FGF9 prevents pleural fibrosis induced by intrapleural adenovirus injection in mice

Aurélien Justet, Audrey Joannes, Valérie Besnard, Joëlle Marchal-Sommé, Madeleine Jailet, Philippe Bonniald, Jean Michel Sallenave, Brigitte Solhonne, Yves Castier, Pierre Mordant, Hervé Mal, Aurélie Cazes, Raphael Borie, Arnaud A. Maillieux, and Bruno Crestani

Institut National de la Santé et de la Recherche Médicale U1152, Paris, France; Département Hospitalo-Universitaire Fibrosis Inflammation and Remodeling (DHU FIRE), Paris, France; Labex Inflamex, Paris, France; Université Paris Diderot, Sorbonne Paris Cité, Paris, France; Assistance Publique-Hôpitaux de Paris, Hôpital Bichat, Service de Pneumologie A, Paris, France; Institut National de la Santé et de la Recherche Médicale U866, Université de Bourgogne, Dijon, France; Assistance Publique-Hôpitaux de Paris, Hôpital Bichat, Service de Chirurgie Thoracique et Vasculaire, Paris, France; Assistance Publique-Hôpitaux de Paris, Hôpital Bichat, Service de Pneumologie et Transplantation, Paris, France; and Assistance Publique-Hôpitaux de Paris, Hôpital Bichat, Département d’Anatomie Pathologique, Paris, France

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MESOTHELIAL CELLS form a monolayer, known as the mesothelium, that lines the pleural cavity with visceral and parietal surfaces covering the lung and the thoracic wall, respectively (2). Mesothelial cells play critical roles in the maintenance of pleural homeostasis in response to injury, inflammation, and immunoregulation (36). Mesothelial cells are also central cells in pleural repair, secreting inflammatory mediators, chemokines, growth factors, and extracellular matrix components (36). During fetal lung development, mesothelial cells interact with other cell types to regulate the harmonious development of lung structures (55).

Idiopathic pulmonary fibrosis (IPF) is a progressive, irreversible, and lethal lung disease of unknown etiology (3, 26). IPF is characterized by excessive extracellular matrix deposition and leads to severe restrictive lung disease (14). IPF begins in the subpleural region and then extends centrally. Three-dimensional morphometric analysis of the IPF lung suggests that fibroblast foci are the leading edge of a complex fibroblast reticulum that is highly interconnected and extends from the pleura into the underlying parenchyma (8). Several studies suggest that the mesothelial cells may be involved in IPF pathogenesis (11, 34). Mesothelial cells can be induced to undergo mesothelial-to-mesenchymal transition and then differentiate into myofibroblasts (7, 37, 62). Decolgone et al. previously developed an experimental model of pleural fibrosis induced by adenoviral transfer of transforming growth factor-β (TGF-β) in mesothelial cells (11). The authors observed the development of a progressive pleural fibrosis that extended into the lung parenchyma while mesothelial cells underwent mesothelial-to-mesenchymal transition. Similar findings were observed after the intrapleural injection of bleomycin combined with carbon particles in mice (12). This phenotypic change was also observed in vitro with mesothelial cells treated with recombinant TGF-β (37, 41). More recent mouse models of fibrogenic lung injury have also supported this observation by showing that mesothelial cells invade the lung parenchyma and...
FGF9 IN PLEURAL FIBROSIS

Adopt a myofibroblastic phenotype after intratracheal TGF-β1 administration, leading to fibrosis (62). Most importantly, immunohistochemical analysis of human IPF lung sections showed Wilms tumor-1 (WT-1)-positive mesothelial cells in the pleura and lung parenchyma (62). Collectively, these studies indicate potential contributions of pleural mesothelial cells as a source of myofibroblast in IPF.

IPF is characterized by the reactivation of key developmental pathways involved in lung development such as the Gli/hedgehog pathway (5, 33), the canonical/noncanonical WNT pathway (22, 27), or the fibroblast growth factor (FGF)/FGFR receptor (FGFR) pathway (20, 24, 48). The FGF pathway comprises 22 members acting on five FGF receptors (FGFR1, FGFR2, FGFR3, FGFR4, and FGFR1L) (39). Alternative splicing in the extracellular IgIII loop generates either a IIIb isoform (preferentially expressed by epithelial cells) or a IIIc isoform (preferentially expressed by mesenchymal cells) (16), which differ in their ligand-binding affinity and tissue distribution. FGF/FGFR signaling is essential for all stages of lung morphogenesis and actively contributes to epithelium-mesenchymal interactions (43, 54). In particular, FGF9 has an important role in lung ontogenesis (56). Interestingly, FGF9 is expressed in mesothelium and epithelium and controls epithelial branching and mesenchymal proliferation (58), mainly during the pseudoglandular stage of embryonic lung development. Mice homozygous for a targeted disruption of Fgf9 show lung hypoplasia and early postnatal death (7). Fgf9−/− lungs present reduced mesenchyme and decreased airways branching (7).

Recently, we showed that FGF9 had anti-apoptotic and promigratory properties on human lung fibroblast and maintained fibroblasts in an undifferentiated state. These effects were partially driven by FGFR3 (24). Expression of FGF9 by mesothelial cells and the biological effect of FGF9 on mesothelial cells have never been addressed.

In the current study, we asked whether FGF9 expression by mesothelial cells could participate in the pleural repair process. We determined the pattern of expression of FGF9 and FGFR by mesothelial cells in the normal human and mouse lungs as well as in IPF lung. We next overexpressed human FGF9 in vivo in mesothelial cells, using a recombinant adenovirus injected in the pleural cavity, and we determined the effect of FGF9 in the mesothelial repair process in vivo. Finally, we examined the effect of FGF9 on mesothelial cell properties in vitro. Our results demonstrate for the first time that FGF9 has potent anti-fibrotic effects in the pleura in vivo and inhibits differentiation and migration in vitro.

MATERIALS AND METHODS

Human lung samples. IPF lung samples were obtained from patients undergoing open lung biopsy or at the time of lung transplantation (n = 39; median age, 59.3 yr; range, 50.7–69.9 yr). IPF was diagnosed according to American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association criteria, including histopathological features of usual interstitial pneumonia (9, 44). Lung samples obtained after cancer surgery, away from the tumor, were used as controls; normality of control lungs was verified histologically (n = 36 patients; median age, 62.2 yr; range, 30–82.7 yr). Some of these patients were included in a previous work (24). This study was approved by the local ethics committee (CPP Ile de France 1, no. 0811760). Written informed consent was obtained from all subjects.

Recombinant adenovirus. Recombinant nonreplicative and nonintegrative adenoviruses were obtained from Applied Biological Materials (Teddington, UK). The sequence of control (AdCont) and recombinant (AdFGF-9) adenovirus (BC103978) differs only in the human FGF-9 gene inserted in the recombinant virus. This sequence remains empty in the control adenovirus. The viruses were amplified on HEK293 cells and purified by centrifugation. The final product was desalted, assayed spectrophotometrically, and then stored at −80°C until use (32).

Animal treatment. C57BL/6N mice from Janvier Laboratories (Le Genest Saint Isle, France) were treated in accordance to the guidelines of the French Ministry of Research. All experiments were approved by the animal ethical committee of Université Paris-Diderot (agreement no. C75–18–01). Adenovirus (100 µl, 1 × 10⁶ plaque-forming units/ml) or saline serum was injected in the pleural cavity as described by Decologne et al. (11). Mice were euthanized 3, 5, 14, and 25 days after adenoviral injection. Lung, pleural lavage and bronchoalveolar lavage (BALF) fluids were collected as previously described (12, 33).

Morphological analysis. Hematoxylin-eosin and picrosirius staining was performed to evaluate the morphology of the lung. The slides were scanned using a motorized microscope coupled to Calopix acquisition software (TRIBVN, Châtillon, France). The whole area of each pleural section was manually delineated. The pleural thickness was calculated by dividing the total pleural area by the length of the pleura. A semiquantitative analysis of intrapleural collagen by picrosirius stain was performed as previously published by Charpin et al. (4).

Immunohistochemistry. The paraffin-embedded sections were treated as previously described (33). Primary antibodies for immunohistochemistry were anti-FGF9 (clone C-19; Santa Cruz Biotechnology, Santa Cruz, CA); anti-WT-1 (clone C-19; Santa Cruz Biotechnology); anti-FGFR1 (ab10646), anti-FGFR2 (ab10648), anti-FGFR3 (EPR2305) (ab)37084, all Abcam, Cambridge, MA); anti-FGFR4 (HPA027369; Sigma-Aldrich), anti-α-smooth muscle actin (SMA) (clone 1A4; Sigma-Aldrich), anti-vimentin (clone V9; Sigma-Aldrich), anti-collagen I (Southern Biotechnology Associates, Birmingham, AL), and anti-fibronectin (ab 2413). To test the specificity of immunostaining, primary antibodies were replaced by an isotype-matched control. For semiquantitative assessment of anti-Ki-67 (Bio-Science, San Diego, CA), the positive cells and the total number of cells (nuclei) were enumerated in the lung, excluding the vessels and bronchi, using Calopix Software (TRIBVN). For the semiquantitative analysis of pleural staining in five IPF patients and five other controls, three of us, including one pathologist (AC) independently quantified the staining (from 0 to 3+). Species source and species cross-reactivity of FGF9 and FGFR antibodies are given in Table 1.

Cell culture. Rat lung mesothelial cells (6/4-RM-4 cell line; ATCC-CRL216) were obtained from ATCC (LGC Standards; Molsheim, Scientific, Rochester, NY), supplemented with 15% fetal bovine serum (GIBCO), and grown in DMEM-F-10 (GIBCO). Thermostabilized fibroblast growth factor; FGFR, fibroblast growth factor receptor; WT-1, Wilms tumor-1.

Table 1. Species source and species cross-reactivity of FGF9 and FGFR antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species Source</th>
<th>Species Reactivity</th>
<th>Isotype</th>
<th>Ref. No.</th>
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<tr>
<td>FGF9</td>
<td>Goat</td>
<td>Mouse, rat, human</td>
<td>IgG</td>
<td>(29)</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Rabbit</td>
<td>Mouse, rat, human</td>
<td>IgG</td>
<td>(49)</td>
</tr>
<tr>
<td>FGFR2</td>
<td>Rabbit</td>
<td>Mouse, rat, human</td>
<td>IgG</td>
<td>(47)</td>
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<tr>
<td>FGFR3</td>
<td>Rabbit</td>
<td>Human</td>
<td>IgG</td>
<td>(24)</td>
</tr>
<tr>
<td>FGFR4</td>
<td>Rabbit</td>
<td>Human</td>
<td>IgG</td>
<td>(42)</td>
</tr>
<tr>
<td>WT-1</td>
<td>Rabbit</td>
<td>Human, mouse, rat, avian</td>
<td>IgG</td>
<td>(25)</td>
</tr>
</tbody>
</table>

FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; WT-1, Wilms tumor-1.

Downloaded from journals.physiology.org/journal/ajplung (052.011.211.149) on June 9, 2020.
RM-4 cells were treated for 48 h with human recombinant FGF9 (20 ng/ml) (R&D Systems, Minneapolis, MI), in the presence of heparin chocky (100 μg/ml) because heparin is essential for FGF signaling (45, 57). Heparin alone was used as a control condition.

For immunoblot analysis and mRNA analysis, mesothelial cells were plated in six-well plates for 24 h and then starred in DMEM-F-10 with 1% fetal calf serum for 16 h before stimulation. For TGF-β1-induced mesothelial differentiation experiments, serum-starved mesothelial cells were cultured for 1 h with FGF9 (20 ng/ml), and then media was removed and TGF-β1 (5 ng/ml) (R&D Systems) was added for the next 48 h with FGF9 (20 ng/ml).

For cell growth assay, 4/4RM-4 cells were cultured for 48 h with FGF9 (20 ng/ml) in medium containing 1% fetal calf serum. The proliferation rate of fibroblasts was assessed by cell counting and cell viability with the use of a V-Cell counter (Beckman Coulter, Villepinte, France). Cell viability was assessed by Trypan blue exclusion assay.

For FGFR3 knockdown experiments with CRISPR-Cas9 plasmid, a 24-h incubation at 37°C. FGF9 was determined from BALF and pleural fluid supernatants using an ELISA kit (R&D Systems) according to the recommendations of the manufacturer. The detection range of this assay is 62.5–4,000 pg/ml. In preliminary experiments, we observed that this assay detected both human and murine FGF9.

### Table 2. Taqman gene expression assay

<table>
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<th>Gene</th>
<th>Assay ID</th>
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<tr>
<td>FGF9</td>
<td>Hs00181829_m1</td>
</tr>
<tr>
<td>βm</td>
<td>Hs0187842_m1</td>
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<td>Fgfr9</td>
<td>Mm00442795_m1</td>
</tr>
<tr>
<td>βm</td>
<td>Mm00437762_m1</td>
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Microglobulin (β2m) transcripts were used as an endogenous RNA control. FGF9 and FGFR mRNA were quantified by TaqMan Gene Expression Assays (Life technologies, Saint Aubin, France), and collagen, actine-α2 (Acta2), fibronectin, vimentin, TGF-β1 (Tgfβ1), plasmogen activator inhibitor-1 (Pai-1), and connettive tissue growth factor (Ctgf) were quantified with SybrGreen real-time PCR master mix (see Tables 2 and 3 for primers) with use of PCR ABI 7500 (Applied Biosystems, Carlsbad, CA).

### Table 3. Primer sequences for quantitative PCR

<table>
<thead>
<tr>
<th>Mice</th>
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<th>Reverse</th>
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<tr>
<td>acta-1</td>
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<td>Acta-2</td>
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<td>5'-GTTGACCTGCGTCTTCTGCTG-3'</td>
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<tr>
<td>Ctgf</td>
<td>5'-GAGTGCTGCTGCTGAGAAGATGG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5'-GTTGGCTGAGCAGAAGATGG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>Pai-1</td>
<td>5'-TGGGCTGCTGCTGAGAAGATGG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>Vimentin</td>
<td>5'-ATGACCTGCGTCTTCTGCTG-3'</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>Tgfβ</td>
<td>5'-ACTGCTGCTGCTGAGAAGATGG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>WT-1</td>
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<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
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</table>

<table>
<thead>
<tr>
<th>Rat</th>
<th>Forward</th>
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<tr>
<td>Fgfr1IIb</td>
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<td>5'-CAATGCTGCTGCTGCTGCTG-3'</td>
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<tr>
<td>Fgfr1IIC</td>
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<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
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<tr>
<td>Fgfr2IIb</td>
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<td>Fgfr3IIC</td>
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<td>Fgfr4</td>
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<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
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<td>α-col-1</td>
<td>5'-GAGGCCGCTGAGAAGATGGTGAG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>α-col-1</td>
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<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5'-GAGGCCGCTGAGAAGATGGTGAG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>Acta-2</td>
<td>5'-GAGGCCGCTGAGAAGATGGTGAG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
<tr>
<td>βm</td>
<td>5'-GAGGCCGCTGAGAAGATGGTGAG-3T</td>
<td>5'-GAGGACAGATGCTGATGGAT-3'</td>
</tr>
</tbody>
</table>

βm, β2-microglobulin; Ctgf, conneteive tissue growth factor; Pai-1, plasmogen activator inhibitor-1; Tgfβ, transforming growth factor-β; col-1, collagen 1; Acta2, actine-α2.
Mann Whitney U-test was used for comparisons between groups. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

*FGF9 was expressed by mesothelial cells in IPF.* We examined FGF9 and FGFR expression by mesothelial cells in human lung samples obtained from control and IPF patients by immunohistochemistry (Fig. 1A), and we performed a semiquantitative analysis of pleural staining in five IPF samples and five controls (Table 4). In IPF, mesothelial cells were strongly positive for FGF9, FGFR3, and FGFR1. In controls, FGF9 was never detected while FGFR3 was detected in 3/5 samples and FGFR1 in 5/5 samples. FGFR2 and FGFR4 were not detected in control and IPF pleural mesothelial cells (Fig. 1A and Table 4).

We then assessed FGF9 and FGFR immunolocalization in normal mouse pleura. Only a few mesothelial cells were positive for FGF9 in the normal pleura (Fig. 1B). With regards to FGF9, FGFR3 was strongly detected in pleural mesothelial cells, compared with FGFR1 and FGFR2, which were present at a weaker level. Meanwhile, FGFR4 was not detected in mouse mesothelial cells (Fig. 1B).

*FGF9 expression kinetics in vivo after adenoviral infection.* Because FGF9 expression was associated with activated mesothelial cells in human lung fibrosis (Fig. 1A), we undertook to investigate the effects of FGF9 overexpression on mouse mesothelial cells in vivo. To characterize and to validate our model of FGF9 overexpression, we first focused on FGF9 expression kinetics at both mRNA and protein levels. Thus, lungs were harvested at various time points after adenovirus administration in the right pleural space. We measured human and murine FGF9 mRNA expression in whole lung homogenates. As expected, human FGF9 mRNA was not detected after AdCont injection, whereas human FGF9 mRNA was detected at all time points in the animals receiving AdFGF9 (Fig. 2A). Human FGF9 mRNA level peaked on day 3 and decreased from days 5 to 14 (Fig. 2A).

Interestingly, adenoviral infection of the pleural cavity induced the expression of murine Fgf9 mRNA, which peaked on day 5 and persisted on day 14 (Fig. 2A). The magnitude of the response was similar in control and recombinant adenovirus. Murine Fgf9 mRNA was not detected in mice injected with saline (data not shown).

We measured FGF9 protein level by ELISA in the pleural lavage fluid at days 3, 5, and 14 after adenovirus injection. FGF9 was not detected on day 3, was maximal on day 5, and remained slightly increased on day 14 after adenoviral injec-

**Table 4. Semiquantitative analysis of FGF9 and FGFR3 staining by mesothelial cells**

<table>
<thead>
<tr>
<th>Mesothelial Cell Staining</th>
<th>Controls</th>
<th>IPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF9</td>
<td>Absent</td>
<td>Positive in 5/5 (+++)</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Absent</td>
<td>Positive in 5/5 (+++++++)</td>
</tr>
<tr>
<td>FGFR2</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>FGFR3</td>
<td>Positive in 3/5 (+)</td>
<td>Positive in 5/5 (+++++++)</td>
</tr>
<tr>
<td>FGFR4</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

IPF, idiopathic pulmonary fibrosis.
A. Human FGF9 mRNA

- Relative human FGF9 mRNA ratio
- AdFGF9 vs. AdCont

B. Murine Fgf9 mRNA

- Relative murine Fgf9/β2m mRNA ratio
- AdFGF9 vs. AdCont

C. Pleural fluid FGF9 concentration

- FGF9 concentration (pg/ml)
- D14 vs. D3 vs. D5
- AdFGF9 vs. AdCont

D. Pleural thickness (µm)

- D3 vs. D5 vs. D14
- AdFGF9 vs. AdCont

E. FGF9 IN PLEURAL FIBROSIS

- Human FGF9 vs. Murine Fgf9
- Isotype

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tion (Fig. 2B). FGF9 was never detected in untreated mice or in mice receiving an intrapleural injection of saline solution (data not shown). FGF9 concentration was twofold higher in the animals receiving the AdFGF9 compared with the AdCont group (Fig. 2B). These data demonstrate that AdFGF9 infection induces a moderate but still measurable increase in soluble FGF9 level in the pleural space.

To identify the source of FGF9, we performed FGF9 immunostaining in naïve mice and in intrapleurally injected mice with AdFGF9 and AdCont (Fig. 2C). In naïve mice, alveolar epithelial cells were immunoreactive to FGF9, whereas mesothelial cells were not. Consistently with FGF9 mRNA expression kinetics, mesothelial cells were strongly positive in the AdFGF9 group and faintly positive in the AdCont group at day 5. At day 14, some mesothelial cells were still immunoreactive for FGF9 in the AdFGF9 group while they were negative in the AdCont group (Fig. 2C).

**FGF9 prevented adenosiviral-induced pleural remodeling.** Histology revealed that intrapleural injection of adenosivirus induced marked pleural changes, with a pleural thickening starting at day 5 with a maximum on day 14 in the animals receiving the control adenovirus (Fig. 2C). These changes were spontaneously resolved on day 28 (data not shown). Although limited remodeling of the pleura was observed at day 5 in the animals treated with AdFGF9, the pleura displayed a normal morphology at day 14 in the animals treated with the FGF9 adenovirus (Fig. 2C). Measure of pleural thickness confirms these observations, showing a significant decrease of pleural thickness at day 14 in the AdFGF9 group compared with the AdCont group (Fig. 2D). Interestingly, the subpleural lung showed limited changes with no evidence of fibrosis (data not shown).

We quantified extracellular matrix components in animals treated with control or FGF9 adenovirus. Morphological analysis revealed that control adenoviral infection was associated with the accumulation of collagen (Fig. 3, A and D) and fibronectin (Fig. 3, B and D) in the thickened pleura associated with an increased expression of a1Col1, a1Col3, and fibronectin mRNA (Fig. 3C). Most importantly, these changes were prevented by FGF9 overexpression as assessed by histomorphometry (Fig. 3, A and B), mRNA expression (Fig. 3C), and protein content analysis (Fig. 3D).

**FGF9 prevented accumulation of myofibroblasts in remodeled pleura upon adenosivirus transduction in vivo.** We used immunohistochemistry to characterize the phenotypic changes of pleural cells induced by adenosivirus infection. Control adenoviral infection was associated with an increase of α-SMA (Fig. 4A) and vimentin-positive (Fig. 4B) cells in the remodeled pleura at day 14. Most importantly, FGF9 overexpression completely prevented these cellular changes (Fig. 4, A and B). However, at the mRNA level, we did not observe any change of total lung expression of Vimentin or Acta2 mRNAs between AdCont and AdFGF9 groups, suggesting the phenotypic change were restricted to pleura (Fig. 4, A and B).

**WT-1 expression by mesothelial cells was assessed by immunostaining and mRNA expression.** WT-1 is known to regulate mesothelial cell plasticity (25). In naïve mice, most mesothelial cells did express WT-1 as assessed by immunostaining (data not shown). On day 14, some WT-1 cells were observed at the surface of the remodeled pleura in AdCont and AdFGF9 groups, with a similar pattern between groups (Fig. 4C). Similarly, WT-1 mRNA level was similar in both groups on day 14 (Fig. 4C).

Pleural remodeling induced by adenosivirus infection was associated with an increased expression of key soluble factors at the mRNA level involved in myofibroblast differentiation (17, 18, 38, 50), such as Tgfb1, Ctgf, and Pai-1 (Fig. 4D). Conversely, FGF9 overexpression significantly decreased Ctgf and Pai-1 mRNA levels on day 14 (Fig. 4D), while no significant difference was observed with regards to Tgfb1 mRNA.

**FGF9 inhibited cell proliferation and apoptosis in remodeled pleura after adenosivirus transduction in vivo.** To elucidate the mechanisms associated with the in vivo protective effect of FGF9 overexpression on pleural remodeling, we evaluated the proliferation and apoptosis in remodeled pleura.

The number of Ki-67-positive cells (a marker of cell proliferation) was strongly increased in the remodeled pleura after control adenosivirus infection, and this was again prevented by FGF9 overexpression (Fig. 5A).

We next evaluated the apoptosis process in AdCont animals compared with AdFGF9 ones. We observed that the apoptotic process was activated in the animals infected with the control adenovirus, with an increased expression of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) as assayed by Western blot. FGF9 overexpression was associated with decreased levels of cleaved caspase-3 (day 5) and cleaved PARP (day 14) compared with control adenovirus (Fig. 5B), pointing to an inhibition of the apoptotic process.

**In vitro, FGF9 decreased migration of mesothelial cells in vitro.** Altogether, our in vivo results suggest that FGF9 promotes early repair of the mesothelium after adenosivirus injury. To decipher the molecular mechanisms involved in FGF9 response, we studied the effect of exogenous recombinant human FGF9 on rat lung mesothelial cells (6/4-RM-4 cell line) in vitro.

We first analyzed the pattern of FGF receptor expression by mesothelial cells in vitro. The mesenchymal-associated IIIc FGR isoforms were the most detected, and the FGRHIIIc was strongly expressed compared with FGFR2IIIc and FGFR1IIIIC (Fig. 6A), whereas FGFR4 was not detected. However, 6/4-RM-4 mesothelial cells also expressed the epithelial IIIb isoform.
** McGill University and Royal Victoria Hospital, Montreal, Canada **

** ICU and Critical Care Medicine, McGill University, Montreal, Canada **

** McGill University and Royal Victoria Hospital, Montreal, Canada **

** McGill University and Royal Victoria Hospital, Montreal, Canada **

** McGill University and Royal Victoria Hospital, Montreal, Canada **
Next, we assessed the effect of FGF9 (20 ng/ml) on mesothelial cells. We observed that FGF9 strongly stimulated ERK phosphorylation and decreased pSTAT3 phosphorylation in mesothelial cells (Fig. 6B). These results indicated that 6/4-RM-4 mesothelial cells did respond to FGF9 stimulation in vitro.

Our in vivo results clearly indicate that FGF9 overexpression in mice pleura affects mesothelial cell proliferation and apoptosis. However, FGF9 did not modulate mesothelial cell proliferation in vitro over 24–96 h of stimulation (Fig. 6C). Migration contributes to mesothelial cell dispersion in lung parenchyma and may participate to subpleural fibrosis (62). We tested the effect of FGF9 on mesothelial cell migration in a modified Boyden chamber assay. FGF9 decreased the cell migratory capacities by 50% (Fig. 6D).

FGF9 decreased basal differentiation and partially prevented TGF-β-induced differentiation of mesothelial cells in vitro. Mesothelial-to-mesenchymal transition has been identified as a profibrotic cellular pathway in the lung. In this process close to epithelial-to-mesenchymal transition, mesothelial cells lose their mesothelial characteristics and acquire mesenchymal properties. TGF-β is a well-characterized inducer of mesothelial-to-mesenchymal transition (37).

We assessed mesothelial and mesenchymal markers in mesothelial cells cultured in vitro with FGF9 (20 ng/ml) for 48 h with or without TGF-β1 (5 ng/ml). In the absence of TGF-β, FGF9 decreased collagen-1 and α-SMA protein content while vimentin and WT-1 were unchanged (Fig. 6, E, F, and G). As expected, TGF-β1 increased collagen-1, α-SMA, and vimentin and decreased WT-1 content in mesothelial cells (Fig. 6, E, F, and G). FGF9 prevented TGF-β1-induced collagen-1 and α-SMA protein upregulation but did not affect vimentin expression or WT-1 expression (Fig. 6, E, F, and G).

Taken together, our results indicate that FGF9 modulates mesothelial plasticity and partially prevents the mesothelial-to-mesenchymal transition process in vitro.

FGF9 effects were partially driven by FGFR3 in 4/4-RM-4 mesothelial cells. FGFR3 is the main receptor for FGF9 response (40, 59). To determine the role of FGFR3 in FGF9 response in vitro, we knocked down Fgfr3 in rat mesothelial cells with CRISPR-Cas9 gene editing assay. We obtained a population of mesothelial cells with 50% Fgfr3 inhibition at the mRNA level. We observed that the effect of FGF9 on collagen-1 and α-SMA protein content was partially blocked in Fgfr3 CRISPR-Cas9 mesothelial cells (Fig. 7). These data indicate that FGFR3 partially drives the effect of FGF9 on mesothelial cell differentiation in vitro.

**DISCUSSION**

FGF9 is implicated in the communication between epithelium and mesenchyme during fetal lung development (56, 58). Indeed, FGF9 is the only FGF expressed by the mesothelium during the lung pseudoglandular stage, and FGF9 controls mesenchymal cell proliferation. Recently, we reported that FGF9 played a role in IPF as an anti-apoptotic and promigratory growth factor on human lung fibroblast and maintained fibroblasts in an undifferentiated state (24). In the current study, we observed that FGF9 and its preferential receptor, FGFR3, were strongly expressed in IPF pleura. We demonstrated that in vivo intrapleural FGF9 overexpression prevented pleural remodeling, impaired myofibroblastic differentiation of pleural mesothelial cells, and inhibited mesothelial proliferation and apoptosis induced compared with intrapleural injection of a control adenovirus. In vitro, we demonstrated that FGF9 modulated mesothelial plasticity and partially prevented myofibroblastic differentiation of mesothelial cells induced by TGF-β1. Finally, we demonstrated that FGF9 effects on mesothelial cells were, at least partially, driven by FGFR3.

To our knowledge, this is the first attempt to characterize in depth FGF9 expression in vivo, in control and IPF human mesothelial cells, mouse lung pleura, as well as in vitro in a rat mesothelial cell line. We observed that FGFR3 and FGFR1 were the most expressed receptors by pleural mesothelial cells, in mice and humans, in control and IPF tissue. MacKenzie et al. reported in a recent work a strong expression of FGF1 and of c-isoform of FGF1R2/3 in the fibrotic region (30). In our study, we observed an increased expression of FGFR3 by mesothelial cells as assessed by immunohistochemistry (Fig. 1). In mice, FGF9 expression and localization were not affected by adenoviral infection (data not shown). FGFR3 is the main receptor for FGF9, but FGFR1 is also a target for FGF9 (40, 59). Coffey et al. previously detected FGF9 expression by mesothelial cells in IPF (6). This is an important feature that fits with the general observation that IPF is characterized by a reactivation of signaling pathways involved in lung development (5, 10, 22, 27, 33).

Plural fibrosis affects patients suffering from complicated parapneumonia pleural effusions, empyema, or asbestosis-related injury. This thickened pleura contributes to decreased lung volume, decreased lung compliance and led to lung restriction (23, 51). Pleural mesothelial cells undergo a process called mesothelial-mesenchymal transition. During this process, mesothelial cells acquire a myofibroblast-like phenotype characterized by a change of morphology, an increased expression of myofibroblast marker such as α-SMA, and an increase of matrix protein expression such as collagen-1. To our knowledge, pleural remodeling induced by the intrapleural injection of a control adenovirus has not been previously described. Interestingly, the pleural alterations observed in our study present some similarities, but with less intensity, to those described in experimental pleural fibrosis induced by adenoviral gene transfer of TGF-β to the pleura (11) or with coinstillation of bleomycin and carbon particles (12). However, these alterations are spontaneously resolutive in the current...
Fig. 4. FGF9 prevented myofibroblast differentiation in the pleura and decreased profibrotic mediator expression in vivo. Immunolabeling and quantitative PCR of α-smooth muscle actin (SMA, A), vimentin (B), and Wilms tumor-1 (WT-1, C) on day 14 in treated animals (scale bar, 50 μm). Mesothelial cells strongly expressed SMA and vimentin in AdCont-treated animals, whereas the AdFGF9-treated animals were protected. Mesothelial cells expressed similar levels of WT-1 in both groups (scale bar, 50 μm). D: quantitative PCR analysis of mRNA levels for transforming growth factor-β (TGF-β), CTGF, and plasminogen activator inhibitor-1 (PAI-1) on days 3, 5, and 14. Levels are relative to that of β2m; 6–9 mice/condition, *P < 0.05 and **P < 0.01.
model. Recently, another study also reported that a fibrotic process could be triggered upon adenoviral transduction in vivo. Zhou et al. demonstrated that the intratracheal instillation of a high dose of adenoviral vectors was sufficient to induce lung injury with an inflammatory cell influx, increased TGF-β concentration in bronchoalveolar lavage, and subsequent fibrosis in mice (61).

One limitation of our study is the use of an empty adenovirus as a control. An adenovirus containing a nonsense sequence could have been a better control. However, except for the sequence of the human FGF9 gene insert in the recombinant adenovirus, both adenoviruses were identical and they induced a similar inflammatory reaction at days 3 and 5, as assessed by pleural cellularity (data not shown) and pleural thickening (Fig. 2, C and D). Interestingly, the AdFGF9 virus induced a mild overexpression of FGF9. Indeed, we only observed a twofold higher intrapleural concentration of FGF9 in the AdFGF9 group compared with the AdCont group (Fig. 2A). Considering the difference of pleural morphology at day 14 between the two groups, this result suggests a very strong biological effect of FGF9. However, different factors such as the dilution associated with pleural lavage technique and the ability of heparan sulfate to bind FGF9 may have led to an underestimation of FGF9 intrapleural concentration. Indeed, the mesothelial cells secrete surface glycosaminoglycans, predominantly hyaluronan (35), able to sequestrate FGF but also to modulate and increase FGF/FGFR affinity (31, 45, 53).

We observed that the adenoviral infection of the pleural cavity induced the expression of FGF9 in mice injected with the control adenovirus. It is known that FGF9 may be produced in other tissues after injury. In a model of peritonitis induced by thioglycollate in mice, Lam et al. observed an increased production of FGF9 in the peritoneal fluid and plasma (28). Antoine et al. observed overexpression of FGF9 by stellar liver

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Fig. 5. FGF9 inhibited mesothelial proliferation and apoptosis activation in vivo. A: Ki-67 immunolabeling at day 14 in AdCont- and AdFGF9-treated animals. Graph column represents the quantification of positive Ki-67 cells, using Calopix software, ***P < 0.001. B: Representative Western blot analysis of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) in AdCont- and AdFGF9-treated animals. Graph column represents the quantification of Western blot. Protein levels were quantified and normalized with β-tubulin (β Tub) loading control and expressed as fold induction compared with control. *P < 0.05.
cells after exposition to carbon tetrachloride, as an acute liver injury model (1). Zheng et al. observed an increased expression of FGF9 in the skin after laser-induced injury (60). In our model, we cannot assess the role of mesothelial cells and inflammatory cells in FGF9 accumulation in the pleural space, since we were not able to confidently detect FGF9 in mice.

We showed that pleural FGF9 overexpression was associated with a decrease in mRNA expression of profibrotic factors such as CTGF and PAI-1, an inhibition of the accumulation of myofibroblasts as well as extracellular matrix components such as collagen and fibronectin in the thickened pleura. This observation was confirmed in vitro where FGF9 inhibited the acquired expression of a myofibroblastic phenotype by mesothelial cells. Part of the protective effect of FGF9 may be the result of an interaction with TGF-β. Indeed, a cross talk between FGF9 and TGF-β signaling has been previously reported in the small intestine where FGF9 is known to play a key role in fetal development. In that tissue, FGF9 targets a subpopulation of mesenchymal stem cells and downregulates TGF-β signaling through the production of TGF-β inhibitors (15). Similarly, in human lung fibroblasts, we showed that FGF9 prevented TGF-β1-induced myofibroblast differentiation (24). Karki et al. demonstrated that WT-1 regulated mesothelial cell plasticity and that its loss of expression would lead to mesothelial-to-mesenchymal transition (25). Using WT-1 immunolabeling, we observed a decreased expression of WT-1 by mesothelial cells in the remodeled pleura at day 14, without difference between the AdCont and AdFGF9 groups (Fig. 4C). In vitro we observed that TGF-β1 induced a decrease of WT-1 expression (Fig. 6G), whereas FGF9 did not influence its expression.

We observed that FGF9 overexpression was associated with decreased mesothelial cell proliferation and reduced apoptosis in vivo. In vitro, FGF9 had no effect on cell proliferation of the immortalized rat 6/4-RM-4 mesothelial cell line. This apparent discrepancy may reflect the complexity of the in vivo milieu where multiple mediators may interact to produce a given effect. Interestingly, FGF9 was identified as a survival promoter of germ cells in the fetal testis (6, 7, 13) and as promoter of malignant transformation in gastric cancer (46). The antiapoptotic properties of FGF9 and its overexpression in IPF could contribute to the high incidence of lung cancer in this population. Resistance to apoptosis has been identified as a cause of the increased pool of fibroblast and myofibroblast in the fibrotic lung (50). Recently our group demonstrated that FGF9 promoted survival of lung fibroblast in vitro (24). Alternatively, FGF9 may be enhancing adenovirus-induced DNA damage, since other FGFs, such as FGF2 (21) or FGF10 (52), have been shown to act against oxidative stress.

The acquisition of migration properties is a key feature in the mesothelial-to-mesenchymal transition process. Zolak et al. and Decolgone et al. have demonstrated that transdifferentiated mesothelial cells could enter in the lung parenchyma and participate in the fibrotic process and increase the myofibro-
blast pool (11, 62). In this study, we observed that FGF9 decreased the migration of mesothelial cells in vitro. Our team previously reported that FGF9 enhanced human lung fibroblast migration in vitro. This divergent result could result from the differential expression of FGFR by different cell types, mesothelial cells expressing both epithelial and mesenchymal isoforms as assessed by PCR (Fig. 6A). Indeed, FGF9 can activate the mesenchyme-specific "c"-splice forms of FGFR and has also the unique ability to activate epithelial FGFR signaling after binding IIIb isoforms (58). Alternatively, this apparent discrepancy in the effect of FGF9 on mesothelial cells and fibroblasts may just reflect the positive and preventative effects of FGF9 related to resolution of damage, typical of other FGFs (19, 48), whereas in the IPF context, the overexpression of FGF9 may represent failed attempts to resolve overwhelming damage. Evaluating mesothelial repair in FGF9-deficient mice would be critical to further confirm the role of FGF9 in pleural homeostasis; however, these mice were not available to us.

In conclusion, we observed that the injection of an adenovirus in the pleural cavity induced transient fibrotic remodeling of the pleura. This phenomenon was prevented by the pleural overexpression of FGF9. These results suggest that the expression of FGF9 by mesothelial cells is a physiological response to injury and that FGF9 has anti-fibrotic properties promoting mesothelial repair after infectious injury, but is not sufficient to resolve overwhelming damage in IPF.

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Present address for A. Joannes: INSERM U1085, IRSET Institut de Recherche sur la Santé, l’Environnement et le Travail, Université de Rennes-1, France.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


