Animal Models of Fibrotic Lung Disease

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Interstitial lung fibrosis can develop as a consequence of occupational or medical exposure, as a result of genetic defects, and after trauma or acute lung injury leading to fibroproliferative acute respiratory distress syndrome, or it can develop in an idiopathic manner. The pathogenesis of each form of lung fibrosis remains poorly understood. They each result in a progressive loss of lung function with increasing dyspnea, and most forms ultimately result in mortality. To better understand the pathogenesis of lung fibrotic disorders, multiple animal models have been developed. This review summarizes the common and emerging models of lung fibrosis to highlight their usefulness in understanding the cell–cell and soluble mediator interactions that drive fibrotic responses. Recent advances have allowed for the development of models to study targeted injuries of Type II alveolar epithelial cells, fibroblastic autonomous effects, and targeted genetic defects. Repetitive dosing in some models has more closely mimicked the pathology of human fibrotic lung disease. We also have a much better understanding of the fact that the aged lung has increased susceptibility to fibrosis. Each of the models reviewed in this report offers a powerful tool for studying some aspect of fibrotic lung disease.

Keywords: fibrosis; collagen; fibroblast; aging; cytokines

CLINICAL RELEVANCE

This review summarizes common and recently developed animal models of lung fibrosis. It highlights the pathologic features of these models, and will aid basic science and translational science investigators in choosing the most appropriate model system to be used for their own fibrosis research.

Interstitial lung disease is often associated with the development of chronic fibrosis. These diseases are characterized clinically by progressive dyspnea, cough, restrictive physiology, and impaired gas exchange. Humans manifest many types of fibrotic lung disease (1). Among the diffuse parenchymal lung disorders (DPLDs) are diseases of known cause (e.g., drug-related, environmental exposures, or those associated with collagen vascular disease), the idiopathic interstitial pneumonias (IIPs), the granulomatous DPLDs (e.g., sarcoidosis), and rare noncategorized diseases, such as lymphangioleiomyomatosis. Idiopathic pulmonary fibrosis (IPF) is the most common disease within the category of IIPs, and is histopathologically identified as usual interstitial pneumonia (UIP). Additional diseases within the IIP category include desquamative interstitial pneumonia, respiratory bronchiolitis interstitial lung disease, acute interstitial pneumonia, cryptogenic organizing pneumonia, lymphocytic interstitial pneumonia, and nonspecific interstitial pneumonia (NSIP). IPF carries a poor prognosis, with a mean survival time of less than 5 years after diagnosis (2–5). Biopsies from a single patient can show heterogenous patterns consistent with both UIP and NSIP (4, 6, 7), suggesting that NSIP shares common pathogenic mechanisms with UIP.

Diagnoses of patients with IPF who do not exhibit classic high-resolution computed tomography scan changes are confirmed by histopathologic evaluations of surgical lung biopsies, which demonstrate the pattern of UIP. Hallmark features of UIP include epithelial cell hyperplasia, basement membrane denudation, alveolar consolidation, and fibroblastic foci in a pattern that is spatially and temporally heterogeneous (8). Attempts to understand the pathogenesis of IPF and other fibrotic lung disorders have relied on animal models. Unfortunately, no animal model exhibits progressive disease, and most animal models do not fully recapitulate the histologic pattern of UIP or other interstitial lung diseases. This, however, does not mean animal models are dispensable for research in pulmonary fibrosis. Traditional animal models of lung fibrosis have generated important insights into the pathobiology of lung injury, inflammation, and fibroproliferation (9). In addition, animal models have recently been refined to reflect better the known pathogenic mechanisms in IPF. Although it is appreciated that the spontaneous development of fibrosis in other species (e.g., cats, horses, and dogs) (10–12) can be instructive for comparative medicine, the most tractable models for studies of pathogenesis involve rodents. Many traditional and newly developed models of experimental lung fibrosis offer opportunities to study cell–cell interactions and the soluble mediators driving pathologic fibrotic remodeling. A better understanding of these pathways in experimental models provides a critical approach to identifying novel targets to assess and validate in human studies. We will review several established and newer models of lung fibrosis, and highlight particularly useful aspects of each. We include environmental and genetic models because these offer insights into the development of fibrosis by known causes, and also shed

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light on the pathogenesis of intractable fibrotic diseases such as IPF. In addition, we include discussions on animal models of interstitial fibrosis secondary to specific risk factors for acute lung injury (ALI) and acute respiratory distress syndrome (ARDS).

ANIMAL MODELS REPLICATING IPF

Asbestosis

Asbestosis continues to be an important fibrotic lung disease in exposed humans. Human asbestosis and IPF are similar in their gross distribution, and share a common UIP histopathology (13, 14). In terms of their main difference, asbestosis involves fewer myofibroblastic foci than does IPF, and this may explain the slower progression of asbestosis compared with IPF (14). Asbestosis is distinguished from IPF by histologic findings of asbestos bodies in the lungs, indicating the cause of disease. These features are largely recapitulated in inhalation models in animals, and these models are useful for understanding the pathogenesis of both asbestosis and UIP. An intratracheal instillation of asbestos fibers promotes the rapid development of fibrosis with a single administration. One caveat about intratracheal models involves the distribution between lung lobes, which can be uneven, and the pattern of fibrosis tends to be central rather than subpleural, as is more prominent in the inhalation model. However, the ability to visualize asbestos fibers histologically can confirm an appropriate deposition, and can be used to locate areas where fibrosis is likely to develop. As indicated, inhalation models develop a more peripheral pattern of disease, but they require special inhalation chambers and the time for disease development can be long, especially if using nonamphibole (chrysotile) fibers. All asbestos should be baked before *in vivo* instillation to destroy common contamination by lipopolysaccharide. The intratracheal models with amphibole fibers show fibrosis by Day 7, which is mature by Day 14 (Figure 1). Inhalation models may take over a month to develop fibrosis. The fibrosis that does develop is persistent and may be progressive with large enough dosing, especially when using amphibole fibers. The deposition of asbestos fibers specifically induces oxidative stress and alveolar epithelial cell injury (15). Macrophages, lymphocytes, eosinophils, and neutrophils have also been implicated in promoting injury in these models. Murine asbestosis comprises one of the few animal models to develop fibrotic foci, and therefore is particularly useful for understanding the development of this pathologic lesion. Table 1 summarizes the pathogenic features that are especially well studied, using the asbestos model and other models of lung fibrosis.

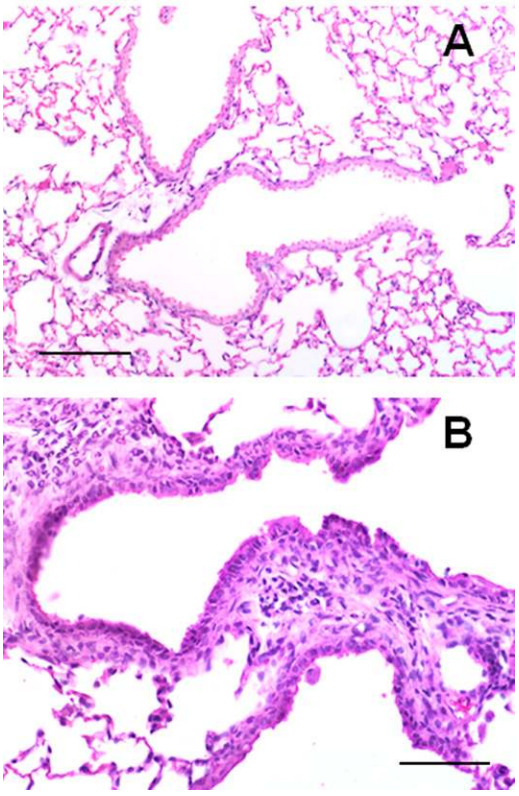

Figure 1. Asbestos induced pulmonary fibrosis. (*A*) Control lung shows normal terminal bronchi and alveolar parenchyma. (*B*) Intratracheal instillation of crocidolite asbestos induces robust peribronchial fibrosis with extension into the adjacent alveolar parenchyma (14 d after exposure). *Scale bars* = 50 μm.

Silica

The instillation of silica into murine lungs results in the development of fibrotic nodules that resemble simple silicotic nodular fibrosis, which develops in humans after some occupational exposures (16), but are generally more cellular than the fibrotic nodules seen in humans. Silica can be delivered to rodents via aerosolization (17), intratracheal administration (18), or oropharyngeal aspiration (19). The fibrotic response to silica instillation is strain-dependent. C3H/HeN, MRL/MpJ, and NZB mice are all susceptible in the aerosolized model, whereas Balb/c mice show little response (17). Similarly, C57Bl/6 mice are more susceptible than CBA/J mice after intratracheal silica (20). Silica is retained in the lung, and the response is characterized by a persistent, toxic, and inflammatory response. Fibrotic nodules develop around silica deposits, and silica particles are easily identified both histologically and by polarization microscopy. The intratracheal models are easier and more cost-efficient, whereas inhalation models more closely mimic human exposure but take longer to develop (40–120 d) (18) than the intratracheal models (14–28 d) (19). Care must be exercised when choosing formulations of silica particles, because different formulations vary widely in their potency of stimulating fibrotic responses. Silica should be baked before *in vivo* instillations, to destroy contamination by lipopolysaccharide. One important feature of silicosis involves the strong influence of macrophage NACHT, LRR, and PYD domains-containing protein 3 (NALP3) inflammasome activation (21), making it an important model for studying the innate immune regulation of lung fibrotic responses. Figure 2 demonstrates the histopathologic findings in the silica model.

Bleomycin

Among currently applied models of experimentally induced pulmonary fibrosis, the administration of bleomycin is used most frequently (9, 22). The recognition that bleomycin could result in pulmonary fibrosis in humans led to its use in experimental models, and for four decades it has been the most commonly applied model of experimental lung fibrosis. It has been used in multiple animals, including mice, rats, guinea pigs, hamsters, rabbits, dogs, and primates, but mice are most common (9, 23). Furthermore, it has been delivered by multiple methods, including intratracheal, intraperitoneal, subcutaneous, intravenous, and inhalational (9, 23). Although each method of delivery has its own strengths and weaknesses, the intratracheal delivery of bleomycin has emerged as the most frequent route (22). Whatever the route of administration, the delivery of bleomycin results in direct cell

TABLE 1. PATHOGENIC MECHANISMS IN ANIMAL MODELS OF LUNG FIBROSIS

Model	Pathologic Features
Asbestos	Epithelial cell injury
	Fibroblastic foci
	Macrophage oxidative stress
	Ability to visualize fiber deposition
Silica	Ability to visualize particle deposition
	Macrophage NALP3 inflammasome activation regulates disease development
Bleomycin	Direct cell injury via DNA damage
	Initial site of injury can be determined via method of delivery (intravenous or intraperitoneal vascular endothelium, intratracheal alveolar epithelial cells)
	AEC hyperplasia in repetitive dosing model
	Resolution in single-dose model
FITC	AEC injury
	Vascular leak
	Ability to visualize injured areas of the lung
Age-dependent fibrosis	Epithelial stress in response to injury
	Fibroblasts in aged lungs are poised to respond well to TGF-β signaling
	Fibroblast loss of Thy-1, but gain of EDA-fibronectin
	Natural infections with herpesvirus cause fibrosis in aged, but not young, mice
TGF-β overexpression	AEC and airway epithelial cell injury
	Epithelial–mesenchymal transition
	Fibrosis develops in absence of significant inflammation
Cytokine overexpression	Opportunities to study cross-regulation of the cytokine with TGF-β activation and responsiveness
Familial IPF models	AEC ER stress responses
	Opportunities to study "two-hit" models of pathogenesis
Targeted Type II AEC Injury	Directed injury of Type II AECs
	Allows studies of how other cell types respond to directed Type II AEC injury
	Pattern of fibrosis involves interstitial rather than alveolar consolidation
Direct forms of lung injury (acid/hyperoxia)	Allows studies of hypoxemia, permeability injuries, and effects of hyperoxia
	Models fibroproliferative changes seen with ALI and ARDS
Radiation	AEC injury
	Vascular remodeling (reminiscent of pulmonary arterial hypertension)
	Mesenchymal stem cells regulate repair responses
Humanized mouse models	Opportunity to study phenotype of human IPF fibroblasts *in vivo*
	Studies of how fibroblast-autonomous alterations affect other lung cell types are possible
	Currently does not allow studies of immune cell regulation of disease development

Definition of abbreviations: AEC, alveolar epithelial cell; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; EDA, extra domain A containing fibronectin; FITC, fluorescein isothiocyanate; IPF, idiopathic pulmonary fibrosis; NALP3, NACHT, LRR, and PYD domains-containing protein 3; TGF-β, transforming growth factor–β; Thy-1, thymocyte differentiation antigen 1.

injury through the induction of DNA strand breaks, the generation of free radicals, and the induction of oxidative stress (24). Cell necrosis and/or apoptosis follow, with subsequent inflammation and the development of fibrosis (9). When delivered systemically (intravenously, intraperitoneally, or subcutaneously), the initial site of injury is the pulmonary vascular endothelium, which is thought to reflect similar processes at play in humans affected by bleomycin-induced pneumonitis (25). With this initial endothelial cell damage, the drug can then gain access to the alveolar epithelium to induce damage. In contrast, with lung-specific delivery, primary alveolar epithelial cell (AEC) injury from bleomycin is the inciting event (9, 22, 26). The delivery of bleomycin directly to the airways can be accomplished by direct intratracheal injections after surgical neck cutdown (27, 28), by injections of dry powder (29), or by endotracheal intubation, which allows for repeated dosing. One repeated dosing regimen administered 0.04 of a unit of bleomycin every other week for eight doses (30). This route of delivery offers the advantage of mimicking a repetitive or chronic injury with more robust fibrosis noted than with single doses. Neutrophilic inflammation is attenuated later in the repetitive dosage model, compared with single dose. AEC hyperplasia is prominent in areas of fibrosis, and fibrosis remains prominent (as does AEC hyperplasia) 20 to 30 weeks after the final bleomycin dose in the eight-dose, every-other-week model (22, 30).

For the systemic delivery of bleomycin, a common intravenous dosing regimen administers 20 mg/kg twice weekly for 4 to 8 weeks, resulting in initial endothelial cell injury followed by epithelial cell injury, inflammation, and fibrosis. Fibrosis is first noted by Week 4, with progression through Week 12 for mice treated for 8 weeks (31). For intraperitoneal injections, one dosing regimen administers bleomycin at 0.035 U/g twice weekly, and lungs are subsequently harvested 33 days after the first dose of bleomycin (32), a time point at which fibrosis is prominent. Another subcutaneous dosing regimen administers 0.05 mg bleomycin three times a week for 4 weeks (33). Bleomycin can also be delivered via miniosmotic pumps, providing another means of subcutaneous delivery (34).

Bleomycin can induce fibrosis in a relatively short time period (2–4 wk in an intratracheal model, and 4–12 wk in a systemic delivery model). The histopathology is not fully consistent with UIP, and by most accounts, the single-dose model is thought to resolve over time, although reports are conflicting (26, 35–39). Because the bleomycin model resolves in some cases, this offers an opportunity to study the natural resolution of fibrosis. The response to bleomycin is strain-dependent, with C57Bl/6 mice demonstrating more susceptibility than Balb/c mice. The inflammatory component is considerable within the first week. Interventions that interrupt the inflammatory phase are often protective (24). It is recommended that when using the bleomycin model to study the effects of an antifibrotic therapeutic agent, it is best if the therapy is delivered after the inflammatory phase (at least 7 d after bleomycin) for assessment (24). Multiple cell types have been shown to be involved in the development of bleomycin-induced fibrosis, including Type I and Type II AECs, fibroblasts, myofibroblasts, fibrocytes, macrophages, lymphocytes, neutrophils, endothelial cells, pericytes, airway epithelial cells, and

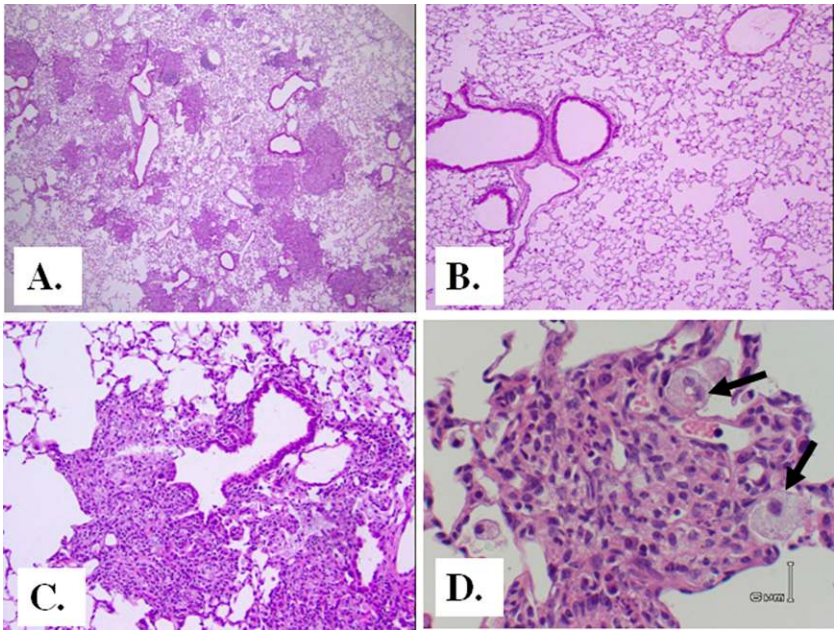

Figure 2. Intratracheal silica injury leads to multinodular fibrosis (*A*), predominantly in the alveolar parenchyma and terminal bronchioles (*C*). Activated macrophages are predominant in these lesions (*D*, *arrows*). (*B*) Control lung section.

stem/progenitor cells. Among the major advantages of repetitive dosing models, the site of initial injury can be controlled via the delivery route, and the histopathologic changes are similar to those in human IPF (22). Figure 3 demonstrates the typical histologic patterns seen after various routes of bleomycin administration.

Fluorescein Isothiocyanate

Fluorescein isothiocyanate (FITC) has also been used as a model of lung injury leading to fibrosis (40). The intratracheal delivery of FITC to the lungs results in alveolar and vascular permeability, culminating in lung fibrosis within 14 to 21 days (9, 41). In what is perhaps the most useful aspect of the model, FITC conjugates to parenchymal proteins and remains persistently localized to the areas of initial injury. This allows investigators to use immunofluorescence to localize areas of injured lungs (9). Fibrosis has been shown to correlate closely with areas of FITC deposition (9), as shown in Figure 4. In terms of pathogenesis, FITC is associated with acute lung injury and the development of edema and inflammation (including neutrophils), followed by the development of fibrosis. Doses ranging from 0.007 mg per gram body weight dissolved in PBS to an intratracheal delivery of 50 μl of a 1.4-mg/ml solution have been used in mice (28, 40, 42). Advantages of the model include a robust effect in both Balb/c and C57Bl/6 mice and durable fibrotic response, lasting for months (9). The FITC model has been shown to be dependent on Th2 cytokines (IL-13) (43), and is also regulated by the

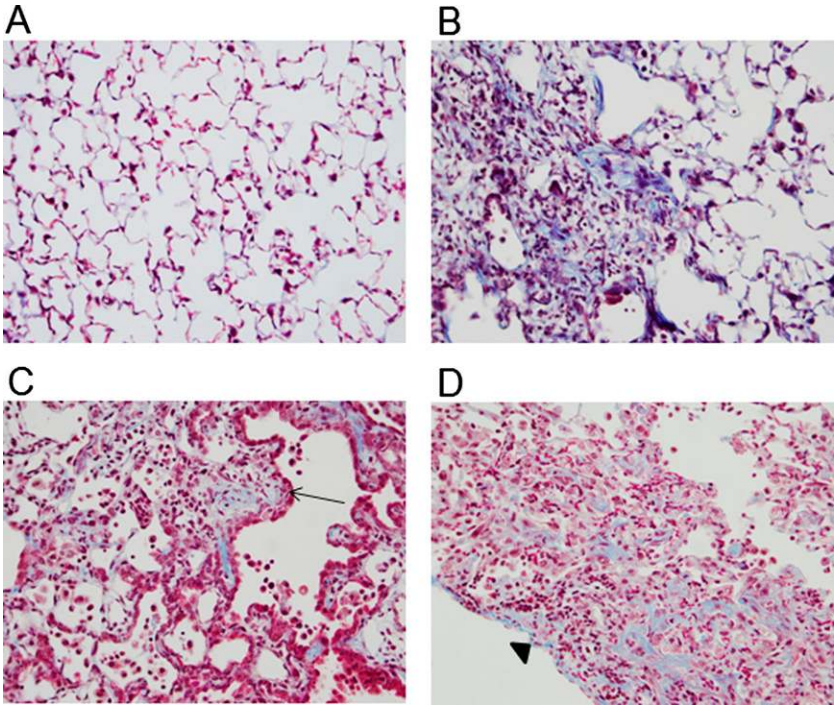

Figure 3. Bleomycin can be administered in different manners to induce lung fibrosis in mice. (*A*) Trichome blue–stained lung sections from normal wild-type C57BL/6 mice. (*B*) Lung section at 3 weeks after 0.08 of a unit of intratracheal bleomycin demonstrates the development of an area of fibrosis typically seen with this model. (*C*) Lung section from a mouse at 2 weeks after the eighth biweekly repetitive intratracheal 0.04-unit bleomycin dose. This repetitive intratracheal model not only induces prominent lung fibrosis, but also results in regions with prominent alveolar epithelial cell (AEC) hyperplasia lining areas of fibrosis. *Arrow* points to hyperplastic AECs. (*D*) Lung section from a mouse harvested on Day 33 in a twice-weekly intraperitoneal 0.035-U/g bleomycin study. With systemic delivery modalities such as intraperitoneal injections, fibrosis develops prominently in the subpleural regions. *Arrowhead* points to pleural edge. All sections, magnification, ×400.

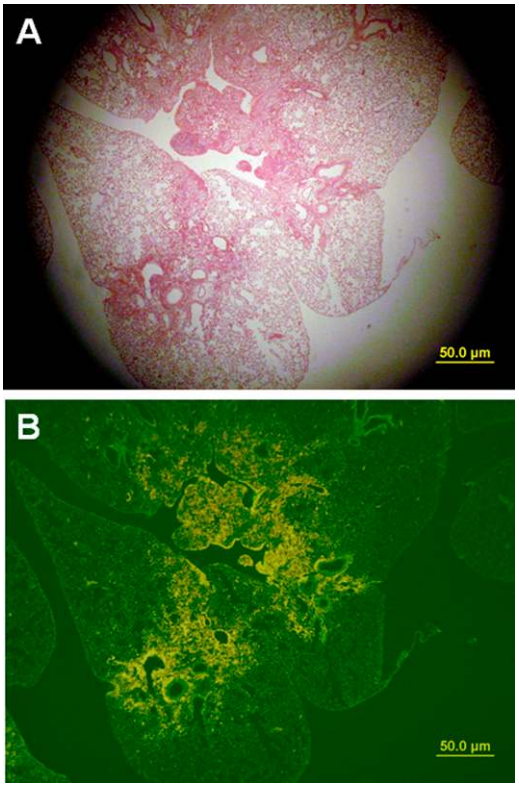

Figure 4. Fibrosis develops in areas of fluorescein isothiocyanate (FITC) deposition. Serial lung sections were prepared on Day 21 after FITC intratracheal instillation in C57Bl/6 mice. Hematoxylin-and-eosin staining shows patchy areas of fibrosis and consolidation (*A*), which line up well with areas of FITC deposition, as seen in the immunofluorescent image captured of a serial lung section (*B*).

chemokine (C-C) motif ligand (CCL12)-mediated recruitment of fibrocytes in response to the lung injury (44). This model also has its disadvantages. They include the stipulation that the solution must be made fresh each time, and its efficacy can vary, depending on the lot of FITC used and the size of the particle created via sonication. Longer periods of sonication, resulting in smaller particle sizes, can increase the acute toxicity leading to early death from lung injury (9).

Age-Related Models of Lung Fibrosis

Progress has been made in identifying models that demonstrate an age-dependent increase in fibrosis, more closely mimicking the pathogenesis of human IPF. For example, recent studies have shown that older mice are more susceptible than younger mice to bleomycin-induced injury (45), and that aged male mice may be more sensitive than female mice in the same model (46). This is especially intriguing because IPF also has a male predominance (8). Moreover, senescence-prone mice develop more severe fibrosis in response to bleomycin than do senescence-resistant mice (47). The transgenic deletion of genes for the receptor of the advanced glycation end products ($RAGE^{-/-}$ mice) (48) or relaxin ($relaxin^{-/-}$ mice) (49) results in the spontaneous age-related development of lung fibrosis. In $relaxin^{-/-}$ mice, males developed worse age-dependent fibrosis than did female mice (49). An age-dependent development of fibrosis has also been noted in murine models of Hermansky-Pudlak disease (50). Finally, the infection of aged, but not young, mice with γ-herpesvirus–68 results in the development of lung fibrosis, which is associated with epithelial cell endoplasmic reticulum stress and increased transforming growth factor–β (TGF-β) signaling in fibroblasts (51, 52). This is particularly intriguing because it suggests that a common viral infection may be able to promote the development of fibrosis in aged individuals. This could conceivably involve a fibrotic response to a new infection or to a reactivation of a previous herpesvirus infection. Interestingly, γ-herpesvirus (both as a latent and as a lytic infection) has been shown to augment the fibrosis caused by FITC or bleomycin (53, 54). Patients with IPF tend to harbor herpesviral genomes within their lung tissue, whereas this is uncommon in normal individuals (55–58). This may indicate that certain individuals are genetically inclined to harbor virus in their lung epithelium, which may lead to reactivation and fibrosis as they age. In addition, fibroblasts from aged mice display decreased thymocyte differentiation antigen 1 (Thy-1), a hallmark of human myofibroblasts (59), and lungs from aged mice display an increased expression of matrix metalloproteinase–9 and extra domain A (EDA)-containing fibronectin (45), a molecule associated with TGF-β activation and responsiveness in myofibroblasts (60). Many cellular processes involved in fibrosis likely operate differently in aged versus young lungs. As such, there is enthusiasm for promoting the use of aged mice in all of these models of lung fibrosis. Of course, in terms of the downside of this strategy, aged animals are not readily available to all investigators, and the costs associated with housing an animal until the appropriate age could be prohibitive. Another difficulty with the model involves choosing the appropriate age. With the bleomycin model, studies have been published on 24-month-old (45), 15- to 18-month-old (51), and 12-month-old mice (46) to show the differential effects of age. Additional studies are needed.

CYTOKINE OVEREXPRESSION MODELS

The overexpression of cytokines, including TGF-β, TGF-α, IL-13, TNF-α, and IL-1β, using both gene-transfer and transgenic approaches, results in lung fibrosis. We will briefly detail these important models of cytokine overexpression.

TGF-β Overexpression

TGF-β is a potent profibrotic cytokine that is elevated in most forms of lung fibrosis (61). As such, models that rely on the overexpression of TGF-β are especially relevant for dissecting the downstream signaling pathways involved in multiple cell types. The overexpression of TGF-β can be achieved by adenoviral delivery (62) or by doxycycline-regulated transgenic expression in epithelial cells (63). In the original rat models of adenoviral overexpression, significant elevations of active TGF-β were seen in the lung by Day 1, and reached a peak concentration of 13 ng/ml by Day 7. Notably, the concentration of latent TGF-β also peaked in these experiments around Day 7 at a concentration of 72.3 ng/ml, suggesting that the expression of the constitutively active transgene induced endogenous production. The expression of TGF-β in the lung was accompanied by mononuclear cell infiltration (Days 3–7), followed by the development of alveolar consolidation. Lung collagen concentrations increased 2-fold by Day 14 (62). Subsequent to these original reports, adenoviral vectors have been used to demonstrate a dose-dependent increase in fibrosis among mice. Interestingly, this model was also strain-dependent, in that C57Bl/6 mice demonstrated increased responsiveness to TGF-β overexpression, compared with Balb/c mice (64). Other features of this model include epithelial cell apoptosis and changes in soluble mediators that mimic human disease (63). Adenoviral-mediated TGF-β overexpression is also unique in that it leads to persistent scarring, which may more closely mimic the fibrotic changes

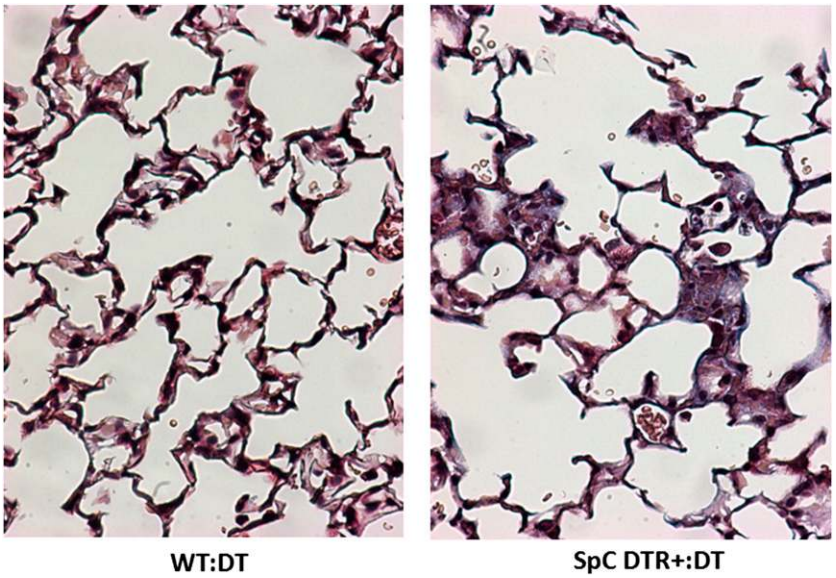

Figure 5. Targeted Type II AEC injury induced fibrosis. Trichrome-stained sections of lungs from wild-type (WT) or surfactant protein–C–diphtheria toxin receptor (SpC DTR) mice harvested on Day 28 after diphtheria toxin (DT; 100 μg/kg) treatment on Days 0–14. *Magnification*: ×400.

that occur late in IPF. In the doxycycline-inducible, club cell (Clara cell) 10 (CC10) promoter–driven model of TGF-β overexpression, the treatment of mice with doxycycline resulted in a rapid up-regulation of TGF-β (~ 1 ng/ml) within the lungs at 12 hours. Induction over the course of 2 months resulted in an approximately 2-fold increase in lung collagen concentrations (63). Although not progressive *per se*, the degree of fibrosis continued to worsen over the duration of the doxycycline exposure. In these models, the overexpression of TGF-β has been associated with airway and alveolar cell apoptosis, myofibroblast accumulation, and the induction of the epithelial-to-mesenchymal transition. Studies of the overexpression model have also elucidated the contributions of alternatively activated macrophages to disease development (65).

TGF-α Overexpression

TGF-α, similar to TGF-β, is also overexpressed in the lungs of patients with IPF (66). The overexpression of TGF-α in the lung epithelium results in lung fibrosis in rodent models (67, 68). In addition, TGF-α overexpression results in pulmonary hypertension, and therefore is instructive in elucidating the pathogenic changes of the vascular architecture. This model illustrates the importance of regulatory crosstalk between lung epithelial cells and mesenchymal cells. For example, the overexpression of TGF-α in the lung epithelium results in a persistent up-regulation of the mitogen-activated protein kinase kinase (MEK)/extracellular regulated kinase signaling pathway in lung mesenchyme (69). When the inducible expression of TGF-α is discontinued, lung remodeling is partly reversed, allowing for studies of the mediators involved in this reversal process (67).

IL-13

The lung-specific overproduction of IL-13 has been accomplished through a transgenic approach in which the IL-13 gene is constitutively expressed by the CC10 promoter. These transgenic mice develop both airway and parenchymal fibrosis (as measured by trichrome staining and Sircol assay) (70). Although the time course of collagen accumulation in this model is not well characterized, the development of airway and parenchymal scarring is mediated by an increase in TGF-β activity. The requirement for TGF-β activity in modulating the profibrotic effects of IL-13 was established by demonstrating a decrease in collagen accumulation after treatment with a soluble TGF-β receptor. Furthermore, the administration of aprotinin, a serine protease inhibitor, to IL-13–overexpressing mice decreased TGF-β activity and limited scarring (70).

IL-1β

A single administration of an adenoviral vector containing the IL-1β gene was also shown to induce lung fibrosis (71). Compared with rats treated with a control vector, the instillation of 5×10^8 plaque-forming units (pfu) of adenoviral (Ad). IL-1β resulted in a progressive increase in lung collagen, as measured by hydroxyproline from Day 21 to Day 60. The administration of the IL-1β vector also resulted in an early neutrophilic inflammatory response in bronchoalveolar lavage fluid and an increase in lung macrophages, and this influx in inflammatory cells was evident on lung histology. By Day 14, lung histology also revealed an increase in α–smooth muscle actin (α-SMA)–positive cells within fibroblast foci. Of note, IL-1β overexpression also increased TGF-β concentrations, TGF-β activity, and platelet-derived growth factor (PDGF) concentrations, suggesting they are potential downstream mediators for the profibrotic effects of IL-1β (71).

TNF-α

The adenoviral-mediated gene transfer of TNF-α has also been found to induce lung fibrosis (72). Similar to IL-1β, the overexpression of TNF-α resulted in an early inflammatory response, with an influx of neutrophils, macrophages, and lymphocytes. Although the extent of collagen accumulation was not quantified in this initial study, lung sections demonstrated an accrual of α-SMA–positive cells that were present as early as Day 7, and that had increased in number on Day 14. The gene transfer of TNF-α was also associated with an increase in TGF-β. Although this study implicated TNF-α as a profibrotic molecule, a follow-up study of transgenic mice that overexpressed this cytokine off the surfactant protein–C promoter suggested that it may exert antifibrotic properties in the setting of a second insult with bleomycin or TGF-β (73). Interestingly, in the study showing an inhibitory effect of TNF-α overexpression, concentrations of the antifibrotic mediator prostaglandin E_2 in the bleomycin-injured lungs were increased, suggesting a mechanism of protection against fibrosis. These studies point out the complex

role that TNF-α may play in both promoting and inhibiting lung fibrosis.

FAMILIAL MODELS OF IPF

Over the past decade, reports have linked cases of familial interstitial pneumonia (FIP) to mutations in four genes: surfactant protein–C (*SFTPC*) (74–76), surfactant protein–A2 (*SFTPA2*) (77), telomerase reverse transcriptase (*TERT*) (78, 79), and telomerase RNA component (*TERC*) (78, 79). Depending on the particular reports, FIP may account for anywhere from 2–20% of cases of IPF (32, 80–82). Furthermore, these four genes probably account for 15–20% of cases of FIP (83). Thus, the primary underlying genetic defects in most cases of FIP, which is most often encountered in an autosomal dominant inheritance pattern (84), have yet to be determined. In addition to searches for rare alleles underlying cases of FIP, common alleles may also be important, serving as risk factors for disease development. In 2011, a common allele in the promoter of mucin 5B (Muc5B) was found to be significantly more frequently encountered in cases of both FIP and sporadic IPF (85). In another genetic correlate of interest, some forms of Hermansky-Pudlak syndrome (HPS), an autosomal recessive disorder, lead to pulmonary fibrosis, which is often the cause of death in these subtypes (86, 87).

The promise behind an improved understanding of FIP genetics is based not only on the fact that individual genes may be important to specific families, but also that key pathways may be identified that could be important to the pathogenesis of IPF in general (83). As such, the identification of *SFTPC* mutations (as well as *SFTPA2* mutations) suggests that endoplasmic reticulum (ER) stress responses may be important in the alveolar epithelium, and indeed ER stress markers have been shown to be prominent in AECs during IPF in general, even in the absence of known surfactant protein mutations (88, 89). In a similar fashion, telomere shortening is prominent in IPF in general, even among populations without defined *TERT* or *TERC* mutations (90, 91). Thus, delineating the mechanisms at play in FIP may help considerably in improving our ability to understand the pathogenesis of IPF. As such, models based on known FIP mutations may serve as paradigms for modeling key aspects of IPF.

SFTPC

SFTPC mutations have been linked to both pediatric and adult interstitial lung disease, including individuals with UIP according to pathologic criteria, and IPF according to clinical definitions (74). Surfactant protein–C (SPC)–deficient mice in a 129/Sv strain do develop mild interstitial lung disease spontaneously, but also express an emphysematous phenotype. Thus, the findings do not recapitulate the pathology noted in human forms of the disease (92). $SPC^{-/-}$ mice in an outbred Black Swiss background have a normal lung appearance, but with enhanced fibrosis after bleomycin (26). In 2011, a model was reported in which a mutant form of *SFTPC* identified in a large FIP family (L188Q *SFTPC*) was expressed in AECs, resulting in ER stress in Type II AECs (88). ER stress alone did not cause disease, but did predispose the mice to bleomycin-induced pulmonary fibrosis, raising suspicion that ER stress leads to a vulnerable AEC population, and that a "second hit" then unmasks the fibrotic tendency (88).

SFTPA2

Mutations in one of the surfactant protein–A genes (*SFTPA2*) have been linked to cases of FIP (77), and *in vitro* modeling suggests that such mutations may cause ER stress, as is seen with *SFTPC* mutations. Specific *SFTPA2* mutations have not been modeled *in vivo*, but studies have revealed greater AEC injury/death and greater lung inflammation in SP-A–deficient mice, compared with control mice (93).

TERT and *TERC*

TERT and *TERC* mutations have been linked to cases of adult FIP and IPF (78, 79). However, no studies have recapitulated a pulmonary phenotype of increased susceptibility to lung fibrosis in these mice that might be expected, given the genetic observations (94). In the bleomycin model, whether single-dose or repetitive, a recent study revealed that despite significant telomere shortening, bleomycin-induced lung fibrosis was similar between telomerase-deficient mice (whether $TERT^{-/-}$ or $TERC^{-/-}$) and control mice (94). In contrast, a separate study presented data that telomerase deficiency was actually protective against bleomycin-induced fibrosis (95). It is hard to know exactly why these mutations are not generating a consistent phenotype in murine models, but one may speculate that these mutations will only be relevant with particular environmental cues that are not adequately modeled by bleomycin.

HPS

Some types of HPS develop pulmonary fibrosis, and this manifestation often leads to pulmonary insufficiency and death (86). HPS is characterized by trafficking defects in different cell populations, and in the lung, the Type II AEC appears to be the culprit cell. For each human form of HPS, a mouse correlate exists, and accordingly, those that are fibrosis-prone in humans also experience greater bleomycin-induced lung fibrosis (87, 96). Similar to *SFTPC* and *SFTPA2*, HPS mutations point to the role of the alveolar epithelium in disease pathogenesis, and provide suggestive evidence for a "two-hit" model of fibrotic pathogenesis. As new genetic links to disease are identified, either as rare variants in familial cases or as common variants in population studies, mouse modeling will hold promise in dissecting the pathways pertinent to disease development.

TARGETED TYPE II CELL INJURY

Because IPF is known to involve AEC injury and hyperplasia, a model was recently developed to target injury specifically to alveolar Type II cells. This model involves the expression of the diphtheria toxin receptor (DTR) under the control of a Type II AEC promoter (surfactant protein–C) (97). In this manner, the repetitive delivery of diphtheria toxin (DT, administered daily for 14 d intraperitoneally) injures AECs specifically, induces a hyperplastic proliferative response, and results in a model of interstitial thickening that bears similarity to the changes noted in IPF (Figure 5). The development of fibrosis is evident by Day 21 and persists through Day 28. At a dose of 8.0 μg/kg, DT-treated DTR-expressing mice demonstrate an approximately 2-fold increase in lung collagen content (compared with untreated mice and wild-type mice treated with DT) and a mortality rate of 25% by Day 21. Higher doses of DT increase mortality (a dose of 10 μg/kg causes 50% mortality by Day 21), without substantially exacerbating the accumulation of lung collagen. The development of a lung-scarring response is associated with weight loss and ruffled fur. This model is also interesting because a selective inflammatory response, composed mostly of lymphocyte antigen $(Ly)6C^{high}$ monocytes and exudative macrophages that exhibit a phenotype of alternative activation, is associated with the development of fibrosis (98). This model is particularly useful for studying the

downstream pathways that translate epithelial cell–specific defects into fibrosis. In this regard, targeted epithelial injury results in the significant up-regulation of plasminogen activation inhibitor–1, a known profibrotic mediator (98).

MODELS REPLICATING INTERSTITIAL FIBROSIS AS A RESULT OF DIRECT LUNG INJURY

Interstitial fibrosis as a result of severe acute lung injury (ALI)/ARDS in human subjects is a devastating complication, resulting in significant secondary pulmonary hypertension and high mortality. The development of associated multifactorial pulmonary vasculopathy in ARDS is an independent risk factor for mortality (99). The risk factors for ALI/ARDS include a multitude of direct pulmonary injuries (such as aspiration or lung contusion) and indirect factors (e.g., sepsis and pancreatitis) (100). Most of the animal models to study this phenomenon are related to direct injuries, and a few of these models will be discussed.

Acid Instillation–Associated Fibroproliferative Lung Injury

Acid instillation recapitulates many clinical features of ALI, and with certain modifications of delivery and/or recovery, rodents can survive to develop interstitial thickening. This type of model mimics the fibroproliferative phase of ARDS. For this model system to be used to study fibrosis, modifications (e.g., a fluid bolus, supplemental oxygen, and careful monitoring to be assured of surviving the procedure) are imperative because without them, the animals die of lung injury before the development of lung scarring. This model recapitulates the hypoxemia that is clinically relevant in ARDS. This model can be performed in hamsters as well as rats and mice (101, 102). Epithelial cell responses (secretory cell metaplasia) have been characterized in hamsters in response to three different types of acid instillation, and these cellular changes are long-lasting (up to 17 wk) (103). Although some investigators have used rodent ventilators to provide supplemental oxygen, a recent study provides a modification that uses a box with humidified supplemental oxygen (with an inspiratory oxygen fraction reduced gradually from 1.0 to 0.21) to improve survival and allow for studies of the fibroproliferative phase after ALI. This modification should be widely accessible to most laboratories (104) wanting to study fibroproliferation as a result of ALI/ARDS. Similar models in both rats and mice have used a combination of acid and particulate matter to mimic clinically relevant aspiration events in human subjects (101, 105). A combination insult of acid with particulate matter induces synergistic and progressive lung injury, and therefore is likely to result in severe interstitial pulmonary fibrosis (101).

Radiation-Induced Fibrosis

Radiation-induced fibrosis is a clinically relevant injury to the respiratory tract that results in the development of fibrosis. Susceptibility to this injury is genetically based in mice, and most notably C3H/HeJ and CBA/J mice are resistant in comparison with the more susceptible C57Bl/6 strain (106). A single dose of 12 to 15 Gy of total body irradiation can result in lung fibrosis by 20 weeks (107). However, if other organs are not shielded, fibrotic responses will be systemic. Regimens to deliver thorax-limited radiation are commonly applied to avoid this complication, and these models result in fibrosis by 24 weeks (108, 109). The inflammatory responses appear to dictate the severity of lung remodeling, but overall, this form of lung fibrosis is associated with low mortality (106). Regarding a major feature of this model, the pulmonary vascular remodeling that occurs is reminiscent of pulmonary arterial hypertension (110). The pathobiology of irradiation-induced fibrosis is believed to involve free radical–mediated DNA damage and the induction of TGF-β. This was one of the first models used to demonstrate a reparative role for bone marrow mesenchymal stem cells (111).

Lung Contusion–Induced Fibrosis

An understudied area of fibrotic lung disease involves the development of fibrosis after traumatic injury. During the past decade, multiple small-animal models of lung contusion–induced fibrotic injury have been established to fill this gap (102, 105, 112, 113). Using rats, a bilateral lung contusion without associated cardiac injury is induced in anesthetized animals by dropping a 0.3-kg hollow cylindrical weight onto a mobile plate with a precordial shield (102). The height from which the cylinder is dropped can be changed, to generate an impact energy of 2.0–2.45 Joules. The model results in the development of lung contusion characterized by hemorrhagic injury, alveolar disruption, and a subsequent neutrophilic inflammatory response, peaking by 48 hours after injury (112). This injury is neutrophil-dependent and is characterized by severe hypoxia that resolves over a period of 48 hours. A similar murine model of unilateral closed-chest injury, using a cortical-contusion impactor, was first developed in 2007, and is currently being used with various transgenic animals (113). These rodents go on to develop a form of fibrosis seen with bronchiolitis obliterans organizing pneumonia by 7 d after the initial insult (112). Figure 6 depicts representative hematoxylin-and-eosin staining of a rat lung on Day 7 after contusion injury. This model may be especially useful for understanding how acute inflammatory and injury responses can lead to fibrotic outcomes.

HUMANIZED MODELS OF LUNG FIBROSIS

Because none of the animal models of experimental fibrosis fully recapitulate IPF pathology, recent attempts have been made to "humanize" these models. The best-characterized humanized model involves the intravenous instillation of human IPF fibroblasts into immunodeficient nonobese diabetic/severe combined immunodeficiency (NOD/SCID/beige) mice (114, 115). These mice lack many features of innate and adaptive immune response, thus allowing for the growth of human cells in the lung. However, the lungs of these mice show no evidence of fibrotic pathology before the instillation of IPF fibroblasts. Thus, this model has provided important insights into the pathogenic

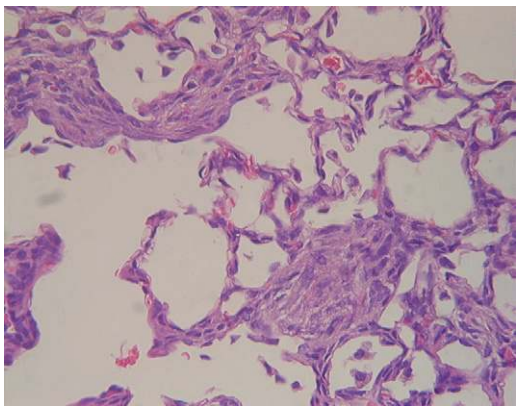

Figure 6. Lung contusion induced fibrosis. Hematoxylin and eosin–stained lung sections from a rat on Day 7 after contusion injury show the development of fibrotic lesions, especially around bronchioles. *Magnification*: ×400.

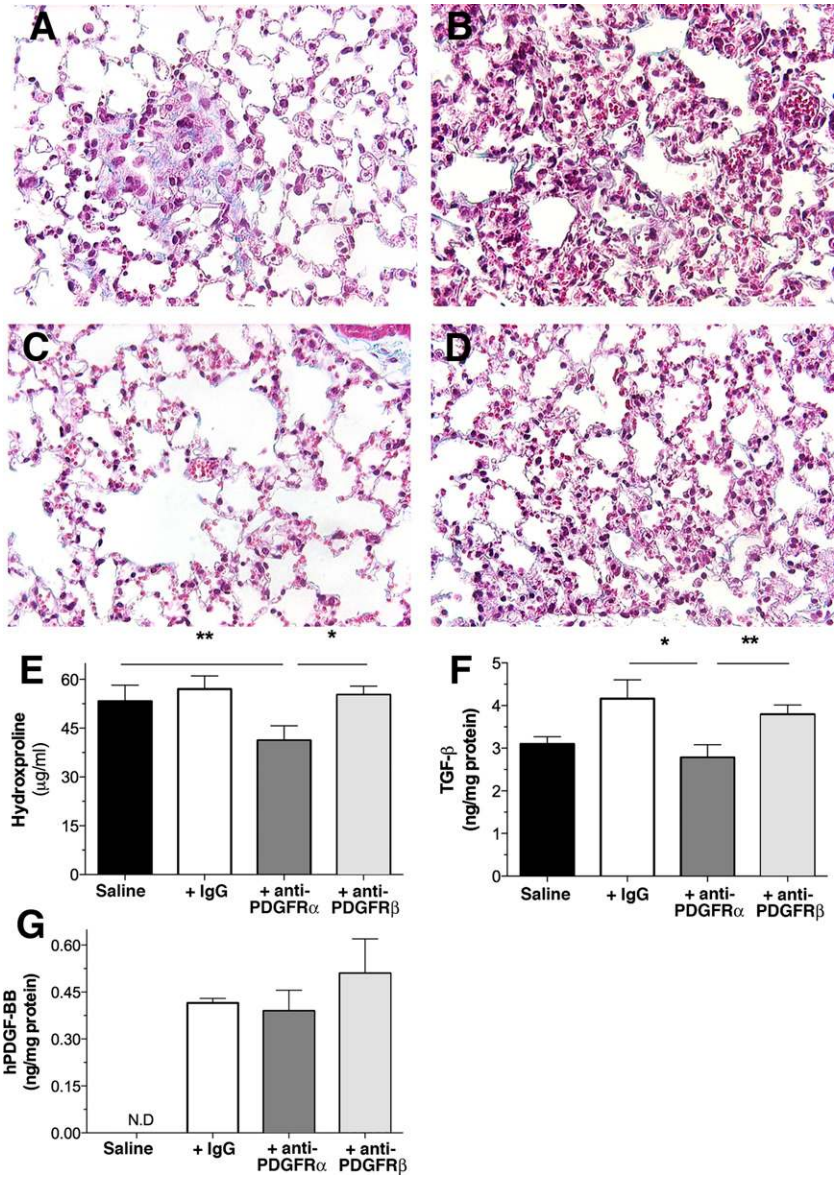

Figure 7. Humanized model of lung fibrosis. Pulmonary fibroblasts were grown from idiopathic pulmonary fibrosis (IPF) surgical lung biopsies and labeled with PKH26. After the PKH26 labeling, 1 ml of the fibroblast suspension and PKH26 dye solution containing 1×10^6 fibroblasts were then injected intravenously into each mouse, using a 26-gauge needle and tuberculin syringe. The histological appearance of the lungs of these mice on Day 63 after injection is shown in *A* (trichrome staining; original magnification, ×40). The effects of a human IgG1-λ isotype control (anti–chi-lysozyme–MOR03207), anti-PDGFRα monoclonal antibody (mAb) (IMC-3G3; ImClone, Summerville, NJ), or anti-PDGFRβ antibody (IMC-2C5; ImClone) were examined using histological (trichrome staining) (*B*) IgG1, (*C*) anti-PDGFRα mAb, and (*D*) anti-PDGFRβ mAb (original magnification, ×40), biochemical analysis (hydroxyproline; *E*), and transforming growth factor–β and PDGF-BB (ELISA; *F* and *G*, respectively) analyses for the presence of pulmonary fibrosis, performed at various times after the intravenous injection of human fibroblasts. All antibodies and control IgG were administered at 1 mg per mouse every other day, beginning on Day 35. Groups of mice were killed on either Day 49 or Day 63. All data shown in *E–G* represent means ± SEMs for groups of $n = 5$ mice. *$P \leq 0.05$. **$P \leq 0.01$. PDGFR, platelet-derived growth factor receptor; hPDGFR, human platelet-derived growth factor receptor.

potential of IPF fibroblasts. The instillation of IPF fibroblasts, but not of fibroblasts from normal lungs, results in focal fibrotic alveolar remodeling (114, 115), such as that seen in Figure 7A, but no fibrotic changes in other organs. Interestingly, although human fibroblasts directly contribute to the pathologic remodeling in the mouse lung, these cells also activate murine epithelial cells and fibroblasts to undergo pathologic remodeling and proliferation. This is perhaps the best evidence to date for a pathologic phenotype that is fibroblast-autonomous. This model offers advantages in that the transferred fibroblasts can be easily labeled with cell-permeable dyes to follow trafficking, and the development of the fibrosis is relatively rapid (occurring within 30–35 d after injection) and persists for months. This model also offers an opportunity to study cells derived from multiple patients, thus providing insights into IPF heterogeneity in fibroblast phenotypes. Finally, this model has created the opportunity to explore antifibrotic agents with human specificity. As shown in Figures 7B–7G, the pathologic remodeling in this model can be markedly attenuated after the administration of a monoclonal antibody (mAb) directed against human platelet-derived growth factor receptor (PDGFR)–α (Figure 7C), but not PDGFR-β (Figure 7D), compared with an IgG control (Figure 7B). The antifibrotic effect of the anti-PDGFRα mAb in this model was confirmed by hydroxyproline analysis (Figure 7E). Although the PDGFRα mAb treatment significantly reduced human TGF-β protein expression, it exerted no effect on human PDGF-BB expression in whole-lung samples compared with the other treatment groups. This model also involves the disadvantage of modeling the development of fibrosis in the absence of immune cells, which is not what occurs in humans. This model involves the further disadvantage that these immunodeficient mice are quite expensive and require specialized housing. Expanded analyses of such humanized mouse models, including mice with humanized immune systems (e.g., bone marrow, liver, thymus [BLT] mice) (116), may offer unique insights in future studies.

EVALUATION OF EXPERIMENTAL MODELS

Each experimental model of lung fibrosis includes its own timeline for best evaluations of fibrotic responses. Nonetheless, they share a common need for robust and accurate evaluations of lung fibrosis. Importantly, when analyzing outcomes in all of these models, care must be taken to ensure the changes in collagen

expression are truly caused by fibrosis. Appropriate analyses of experimental lung fibrosis should combine biochemical measures of matrix deposition (e.g., hydroxyproline assays, Sircol assays, or Western blots) with histologic evaluations of the pathology associated with those changes. For example, collagen accumulation has been noted in emphysema models (117), but the pathology is clearly different from that of IPF. Thus, to provide both biochemical and histologic analyses is important for a full understanding of how modulations of cell–cell interactions and soluble mediators may be affecting fibrosis. Additional modalities to complement histologic and biochemical evaluations include evaluations of lung mechanics and lung imaging. Unfortunately, these techniques alone are not sufficient for delineations between lung inflammation and lung fibrosis. However, we hope that small-animal imaging techniques will continue to evolve and allow live-animal imaging that permits detailed evaluations of interstitial lung disease in these models.

CONCLUSIONS

In conclusion, recent advances have led to more persistent models of experimental fibrosis and have created systems to allow for studies of targeted epithelial injury, fibroblast-specific alterations, inflammatory cell modulations of fibrosis, and epithelial–mesenchymal crosstalk. Although these models still do not recapitulate all features of IPF pathogenesis, they allow for specific analyses of signaling pathways and interactions among various cell types. More persistent models contain the further advantage of enhancing our ability to study mechanisms operative during the fibrogenic stage, and this approach increases the likelihood of translating findings to human disease as well as allowing for the efficacy testing of therapeutics on fibrotic remodeling. This may permit more accurate predictions of which compounds have the capacity to improve outcomes after fibrosis has been established. Taken together, animal modeling provides an important tool as the pulmonary fibrosis research community seeks important clues to the pathogenesis of IPF and other fibrotic lung diseases, while also seeking therapies for these devastating diseases.

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