Expression of Fibroblast Growth Factor 9 in Normal Human Lung and Idiopathic Pulmonary Fibrosis

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Summary

The fibroblast growth factor (FGF) family of signaling ligands contributes significantly to lung development and maintenance in the adult. FGF9 is involved in control of epithelial branching and mesenchymal proliferation and expansion in developing lungs. However, its activity and expression in the normal adult lung and by epithelial and interstitial cells in fibroproliferative diseases like idiopathic pulmonary fibrosis (IPF) are unknown. Tissue samples from normal organ donor human lungs and those of a cohort of patients with mild to severe IPF were sectioned and stained for the immunolocalization of FGF9. In normal lungs, FGF9 was confined to smooth muscle surrounding airways, alveolar ducts and sacs, and blood vessels. In addition to these same sites, lungs of IPF patients expressed FGF9 in a population of myofibroblasts within fibroblastic foci, hypertrophic and hyperplastic epithelium of airways and alveoli, and smooth muscle cells surrounding vessels embedded in thickened interstitium. The results demonstrate that FGF9 protein increased in regions of active cellular hyperplasia, metaplasia, and fibrotic expansion of IPF lungs, and in isolated human lung fibroblasts treated with TGF-β1 and/or overexpressing Wnt7B. The cellular distribution and established biologic activity of FGF9 make it a potentially strong candidate for contributing to the progression of IPF. (J Histochem Cytochem 61:671–679, 2013)

Introduction

Idiopathic pulmonary fibrosis (IPF), or usual interstitial pneumonia (UIP), is a devastating disease characterized by the ineffective repair of damaged pulmonary epithelium due to its failed differentiation. The results are epithelial hyperplasia and metaplasia, rapid expansion of a heterogeneous population of myofibroblast-like cells, and a fibrous extracellular matrix (ECM) with progressive loss of normal respiratory architecture and function (Schwartz et al. 1994; Green 2002). The hallmark lesions of this failed restructuring are fibroblastic foci (FF), indicative of progressive disease, which are believed to represent a complex, interconnected reticulum extending from the pleural surface into the lung parenchyma (Cool et al. 2006). Early lesions often appear highly cellular, with subepithelial FF adjacent to normal pulmonary architecture (Katzenstein and Myers 1998; Katzenstein et al. 2002). Other characteristics include honeycomb change and traction bronchiectasis. Its progression leads to end-stage fibrosis with diminishing pulmonary structure and function (Kapanci et al. 1995; Katzenstein and Myers 1998; Katzenstein et al. 2002).

A number of growth factors are believed to play key roles in the cellular and ECM changes characteristic to IPF.

Among them, TGF-β is well recognized for its regulatory contribution to fibrogenic processes in IPF (Chilosi et al. 2003; Fernandez and Eickelberg 2012) by promoting the development of myofibroblasts and influencing the expansion of fibrillar collagen and other ECM components, in many cases modulated by cooperative Wnt signaling (Broekelmann et al. 1991; Scotton and Chambers 2007; Salazar, Lankford, and Brody 2009). Previous studies have demonstrated that Wnt7B mRNA is upregulated in IPF lungs (Konigshoff et al. 2008) and is histochemically localized in discrete sites including the ECM and cells within FF of IPF lungs (Meuten et al. 2012). Furthermore, one of its downstream signaling targets, β-catenin, has also been localized histochemically in FF (Chilosi et al. 2003). Wnt7B is an important signaling glycopeptide that coordinates proliferation of adjacent epithelium and mesenchymal cells (Rajagopal et al. 2008) and regulates vascular growth during early lung development (Shu et al. 2002). Accordingly, it has been proposed to have a potentially significant impact on the progression of IPF in the adult lung (Morrisey 2003; Meuten et al. 2012).

Fibroblast growth factor 9 (FGF9) is expressed by pulmonary epithelium and mesothelium during the pseudoglandular stage of lung development (Colvin et al. 1999). Fgf9$^{-/-}$ mice have severely impaired lung development and do not survive (Colvin et al. 2001; White et al. 2006). FGF9 and Wnt7B/β-catenin signaling pathways have been shown to coordinately control critical communication between mesothelial-mesenchymal and epithelial-mesenchymal compartments during lung development, resulting in epithelial growth and mesenchymal expansion (Yin et al. 2008; Yin, Wang, and Ornitz 2011). This demonstrated relationship coupled with our recent observations of epithelial, mesothelial, and fibroblast expression of Wnt7B in adult IPF lungs (Meuten et al. 2012) raised the question of FGF9's potential involvement in interstitial expansion during fibrosis in the adult lung. Results presented here show strong expression of FGF9 in airway and vascular smooth muscle, endothelium, mesothelium, a population of fibroblasts within fibroblastic foci, hyperplastic and metaplastic epithelium of airways and alveoli, and smooth muscle cells associated with vessels embedded in thickened interstitium.

Materials & Methods

Immunostaining

Tissue blocks of formalin-fixed lung tissue samples were obtained from the Lung Tissue Research Consortium (LTRC). The samples had previously been placed into three groups: (1) patients with forced vital capacities (FVCs) >80% (normal, or no specified major or minor diagnosis, n=3); (2) patients with FVCs between 50% and 80% [major final clinical diagnosis as interstitial lung disease (ILD, n=8) and minor final clinical diagnosis as usual interstitial pneumonia (UIP)/idiopathic pulmonary fibrosis (IPF)]; and (3) patients with FVCs <50% (major final clinical diagnosis of ILD and minor final clinical diagnosis as UIP/IPF, n=8). No other patient identifiers were provided, and their anonymity and confidentiality were preserved. The study was approved by the North Carolina State University Institutional Review Board. Blocks were sectioned and stained with hematoxylin and eosin (H&E) and examined by a board-certified pathologist to independently confirm/reclassify initial clinical diagnoses (Meuten et al. 2012).

Sections were treated with citrate buffer for antigen retrieval and treated with a polyclonal goat anti-human FGF9 antibody (AF-273-NA, lot UZ03; R&D Systems, Minneapolis, MN) at a concentration of 5 μg/ml for 2 hr at room temperature. The immunogen was a recombinant human FGF9, whose specific IgG was purified by affinity chromatography, according to the manufacturer. A polyclonal goat anti-Wnt7B antibody (sc-26363, lot I0205; Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 2 μg/ml and incubated overnight at 4C was raised against a peptide mapping near the C-terminus of human Wnt7B and was affinity purified, according to the manufacturer. A mouse monoclonal anti-smooth muscle actin antibody (A5228, lot 110M4795; Sigma, St Louis, MO) was used at a concentration of 5 μg/ml for 2 hr at room temperature. The antibody to α-smooth muscle actin (mouse IgG2a isotype) was derived from a 1A4 hybridoma produced from mice immunized with the NH$_2$ terminal synthetic decapeptide. The isotype was determined by double diffusion immunoassay.

Primary antibody treatment was followed by multiple washes, incubation with peroxidase-labeled secondary antibodies, according to the instructions of the manufacturer (Dako LSAB+SystempHRP; Dako Laboratories, Carpinteria, CA), and visualization with the NovaRED Kit for Peroxidase, according to the directions of the manufacturer (Vector Laboratories; Burlingame, CA). Control samples substituted normal goat serum, mouse ascites, or heat-inactivated goat anti-Wnt2 antibody (AF3464, lot XjH0312061; R&D Systems) for the primary antibody at an equivalent protein concentration. Sections were counterstained with methylene blue or Celestine blue and routinely mounted. When possible, adjacent or parallel sections were used to compare localization of different target molecules in the same site. Slides were evaluated and photographed on an Olympus BX-40 microscope with a DP-25 digital camera attachment and computerized storage management. Photographs were taken using either a 10X plan achromat objective with a 0.25 numerical aperture and 10.50-mm working distance or 40X plan achromat objective with 0.65 numerical aperture and 0.57-mm working distance.

Cell Preparations

Human AT2 (hAT2) cells were isolated from organ donor lungs provided by the Tissue Procurement and Cell Culture Core of the Cystic Fibrosis/Pulmonary Research and Treatment Center (the Core) at the University of North Carolina at Chapel Hill (UNC). Organ donor lungs not suitable for transplantation but still useful for cell harvest were obtained by the Core through the National Disease Research Interchange (Philadelphia, PA). All human materials were handled per protocols approved by the UNC Institutional Committee on the Protection of the Rights of Human Subjects (IRB) and strict procedures were followed to ensure patient confidentiality. Age, sex, and ethnic background were not considered when obtaining specimens and are expected to reflect those of the U.S. population of general organ donors. Uniform consent is not practicable or feasible because donors were deceased (i.e., cadaveric organ donors). For these specimens, consent for research use of tissue was obtained from an authorized representative of the deceased by the organ procurement agency and has been deemed acceptable by the IRB. The waiver does not adversely affect the rights and welfare of the tissue donors because of procedures to ensure subject anonymity. The use of anonymous cadaveric organ donor tissue is considered to be exempt from IRB review. Following isolation (see below), hAT2 cells were maintained in low-glucose DMEM supplemented with 10% FBS and Antibiotic-antimycotic solution (Mediatech; Manassas, VA).

Human airway smooth muscle cells (hASMs) were the generous gift of Dr. Julian Solway, University of Chicago. Cells were cultured in DMEM:F-12 medium (50/50) supplemented with 10% FBS and selected growth factors.

hAT2 and hLF Cell Isolation and Treatments

Primary alveolar cells were isolated according to a scaled-up, modified version of the original Dobbs procedure (Dobbs, Williams, and Brandt 1985; Apparao et al. 2010), fibroblast-depleted using an anti-Thy-1 antibody (AS02; EMD Chemicals, Gibbstown, NJ) and pan-mouse IgG Dynabeads (Invitrogen; Carlsbad, CA), as previously described (Zhang, Newman, and Sannes 2012), and seeded in low-glucose DMEM/10% FBS on rat-tail collagen-coated tissue culture dishes. After a medium change the next day, cells were cultured for 24 hr (for AT2) or 10 to 14 days (for AT1). Human lung fibroblasts (hLFs) were grown out from non-fibroblast-depleted lung cell isolates and purified by two sequential passages.

To model in vivo conditions and potentially enhance FGF9 expression, some hLF cells were adenovirally transduced with either CMV-LacZ (control) or CMV-Wnt7B (Wnt7B overexpression) and/or treated with 10 ng/ml TGF-β1 for 48 hr prior to protein isolation. These two growth factors are present in or near fibroblastic foci (Khalil et al.

1996; Meuten et al. 2012) and could be expected to modulate FGF9 expression.

Western Blot Analysis

Equal amounts of proteins were subjected to electrophoretic separation in MOPS running buffer under reducing conditions at 200 V for 60 min on NuPage 4% to 12% Bis-Tris gels using the Novex X-Cell II system (Invitrogen). Proteins were transferred to nitrocellulose membranes and blocked with Tris Buffered Saline containing 0.1% Tween20 (TBS/T)/5% milk (TBS/T/milk) for 1 hr. Blocked membranes were incubated in TBS/T and 5% BSA (TBS/T/BSA) with primary antibodies to FGF9 [goat anti-human FGF9 antibody (AF-273-NA, lot UZ03; R&D Systems)] and GAPDH (sc-32233; Santa Cruz Biotechnology) overnight at 4C with agitation or Actin-HRP (sc-1615-HRP; Santa Cruz Biotechnology). As a specificity control, the FGF9 antibody was pre-absorbed overnight with an 8-fold excess of recombinant (rh) FGF9 (23kDa; R&D Systems), and this pre-absorbed antibody was used in parallel with the anti-FGF9 on a duplicate blot. After washing in TBS/T, blots were incubated in TBS/T/milk containing secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology; Danvers, MA), when needed, for 2 hr with agitation. Bands on the membrane were detected by chemiluminescence using SuperSignal West Pico or Dura substrate (Pierce Biotechnology; Rockford, IL) and visualized by autoradiography.

Results

In normal human lung, detection of FGF9 by immunohistochemistry was limited to smooth muscle surrounding airways, alveolar ducts and sacs, and blood vessels (Figs. 1A and 1B). Parallel sections treated with pre-immune serum and appropriate secondary antiserum and immunolabeling sequence were non-reactive and lacked label contrast (Fig. 1C). In patients with IPF, FGF9 reactivity was again identified in smooth muscle associated with airways and blood vessels but, notably, in fibroblasts within fibroblastic foci (FF) (Fig. 1D) as well as throughout a larger subpopulation of readily detectable interstitial fibroblasts (Fig. 1D). The specificity of the FGF9 antibody was confirmed by western blot analysis of a recombinant FGF (23 kDa) probed with anti-FGF9 after pre-absorption with an 8-fold excess of rhFGF9 (Fig. 2A). In addition, FGF9 expression was enhanced in hFLs by adenovirally transduced CMV-LacZ and CMV-Wnt7B and up-regulated by TGF-β1 treatment (Fig. 2B). In an identical blot probed with anti-FGF9 pre-absorbed with rhFGF9, the FGF9 band was absent (Fig. 2C). Further, isolated hFLs and hASMs cultured for 96 hr expressed FGF9 that was detectable by western blot analysis (Fig. 3).
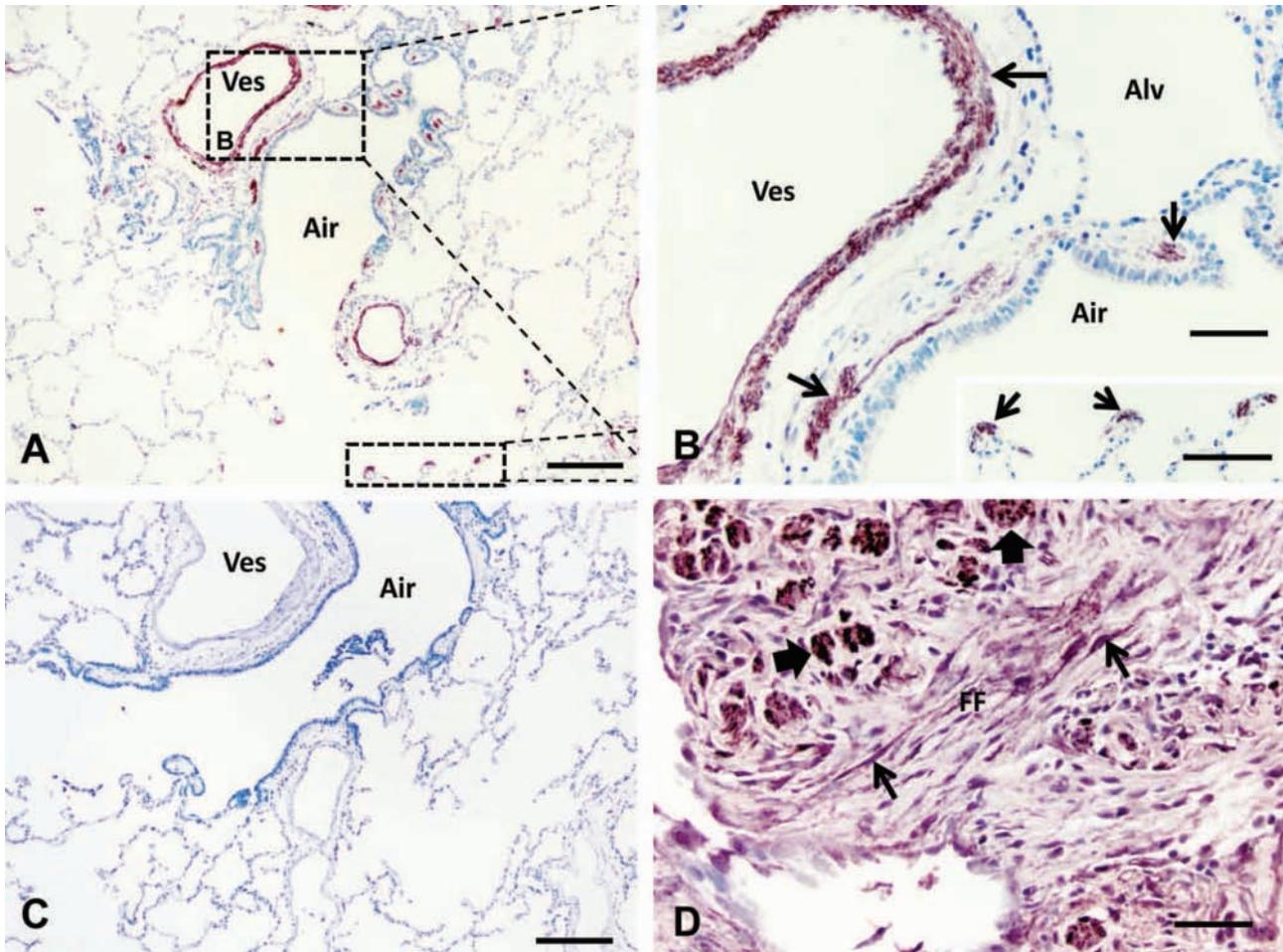
Figure 1. (A, B) Normal human lung tissue treated for the localization of fibroblast growth factor 9 (FGF9) by immunohistochemistry showed reactivity in smooth muscle (thin arrows) surrounding airways (Air), alveolar ducts and sacs (inset, B), and blood vessels (Ves). Airway and alveolar (Alv) epithelium had no detectable reactivity. (C) Pre-immune serum immunohistochemical controls were free of distinguishing reactivity. (D) In patients with idiopathic pulmonary fibrosis, FGF9 reactivity was found in smooth muscle associated with airways and blood vessels (thick arrowheads) and in myofibroblasts within fibroblastic foci (FF; thin arrows). Bars A, C = 200 µm; bars B, D = 100 µm.

Separate parallel sections of IPF lungs immunostained for markers of FF (Wnt7B) (Meuten et al. 2012) and for myofibroblasts and smooth muscle [α-smooth muscle actin (αSMA)] (Kapanci et al. 1995; Ohta et al. 1995) were then immunostained for FGF9 to define potential intersecting reactivity with established markers of site-specific activities. In Fig. 4, panels A and B, immunostaining for Wnt7B is observed in FF of patients with IPF, as shown previously (Meuten et al. 2012). Less intense reactivity was seen in smooth muscle bundles or surrounding small vessels (Fig. 4B). Immunohistochemical control treatments of parallel sections were free of specific staining (Fig. 4C). Parallel sections from the same tissue block depicted in panels A through C but stained for αSMA identified myofibroblasts within FF and bundles of smooth muscle (Figs. 4D and 4E), while histochemical controls of parallel sections of adjacent
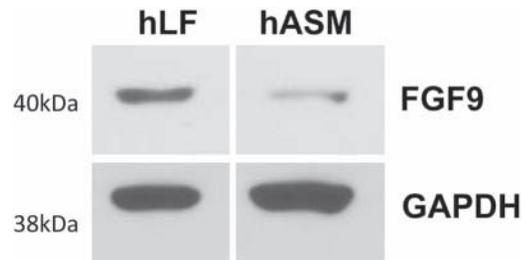
regions were free of specific staining (Fig. 4F). Similarly, FGF9-stained parallel sections demonstrated smooth muscle cell reactivity and identified a subpopulation of myofibroblasts within FF (Figs. 4G and 4H), while parallel control sections of adjacent regions were free of specific staining (Fig. 4I).

In other FF, there was more intense immunoreactivity for FGF9 in larger numbers of fibroblasts/myofibroblasts (Figs. 5A–D). This often reflected a level of difference between FF even in the same section rather than differences between tissue samples, different patients, and/or the originally diagnosed disease severity. In addition, FF were often associated with FGF9-positive, hypertrophic alveolar epithelium (Figs. 5A and 5C) as well as fibroblasts/myofibroblasts within interstitium lacking definitive FF near dysplastic airway epithelium (Fig. 5D). Notably, in many cases, smooth muscle
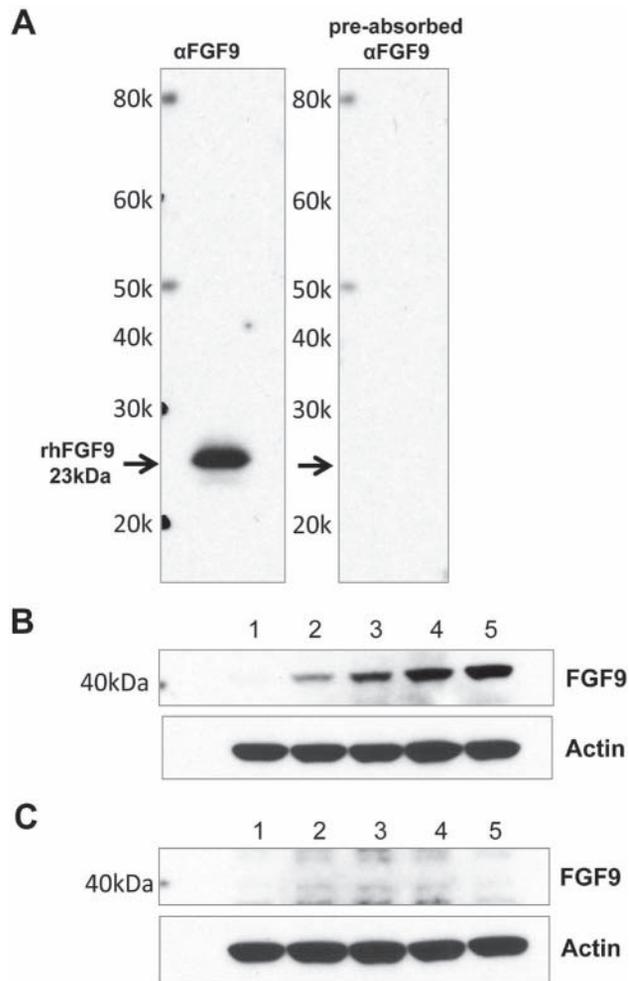
Figure 2. (A) Western blot analysis of recombinant human fibroblast growth factor 9 (rhFGF9) protein (0.5 ug; 23 kDa, arrow) probed with anti-FGF9 (left panel), or with anti-FGF9 pre-absorbed with an 8-fold excess of the immunizing peptide (rhFGF9) and probed with anti-FGF9 (right panel, arrow). Thermo SuperSignal Molecular Weight Markers, each of which contains an IgG-binding site, run at the stated molecular weight at the left of each panel. (B) FGF9 in human lung fibroblasts (hLF) untreated (1), CMV-LacZ (2), CMV-LacZ + TGF-β1 (3), CMV-Wnt7B (4), and CMV-Wnt7B + TGF-β1 (5). Note that, under our conditions, FGF9 runs at 40 kDa (molecular weight marker on left of panel). (C) Identical western blot as in (B), except that the anti-FGF9 was pre-absorbed with an 8-fold excess of rhFGF9.

Figure 3. Western blot analysis of isolated human lung fibroblasts (hLF) and human airway smooth muscle cells (hSMC) cultured for 96 hr showing expression of FGF9 at the expected 40 kDa molecular weight.

cells that appeared dissociated from their usual location within bundles were observed in thickened and distorted interstitial regions of IPF lungs (Fig. 5D). They were identified by their unusually long lengths compared to the shorter myofibroblasts (Fig. 5D). Additionally, a subpopulation of interstitial blood vessels was circumscribed with FGF9-reactive cells that were presumed to be either a single layer of smooth muscle or unusually large pericytes (Figs. 5A and

5C). Similar/related cells were αSMA-positive (Figs. 4F and 5C) but not reactive for Wnt7B (data not shown).

Discussion

In normal human lungs, FGF9 immunohistochemical reactivity was principally confined to smooth muscle surrounding airways, alveolar ducts and sacs, and blood vessels. These results were confirmed by protein expression of normal adult lung fibroblasts and smooth muscle cells with 96 hr in culture (Fig. 3). Importantly, isolated hLFs treated with TGF-β1 or adenovirally transduced for overexpression of Wnt7B showed significant increases in FGF9 expression. This would follow with the proximity of myofibroblasts in FF to the TGF-β1 produced by epithelium (Khalil et al. 1996) and Wnt7B in epithelium and FF (Meuten et al. 2012).

One of the emerging concepts in the pathogenesis of IPF is that it is a disease of derangement/dysregulation of epithelial-mesenchymal cross-talk (Coward, Saini, and Jenkins 2010). There is good evidence that this cellular instability manifests in the interplay of TGF-β and Wnt signaling. Recent work from our laboratory has demonstrated the immunolocalization of Wnt7B in myofibroblasts and ECM of FF near dysplastic airway and alveolar epithelium, discrete subepithelial, basement membrane-associated regions, and interstitium (Meuten et al. 2012). Quantitation of the distribution and size of Wnt7B-reactive sites indicated that the group of subepithelial regions less than $50\mu m^2$ were the most numerous, and it was speculated that they could represent evidence of a specific Wnt signal capable of influencing expansion of interstitial cells and ECM, perhaps indicative of early developing FF (Meuten et al. 2012). Wnt7B's exact functions in the adult lung and IPF are not known, but in the developing lung it is required for mesenchymal proliferation and vascular development (Shu et al. 2002). A recent study has shown that, in early lung development, Wnt7B modulates FGFR expression in mesenchymal cells and thus affects their responsiveness to FGF9. In turn, FGF9 controls the proliferative activity of mesenchymal cells and the expansion of
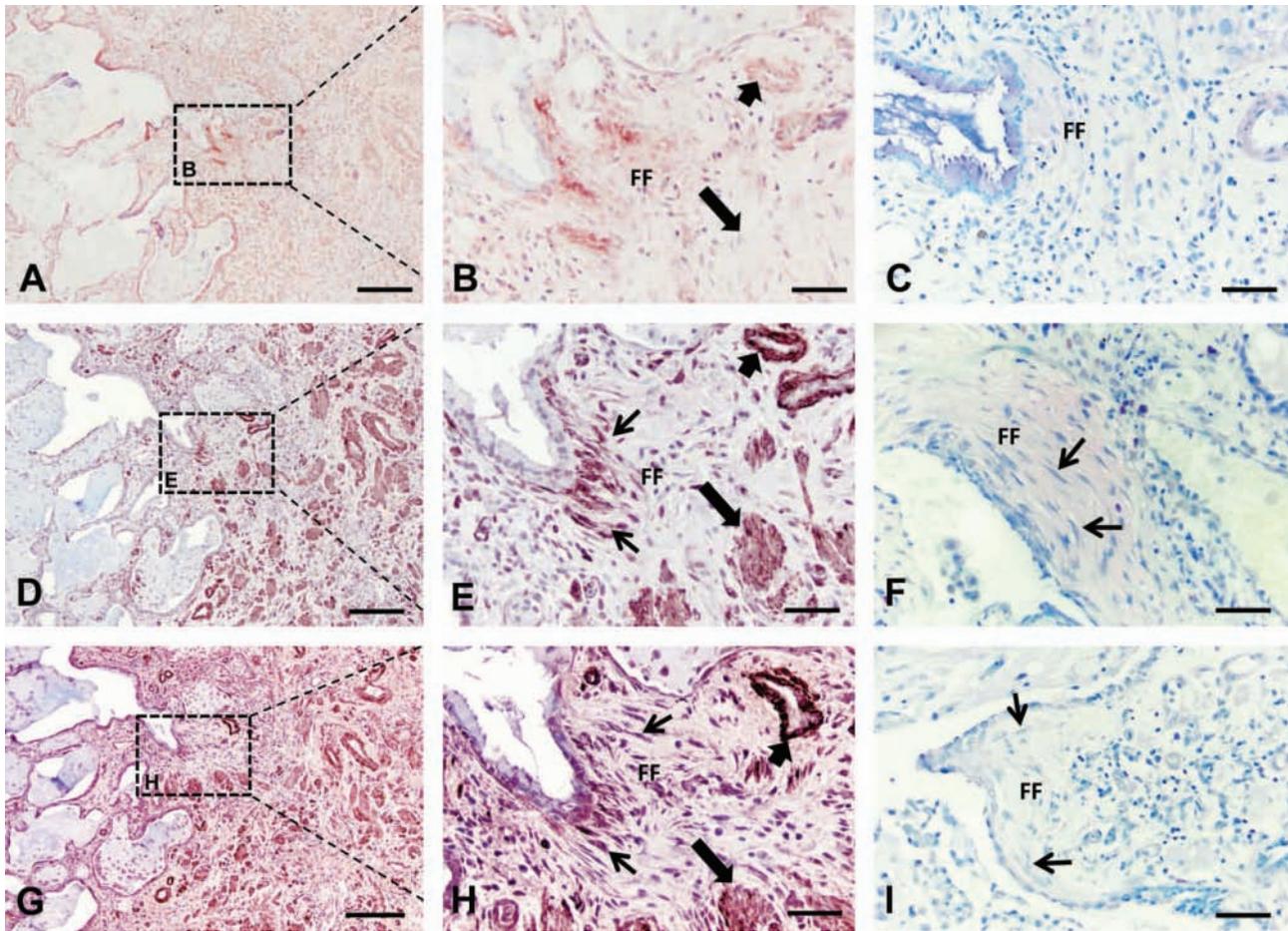
Figure 4. (A, B) Immunostaining for Wnt7B is observed in fibroblastic foci (FF) of patients with idiopathic pulmonary fibrosis, with no detectable reactivity in smooth muscle bundles (thick arrow) but light reactivity in pericytes surrounding small vessels (thick arrowheads). (C) A heat-inactivated goat anti-Wnt2 antibody substituted for the Wnt7B antibody was free of distinguishing reactivity in a parallel section, as noted in identifiable FF. (D, E) Parallel sections from the same tissue blocks depicted in (A) through (C), stained for αSMA, identified myofibroblasts within FF (thin arrows) and bundles of smooth muscle (thick arrow) or pericytes (thick arrowheads). (F) Control section in which normal ascites substituted for the primary antibody was free of distinguishing reactivity observed in (D) in nearby regions of parallel sections. FF, though metachromatic with methylene blue, and their complement of myofibroblasts (arrows) are not reactive for αSMA. (G, H) Similarly, FGF9-stained parallel sections had smooth muscle cell bundle reactivity (thick arrows), smooth muscle or pericytes surrounding small vessels (thick arrowheads), and myofibroblasts within FF (thin arrows). (I) Control section in which normal goat serum was substituted for the primary antibody was free of distinguishing reactivity seen with FGF9 antibody in nearby regions of parallel sections, as noted by absence of reactivity in these sites, FF, and their complement of myofibroblasts. Bars A, D, G = 200 μm; bars B, C, E, F, H, I = 100 μm.

mesenchyme while promoting epithelial branching activity as the lung grows peripherally (Yin, Wang, and Ornitz 2011). It has been suggested that aberrant reactivation of developmental pathways contributes to the pathogenesis of IPF (Konigshoff et al. 2008; Konigshoff and Eickelberg 2010). It follows, therefore, that the distribution of reactive sites for Wnt7B and FGF9 in FF, epithelium, and myofibroblasts in lungs of IPF patients could be indicative of a reactivation of developmental pathways, which progresses with the previously demonstrated directional expansion of FF from the periphery toward the hilus (Cool et al. 2006). It therefore may not be surprising that treatment of normal hLFs with TGF-β1 and/or adenovirally inducing overexpression of Wnt7B results in a significant increase in FGF9 expression (Fig. 2B). Moreover, the interactions of these three potent signaling factors combined would constitute a powerful driving force in the growth and expansion of FF, leaving in its wake a heterogeneous mixture of myofibroblasts and the distorted, overabundant ECM characteristic of IPF.
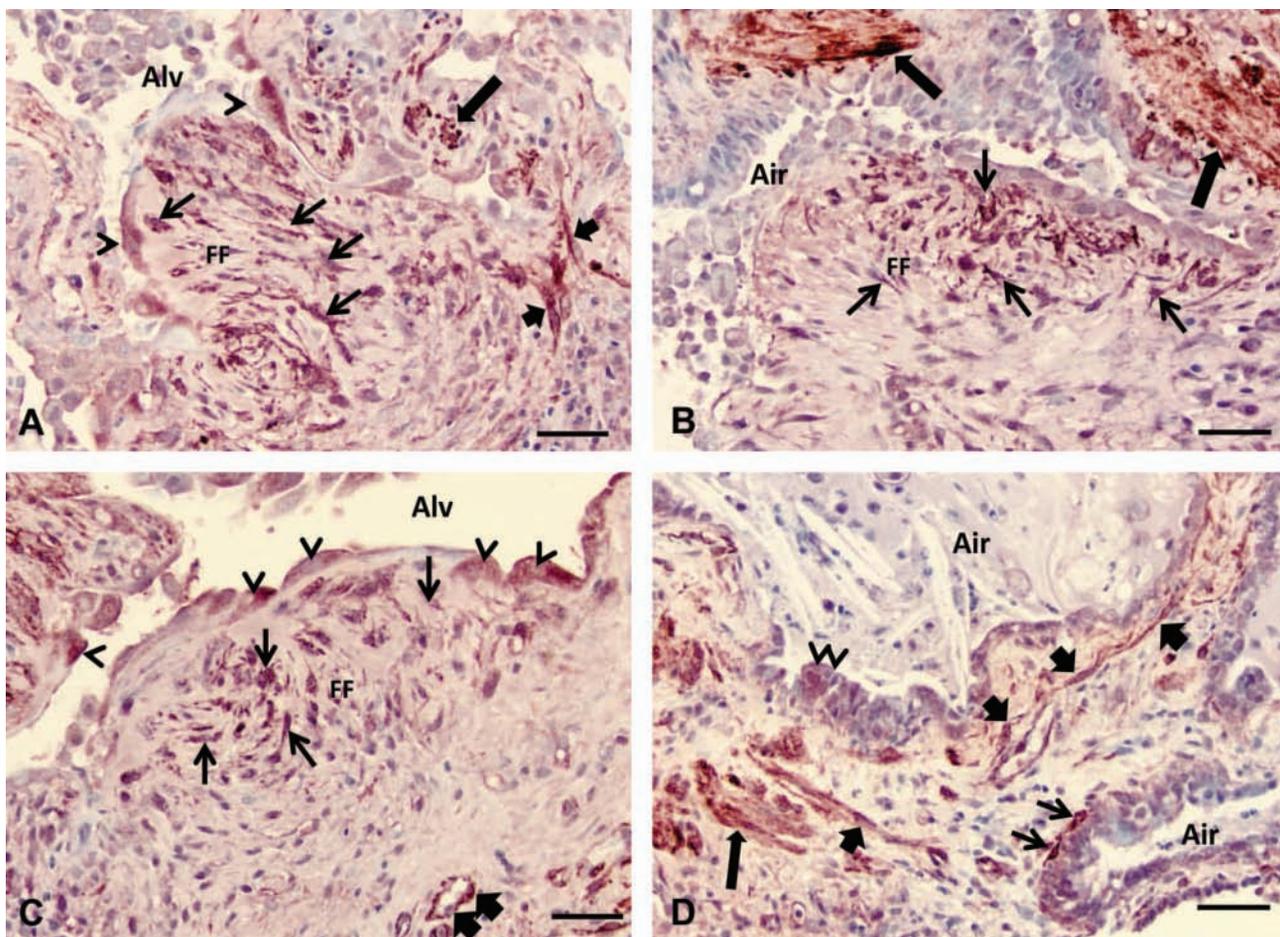
Figure 5. (A–D) Immunostaining for fibroblast growth factor 9 (FGF9) was observed in fibroblastic foci (FF) associated with alveoli (Alv) or interstitium near airways (Air) and indicated that many were associated with high levels of immunoreactive fibroblasts/myofibroblasts (thin arrows). These were often lined with FGF9-positive, hyperplastic/metaplastic epithelium (thin arrowheads), as well as dysplastic airway epithelium (double thin arrowheads). Notably in many cases, smooth muscle cells that appeared in bundles (thick arrows) or dissociated into smaller groupings or single cells (thick arrowheads) were observed in thickened and distorted interstitial regions of idiopathic pulmonary fibrosis lungs. They were identified by their unusually long lengths compared to the shorter myofibroblasts (thin arrows). Additionally, a subpopulation of interstitial blood vessels circumscribed with FGF9-reactive cells (double thick arrowheads), presumed to be either a single layer of smooth muscle or possibly large pericytes, were also αSMA-positive but not reactive for Wnt7B (data not shown). Bars A–D = 100 μm.

Using antibodies to alpha and gamma smooth muscle actins, Ohta et al. (1995) have demonstrated that smooth muscle and smooth muscle-like cells, distinct from those associated with airways and vessel walls, were increased in IPF and found as hypertrophic or hyperplastic cells in clusters, as well-organized or atypical, loosely arranged bundles, or as single cells distributed throughout fibrotic parenchyma. These descriptions closely parallel our observations of FGF9-positive cells interpreted here to be smooth muscle (Figs. 4H, 4I, 5B, and 5D). Ohta et al. (1995) also showed that some of these cells were pro-collagen 1-positive, indicating their capacity to biosynthesize ECM. Their positive immunoreactivity for Wnt7B (Meuten et al. 2012) and FGF9 further link these cells with potential expansion of ECM, both, as we propose, in IPF and as seen in early lung development (Yin, Wang, and Ornitz 2011).

Additionally, angiogenesis has been recognized as a characteristic of IPF (Keane et al. 2006), and cells interpreted to be smooth muscle cells or pericytes surrounding the peripheries of small to intermediate-size vessels adjacent to FF were FGF9- and SMA-positive (Figs. 4E, 4H, and 5C). Interestingly, FGF9 has been shown to be upregulated in smooth muscle cells as they assemble into layered cords (Frontini et al. 2011) and as they proliferate at sites of neovascularization (Agrotis et al. 2004).

In summary, new data are presented describing gene and protein expression of FGF9 for the first time in adult human lungs and lung cells and localizing sites of its

immunoreactivity in lungs of patients previously diagnosed with IPF. FGF9 immunoreactivity was found in regional sites and cells previously shown to express Wnt7B, which raises the possibility that their established unique interactions during early lung development could be reactivated in IPF to control expansion of fibroblastic cells and ECM. Furthermore, FGF9 expression by smooth muscle cells in bundles or dispersed individually could be expected to add further stimulus to the pro-fibrogenic milieu of the IPF lung. Closer examination of specific cell–cell and cell–ECM interactions are under way to fully identify the mechanisms triggering these complex relationships.

Acknowledgments

Declaration of Conflicting Interests

Funding

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