of these abnormal epithelial phenotypes in areas that normally contain predominately type I epithelial cells is suggestive of a failure of normal reepithelialization after injury [36].

While AEC injury could be consequent to an ongoing, aberrant fibrotic response, several lines of evidence suggest that AEC injury may be a primary driver of the non-resolving reparative response. For example, in IPF alveolar epithelial cell apoptosis is found in areas without significant interstitial fibrosis, suggesting that this process may be a primary inciting factor [33].

Genetic susceptibility may predispose to AEC injury and failure of reepithelialization Further support for the concept of AEC-driven fibrosis comes from studies that have identified genetic alterations that confer susceptibility to AEC injury and apoptosis and promote aberrant AEC function. Several rare mutations in surfactant protein C (SP-C), a protein produced by the type II AEC cells, have been identified in patients with the familial form of pulmonary fibrosis [37, 38], which can have an identical histopathology to sporadic IPF. These mutations result in misfolding and altered processing of SP-C by type II AEC cells, leading to deficient expression and secretion, ER stress, and apoptosis [38–40]. Mice with germline deletion of SP-C develop interstitial lung disease as adults [41], suggesting a causal relationship for disordered SP-C biology. Rare mutations in surfactant protein A2 have also been identified in patients with familial pulmonary fibrosis (FPF) and in rare cases of sporadic IPF [42, 43]. These mutations result in a similar defect in protein stability, defective secretion, and a subsequent increase in ER stress-associated signaling [44].

The interaction of aging with AEC injury may be a very important etiologic factor in the development of IPF. Telomeres are multi-protein structures that cap the end of chromosomes and prevent their degradation. Chromosomal telomere shortening occurs with cell division and aging and is associated with the development of cell senescence and susceptibility to apoptosis [45]. Telomerase is present in progenitor cells, where it counteracts telomere shortening, thereby preserving proliferative potential [46]. Diseases of disrupted telomere homeostasis such as dyskeratosis congenita are characterized by short telomeres, premature graying, bone marrow failure, and the development of pulmonary fibrosis [47]. Several mutations in the two components of telomerase, telomerase reverse transcriptase

(hTERT) and the RNA component of telomerase (TERC) [48, 49], have been identified in patients with FPF. When compared to age-matched family members without the mutation, family members with TERT and TERC loss-of-function mutations had shorter telomeres and increased risk for the development of pulmonary fibrosis [48]. The presence of these mutations was associated with a penetrance of pulmonary fibrosis of 40% in the affected individuals [50]. However, the original mutations identified were only identified in a small percentage of patients with sporadic IPF [51]. Subsequent studies of patients with FPF have identified mutations in several additional proteins associated with telomere homeostasis (RTEL, PARN, dyskerin, and TINF2) [52–56].

The concept of telomere length-dependent susceptibility to alveolar epithelial injury and the development of pulmonary fibrosis are supported by the identification of short telomeres as an independent risk factor for the development of the sporadic IPF [57]. A genome-wide association study (GWAS) has identified a common single nucleotide polymorphism (SNP) in the hTERT gene that confers risk for the development of IPF [58], and whole exome sequencing of a cohort of sporadic IPF patients found mutations in TERT, RTEL1, and PARN that may be responsible for 11% of IPF [50].

Linkage analysis of a cohort of Finnish families with FPF identified the gene ELMO domain containing 2 (ELMOD2) [59, 60] as a candidate gene associated with the development of pulmonary fibrosis. ELMOD2, which is normally expressed in epithelial cells and macrophages of the lung, had significantly decreased expression in lungs of IPF patients. ELMOD2 may play a role in the response of epithelial cells and macrophages to viral infection [59], potentially linking an environmental and genetic trigger in this disorder.

Two large GWAS investigations have identified a SNP in the promoter region of the mucin 5B gene (MUC5B) that is strongly associated with the development of familial and sporadic forms of pulmonary fibrosis [61, 62]. The minor (risk conferring) allele is present in 34–37.5% of IPF cases and 9–11% of controls. The presence of homozygosity for the minor allele confers a 10- to 20-fold increase in the risk for developing IPF. MUC5B is present at increased levels in fibrotic areas of IPF lungs, and the mutant allele for this gene is associated with significantly increased expression of MUC5B in lungs of subjects without pulmonary fibrosis when compared to counterparts homozygous for the wild-type allele. This suggests that the discovered SNP results in alterations in gene expression that may contribute to the development of IPF.

GWAS studies have also identified additional polymorphisms that are associated with IPF in the genes of desmoplakin and dipeptidyl peptidase 9 [63], which are involved in epithelial function, and Toll-interacting protein (TOLLIP), which is involved in innate immune responses [64].

Exogenous factors Several exogenous agents that could trigger alveolar epithelial injury are associated with the development of IPF. Gastroesophageal reflux disease is present in up to 90% of patients with IPF [65, 66], and co-existing treatment with proton pump inhibitors has been associated with longer patient survival [67, 68].

Exposure to cigarette smoke is a powerful risk factor for the development of both IPF and FPF [69, 70], and approximately 70% of IPF patients are current or former cigarette smokers [71]. Workplace exposures are less robustly linked [72] but may contribute in a cohort of IPF patients. There is evidence of microsatellite instability in the DNA from IPF lungs [73] which suggests that somatic mutations due to exogenous exposures could account for acquired genetic risk and increase susceptibility to injury and aberrant reparative responses.

Several viruses that are trophic for the lung epithelium have been identified in IPF lungs [74] with the family of herpes viruses having the strongest association. A high prevalence of herpes virus DNA has been identified in the AECs and immune cells of the IPF lung [75–77]. The presence of herpes viral antigens has also been associated with signs of ER stress in the AECs [78, 79], suggesting a possible mechanism of injury triggered by viral infection. Finally, alterations in the bacterial microbiome of the lung are associated with IPF and its progression [80, 81].

These observations provide conceptual evidence that intrinsic epithelial defects may render the epithelial cell susceptible to repetitive injury, possibly from the environmental factors listed above, which could lead to perpetuation of the wound-healing response.

This concept has been experimentally demonstrated by targeted injury to type II AECs in mice via transgenic expression of SP-C-driven diphtheria toxin receptor expression followed by intraperitoneal diphtheria toxin administration. Changes in AEC gene expression and function were present in the transgenic animals, and repeated exposure to diphtheria toxin resulted in the development of alveolar interstitial fibrosis without induction of inflammation [82].

Several potential mechanisms likely account for the development of fibrosis in response to AEC injury. These include failure of reepithelialization with AEC dropout and loss of homeostatic signaling, acquisition of aberrant epithelial phenotypes (including senescence-associated secretory phenotype (SASP) and epithelial-mesenchymal transition (EMT)), and endoplasmic reticulum stress and elaboration of AEC-derived profibrotic soluble mediators (Fig. 7.1).

Failure of normal alveolar reepithelialization Self-limited lung injury is characterized by regeneration of the alveolar epithelium and reestablishment of the normal alveolar epithelial cell layer via proliferation of type II AEC and subsequent transdifferentiation to type I AECs [83–86]. Additional epithelial progenitor populations may contribute to this process [87]. Reepithelialization reestablishes the normal homeostatic function of the epithelium and promotes resolution of the reparative response. IPF is characterized by a failure of reepithelialization with the development of a disordered epithelial layer characterized by proliferation of bronchiolar basilar epithelial cells exhibiting signs of epithelial stress and atypia [88], along with the presence of AECs that exhibit an abnormal, intermediate phenotype with traits of type I and type II cells [36].

Experiments performed in an ex vivo model of hyperoxia-mediated AEC injury support the importance of reestablishing a normal alveolar epithelial cell layer in

regulating fibrotic progression. Lungs that exhibit decreased rates of epithelial cell proliferation develop fibrosis, while lungs that rapidly reepithelialize revert to normal [83]. Similarly, utilizing diphtheria toxin-mediated depletion of airway progenitor (Clara) cells, Perl and colleagues [89] demonstrated that chronic depletion of Clara cells results in incomplete and aberrant reepithelialization of the bronchiolar airway and the development of peribronchiolar fibrosis, while acute depletion, which presumably leaves a reserve of Clara cell progenitors, results in normal reepithelialization and did not lead to fibrosis. Similarly, a fibrotic response results from daily administration of diphtheria toxin to injure SP-C expressing type II AEC in mice [82]. In contrast, repetitive injury to type II AEC every 2 weeks, which allows for recovery of cell populations, did not result in fibrosis [86]. These results suggest that allowing reepithelialization to occur may inhibit the development of fibrosis.

The receptor for advanced glycation end products (RAGE) is a transmembrane receptor that is a specific marker for differentiated type I epithelial cells [90]. The expression of RAGE in type I cells likely plays a role in their differentiation and homeostasis by promoting cell spreading and attachment to the basement membrane [91, 92]. IPF lungs demonstrate abnormally low expression of RAGE [92, 93] that is consistent with the presence of disrupted reepithelialization. Dysfunctional RAGE expression may also play a role in mediating the fibrotic process, however, as RAGE-null mice develop more severe experimental pulmonary fibrosis and spontaneously develop fibrotic-like lesions as they age [93].

Aging is likely an important contributor to deficient reepithelialization. During aging, somatic cells progressively lose telomere length [45, 94]. Loss of telomeres in progenitor populations of AEC contributes to cellular senescence, apoptosis, and diminished replicative capacity, thereby contributing to stem cell exhaustion [95].

Acquisition of aberrant epithelial cell phenotypes In addition to the morphologic alterations in AEC cells visible on histologic specimens from IPF lung, unbiased, single-cell transcriptional profiling of AECs from normal and IPF lung revealed an alteration in AEC phenotypes in IPF, with frequent co-expression of type I AEC, type II AEC, and conducting airway cell markers. This suggested that indeterminate or transitional epithelial phenotypes are common in IPF [96]. Several aberrant AEC phenotypes have been experimentally characterized and are discussed below.

Epithelial-mesenchymal transition (EMT) On a morphologic basis, AECs are present in IPF that have a flattened morphology, which may represent pro-migratory phenotypes that are attempting to reepithelialize the alveolar space after injury [35]. This morphology is similar to epithelial cells that are undergoing EMT. EMT is the process by which epithelial cells lose attributes of full epithelial differentiation (cuboidal shape, apical-basal polarization, cell-cell contacts, epithelial gene repertoire) and take on attributes of mesenchymal cell lineages (spindle morphology, loss of cell contacts, mesenchymal gene expression). EMT is accompanied by the loss of several epithelial markers such as E-cadherin, the acquisition of mesenchymal

markers N-cadherin and vimentin, and the upregulation of transcription factors implicated in EMT, such as Twist, SNAI1 (Snail), and SNAI2 (Slug) [97]. EMT is critical for gastrulation during embryogenesis [98], and epithelial cells that have undergone EMT have an augmented ability to metastasize [99]. Several forms of tissue injury and repair demonstrate the presence of EMT as part of their pathogenesis [100], and deletion of snail protects from the development of hepatic fibrosis [101], suggesting a mechanistic role in the propagation of tissue fibrosis. Tissue sections from established models of experimental pulmonary fibrosis, such as the bleomycin model [102], also demonstrate evidence of EMT [103–105], and lung tissue from patients with IPF demonstrates increased expression of Twist and Snail, suggesting the presence of EMT-associated signaling in human IPF and co-localization of epithelial and mesenchymal proteins within the same cell [105–108]. Single-cell sequencing of AECs also identified a population of cells that displayed co-expression of mesenchymal and AEC lineage markers [96]. These data suggest that EMT and associated signaling is present in IPF, and this may be the source of significant profibrotic signals.

Regulation of EMT during development is mediated, in part, by family members of the transforming growth factor-β superfamily of cytokines [98], which includes TGF-β1, TGF-β2, TGF-β3, and bone morphogenetic proteins (BMPs). TGF-β/BMP balance is important in the development of the mesodermal/epithelial compartment during development and regulates EMT [109, 110]. TGF-β induces EMT in both developmental and fibrotic contexts [111] and is a potent inducer of EMT in ex vivo epithelial cell cultures [112], although cell-contact and integrin-mediated signaling can modify this response [113, 114]. Several BMPs are implicated in the reverse process of mesenchymal to epithelial transition and can antagonize TGF-β-dependent signaling. Interestingly, the expression of two of these BMPs, BMP-2 and BMP-4, is altered in IPF [28], and the inhibitor of BMP signaling, gremlin, is increased in IPF lungs [115], implicating dysregulated TGF-β/BMP signaling balance in the pathogenesis of the disorder.

Senescence-associated secretory phenotype (SASP) Aging can intersect with injury to alter AEC phenotype via the induction of cell senescence. Whereas cell senescence plays an important embryologic and antineoplastic role in health, acquisition of a hypoproliferative, secretory phenotype SASP may be deleterious in certain contexts of tissue repair. Induction of lung injury results in the induction of cell senescence [116], aged mice have increased numbers of senescent cells in their lungs [117], and AEC in IPF lungs display markers of senescence [118, 119]. The SASP is associated with the elaboration of profibrotic mediators and matrix proteins that may perpetuate the fibrotic response [120]. Senescent cells are prone to persist in remodeled tissue, thereby contributing to the lack of resolution of the wound-healing response. Thus, these cells may be an attractive target for antifibrotic therapies.

Endoplasmic reticulum (ER) stress and disrupted proteostasis Type II AECs are highly metabolically active cells that continuously secrete proteins, including

surfactant proteins, into the alveolar space. To maintain this high level of secretory function, highly developed protein processing machinery is required. The ER is the subcellular site of initial posttranslational processing of secreted proteins. When an imbalance exists between the ability of the ER to sufficiently process the requisite amount of proteins to maintain cell homeostasis, there is activation of the unfolded protein response (UPR), and under certain conditions activation of pro-apoptotic pathways may occur. This pathway can be activated by the expression of misfolded surfactant proteins that are implicated in familial forms of pulmonary fibrosis [44, 121]. ER stress and chronic aggregation of misfolded proteins are present in AEC of lungs from patients with sporadic IPF, which is independent of known genetic defects [78]. Chaperone proteins serve an important function in protein folding in the ER. Loss of HSP70, an important chaperone protein, has been observed in IPF lung and may contribute to the activation of the UPR [122]. The ER stress markers, ATF4, ATF6, and CHOP, are preferentially localized to the epithelial cells of patients with sporadic IPF, in contrast to normal lungs or lungs with chronic obstructive pulmonary disease [123]. These changes are often localized to areas with significant fibrosis and co-localize with markers of apoptosis, suggesting a role in this process [123]. Furthermore, ER stress and UPR activation can also drive AECs toward a more mesenchymal morphology and gene expression repertoire (EMT) [124].

Aging contributes to the development of ER stress [125], potentially increasing the susceptibility to repetitive epithelial cell injury. A significant percentage of proteins are misfolded under normal conditions [126], and aging results in derangements in proteostasis with increases in misfolded proteins and oxidative damage. This leads to the accumulation of misfolded proteins with activation of the UPR.

With increases in misfolded proteins under conditions of cellular stress and with aging, the cell may try to compensate via catabolism and clearance of these proteins. Clearance of misfolded proteins occurs via the cell-regulated process of autophagy. Autophagy is a homeostatic function of the cell that allows for the degradation of proteins and thereby participates in maintaining proteostatic balance. Autophagy is a highly regulated process that leads to the formation of a specialized subcellular complex called the autophagosome. Unfortunately, aging is associated with deficient autophagy [127], and the IPF lung has evidence of abnormal autophagy [128, 129]. Aged mice have deficient autophagic responses to lung injury with a disproportionate targeting of mitochondria for autophagy (mitophagy), which can result in a further decrease in metabolic fitness [130]. Profibrotic signaling emanating from TGF-β inhibits autophagy via signaling through mTORC1, beclin1, and LC3 [131, 132], and genetically targeting a key component of autophagosome formation (ATG4B) results in accentuation of the fibrotic response in mice [133].

In addition to disproportionate loss of mitochondria due to mitophagy, aged AEC have increased amounts of dysmorphic and dysfunctional mitochondria [134]. Broad measures of mitochondrial fitness, such as ATP production, are also decreased with age. Mitochondrial dysfunction in aged animals is associated with increases in oxidative stress [135] and can lead to apoptosis of type II AEC [136]. Loss of sirtuin-3, a primary mitochondrial deacetylase that regulates mitochondrial integrity, results in increased AEC mitochondrial DNA damage and apoptosis [137].

As with aged cells, AECs in IPF lung have an increase in enlarged and dysmorphic mitochondria, which is associated with increased ER stress. In aged and IPF type II AEC, there is a decrease in the expression of protein phosphatase and tensin homolog-induced putative kinase 1 (PINK1), a kinase involved in the maintenance of mitochondrial homeostasis [134]. Loss of PINK1 in mice is associated with the induction of AEC ER stress and apoptosis, along with the development of spontaneous pulmonary fibrosis [134, 138].

Loss of homeostatic signaling and epithelial-mesenchymal cross talk Type II AECs maintain normal alveolar homeostasis via the production of surfactant, the regulation of fluid balance, and the interaction with other structural cells of the alveolus [139, 140]. Under normal conditions, AECs have an inhibitory effect on fibroblasts [141]. Thus, AEC dropout and failure to normally reepithelialize the airspace in IPF may lead to loss of inhibitory signaling from the AEC to the mesenchyme. One potential mediator of mesenchymal inhibition is prostaglandin E2 (PGE2). PGE2 is a product of cyclooxygenase and prostaglandin E synthases that is produced by local alveolar epithelial cells, monocytes, and other structural cells of the lung [142, 143]. PGE2 has shown to have an inhibitory effect on fibroblast proliferation [144, 145], migration [146], and collagen synthesis [147, 148]. In IPF, levels of PGE2 are decreased in bronchoalveolar lavage (BAL) [149], and EP2 prostaglandin receptor expression and signaling in fibroblasts are diminished [150]. Thus, AEC injury may result in the loss of the PGE2 production by AECs, leading to fibroblast activation during pulmonary fibrosis.

The WNT/β-catenin signaling pathway has been implicated in mediating altered epithelial cell function during lung injury and fibrosis. WNT/β-catenin signaling mediates branching morphogenesis during lung development and the maintenance of progenitor cells [151]. WNT proteins are secreted glycoproteins that can signal in a paracrine or autocrine fashion through their receptors (Frizzled proteins) and co-receptors (LRPs) to stabilize β-catenin, leading to its nuclear translocation. In the adult lung, WNT/β-catenin signaling is involved in epithelial cell proliferation, differentiation, and cell-cell adhesion in the lung [151, 152]. A common finding from recent unbiased gene expression screens of lung tissue from patients with IPF is that many developmental pathways are upregulated, including markers of the WNT/β-catenin pathway [28, 153–155]. The WNT genes, WNT2 and WNT5a, and the WNT receptors, Frizzled 7 and Frizzled 10, are increased in the lungs of patients with IPF [28, 153, 156]. Patients with IPF have increases in nuclear localization of β-catenin in the hyperplastic epithelium adjacent to fibrotic lesions [157] as well as increased phosphorylation of the Wnt/LRP receptors, suggesting activation of this pathway [158]. Consistent with the role of this pathway in pulmonary fibrosis, several WNT-/β-catenin-dependent genes are upregulated in IPF [154, 155, 159], and disruption of signaling via the WNT target gene, WNT-induced signaling protein (WISP), inhibited both markers of EMT and the development of fibrosis in response to bleomycin [159].

Finally, AECs are an important source of profibrotic mediators that can signal to the surrounding mesenchyme resulting in fibroblast recruitment and induction of

matrix production [36]. Several profibrotic growth factors are localized to the epithelial cells in IPF including TGF-β1 [160, 161], platelet-derived growth factor (PDGF) [162], monocyte chemoattractant protein-1 (MCP-1) [163], connective tissue growth factor (CTGF) [164], endothelin-1 [165], and tumor necrosis factor-α (TNF-α) [160, 166, 167]. AECs are also the source of several matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) that are implicated in IPF pathogenesis [168]. Experimentally, the induction of ER stress in AEC is associated with increased secretion of TGF-β [169], linking aberrant signaling from ER stress with a mechanism by which fibrotic responses can be promoted.

The Provisional Matrix and Coagulant Balance

The fibroblastic foci of UIP are found on the luminal side of the alveolar basement membrane in association with disruptions in the basement membrane [6]. These structures are morphologically analogous to the fibroblast collections that organize fibrinous alveolar exudates during the fibroproliferative phase of acute lung injury and the Masson bodies of organizing pneumonia. IPF lungs demonstrate evidence of endothelial injury, with swelling of endothelial cells and reduplication of the endothelial cell capillary basement membrane [170] and increased trans-endothelial permeability [171]. Interestingly, the degree of capillary permeability in IPF also correlates with prognosis [171, 172]. Animal models of pulmonary fibrosis indicate that vascular leak may be an important driver of the fibrotic response [173]. These observations suggest that the initial injury to the alveolar epithelial cell layer in IPF is accompanied by the exudation of serum-derived factors into the alveolar airspace to form the provisional matrix [6, 174]. Alveolar epithelial cells and macrophages express tissue factor [175, 176], which interacts with coagulation factors present in the alveolar exudate and activates the extrinsic coagulation pathway. Activation of the coagulation cascade results in the generation of thrombin, and subsequent thrombin-mediated conversion of serum-derived fibrinogen to fibrin forms the provisional matrix [177]. The provisional matrix also contains serum-derived fibronectin [6, 170] and growth factors, such as PDGF, facilitating subsequent fibroblast recruitment, migration, and matrix organization [178] (Fig. 7.2).

Stabilization of the nascent fibrin-containing provisional matrix in healing wounds would be predicted to require the presence of an increased procoagulant balance, as normal lung tissue expresses proteases such as the plasminogen activator, urokinase, that promote local fibrinolysis [179]. Immunohistochemical staining of IPF lungs demonstrates the deposition of fibrin localized in the alveolar space in areas adjacent to the epithelial cell layer [180], and BAL samples from patients with IPF demonstrate increased levels of plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2) and a reduction in urokinase activity [176, 181, 182], suggesting the presence of increased procoagulant balance.

Increased procoagulant activity also contributes to profibrotic signaling via the entrapment of serum-derived mediators present within the provisional matrix, forming a reservoir of growth factors that can be activated as the provisional matrix is remodeled [183]. The importance of procoagulant signaling is supported by studies in experimental models of pulmonary fibrosis. PAI-1-deficient mice are protected from the development of fibrosis, and the fibrotic response is potentiated by transgenic overexpression of PAI-1 [184].

Products of activation of the coagulation cascade, such as thrombin, also act as growth factors for fibroblasts. Thrombin is produced from the conversion of prothrombin to thrombin by Factor Va and Factor Xa and can signal through proteinase-activated receptors (PAR) found on epithelial cells and fibroblasts in the lung. Thrombin signaling occurs via proteolytic activation of its high-affinity receptor, PAR-1, leading to the expression of profibrotic cytokines, activation of TGF-β, and myofibroblast differentiation [177]. Germline deletion of the PAR1 receptor is protective against the development of bleomycin-induced pulmonary fibrosis [185]. Other coagulation proteinases may play a role in coagulation-dependent signaling as well. Factor X co-localizes to the alveolar epithelia of IPF lungs and can signal via PAR-1 [186]. Factor VIIa is also found in abundance on tissue biopsies from IPF lung, and in combination with tissue factor, Factor VIIa can mediate PAR-2-dependent proliferation of fibroblasts [187].

Despite the robust evidence supporting a key role for coagulation balance in the pathogenesis of fibrosis, a recent, large randomized clinical trial of systemic anticoagulation with warfarin for patients with IPF did not show a benefit, and the trial was terminated before completion due to increased deaths in the treatment arm [188]. Nonetheless, pharmacotherapy directed at specific coagulation cascade targets and coagulation-associated signaling remains a potential strategy for therapy.

Myofibroblasts: Effector Cells of Fibrosis

Concept of the myofibroblast The primary effector cell for connective tissue remodeling is the myofibroblast, a mechanically active, matrix-producing mesenchymal cell with distinct morphologic features that differ from normal resident fibroblasts. Myofibroblasts are characterized by the presence of large, bundled microfilaments and enlarged focal adhesions [189], and myofibroblast differentiation has been historically defined by the expression of both contractile proteins, such as the α-isoform of smooth muscle actin (α-SMA), and matrix proteins, such as collagens and the extra type III domain A (ED-A) splice isoform of fibronectin [190]. Myofibroblasts expressing α-SMA are not thought to be present in the normal tissue of the lung, although niche populations of microfilament containing α-SMA(−) myofibroblasts have been identified [191]. In contrast, α-SMA(+) myofibroblasts are invariably found in granulation tissue of wounds [192] and in scarring diseases that occur in other organs [193, 194]. Myofibroblasts act as central mediators of connective tissue remodeling via their production of matrix proteins,

pro- and anti-proteinase proteins, and modulation of matrix organization and tension [189, 195, 196]. Their presence in the lung is associated with the formation of a dense collagen matrix and progression of pulmonary fibrosis [197].

Origins of myofibroblasts The potential origins of myofibroblasts are diverse with several cellular precursors implicated in the expansion of the myofibroblast population during tissue fibrosis [189]. These include the resident fibroblasts of the alveolar interstitium, mesenchymal progenitor populations [198], alveolar epithelial cells that have undergone EMT, and circulating, bone marrow-derived progenitors that are termed "fibrocytes" [199].

Fibrocytes are circulating progenitor cells that express the hematopoietic surface antigens, CD34 and CD45, along with the fibroblast-associated proteins such as collagen I (Col I), collagen III, and collagen IV [200]. The cells were originally identified in a model of dermal wound healing [201] and are derived from bone marrow precursors [202]. Subsequently, studies using chimeric mice and bone marrow precursors tagged with green fluorescing protein (GFP) demonstrated the accumulation of GFP+, Col I+ cells in their lungs after the induction of bleomycin-induced pulmonary fibrosis [200, 203]. Fibrocytes express the chemokine receptor CXCR4, and fibrocyte recruitment to the lung is dependent on the CXCR4 receptor ligand, CXCL12 [204]. Several other studies in murine models of pulmonary fibrosis have demonstrated that circulating fibrocytes can express additional fibroblast-associated markers (S100A, vimentin, α-SMA) in association with their recruitment to the lung [104, 200, 204, 205]. However, conflicting data exist as to the potential of these cells to contribute to the myofibroblast (α-SMA expressing) population in vivo, with several studies demonstrating no evidence of an α-SMA+ fibrocyte population during experimental fibrosis [203, 206] and an inability of fibrocytes to express α-SMA [207]. Regardless of the ability of fibrocytes to become "fully differentiated" myofibroblasts, they may promote fibrosis via other paracrine effects, such as the production of profibrotic cytokines [208]. Fibrocytes and elevations in CXCL12 are present in the blood of patients with IPF [209] as well as in ex vivo preparations of lung specimens from patients with IPF [210]. Elevations in circulating fibrocytes are a marker of disease progression in human IPF [211], and neutralizing antibodies against CXCL12 ameliorate bleomycin-induced pulmonary fibrosis [204].

An additional hypothesized source of myofibroblasts is through the process of epithelial-mesenchymal transition. As previously discussed, substantial evidence supports the presence of aberrant epithelial signaling, including EMT-associated signaling, in IPF and experimental pulmonary fibrosis [108, 212]. Additionally, lineage marking techniques that can broadly label distal airway and AECs during gestation provide evidence that epithelial cells can express markers of mesenchymal cells during experimental lung fibrosis [103, 104]. In contrast, a more restricted lineage marking strategy of adult type II AEC cells or terminal bronchial epithelial cells found that no α-SMA+ cell population derives from these epithelial lineages in the bleomycin model of pulmonary fibrosis [85]. Additionally, in vitro work has demonstrated limitations in the ability of lung epithelial cells to contribute to the

collagen organization that comprises a stiff, remodeled matrix [213]. Discrepancies between these studies could be explained by technical differences in marking techniques or the presence of a discrete epithelial progenitor population that evaded lineage marking in the adult murine lung that could differentiate into type II cells or undergo EMT directly in response to injury [214]. Recent evidence supports the existence of such a population [215]. However, single-cell sequencing of IPF and normal lung found several abnormal epithelial cell populations in the lung, but no evidence of a population strongly co-expressing epithelial and myofibroblast markers was observed [96, 216]. Thus, while EMT-associated signaling programs are present in pulmonary fibrosis and appear to mediate important profibrotic cross talk between the epithelial and mesenchymal compartments, it is unlikely that epithelial-derived cells are a significant contributor to the contractile and matrix-producing cells of the parenchyma in pulmonary fibrosis.

These data suggest that the resident mesenchymal precursor cell population within the lung remains a predominant source of myofibroblasts during tissue fibrosis. The resident mesenchymal precursor population is a mixed population of several different mesenchymal cell subtypes important for the normal homeostatic maintenance and turnover of the lung connective tissue scaffold, and this cell population can proliferate and expand in response to injury [85, 140, 191, 217]. Upon exposure to profibrotic signals, such as cell, serum, or matrix-derived TGF-β, these cells can differentiate into myofibroblasts [218].

Upon expansion in response to pulmonary injury and fibrosis, the fibroblast population exhibits significant heterogeneity with the presence of several different subphenotypes [219, 220]. Myofibroblasts are defined by the expression of α-SMA and collagen production, but a significant subset lacks the cell surface marker Thy-1 [221], which correlates with a more fibrotic myofibroblast phenotype [222]. Xia and colleagues were able to isolate mesenchymal cell progenitors that shared features with mesenchymal stem cells but had retained differences in profibrotic features [198]. These profibrotic features were associated with the expression of the calcium-binding protein S100A4 [223]. Single-cell sequencing-based characterization of fibroblast populations in murine models of pulmonary fibrosis also demonstrates heterogeneity in the mesenchymal cell population that contributes to the fibrotic milieu [224, 225].

Aberrant fibroblast behavior Deranged fibroblast biology likely plays an important role in the propagation of pulmonary fibrosis by enabling a disproportionate and non-resolving fibrotic response to epithelial injury. Populations of lung fibroblasts isolated from patients with IPF demonstrate differences in global gene expression [226], proliferative capacity [46, 227], resistance to apoptosis [228], anchorage-independent growth [229], and deficits in translational control [230] when compared to normal lung fibroblasts.

The putative mechanisms mediating some of these disordered functions have begun to be elucidated. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid/protein phosphatase that can act as a tumor suppressor via inhibition of phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. Levels of PTEN

are nearly absent in the fibroblastic foci of IPF lungs and in ex vivo IPF fibroblast cultures, which stands in contrast to normal lung tissue and fibroblasts [227, 231]. Disordered PTEN activity in IPF fibroblasts conveys an abnormal proliferative response to polymerized collagen matrices via increases in PI3K/Akt signaling, and PTEN-deficient mice develop an accentuated fibroproliferative wound-healing response and more severe bleomycin-induced pulmonary fibrosis [227].

Caveolin-1 (cav-1) serves as a scaffolding protein and can inhibit the responses to growth factor signaling [232, 233]. The fibroblastic foci of IPF lungs lack cav-1 staining, and cav-1 expression by fibroblasts decreases in response to TGF-β. In contrast, overexpression of cav-1 disrupts TGF-β signaling and matrix protein induction, and its overexpression attenuates bleomycin-induced pulmonary fibrosis [234]. The loss of caveolin-1 in IPF myofibroblasts also results in decreases in PTEN expression [169].

Finally, myofibroblasts from IPF lungs manifest deficits in response to the anti-fibrotic cytokine, PGE2 [150], and the mechanism mediating PGE2 "resistance" has been linked to decreased expression of the PGE2 receptor, EP2, by IPF myofibroblasts [235].This is partially due to hypermethylation of the promoter region for the EP2 receptor, which leads to decreased EP2 expression [236].

Just as AECs demonstrate age-related changes that can contribute to the propagation of tissue fibrosis, resident lung fibroblast behavior can change with age. Age-related senescent fibroblasts are found in IPF lung, have increased secretion of inflammatory mediators, and can become resistant to apoptosis, thereby perpetuating the fibrotic response [216, 237, 238]. Aged-matched fibroblasts from IPF lung also have an observed increase in mitochondrial dysfunction, disrupted autophagy, and mitophagy [131, 238, 239].

Paracrine Mediators of Tissue Fibrosis

Growth factors TGF-β was one of the first cytokines implicated in the normal wound-healing response [240], and it plays a central role in the pathobiology of tissue fibrosis [241–243]. Patients with IPF have increased immuno-localization of TGF-β in epithelial cells, macrophages, and myofibroblasts in areas of active fibrosis (fibroblastic foci) [244, 245]. Inhibition of TGF-β signaling protects against fibrotic progression in experimental models of pulmonary fibrosis [241, 246, 247].

TGF-β is secreted as a latent protein that is dimerized and forms a complex with latent binding protein-1 (LTBP-1) via its latency-associated peptide (LAP) [248]. As part of this complex, it is tethered to matrix elements, such as fibrillin and fibronectin [249], and is unable to activate the TGF-β receptor on neighboring cells [250]. Activation of latent TGF-βs may occur via direct proteolytic cleavage by several proteinases, including MMP-2 and MMP-9, or via interactions with α_v-containing integrins [251]. In the lung $\alpha_v\beta_6$ integrins expressed on the surface of epithelial cells bind the LAP of the latent TGF-β complex and facilitate its activa-

tion by G-protein-coupled receptor agonists such as thrombin and lysophosphatidic acid [252, 253] via the generation of cell-mediated mechanical tension. The application of tension to the $\alpha_v\beta_6$ integrin releases TGF-β from the latent complex, thereby allowing it to interact with its cognate receptor complex on the surface of adjacent cells (such as fibroblasts) [254].

The TGF-β receptor complex is a heterodimer comprised of a TGF-β type I receptor (TGF-βR1) and a type II receptor (TGF-βR2) with TGF-βR1 having serine-threonine kinase activity. Upon activation, TGF-βR1 phosphorylates receptor-activated SMAD effector proteins (SMAD2 and SMAD3) resulting in association with the common mediator SMAD (SMAD4) and translocation to the nucleus with activation of SMAD target genes. Signaling via this pathway appears to be critical during fibrogenesis, as SMAD3 null mice are protected from experimental pulmonary fibrosis [241], and depletion of the high-affinity type II TGF-beta receptor in resident fibroblasts inhibits experimental pulmonary fibrosis [255].

TGF-β receptor activation also results in the activation of several noncanonical signaling pathways that promote myofibroblast differentiation and resistance to apoptosis. Activations of mitogen-associated kinase pathways [256] that include TGF-activated kinase [257], PTEN/PI3kinase/Akt [258], focal adhesion kinase [259, 260], the tyrosine kinase c-Abelson [261], the small GTPase rho/cytoskeletal-dependent signals [262, 263], oxidant-mediated signaling [264], and others have been identified as downstream targets of TGF-β. Activation of these pathways results in cell shape change and the regulation of gene programs mediating fibroblast phenotype and survival [265, 266].

Functionally, TGF-β results in pleotropic effects that promote a coordinated fibrotic response. Treatment of AECs with TGF-β can result in the induction of apoptosis or the induction of EMT, depending on the matrix substrate to which they are exposed [103, 113]. In fibroblasts, TGF-β results in myofibroblast differentiation [218], apoptosis resistance [266], and marked upregulation of the expression of matrix components [190, 267, 268]. Finally, TGF-β mediates the epigenetic regulation of gene expression via the induction of several microRNAs that mediate the fibrotic response, and these microRNAs are also differentially regulated in IPF and include mir-21 and let-7d [269, 270].

Lysophosphatidic acid (LPA) is a lipid-derived mediator that can be produced by platelets, membrane phospholipids, and lung surfactant [271], and LPA signals through several G-protein-coupled receptors to exert its biologic effects. In the context of pulmonary fibrosis, LPA appears to promote the fibrotic response via induction of epithelial cell apoptosis [272], increased endothelial cell permeability [173], and increased fibroblast migration [173, 273] and survival [272]. Elevated levels of LPA have been found in BAL fluid from patients with IPF, and LPA_1 receptor knockout mice are protected from the development of pulmonary fibrosis [173].

Multiple other growth factors including endothelin-1 [274], angiotensin II [275], PDGF [276], and transforming growth factor-α [277] have been identified as playing a role in the fibrotic response, are implicated in IPF pathogenesis, and may serve as targets for therapy.

Immune cells and inflammatory mediators Early studies in IPF lungs identified significant alteration in levels of several cytokines and chemokines typically involved in mediating the inflammatory response. Despite the lack of therapeutic benefit to broad immunosuppression in IPF, inflammatory cells and their associated signaling may still play a role in the pathobiology of IPF, potentially via the modulation of the fibrotic response. BAL neutrophilia is associated with a worse prognosis [278], and mRNA profiling of IPF monocytes reveals upregulation of several markers of macrophage activation [279]. Macrophage populations appear to be important in mediating the wound-healing response, in part by contributing soluble factors to fibroblast activation [145]. The inflammatory cytokines, TNF-α and interleukin-1β (IL-1β), are both localized to epithelial cells at sites of fibrosis in IPF [167, 280] and are released by alveolar macrophages obtained from patients with IPF or asbestosis [281]. Similarly, a downstream target of IL-1β, interleukin-17A (IL-17A), is increased in the BAL fluid of patients with IPF and mediates the fibrotic response to bleomycin in a murine model [282]. Markers of the Th2 immune response, including interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13), also have been found in increased levels in the interstitium of patients diagnosed with cryptogenic fibrosing alveolitis [283]. IL-13 plays a key role in inducing Th2 responses in the lung in chronic inflammation [284], and IL-13 levels and IL-13 receptor expression correlate with disease severity [285].

Chemokines play a role in IPF via the recruitment of monocytes, leukocytes, and fibrocytes to the injured lung, and chemokines are involved in the angiogenic remodeling that occurs in fibrotic lung disease. CCL-12 and its receptor CXCR4 are strongly implicated in fibrocyte recruitment to the lung [200] along with MCP-1 and its receptor CCR2 [286]. Macrophage inflammatory protein-1α (MIP-1α) and MCP-1 are increased in tissue and BAL [287–292] in human IPF and likely participate in macrophage recruitment, which can amplify the fibrotic response via production of profibrotic cytokines and recruitment of additional inflammatory cells via chemokines [293]. Production of CCL-18 by macrophages has also been implicated in the progression of pulmonary fibrosis, and circulating levels of CCL-18 correlate with survival in IPF [294]. Conversely, macrophages may facilitate resolution of the fibrotic response via phagocytosis of apoptotic cells and the production of matrix metalloproteinases [284]. Alternatively activated macrophages, which represent the majority of macrophages in IPF lungs, may play a role in this process, as depletion of this cell cohort attenuates the fibrotic response in bleomycin-induced pulmonary fibrosis [295].

Several unbiased assessments of genetic alterations in pulmonary fibrosis have renewed interest in derangements in innate immune signaling in IPF. TOLLIP is a key signaling component of the innate immune system and has a single nucleotide variant (rs5743890) that is associated with both susceptibility to developing IPF and worse outcome [64]. In addition, polymorphic variants in Toll-like receptor 3 and 9 have also been identified as risk factors for IPF progression [296, 297]. The innate immune system may play an important role in the response to injury via recognition of danger- and pathogen-associated molecular patterns (DAMPs and PAMPs) by

pattern recognition receptors. Alteration of the lung microbiome in IPF could be an important source of PAMPs with subsequent activation of the innate immune system and increased mortality risk [81, 298], while cell debris, collagen fragments, and mitochondrial DNA may play a role as DAMPs in the injured and fibrotic lung.

With respect to the adaptive immune system, reduction in the T-cell regulatory genes, CD28, ICOS, and the tyrosine kinases LCK and ITK is predictive of poor outcome in IPF [299, 300]. GWAS-based studies have identified an association between the human leukocyte antigen (HLA) region and the development of fibrotic idiopathic interstitial pneumonias [301]. A recent proteomic analysis of IPF lung identified the presence of a plasma B-cell population that was not seen in normal lung [302].

Despite the abundance of inflammatory mediators and immune cell types that have been implicated in IPF pathogenesis, much work remains to be done to determine how these varied pathways intersect with other components of the fibrotic process. An improved understanding of these interactions may allow for a more rational approach to targeting these pathways for therapeutic benefit in the future.

Tissue Remodeling and Failure to Resolve the Wound

Angiogenesis, the formation of new blood vessels, is an important component of the wound-healing response in several tissue beds. In dermal wounds the angiogenic response potentiates the influx of inflammatory mediators that participate in the tissue remodeling process. Insofar as the pathobiology of IPF is an extrapolation of many of the mechanisms that mediate other forms of wound healing, it would not be surprising to detect an angiogenic response. Indeed, pathologic evaluation of the IPF lung has demonstrated areas of neovascularization as well as the presence of pulmonary-systemic anastomoses that are often seen in a subpleural location [303]. Additionally, circulating levels of the angiogenic cytokines, interleukin-8 (IL-8/CXCL8) and endothelin-1, are elevated in patients with IPF compared to normal controls and correlate with disease progression [304].

However, there is significant spatial heterogeneity of neovascularization and vascular density in IPF tissue biopsies when compared to normal lungs. When carefully quantified using endothelial cell markers, the level of neovascularization present within an area of IPF lung is inversely correlated with degree of parenchymal fibrosis in that area [305–307]. Furthermore, complete vascular obliteration is often seen in areas of dense parenchymal fibrosis. Most often, areas of neovascularization are present adjacent to intact AECs, suggestive of an angiogenic response that attempts to reestablish the normal alveolar/capillary interface [306]. This suggests significant spatial heterogeneity to the angiogenic response in IPF with areas of angiogenic signaling alternating with areas defined by angiostatic signaling.

Corroborating these observations, the angiostatic cytokine, endostatin, was found to be elevated in the serum of IPF patients [308], while serum levels of vascular endothelial growth factor (VEGF) have been observed to be decreased.

Clarifying the issue significantly, it has been shown that local VEGF expression is absent in areas of dense fibrosis, while the angiostatic protein, pigment epithelium-derived factor (PEDF), which is a VEGF antagonist, has increased expression in the fibroblastic foci of IPF lungs [309]. PEDF is a TGF-β target gene, suggesting that the local environment of the fibroblastic focus is characterized by an angiostatic environment. Whether the angiostatic environment of areas of fibrosis is cause or consequence of the fibrotic response is unclear. Similarly, the role of the scattered areas of neovascularization in adjacent lung tissue remains undetermined.

Role of matrix remodeling on progression of fibrosis The normal matrix environment is maintained by the constant and tightly regulated control of cell activation, matrix production, and extracellular matrix (ECM) proteolysis in order to maintain "normal" lung architecture [5]. As tissue fibrosis proceeds, matrix organization is severely altered with increased accumulation of multiple matrix components that include extra domain-A (EDA) fibronectin, hyaluronic acid, and collagen isoforms. In response to TGF-β, other growth factors, and environmental cues, collagen synthesis is induced and secreted by fibroblasts and myofibroblasts. Collagens are secreted as a soluble promolecule that subsequently self-assembles to form insoluble collagen fibrils that are relatively resistant to degradation by proteases [168]. Studies of the collagen content of IPF lungs have demonstrated that collagen III is the primary component in areas of alveolar septal fibrosis with collagen I predominating in areas of mature fibrosis [310, 311].

Extracellular matrix turnover is tightly regulated by several families of proteinases and their respective inhibitors [22]. Matrix metalloproteinases (MMPs) comprise a family of proteinases that can target collagen and other matrix components for degradation. Given the role of these molecules in maintaining the balance of matrix molecules during normal tissue homeostasis, a defect in the balance of these factors might be expected in disorders such as IPF that are characterized by matrix accumulation. In line with this expectation, several tissue inhibitors of metalloproteinases (TIMPs) are locally expressed in pulmonary fibrosis [312], and overall collagenase inhibitory activity is elevated in IPF patients when compared to controls [313]. However, total collagenase activity is increased in IPF as well [314], and several matrix metalloproteinases including MMP-1, MMP-2, and MMP-7 have been identified as highly enriched genes in the tissue from IPF lungs [153, 315]. Interestingly, an assessment of global gene expression in IPF lungs found a strong bias toward increased protease expression, which supports a net degradative environment [316]. Given this observation, the effects of spatial localization of protease/antiprotease expression likely predominate over global assessments of protease/antiprotease "balance."

Analysis of MMP expression demonstrates the importance of spatial localization in IPF. MMP-1 is increased in IPF [153] and localizes to the alveolar epithelium [312] rather than the fibroblastic focus, where it participates in the processing of cytokines, which stands in contrast to its role in collagen fibril degradation [168]. MMP-7 (matrilysin) is a highly upregulated gene in IPF lungs when compared with

control samples, and the degree of MMP-7 elevation in BAL fluid from IPF patients correlates with survival [172]. MMP-7 also localizes to the alveolar epithelial cells [154], but it has diverse roles that are relevant to tissue remodeling, and these roles are distinct from its degrading effect on matrix proteins. In particular, MMP-7 can activate other MMPs, regulate TGF-β activation, and activate osteopontin [155, 317]. MMP-2 is a gelatinase that targets collagen IV as a substrate [318], and it is increased in the BAL fluid from IPF patients [312] and has been localized to AECs [319, 320], where it may contribute to alveolar basement membrane degradation. MMP-9 is also expressed by epithelial cells and inflammatory cells [321], has increased expression in patients with IPF [322], and has been associated with increases in endothelial permeability, neutrophil activation, and rapidly progressive disease [172, 323]. TIMPs also have differential localization with TIMP-2 predominating in the fibroblastic foci, where it may facilitate matrix stabilization and accumulation [312].

MMPs can also modify the matrix remodeling response via the cleavage of matrix proteins, which yields fragments that can act as cell signaling ligands [22]. Additionally, MMPs and TIMPs can themselves mediate profibrotic signaling via proteolytic activation of growth factors, chemokines, and shedding of membrane-associated ligands [318]. These profibrotic effects of MMPs may predominate in IPF, making inferences concerning the net effect of increased MMP expression on matrix accumulation difficult.

Matrix composition and organization plays a key role in modifying cell behavior, and dysregulation of matrix cues has been implicated in various disease states including tumor progression [324]. In the context of IPF, individual ECM components can significantly modify the response to soluble and matrix-derived mediators. For example, primary AECs cultured on fibrinogen or fibrin and treated with TGF-β will undergo EMT, while the same cells when cultured on Matrigel (collagen and laminin) and treated with TGF-β will undergo apoptosis [103]. Myofibroblast differentiation is also dependent on the presence of several matrix cues. EDA-FN is preferentially expressed in healing wounds, and its presence is required for TGF-β-induced myofibroblast differentiation [190]. Mice deficient in this isoform are protected from bleomycin-induced pulmonary fibrosis [325]. De novo expression of the matrix protein, periostin, has been implicated in the fibrotic remodeling that occurs with asthma [326]. Periostin is also highly expressed in the fibroblastic foci and serum of patients with IPF [327], and periostin-deficient mice are protected from bleomycin-induced pulmonary fibrosis [328]. Increased expression of matrix-associated proteoglycans, such as hyaluronic acid, participates in the fibrotic process, which likely occurs via recruitment of inflammatory cells and the facilitation of fibroblast migration through cognate receptors such as CD44 [329]. Thus, while the in vivo details of matrix-dependent signaling are currently lacking, it is likely that altered expression of these and other matrix components facilitate and perpetuate the fibrotic response in IPF.

Incorporation of new matrix elements is not merely a result of haphazard matrix protein accumulation but proceeds in an orderly fashion [21]. Newly synthesized fibronectin is desolubilized by integrin-mediated incorporation [106] and serves as

a scaffold for collagen and other matrix protein deposition [330]. Newly deposited collagen and elastin are cross-linked via the action of tissue transglutaminases and lysyl oxidases [331], which increases tissue stiffness. In IPF, lysyl oxidase 2 (LOXL2) is increased in the fibroblastic foci. However, directly inhibiting its activity using a monoclonal antibody did not prevent progression of IPF. Similarly, tissue transglutaminase 2 expression and activity is upregulated in IPF, and germline knockout of this protein prevents the development of experimental pulmonary fibrosis [332].

Alterations in the biomechanical characteristics of the ECM during fibrosis, such as increased tissue elasticity (stiffness), can independently modify cell behaviors and phenotype determination. Tissue stiffness is quantified by its shear modulus, which is typically determined via atomic force microscopy [333]. Careful determinations have demonstrated that normal lung tissue has a shear modulus of 0.5 kPa, whereas the median shear modulus in fibrotic lung increases to 6 kPa [334]. However, significant spatial heterogeneity of tissue stiffness exists within the fibrotic lung with uninvolved areas retaining a near normal shear modulus but areas of dense fibrosis having a shear modulus that surpasses 15 kPa.

All cell types likely sense and respond to alterations in the biomechanical features of the matrix [335]. The development of tension across a healing wound modifies myofibroblast differentiation [336, 337], and release of this tension leads to the induction of myofibroblast apoptosis [338]. Similarly, stiff matrices induce fibroblast to myofibroblast transition [339, 340], which is accompanied by the augmentation of matrix protein expression [334]. The development of matrix tension and stiffness also modifies cellular responses to TGF-β. TGF-β bioavailability is directed and modified by the transmission of tension to its associated LTBP via α_v-containing integrins [341, 342]. Therefore, myofibroblast differentiation induced by soluble TGF-β requires the development of matrix-derived tension across the cell [343, 344].

Functionally, increases in matrix stiffness that mimic fibrotic lung result in augmentation of traction forces by lung fibroblasts in response to TGF-β, whereas normal matrix stiffness does not [345]. Epithelial cells toggle their response to TGF-β stimulation that is dependent on the matrix stiffness of their environment, undergoing apoptosis on low-stiffness substrates but EMT on high-stiffness substrates [346]. Some matrix stiffness-dependent effects on cells may be durable, as fibroblasts retain the "programmed" behavior imparted by culture on a stiff matrix, even after subsequent prolonged culture on matrix with "normal" stiffness [347]. Similarly, adoptive transfer of lung fibroblasts from patients with pulmonary fibrosis induces the development of fibrotic lung lesions in mice, while those from normal lungs do not [297, 348]. The acquisition of these durable aberrant behaviors from the matrix environment may be due to epigenetic "programming," although this has not been formally demonstrated as of yet.

These observations strongly suggest that the ECM and its cellular constituents participate in a reciprocal signaling fashion during fibrosis that provides a "feed-forward" mechanism that promotes progression of fibrosis. How matrix-derived signaling varies between fibrosis and normal wound healing remains an open area of inquiry.

New Directions and Targets for Therapy

Current strategies to stop IPF progression rely heavily on the mechanistic understanding of fibrosis that has been reviewed thus far (Table 7.1). However, only two therapies (nintedanib and pirfenidone) have had successful Phase III trials that have led to FDA approval for therapy (see Chap. 13). Many additional drugs have been investigated that target known mechanisms of fibrosis, but to date these therapies have either failed to attain the desired endpoint or are still in the developmental pipeline (Table 7.1). New advances in the understanding of IPF pathogenesis will be essential for the next generation of therapies to be developed. The past decade has seen the advent of the use of unbiased GWAS investigations as well as studies of RNA profiles (mRNA, splice isoforms, microRNA, long noncoding RNA), protein expression (proteomics), epigenetic alterations (epigenomics), and metabolic alterations (metabolomics) in human samples from patients with IPF. These analyses have provided investigators with powerful new tools that facilitate pathway discovery for complex disorders such as IPF. Single-cell sequencing has more recently

Table 7.1 Mechanistic targets of therapy for IPF

	Drug		
Mechanistic target	Ineffective	Under evaluation (phase II/III)	Effective
Unknown			Pirfenidone
Receptor tyrosine kinases (VEGFR, PDGFR, FGF)			Nintedanib
Receptor Tyrosine Kinases (PDGFR, DDRs, c-Kit, c-Abl)	Imatinib		
Immune response	Prednisone Azathioprine		
Antioxidant	N-Acetylcysteine		
Coagulation	Warfarin		
TNF-α	Etanercept		
Interferon γ-1b	Interferon γ-1b		
Endothelin-1 receptor	Bosentan Ambrisentan		
LPA1 receptor		BMS-986020	
Autotaxin		GLPG1690	
Interleukin-13	Tralokinumab	Lebrikizumab	
CTGF		Pamrevlumab	
Avβ6 (via TGF-β release)		STX-100	
LoxL2 (matrix cross-linking)	AB0023		
Serum amyloid P (macrophage function)		PRM-151	
Fatty acid receptors (GPR84, GPR40)		PBI-4050	
Microbiome		Co-trimoxazole	

emerged and holds the promise of dramatically improving the resolution of the changes that occur in the underlying cell populations during the induction and progression of pulmonary fibrosis, and this novel technique may inform the discrete function of gene expression changes that are associated with IPF pathogenesis. When coupled with the various mechanistic investigations, these methodologies have opened the door for biomarker development and novel approaches to therapy.

RNA expression profiling Initial investigations of RNA expression focused on mRNA to determine how global expression profiles differed between IPF and normal lung as well as between IPF and other forms of interstitial lung disease (ILD). These studies found increased gene expression of matrix metalloproteinases, developmental signals, adhesion proteins, extracellular matrix proteins, and muscle-related proteins present in IPF lungs, when compared to normal lungs or other ILDs [106, 153, 154, 349]. Subsequent global analysis of the cumulative datasets demonstrated that WNT and TGF-β signaling pathways are highly enriched in IPF lungs [28]. Moreover, recent work has uncovered differences in mRNA expression profiles between sub-phenotypes of IPF and demonstrated distinct patterns of gene expression in IPF patients with secondary pulmonary hypertension [350], those with more progressive IPF [351–353], and patients with acute exacerbations of IPF [354].

In addition to providing insight into disease pathogenesis, a major potential use of gene expression profiling is the development of diagnostic, prognostic, and disease activity biomarkers. Several candidate biomarkers have been identified [355], but validation of these approaches and translation to clinical practice remains a future goal.

Proteomics Several studies have now been completed that have used comparative proteomics to examine lungs from patients with IPF versus lung tissues from untransplanted human donor lungs and found evidence of DNA damage stress responses, UPR, and upregulation of heat-shock proteins in the IPF lung [356, 357] Additionally, deep proteome profiling identified a unique B-cell type that was only present in IPF lungs [302]. Proteomic analysis has also been performed on peripheral blood plasma and identified alterations in host defense, wound healing, and protein phosphorylation in IPF samples, and these investigators were able to identify a minimal gene signature that was highly accurate in differentiating IPF patients from normal controls [358].

Epigenetic Regulation From a mechanistic perspective, the evolving understanding of epigenetic regulation of gene expression has opened a new area of investigation into the pathogenesis of pulmonary fibrosis (see Chap. 9). Epigenetic gene regulation refers to regulation of gene expression that occurs outside of changes in DNA germline coding and occurs via three main mechanisms: histone modifications, DNA methylation, and the effects of noncoding RNAs (microRNAs).

Unbiased oligonucleotide microarray screens to determine microRNA expression profiling have demonstrated that approximately 10% of microRNAs are

differentially regulated in IPF [27]. RNA expression profiles of noncoding RNA have been more recently characterized for IPF with identification of many developmentally associated microRNAs and regulators of TGF-β signaling [359, 360]. The first reports of differentially regulated microRNAs in IPF focused on let-7d [269] and mir-21 [270]. Let-7d is a microRNA that is downregulated by TGF-β and is decreased in the lungs of patients with IPF [269]. Let-7d is localized to the alveolar epithelium in normal lungs, and it is involved in the regulation of EMT [269]. Mir-21 expression is induced by TGF-β, and elevated levels of mir-21 are found in the lungs of IPF patients as compared to controls [270]. In contrast to let-7d, mir-21 localizes to myofibroblasts and is known to mediate many of the effects of TGF-β including the regulation of PTEN expression [361]. Mir-29 is downregulated in pulmonary fibrosis [362], and it is also responsive to TGF-β and matrix-derived mechanical cues [363].

Investigators are now beginning to use transcriptional profiling of individual cell populations using single-cell RNA sequencing, which has now been used to begin to characterize the cell population differences between normal and IPF lung. This technique has identified distinct shifts in the gene expression profiles of epithelial cell populations in fibrotic lung and provided evidence of activation of TGF-β, HIPPO/YAP, WNT, and AKT signaling localized to this compartment [96]. Forthcoming studies looking at other cell populations (immune, mesenchymal, etc.) should provide new insights into cell lineage-specific gene expression changes that are associated with pulmonary fibrosis.

An alternative mode of epigenetic regulation occurs via acquired DNA modifications in somatic cells that can "program" gene expression and pass on this information to daughter cells. One of the most common modifications that can alter gene expression is gene silencing by methylation at CpG islands [364]. Hypo- and hypermethylation of critical genes have been implicated in the development of cancers [365], but only limited investigations have been published in tissue fibrosis until recently. In the context of pulmonary fibrosis, widespread alterations in epigenetic patterning are present in IPF [366, 367], and there is upregulation of DNA methyltransferase 3a in the hyperplastic epithelium of IPF lung [367]. Several fibroblast-related genes exhibit hypermethylation and silencing in fibrosis, including the prostaglandin E2 receptor (PTGER2) [236], IP-10 [368], and Thy-1 [369], while the α-SMA promoter is hypermethylated at several CpG islands in epithelial cells, but decreased methylation was found in fibroblasts [370].

These results suggest that IPF is characterized by severe derangements in the regulatory control of gene expression, and much work remains to be done to achieve an understanding of the origins and implications of many of these observations on the mechanism of disease pathogenesis in IPF. However, several of these technologies have exciting therapeutic and diagnostic potential. The identification of key gene expression profiles may lead to the development of individual biomarkers or gene sets that may obviate the need for a surgical lung biopsy, allow for more precise identification of IPF sub-phenotypes, and identify patients at high risk for disease progression [299, 353, 371]. The identification of key microRNAs involved in IPF may allow for a novel mode of targeting deranged signaling in IPF, as a single

microRNA can target many different genes from divergent signaling pathways. Implementation of this strategy will require a more detailed understanding of the relationship of the downstream signaling pathways along with the development of drug delivery technology that utilizes this novel mode of targeting.

Summary

IPF is a disorder characterized by the presence of extensive alveolar epithelial cell injury accompanied by a robust, non-resolving wound-healing response. Robust investigations in familial cohorts of patients with FPF and sporadic IPF patients strongly suggest that genetic AEC susceptibility to injury (telomeres, MUC5B, etc.) compounded by age-related changes set the stage for exogenous stimuli to chronically injure the AEC, thereby initiating the fibrotic response. AEC injury and failure to reepithelialize the airspace perpetuate the fibrotic response in concert with TGF-β activation and WNT signaling. The reparative response in IPF is characterized by activation of the coagulation cascade, formation of a provisional matrix, and expansion of local progenitor populations for fibroblasts, all of which lead to myofibroblast populations that comprise the fibroblastic focus. Deposition and remodeling of ECM yield a stiffened, fibrotic matrix that feeds forward to perpetuate the fibrotic response. Myofibroblasts of the fibroblastic focus show evidence of resistance to apoptosis, senescence, and metabolic derangements that likely impair the normal resolution of the fibrotic response. Alterations in immune cell phenotype and function also contribute to abnormal repair.

While an effective therapy for IPF remains elusive, approaches to therapy have begun to evolve toward targeted therapies directed at the putative growth factors, receptors, and enzymes for which robust evidence for mechanistic involvement in matrix remodeling has evolved. New approaches that encompass high-throughput but also allow high-resolution assessments of the cell heterogeneity and gene expression in IPF should help identify additional targets for therapies that can halt fibrotic progression and promote reparative responses that restore tissue integrity and function.

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