Fibroblast Growth Factor 2 Is Required for Epithelial Recovery, but Not for Pulmonary Fibrosis, in Response to Bleomycin

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Abstract

The pathogenesis of pulmonary fibrosis involves lung epithelial injury and aberrant proliferation of fibroblasts, and results in progressive pulmonary scarring and declining lung function. *In vitro*, fibroblast growth factor (FGF) 2 promotes myofibroblast differentiation and proliferation in cooperation with the profibrotic growth factor, transforming growth factor- β 1, but the *in vivo* requirement for FGF2 in the development of pulmonary fibrosis is not known. The bleomycin model of lung injury and pulmonary fibrosis was applied to *Fgf2* knockout (*Fgf2*^{-/-}) and littermate control mice. Weight loss, mortality, pulmonary fibrosis, and histology were analyzed after a single intranasal dose of bleomycin. Inflammation was evaluated in bronchoalveolar lavage (BAL) fluid, and epithelial barrier integrity was assessed by measuring BAL protein and Evans Blue dye permeability. *Fgf2* is expressed in mouse and human lung epithelial and inflammatory cells, and, in response to bleomycin, *Fgf2*^{-/-} mice have significantly increased mortality and weight loss. Analysis of BAL fluid and histology show that pulmonary fibrosis is unaltered, but *Fgf2*^{-/-} mice fail to efficiently resolve inflammation, have increased BAL cellularity, and, importantly, deficient recovery of epithelial integrity. *Fgf2*^{-/-} mice similarly have deficient recovery of club cell secretory protein⁺ bronchial epithelium in response to naphthalene. We conclude that FGF2 is not required for bleomycin-induced pulmonary fibrosis, but rather is essential for epithelial repair and maintaining epithelial integrity after bleomycin-induced lung injury in mice. These data identify that

FGF2 acts as a protective growth factor after lung epithelial injury, and call into question the role of FGF2 as a profibrotic growth factor *in vivo*.

Keywords: fibroblast growth factor 2; bleomycin; pulmonary fibrosis; lung injury; alveolar epithelial repair

Clinical Relevance

Fibroblast growth factors (FGFs) and, in particular, FGF2, are implicated in the pathogenesis of pulmonary fibrosis and recovery from airway epithelial cell (AEC) injury; however, the *in vivo* requirement for FGF2 in the development of pulmonary fibrosis is not known. In this study, we have found that mice lacking FGF2 do not show altered levels of fibrosis in response to bleomycin, indicating that FGF2 is dispensable for the generation of bleomycin-induced pulmonary fibrosis. In addition, we show that FGF2 knockout mice have increased mortality and weight loss in response to bleomycin, accompanied by deficient recovery of mature alveolar epithelium and epithelial barrier function. These findings suggest that FGF2 is required for lung epithelial recovery, that pharmacological inhibition of FGF signaling could impair the response to lung injury, and that FGF2 administration or augmentation may have therapeutic benefit for lung injury.

Lung alveolar epithelial cell (AEC) injury can be caused by a variety of insults, including infection, trauma, aspiration, and

sepsis. Chronic AEC injury of unknown origin is central to the pathogenesis of idiopathic pulmonary fibrosis (IPF) (1),

which is characterized by progressive pulmonary scarring and decline in lung function. IPF affects approximately 50,000

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patients annually in the United States, and the median survival is 3–5 years after diagnosis (2). The underlying cause of IPF is unknown, and lung transplantation may be the only viable option for select patients. Profibrotic factors released by damaged epithelium contribute to aberrant activation of fibroblastic foci (2–4). Ultimately, fibroblast proliferation and differentiation to myofibroblasts leads to deposition of collagen-rich matrix, alveolar destruction, and clinically severe fibrosis.

Fibroblast growth factors (FGFs) are implicated in the pathogenesis of pulmonary fibrosis and recovery from AEC injury. The family of FGF proteins is large, with 18 ligands that bind with variable affinity to four signaling receptor tyrosine kinases (FGF receptors [FGFRs]). Several agents, including Nintedanib and Pirfenidone, inhibit FGF signaling and are in clinical trials for IPF (5, 6) or have been recently approved in Europe, Canada, and Japan. Pirfenidone reduces fibrosis in animal models and inhibits increases in transforming growth factor (TGF)- β 1, IL-1 β , and FGF2 (7, 8). Administration of a specific inhibitor of FGFR1 (NP603) inhibits carbon tetrachloride-induced hepatic fibrosis in rats (9), but has not been tested in models of pulmonary fibrosis. A soluble ectodomain of FGFR2c inhibits TGF- β 1-induced primary lung fibroblast proliferation *in vitro*, as well as bleomycin-induced fibrosis *in vivo* (10). This ectodomain inhibits multiple FGFs known to bind to the IIIC splice variant of FGFR2, including FGFs 1, 2, 4, 6, 8, 9, 17, and 18 (11). Despite this, the cell-specific role of FGF signaling in the development of pulmonary fibrosis remains uncertain.

FGF2 is a potent alveolar type II cell mitogen (12), as well as a potent mediator of pulmonary fibroblast proliferation *in vitro* (13). FGF2 is not required for development, as *Fgf2* knockout mice (*Fgf2*^{-/-}) develop near normally, have no reported pulmonary abnormalities, and are fertile (14). In the adult lung, localization of FGF2 is strongest in the epithelium, vascular endothelium, smooth muscle, and epithelial basement membrane (15, 16). FGF2 is required for carbon tetrachloride-induced liver fibrosis (9), and expression is increased in renal fibrosis (17). FGF2 protein is elevated in serum of patients with systemic sclerosis and dermatomyositis, and the level of FGF2 elevation correlates with the presence of

lung fibrosis (18). FGF2 protein levels are also increased in bronchoalveolar lavage (BAL) from patients with IPF (19), and immunohistochemistry for FGF2 localizes to mast cells in IPF lungs (20). Other studies have shown FGF2 staining in IPF lungs localized to the endothelium and areas of angiogenesis (21). In response to bleomycin, immunohistochemical staining of mouse lungs localized FGF2 to inflammatory cells, including macrophages (22, 23) and mast cells (20, 24–26), as well as airway epithelial cells (27–30). Recent studies describe decreased levels of *Fgf2* mRNA in lungs from a large cohort of patients with IPF (31), but the temporal and spatial pattern of *Fgf2* expression and signaling remains unclear in early versus late stages of disease.

In primary human lung fibroblasts, administration of TGF- β 1 induces FGFR expression (32) and FGF2 expression and secretion into culture media (13, 33). Primary lung fibroblasts isolated from rats exposed to peplomycin have increased FGF2 production (34). TGF- β 1 induced extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, and activator protein-1 phosphorylation (13, 33), fibroblast differentiation into myofibroblasts, proliferation (35), and FGF2 production (17), are inhibited by FGF2-neutralizing antibodies, suggesting a cooperative mechanism between FGF2 and TGF- β 1. FGF2, therefore, has been proposed to both facilitate and augment the fibrotic response to TGF- β 1, which is central to the pathogenesis of pulmonary fibrosis. This proposed mechanism, however, has not formally been tested *in vivo* with *Fgf2*^{-/-} mice.

In this report, we show that *Fgf2*^{-/-} mice have significantly increased mortality and weight loss in response to a single dose of inhaled bleomycin, and that *Fgf2*^{-/-} mice have deficient recovery of mature alveolar epithelium and epithelial barrier function. Surprisingly, *Fgf2*^{-/-} mice do not display altered levels of fibrosis, indicating that FGF2 is dispensable for the generation of bleomycin-induced pulmonary fibrosis. These findings also suggest that FGF2 is required for recovery from lung injury, and that widespread pharmacological inhibition of FGF signaling in, for example, cancer therapy or emerging therapies for IPF, could impair lung response to damage, and that FGF2 administration or augmentation may be a potential therapy for lung injury.

Materials and Methods

Animal Care

Mice were housed in a pathogen-free barrier facility and handled in accordance with standard protocols, animal welfare regulations, and the National Institutes of Health guide for the care and use of laboratory animals, all procedures complied with the standards of that document, and all protocols were approved by the Animal Studies Committee at Washington University School of Medicine. Experiments were performed using wild-type or *Fgf2*^{-/-} mice maintained in a C57BL/6;129X1/SvJ hybrid mixed background. *Fgf2*^{+/-} breeding pairs were used to generate *Fgf2*^{-/-} and wild-type control littermates for experiments.

Human IPF Lung Explants

IPF lung tissue samples were obtained from patients with end-stage disease at the time of transplantation under protocols approved by the Washington University Human Research Protection Office. Samples were fixed at room temperature in 10% phosphate-buffered formalin overnight, and subsequently dehydrated in ethanol and xylene, embedded in paraffin, and 5- μ m-thick sections were cut. *In situ* RNA hybridization was performed as described in the MATERIALS AND METHODS in the online supplement.

Bleomycin-Induced Injury and Fibrosis

Adult male and female mice between 8 and 10 weeks of age (20–25 g) were sedated with 2.5% Avertin and treated with a total of 4 mg/kg (6.4 U/kg) of bleomycin (Sigma, St. Louis, MO) in sterile PBS or PBS alone delivered nasally in two 25- μ l divided doses. Mice were monitored daily, weight was measured twice weekly, and mice displaying significant respiratory distress were humanely killed and treated as a death. At 7, 14, 21, or 35 days after treatment, mice were killed with an overdose of a cocktail containing ketamine and xylazine, and lungs were collected for protein or RNA analysis, or lungs were fixed via intratracheal inflation with 10% phosphate-buffered formalin. Samples were then dehydrated in ethanol and xylene, embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin. Additional details are available in the

MATERIALS AND METHODS in the online supplement.

RNA isolation and quantitative real-time PCR

RNA was isolated from whole left lungs via homogenization in trizol, and subsequent purification using RNeasy spin columns (74104; Qiagen, Valencia, CA), with on-column DNA digest, per manufacturer's instructions. Quantitative RT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR thermocycler using Taqman Fast Advanced Master Mix (no. 4444557; Life Technologies, Grand Island, NY) and Taqman gene expression assays (Life Technologies). Additional details are available in the MATERIALS AND METHODS in the online supplement.

Fibrosis Scoring

Ashcroft scoring was performed as described previously by Ashcroft and colleagues (36). Further details are available in the MATERIALS AND METHODS in the online supplement.

Immunohistochemistry

The 5- μ m sections were prepared from paraffin-embedded tissues and deparaffinized with xylene, and immunohistochemistry was performed using standard techniques. Further details are available in MATERIALS AND METHODS in the online supplement.

Statistical Analysis

Significant differences in mean values were calculated using paired Student's *t* tests or one-way ANOVA. Weight loss and mortality were analyzed using log-rank (Mantel-Cox) test and two-way ANOVA. A *P* value of less than 0.05 was considered to be significant.

Results

FGF Signaling and Localization of FGF2 in Response to Bleomycin

A single dose of intranasal bleomycin (4 mg/kg in PBS) or PBS alone was administered to wild-type C57BL/6;129X1/SvJ hybrid mice, and lungs were collected at 7, 14, and 21 days after treatment. ERK1/2 phosphorylation and expression of the transcription factor Ets variant gene 4 (*Etv4*) are increased in response to FGFs, and were used as indicators of FGF signaling. ERK1/2 phosphorylation at 7 and 14 days after bleomycin administration, and *Etv4* mRNA expression at all time points after

Figure 1. Increased fibroblast growth factor (FGF) 2 signaling in response to inhaled bleomycin. (A) A single dose of bleomycin (4 mg/kg) or PBS was administered to wild-type mice, and whole-lung protein extracts were obtained 7, 14, and 21 days after treatment. Western blotting for phosphorylated extracellular signal-regulated kinase (ERK) 1/2, total ERK1/2, and β -tubulin is shown. (B) Densitometric analysis of phosphorylated ERK1/2, normalized to both total ERK1/2 and β -tubulin. Data are presented as mean $\pm$ SEM of fold increase from PBS-treated mice. **P* < 0.05 (*n* = 6–13). (C) Quantitative RT-PCR for Ets variant gene 4 (*Etv4*) was performed from whole-lung RNA isolated from wild-type mice treated with PBS for 7, 14, and 21 days after bleomycin. Ct values were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), and data are presented as mean $\pm$ SEM of fold increase from PBS-treated mice. **P* < 0.05, ***P* < 0.001 (*n* = 5). (D) Western blot of FGF2 in response to bleomycin in whole-lung protein lysates. Samples from FGF2 knockout (*Fgf2*^{-/-}) mice are present as a negative control. Quantitative RT-PCR performed for *Fgf2* from RNA isolated from bronchoalveolar lavage (BAL) cell pellets (E) or whole lung (F) after treatment with PBS or bleomycin. Ct values were normalized to *Gapdh*, and data are presented as mean $\pm$ SEM of fold increase from PBS-treated mice. **P* < 0.05. (*n* = 4). *In situ* hybridization using antisense probes specific for *Fgf2* mRNA was performed on mouse lung sections treated with PBS (G) or bleomycin for 7 days (H) and 21 days (I–K). (K) A magnified view of the area marked in (J). As a negative control, *in situ* hybridization for *Fgf2* mRNA was performed on *Fgf2*^{-/-} mice treated with bleomycin for 21 days (L). Scale bars, 200 μ m.

bleomycin administration were significantly increased (Figures 1A–1C). A trend toward increased Akt/protein kinase B phosphorylation was observed after bleomycin treatment (data not shown). Bleomycin increased FGF2 levels, as detected by Western blot analysis of FGF2 extracted from 2,000 μ g of whole-lung protein using heparin–sepharose beads (Figure 1D). No FGF2 protein was present in *Fgf2*^{−/−} mouse lungs, confirming antibody specificity. *Fgf2* mRNA expression, however, was increased only in cells collected from BAL (Figure 1E), but not in whole-lung lysates (Figure 1F), suggesting that the increased *Fgf2* expression after bleomycin is derived from an inflammatory source. To identify sites of *Fgf2* expression, *in situ* hybridization was performed using digoxin-labeled RNA probes specific for *Fgf2*. In the absence of bleomycin, *Fgf2* mRNA was localized to bronchial and alveolar epithelium (Figure 1G). After bleomycin, epithelial production of *Fgf2* was maintained (Figures 1H and 1I, *arrow*), and additional expression was seen in inflammatory cells (Figures 1J and 1K, *red arrow*). In *Fgf2*^{−/−} mice, no staining was seen (Figure 1L), confirming probe specificity. Expression of *FGF2* in human non-IPF control lungs and IPF explant lungs demonstrated a similar pattern (Figures 1M–1O and Figure E1 in the online supplement). In non-IPF lungs, *FGF2* expression was localized to AECs and occasional alveolar macrophages, whereas, in IPF lungs, expression was confined to epithelial (Figure 1N, *arrow*) and inflammatory cells (Figures 1N and 1O, *red arrow*). In both bleomycin-treated mouse lungs and human IPF explant lungs, very little *Fgf2* expression was detected in fibroblasts or fibroblastic foci (Figures 1I, 1N, and 1O, *arrowhead*). In addition, overall *FGF2* mRNA expression was decreased in IPF lungs relative to non-IPF controls (Figure E1), consistent with decreased inflammation in end-stage usual interstitial pneumonia, the pathologic description of IPF (37, 38).

FGF2 Is Not Required for Pulmonary Fibrosis in Mice

Given the importance of FGF2 for fibroblast proliferation in response to profibrotic stimuli *in vitro*, we hypothesized that

FGF2 would be required for fibrosis *in vivo*. *Fgf2*^{−/−} mice have not been tested in models of acute lung injury or pulmonary fibrosis, although *Fgf1*^{−/−}, *Fgf2*^{−/−} mice have decreased carbon tetrachloride-induced liver fibrosis (39). *Fgf2*^{−/−} mice exhibit no pulmonary functional or histologic abnormalities, and no differences in elastin content, alveolar diameter, as determined by mean linear intercept (Figure E2), or baseline collagen content. To address whether FGF2 is responsible for FGF-dependent pulmonary fibrosis in response to bleomycin, *Fgf2*^{−/−} and littermate wild-type mice were treated with a single dose of intranasal bleomycin (4 mg/kg). *Fgf2*^{−/−} mice showed significantly ($P < 0.001$; wild type, $n = 23$; *Fgf2*^{−/−}, $n = 30$) increased weight loss and mortality (Figure 2) after bleomycin treatment, with a majority of deaths occurring between 14 and 17 days after bleomycin administration. Mice with significant respiratory distress or excessive (>25%) weight loss were humanely killed and recorded as a death, and *post mortem* histologic analysis confirmed extensive alveolitis and fibrosis, with no evidence of extrapulmonary cause of death. Both male and female mice of each genotype exhibited similar weight loss ($P = 0.7$ for wild type; $P = 0.07$ for *Fgf2*^{−/−}) and mortality ($P = 0.2$ for wild type; $P = 0.9$ for *Fgf2*^{−/−}) in response to bleomycin.

We hypothesized that the cause of mortality in *Fgf2*^{−/−} mice was increased pulmonary fibrosis, as a significant increase in fibrosis is known to occur approximately 14 days after inhaled bleomycin. Bleomycin treatment led to a significant increase in histologic fibrosis 21 days after treatment in wild-type mice (Figures 3A and 3C). Surprisingly, the amount of fibrosis observed in *Fgf2*^{−/−} mice 21 days after bleomycin was similar to that seen in littermate wild-type mice (Figures 3B, 3D, and E3). Because of the potential for a significant bias toward surviving mice at the 21-day time point, we also analyzed fibrosis 14 days after bleomycin administration. There were no significant differences between *Fgf2*^{−/−} and wild-type mice in response to bleomycin in Ashcroft fibrosis score 14 or 21 days after

Figure 2. Increased weight loss and mortality in FGF2 knockout mice in response to bleomycin. A single dose of intranasal PBS or bleomycin (4 mg/kg) was administered to *Fgf2*^{−/−} or wild-type (WT) littermate controls. Weight was measured weekly (A), and mortality (or distress requiring that animals be killed) (B) was monitored for up to 21 days after bleomycin. Mortality is expressed as a Kaplan–Meier survival plot. ** $P < 0.001$ compared with wild-type (log-rank [Mantel–Cox] test and two-way ANOVA). $n = 23$ (wild-type), $n = 30$ (*Fgf2*^{−/−}). All data presented are means $\pm$ SEM.

bleomycin administration (Figure 3E), total hydroxyproline 14 and 21 days after bleomycin (Figure 3G), and static compliance 21 days after bleomycin (Figure 3I). Decreasing the bleomycin dose to 2 mg/kg, which decreases the magnitude of weight loss (Figure E4), and does not cause mortality in either wild-type or *Fgf2*^{−/−} mice, did not lead to a significant difference in fibrosis in *Fgf2*^{−/−} mice compared with wild-type mice (Figures 3F and 3H) 21 days after bleomycin administration. It is possible that extending the time of our study to 35 days would have demonstrated increased fibrosis in *Fgf2*^{−/−} mice; however, no difference in histologic fibrosis or hydroxyproline was observed 35 days after bleomycin administration

Figure 1. (Continued). (G–J, L), 400 μ M (K). *In situ* hybridization using antisense probes specific for human *FGF2* was performed on non-idiopathic pulmonary fibrosis (IPF) human lung (M), and human IPF lung explant biopsies obtained at the time of lung transplantation (N and O). Representative images were selected from $n = 5$ IPF explant lungs. Scale bars, 200 μ M. Arrows indicate epithelium; red arrows indicate inflammatory cells; and arrowheads indicate areas of fibrosis. Bleo, bleomycin.

(Figure E4). In addition, changing the route of bleomycin delivery from intranasal to intratracheal did not result in differing amounts of histologic fibrosis (data not shown) or hydroxyproline levels 21 days after bleomycin administration (Figure E4). Expression of the profibrotic cytokine, connective tissue growth factor, was similarly increased in wild-type and *Fgf2*^{-/-} mice after bleomycin (Figure 3J), as was collagen type 1 and fibroblast-specific protein 1, both of which are increased in bleomycin-induced pulmonary fibrosis, and reflect the degree of fibrosis present (Figure E5). The increase seen in collagen type 1 and fibroblast-specific protein 1 expression in *Fgf2*^{-/-} compared with wild-type mice under control conditions was small, and did not result in a difference in baseline collagen accumulation or histologic fibrosis (Figures 3A–3D and 3G).

FGF2 Contributes to Resolution of Inflammation in Response to Bleomycin

Because the overall fibrosis was similar between wild-type and *Fgf2*^{-/-} mice, we hypothesized that the significant difference in mortality could be due to either differences in inflammation or epithelial damage, both of which are known consequences of bleomycin. Histologic analysis of *Fgf2*^{-/-} mice 21 days after bleomycin suggested increased amounts of inflammatory cells in *Fgf2*^{-/-} compared with control lungs. To examine this finding further, BAL cytospin analysis was performed 7, 14, and 21 days after intranasal bleomycin (4 mg/kg). *Fgf2*^{-/-} mice showed a significantly elevated BAL total cell count, macrophage count, and lymphocyte count 21 days after intranasal bleomycin (Figures 4A–4D), but unchanged BAL cellularity 7 and 14 days after bleomycin compared with controls, indicating deficient resolution of lung inflammation. Quantitative RT-PCR from BAL cell pellets revealed a significant ($P < 0.05$, $n = 5$) elevated *IL-6* expression in *Fgf2*^{-/-} mice compared with wild-type controls 21 days after bleomycin (Figure 4E). In addition, immunostaining for CD45 was increased in *Fgf2*^{-/-} mouse lungs 21 days after bleomycin, but not at 7 days (Figure 4F). Although statistically significant, we did not believe that the degree of increased inflammation adequately explained the mortality observed in *Fgf2*^{-/-} mice after bleomycin.

Figure 3. *Fgf2*^{-/-} and wild-type mice have similar fibrosis after bleomycin. A single dose of intranasal PBS or bleomycin was administered to *Fgf2*^{-/-} or wild-type littermate controls. Representative histologic appearance of hematoxylin and eosin-stained lungs 21 days after 4 mg/kg bleomycin in wild-type (A and C) and *Fgf2*^{-/-} (B and D) mice, at 1.2x magnification (A and B) and 10x magnification (C and D). Scale bars, 4 mm (A and B), 600 μ m (C and D). Ashcroft fibrosis score was measured 14 and 21 days after PBS or 4 mg/kg bleomycin (E) ($n = 8$), and PBS or 2 mg/kg bleomycin (F) ($n = 10$). Quantitative hydroxyproline measurements were obtained from left lungs of mice 14 ($n = 4$) and 21 ($n = 12$) days after PBS or 4 mg/kg

Interestingly, the percentage of CD45-negative cells in BALs from *Fgf2*^{-/-} mice 21 days after bleomycin was higher compared with wild-type controls (data not shown), suggesting increased epithelial sloughing in *Fgf2*^{-/-} mice and a potential alteration in epithelial homeostasis.

FGF2 Is Required for Recovery of Epithelium in Response to Bleomycin

FGF2 is required for epidermal and epithelial repair after injury to the skin and retina (40), and other FGFs, such as the FGFR2b ligands, FGF7 and FGF10, promote lung epithelial proliferation and improve recovery of epithelial barrier function after injury (41, 42). It is not known, however, whether this effect is limited to FGF7 and FGF10, or whether endogenous FGF2 is required. Pro-surfactant protein (SP) C (or *Sftpc*)-positive type II AECs are required for surfactant production, and also serve as local progenitor cells that differentiate to type I epithelial cells after distal lung injury (43, 44). Furthermore, inadequate recovery of SPC⁺ AECs in mice with inhibition of FGF signaling in response to hyperoxia-induced lung injury causes respiratory failure and death (45). We therefore hypothesized that the mortality observed in *Fgf2*^{-/-} mice after bleomycin represented a failure to repair damaged epithelium.

To test this, quantitative RT-PCR was performed to evaluate expression levels of selected epithelial markers after bleomycin injury in wild-type and *Fgf2*^{-/-} mice. Both wild-type and *Fgf2*^{-/-} mice demonstrated a significant ($P < 0.05$, $n = 5-6$) decrease in expression of *Sftpc*, SPB (*Sftpb*), E-cadherin (*Cdh1*), thyroid transcription factor-1 (NK2 homeobox 1, or *Nkx2.1*), club cell secretory protein (CCSP, or *Scgb1a1*), and aquaporin 5 (*Aqp5*) at 7 and 14 days after bleomycin treatment (Figures 5A-5F). *Fgf2*^{-/-} mice, however, did not demonstrate a complete recovery of *Sftpc*, *Sftpb*, *Cdh1*, and *Scgb1a1* 21 days after bleomycin. No difference was seen between wild-type and *Fgf2*^{-/-} mice in the Type I AEC marker, aquaporin 5, 21 days after bleomycin (Figure 5F). In addition, expression of *Etv5*, which localizes to AECs and is a transcriptional target of FGF signaling (46, 47), was significantly decreased in both

Figure 4. Prolonged inflammation in *Fgf2*^{-/-} mice compared with wild-type mice after bleomycin. A single dose of intranasal bleomycin (4 mg/kg) was administered to *Fgf2*^{-/-} or wild-type littermate controls, and mice were collected at 7, 14, or 21 days after treatment. Whole-lung BAL was performed, and the total number of cells was counted (A). Cytospin analysis of BALs and manual differential counting for neutrophils (B), macrophages (C), and lymphocytes (D) was performed ($n = 4-6$). Quantitative RT-PCR was performed for IL-6 from RNA isolated from BAL cell pellets (E), and normalized to *Gapdh*. Data presented are fold change from wild-type PBS-treated mice ($n = 5$). (F) Immunohistochemistry for CD45 was performed on wild-type or *Fgf2*^{-/-} lungs 21 days after bleomycin, quantified using ImageJ (National Institutes of Health, Bethesda, MD), and normalized to total tissue area ($n = 5-6$). * $P < 0.05$, ** $P < 0.001$ compared with wild-type PBS control, unless indicated by brackets. All data presented are mean $\pm$ SEM. ns, nonsignificant.

wild-type and *Fgf2*^{-/-} mice 7 and 14 days after bleomycin, and failed to recover expression in *Fgf2*^{-/-} mice 21 days after bleomycin (Figure 5G). This supports a model in which FGF2 signals directly to recovering epithelium.

To determine whether the lack of recovery of *Cdh1* and *Sftpc* mRNA

expression in *Fgf2*^{-/-} mice 21 days after bleomycin corresponded with reduced protein expression, histological sections from lungs 21 days after bleomycin injury were immunostained for E-cadherin and pro-SPC. In areas of injury, E-cadherin and pro-SPC were decreased in *Fgf2*^{-/-} lungs compared

Figure 3. (Continued). bleomycin (G), and 21 days after PBS or 2 mg/kg bleomycin (H) ($n = 3$). (I) Mean static compliance 21 days after PBS or 4 mg/kg bleomycin was measured ($n = 15$). (J) Quantitative RT-PCR performed for connective tissue growth factor (*Ctgf*) from RNA isolated from whole lung after treatment with PBS or 4 mg/kg bleomycin ($n = 4-6$). For all data, * $P < 0.05$, ** $P < 0.001$ compared with PBS control. All data presented are mean $\pm$ SEM. ns, nonsignificant.

Figure 5. Delayed recovery of epithelial gene expression in *Fgf2*^{-/-} mice after bleomycin. A single dose of intranasal bleomycin (4 mg/kg) was administered to *Fgf2*^{-/-} or wild-type littermate controls, and RNA was harvested from lungs at 7, 14, or 21 days after treatment. Quantitative RT-PCR was performed for *Sftpc* (A), *Sftpb* (B), E-cadherin (*Cdh1*) (C), NK2 homeobox 1 (*Nkx2.1*) (D), club cell secretory protein (*Scgb1a1*) (E), aquaporin 5 (*Aqp5*) (F), and *Etv5* (G), and normalized to *Gapdh*. Data presented are fold change from wild-type PBS-treated mice. **P* < 0.05 and ***P* < 0.001 compared with wild-type PBS control, unless indicated by brackets; *n* = 5–8. *Fgf2*^{-/-} mice had a nonsignificant decrease in recovery of *Nkx2.1* 21 days after bleomycin (*P* = 0.07, *n* = 6–8). All data presented are mean ± SEM.

with controls (Figures 6A–6D and 6E). Western blot analysis from whole-lung protein lysates also showed decreased amounts of pro-SPC 21 days (but not 7 or 14 d) after bleomycin in *Fgf2*^{-/-} mice compared with control mice (Figures 6E and 6F).

Abnormal epithelial recovery in *Fgf2*^{-/-} mice may represent a generalized deficit in epithelial repair after injury, or may be unique to bleomycin-induced injury. To test this, *Fgf2*^{-/-} and control mice were treated with a single dose of intraperitoneal naphthalene (250 mg/kg in corn oil) or

corn oil vehicle. No mortality occurred in either genotype, and the amount of CCSP⁺ bronchial epithelial cell depletion, as measured via immunohistochemistry, was similar in wild-type and *Fgf2*^{-/-} mice 3 days after naphthalene (Figures 7A–7D). However, *Fgf2*^{-/-} mice showed significantly (*P* < 0.05, *n* = 4) decreased recovery of CCSP⁺ bronchial epithelium 7 days after naphthalene (Figures 7E–7G), demonstrating that the abnormal epithelial recovery seen after bleomycin is generalizable to other forms of lung injury.

To determine whether decreased recovery of SPC and E-cadherin in *Fgf2*^{-/-} mice after bleomycin contributes to a difference in recovery of epithelial barrier function, epithelial permeability after bleomycin-induced injury was measured. BAL from wild-type and *Fgf2*^{-/-} mice 7, 14, and 21 days after bleomycin (4 mg/kg) or 7 days after PBS administration demonstrated a similar increase in protein levels 7 and 14 days after bleomycin (Figure 8A). However, recovery of wild-type mice corresponded to a decrease in BAL protein levels by 21 days after bleomycin, whereas BAL protein levels remained significantly (*P* < 0.05, *n* = 13) elevated in *Fgf2*^{-/-} mice compared with controls (Figure 8A). As an additional measure of epithelial and endothelial permeability, wild-type and *Fgf2*^{-/-} mice were injected with Evans blue intravenously (1 ml/kg of 3% solution in PBS) 30 minutes before being killed, 21 days after bleomycin treatment. After death, blood, whole lung, and BAL was collected and Evans blue concentration was measured. No difference between wild-type and *Fgf2*^{-/-} mice was seen in whole lung Evans blue (Figure 8B); however, *Fgf2*^{-/-} mice showed a significantly (*P* < 0.05, *n* = 6) higher level of Evans blue compared with controls (Figure 8C), indicating deficient recovery of epithelial barrier function in *Fgf2*^{-/-} mice after bleomycin.

Discussion

In this report, we show that FGF signaling in whole lung is increased in response to intranasal bleomycin administration in mice. Consistent with previous reports (20, 22–26), we also find that FGF2 expression is primarily found in inflammatory cells in both bleomycin-treated mouse lungs and in human IPF explant lungs. Unexpectedly,

Figure 6. Delayed recovery of E-cadherin and surfactant protein (SP) C in *Fgf2*^{-/-} mice after bleomycin. A single dose of intranasal bleomycin (4 mg/kg) was administered to *Fgf2*^{-/-} or wild-type littermate controls. Immunohistochemistry for E-cadherin was performed on wild-type (A) or *Fgf2*^{-/-} (B) lungs 21 days after bleomycin. Immunohistochemistry for pro-SPC was performed on wild-type (C) or *Fgf2*^{-/-} (D) lungs 21 days after bleomycin. Scale bars, 600 μ M (A and B), 400 μ M (C and D). Western blot analysis of pro-SPC (E), with tubulin shown as a loading control. (F) Densitometric analysis of pro-SPC, normalized to tubulin. Data are presented as mean $\pm$ SEM of fold increase from PBS-treated mice. **P* < 0.05 (*n* = 6).

Fgf2^{-/-} mice showed significantly increased weight loss and mortality in response to a single dose of intranasal bleomycin. Analysis of these mice showed that FGF2 is not required for the development of pulmonary fibrosis in response to bleomycin, but is required for recovery of expression of *Sftpc* and E-cadherin, as well as recovery of epithelial barrier function after bleomycin-induced lung injury. These results are in contrast to *in vitro* studies, where, in cultured lung fibroblasts, FGF2 is required for fibroblast proliferation and differentiation in response to TGF- β 1.

FGF2 and Pulmonary Fibrosis

In primary human lung fibroblasts, administration of TGF- β 1 induces FGFR expression (32) and FGF2 expression and secretion into culture media (13, 33). TGF- β 1-induced ERK, c-Jun N-terminal kinase, and activator protein-1 phosphorylation (13, 33), fibroblast differentiation into myofibroblasts, proliferation (35), and FGF2 production (17), are FGF2 dependent, suggesting a cooperative mechanism between FGF2 and TGF- β 1. FGF2, therefore, has been proposed to both facilitate and augment the fibrotic response to TGF- β 1, which is central to the pathogenesis of pulmonary fibrosis. This proposed mechanism, however, has not formally been tested *in vivo* with *Fgf2* knockout mice.

Unexpectedly, we found no difference in development of fibrosis in *Fgf2*^{-/-} mice, and, in fact, found increased mortality, which was not due to increased fibrosis, but rather to impaired epithelial repair. Although our findings argue against FGF2 specifically being required for bleomycin-induced pulmonary fibrosis in mice, they do not argue against FGF signaling via any of the other 17 secreted FGF ligands. FGF9 expression, for example, is increased in areas of active fibrosis in IPF lungs, and is further enhanced by Tgf β 1 and Wnt7B, which are coexpressed in these regions (48, 49). Consistent with prior reports, we observed an increase in downstream FGF signaling in response to bleomycin (10). In addition, nonspecific tyrosine kinase inhibitors, which include FGFRs as targets, decrease bleomycin-induced pulmonary fibrosis in mice, and have shown promising results in treating patients with IPF (5, 6).

Consistent with published data (31), we have found decreased expression of *FGF2* mRNA in human IPF explant lungs

Figure 7. Delayed recovery of club cell secretory protein (CCSP) in *Fgf2*^{-/-} mice after naphthalene. A single dose of intraperitoneal naphthalene (250 mg/kg in corn oil) or corn oil alone was administered to wild-type or *Fgf2*^{-/-} littermates, and mice were collected 3 or 7 days after treatment. Immunohistochemistry for CCSP was performed on wild-type (A, C, and E) and *Fgf2*^{-/-} (B, D, and F) mouse lungs after treatment with corn oil (A and B) or 3 and 7 days after naphthalene (C–F). Scale bars, 600 μ m. CCSP staining was quantified (G), and expressed as percentage of total terminal bronchiole (TB). Data presented are mean $\pm$ SEM. **P* < 0.05 compared with wild-type corn oil control unless indicated by brackets (*n* = 4).

collected at the time of transplantation (Figure E1). Decreased overall expression of *FGF2* in end-stage IPF lungs is likely a result of a lack of inflammation as well as significant loss of lung epithelium, and, given our results, a relative deficiency of FGF2 may correlate with an inability to completely repair damaged epithelium. The underlying contribution of FGF2 to IPF in humans, however, remains unclear, as our data do not show increased fibrosis as a result of FGF2 deficiency, and overexpression of FGF2 in the lung does not cause spontaneous fibrosis *in vivo* (data not shown). Further studies are needed to determine whether expression and activity of FGF2, or other FGF ligands, is related to disease progression or severity of disease in pulmonary fibrosis.

We have also found expression of *Fgf2* mRNA in both mouse and human lung epithelial cells and inflammatory cells, but not in fibroblasts, which is consistent with prior reports (20, 22–30). Our data support a model in which FGF2 expression is increased primarily in inflammatory cells, thus facilitating its delivery to areas of injury. The precise identity of the cell type that produces FGF2, both in response to lung injury as well as in IPF lungs, however, remains unclear. Prior reports have identified mast cells and macrophages as likely sources in IPF (20). In addition, FGF2 protein is known to be sequestered in the basement membrane and extracellular matrix by heparan sulfate proteoglycans (15, 50), and is released from the extracellular matrix in response to injury-induced proteolysis of the matrix (51, 52).

Our studies use bleomycin as a model agent to induce fibrosis. Bleomycin causes pulmonary fibrosis in 1% of patients treated with this chemotherapeutic agent (53). In mice, bleomycin causes alveolar epithelial injury, inflammation, and fibroblast proliferation and differentiation into myofibroblasts. Although bleomycin does not cause lesions characteristic for IPF (fibroblastic foci and honeycombing) in the mouse lung, bleomycin remains a well established and useful model to study underlying mechanisms of AEC injury and subsequent fibroblast activation and fibrosis (54–56). However, differences between mouse and human, and unexplored FGF ligand redundancies, still leaves open the possibility that FGF2 could make a direct contribution to the pathogenesis of human IPF.

Figure 8. Decreased epithelial barrier function in *Fgf2*^{-/-} mice after bleomycin. A single dose of intranasal bleomycin (4 mg/kg) was administered to *Fgf2*^{-/-} or wild-type littermate controls, and mice were collected 7, 14, or 21 days after treatment. (A) Total protein from BAL cell-free supernatants was measured using a bicinchoninic acid protein assay. **P* < 0.05 and ***P* < 0.001 compared with wild-type PBS control, unless indicated by brackets (*n* = 13). (B and C) Mice were injected with an Evans blue solution via tail vein 30 minutes before being killed, and lungs were perfused with PBS via the right ventricle. Evans blue was measured in whole lungs (B) or cell-free BAL supernatants (C) in wild-type and *Fgf2*^{-/-} mice 21 days after bleomycin treatment, and normalized to Evans blue concentration in blood to generate an Evans blue index. **P* < 0.05 (*n* = 6). All data presented are mean ± SEM.

Our results show that *Fgf2*^{-/-} mice have persistent lung inflammation after bleomycin treatment. Although it is likely that this inflammation is a result of insufficient epithelial recovery, it remains unknown whether resolution of inflammation is directly regulated by FGF2. Given that inflammatory cells are the

primary source of FGF2 after injury, the possibility of an autocrine FGF2 feedback signaling mechanism contributing to resolution of inflammation must be considered.

It is also surprising that *Fgf2*^{-/-} mice do not have increased amounts of bleomycin-induced pulmonary fibrosis, as a link between epithelial injury and fibroproliferation in response to bleomycin is well described (57–59). For example, epithelial-specific knockout of β -catenin causes increased epithelial injury in response to bleomycin, and subsequently leads to increased fibrosis (58). FGF2 is required for myofibroblast differentiation and collagen production *in vitro*, and may therefore still be required for a portion of bleomycin-induced pulmonary fibrosis *in vivo*. The increase in profibrotic stimuli seen in *Fgf2*^{-/-} mice, such as inadequate epithelial recovery, prolonged inflammation, and prolonged IL-6 production, may therefore be countered by a decrease in FGF2-dependent fibrosis. Alternatively, the fibrosis observed in *Fgf2*^{-/-} mice may develop through a different mechanism than that observed in wild-type mice. For example, IL-6 is known to directly stimulate fibrosis independently of TGF- β 1 (60, 61), and *Fgf2*^{-/-} mice have prolonged IL-6 expression at late time points after bleomycin.

FGF2 and Lung Injury

FGF2 is protective in the setting of tissue injury, is critical for response to injury in the heart and skin (40, 62), and is up-regulated in acute airway injury (23, 28). Administration of exogenous FGF2 is protective against myocardial infarction (62), and is beneficial for skin wound and ulcer healing (40, 63, 64). Furthermore, *Fgf2*^{-/-} mice have delayed cutaneous wound healing (40), increased emphysema in response to IFN- γ overexpression (65), worsened myocardial ischemia (62, 66), increased hindlimb ischemia (67), and enhanced airway hyperreactivity in response to allergen challenge (68). These findings support our observations that FGF2 is essential for epithelial recovery after lung injury. The precise mechanism by which FGF2 supports lung epithelial repair remains unknown, and is the subject of ongoing studies.

Other FGFs, such as the FGFR2b ligands, FGF7 (keratinocyte growth factor)

and FGF10, stimulate epithelial proliferation and are protective against a variety of experimental models of lung injury when given as a pretreatment (41, 42, 69–73). Inhibition of FGFR2b ligands *in vivo* worsens hyperoxic lung injury (45). The requirement of these specific ligands for bleomycin-induced lung injury, however, has not been demonstrated *in vivo*. In fact, FGF7 knockout mice, and mice in which all FGFR2b ligands were inhibited, showed no difference in weight loss or mortality, and had comparable amounts of fibrosis to wild-type controls in response to bleomycin (S. Bellusci, personal communication). Other FGFRs expressed in the alveolar epithelium, including FGFR3, and, to a lesser extent, FGFR1, may also have an under-appreciated role in lung epithelial repair after injury (74). FGF2 is capable of activating most FGFRs, including the epithelial FGFR1b splice variant and FGFR3 (11, 75), and therefore may provide a critical reparative signal to lung epithelium. Decreased recovery of *Etv5* expression, which is a downstream target of FGF signaling and is localized to AECs in the mouse lung (46), in *Fgf2*^{-/-} mice after bleomycin further supports epithelial-specific FGF2 signaling. Furthermore, we show evidence that *Fgf2*^{-/-} mice also have deficient recovery of CCSP⁺ bronchial epithelium after naphthalene-induced bronchial epithelial injury. Overall, our data suggest that FGF2 may provide an essential endogenous epithelial reparative signal in the setting of lung injury.

Insufficient recovery of the alveolar epithelial barrier is associated with increased mortality in animal models of lung injury (76, 77). E-cadherin expression is specific to epithelial cells, and it is a component of the tight junctions between epithelial cells that maintain epithelial integrity and barrier function after lung injury (78, 79). The impaired recovery of epithelial barrier function *in vivo* in *Fgf2*^{-/-} mice may be a direct consequence of E-cadherin dysregulation in the absence of FGF2 after injury, or may represent indirect consequences of impaired epithelial physiology or regeneration.

Therapeutic Potentials

Our studies suggest that administration of FGF2, or augmentation of FGF2-dependent signaling, may be beneficial in the setting of

lung injury. Recombinant FGF2 is beneficial in multiple models of tissue injury, and has been used to augment recovery from myocardial ischemia, hindlimb ischemia, bone fractures, and skin wounds (62, 66, 68, 80). In the lung, recombinant FGF2 protects from IFN-induced emphysema and airway hyperreactivity in response to allergen exposure (65, 68). Intratracheal delivery of FGF2 transiently increases pulmonary blood flow and reduces experimentally

induced emphysema (81, 82). Overexpression of FGF7 in adult mouse lung epithelium causes pulmonary cystadenomas (83), and intravenous or intratracheal administration causes reversible AEC proliferation (84). Generalized overexpression of FGF2 or systemic administration of recombinant FGF2, in contrast to FGF7, is well tolerated, and does not cause spontaneous pulmonary or cardiac abnormalities (85). In fact, topical administration of FGF2 is

used clinically to treat chronic skin wounds and diabetic ulcers (86–88). Future studies will determine whether FGF2 can protect the lung from injury, and whether administration of FGF2 after injury can augment repair. ■

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