Fibroblast growth factor signaling in myofibroblasts differs from lipofibroblasts during alveolar septation in mice

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Fibroblast growth factor signaling in myofibroblasts differs from lipofibroblasts during alveolar septation in mice. Am J Physiol Lung Cell Mol Physiol 309: L463–L474, 2015. First published July 2, 2015; doi:10.1152/ajplung.00013.2015.—Pulmonary alveolar fibroblasts produce extracellular matrix in a temporally and spatially regulated pattern to yield a durable yet pliable gas-exchange surface. Proliferation ensures a sufficient complement of cells, but they must differentiate into functionally distinct subtypes: contractile myofibroblasts (MF), which generate elastin and regulate air-flow at the alveolar ducts, and, in mice and rats, lipofibroblasts (LF), which store neutral lipids. PDGF-A is required but acts in conjunction with other differentiation factors arising from adjacent epithelia or within fibroblasts. We hypothesized that FGFR receptor (FGFR) expression and function vary for MF and LF and contributes to their divergent differentiation. Whereas approximately half of the FGFR3 was extracellular in MF, FGFR2 and FGFR4 were primarily intracellular. Intracellular FGFR3 localized to the multivesicular body, and its abundance may be modified by Sprouty and interaction with heat shock protein-90. FGF18 mRNA is more abundant in MF, whereas FGF10 mRNA predominated in LF, which also express FGFR1 IIIb, a receptor for FGF10. FGF18 diminished fibroblast proliferation and was chemotactic for cultured fibroblasts. Although PDGFR receptor-α (PDGFR-α) primarily signals through phosphoinositide 3-kinase and Akt, p42/p44 MAP kinase (Erk1/2), a major signaling pathway for FGFRs, influenced the abundance of cell-surface PDGFR-α. Observing different FGFR and ligand profiles in MF and LF is consistent with their divergent differentiation although both subpopulations express PDGFR-α. These studies also emphasize the importance of particular cellular locations of FGFR3 and PDGFR-α, which may modify their effects during alveolar development or repair.

adipocyte; cell differentiation; endosome; fibroblast; PDGF receptor-α

MAMMALIAN PULMONARY ALVEOLI comprise an expansive but thin barrier surface, which facilitates efficient transfer of gases between the air spaces and neighboring capillaries. The area of this specialized surface increases ~3-fold during the first 2 wk of postnatal life in mice and rats, through secondary septation, extension of new alveolar septa away from the primary septa, which arose during late gestation as the alveolar sacs (7). Mesenchymal progenitors expressing Gli1 and PDGFR receptor-α (PDGFR-α) are required, and their absence is marked by severe attenuation of secondary septa and their attendant α-smooth muscle actin (α-SMA) and elastic fibers (6, 25). A prevailing paradigm is that mesenchymal progenitors proliferate and differentiate in a spatially appropriate pattern, in coordination with the epithelium. In addition to the endothelium, three types of differentiated mesenchymal cells reside within the secondary septa, pericytes (which are closely applied to the capillary endothelium), lipid-laden interstitial fibroblasts (F) (termed lipofibroblasts, LF), and myofibroblasts (MF). PDGFR-α is expressed in lower abundance in LF than in MF but not by pericytes (33). Regulation of the divergent differentiation of PDGFR-α progenitors into LF and MF remains incompletely defined but involves Sonic hedgehog and FGF signaling (25) (39). It remains unclear whether FGF signaling defines cell fate or directs differentiation along pre-determined pathways.

The FGFR receptors (FGFRs) comprise a complex family of receptor tyrosine kinases appearing as dimers on the plasma membrane, which undergo autotransphosphorylation of their intracellular domain upon ligand engagement (13). Three of the four mammalian FGFR gene products (FGFR1, FGFR2, and FGFR3) are alternatively spliced, providing variation in the extracellular third immunoglobulin domain, which adapts particular receptors to different cell type-specific functions (18). Most importantly in the lung, differential splicing distinguishes epithelial cells, which bear the IIIb-splice isoform, from mesenchymal cells, which bear the IIIc isoform (17). Twenty-two mammalian FGF genes have been classified into seven families, based on their shared receptor preferences (54). In the lung, the FGF7 and FGF8 families are particularly important and signal respectively to epithelial (FGF7 and FGF10) and mesenchymal (FGF8 and FGF18) cells (49). FGF10 is unique because it also regulates the development of adipocytes and in the lung is produced by LF (38). Although the specificity of signaling is primarily controlled by ligand-receptor interactions, the dynamics and topography of signaling are also regulated by intracellular trafficking (19). After phosphorylation, FGFRs undergo clathrin-dependent internalization and enter the endosomal recycling pathway (2). Endosomal sorting impacts the amplitude and duration of receptor signaling, which primarily proceeds through the phosphoinositide 3-kinase (PI3K)-Akt and Erk-MAP kinase pathways. Sustained Erk-mediated signaling, which has been linked to neurocyte differentiation, is associated with FGF7 serine phosphorylation and delivery to the multivesicular body (MVB) (27). FGFR3 signaling is also sustained by sequestration of the cytoplasmic inhibitors Sprouty-2 and Sprouty-4 (Spry), which, when unrestrained, promote FGFR3 ubiquitination and proteasomal degradation (15). Assignment of FGFRs to particular endosomes can increase ambient concentrations of required substrates or direct them away from the ubiquitination machinery (36). Endosomes can also direct FGFRs to a particular region of the cell, such as the leading edge during migration,
polarizing signaling molecules that regulate the remodeling of cytoskeletal acts and microtubules (5).

Others have shown that FGFR3 and FGFR4 are required for alveolar formation (44). Despite evidence that FGF signaling affects PDGFR-α-expressing lung F, several major questions remain unanswered (39). 1) Which FGF ligand(s) and signaling pathways regulate MF differentiation? 2) Does FGF signaling differentially influence proliferation or differentiation of LF and MF? 3) Do FGF ligands produced by mesenchymal cells signal within the mesenchymal compartment? We have examined F from mice expressing green fluorescent protein (GFP), directed by the endogenous PDGFR-α promoter. Two subpopulations of PDGFR-α-expressing F (GFPlow and GFP<sup>high</sup>) can be distinguished based on the level of PDGFR-α gene expression (20). Our prior work demonstrated that the GFPlow subpopulation is adipocyte like and less proliferative at postnatal day (P)8 (28). The GFP<sup>high</sup> F have characteristics of MF and higher proliferation at P8 (28). We hypothesized that FGF signaling is differentially regulated in these two F populations and that understanding this differential regulation may identify mechanisms regulating F positioning during secondary septal formation.

MATERIALS AND METHODS

Antibodies and reagents. Rat anti-mouse Ki67 (monoclonal TEC3, no. M7249) was from DAKO (Carpinteria, CA); rabbit anti-phospho-p44/42 MAPK (Erk1/2) (no. 4695), rabbit anti-phospho-Akt (Ser473) (no. 4370), rabbit anti-CHMP2B (no. ab33174), and rabbit anti-HSP90 (no. ab178854) were from Abcam (Cambridge, MA); rabbit anti-FGFR4 (CD333) (no. sc-9006), goat anti-FGF18 (no. sc-16830), goat anti-FGFR3 (CD333) (no. sc-1162), and goat anti-mouse-Spry-4 (no. sc-18609) were from Santa Cruz Biotechnology (Dallas, TX); rabbit anti-FGFR2 (CD332) (no. PA5-14651) was from Thermo Scientific (Rockford, IL); rat anti-mouse-CD45-PECy7 (no. 103113) and Pacific Blue-conjugated rat anti-mouse CD45 (no. 103125) were from Bio-Legend (San Diego, CA); rat anti-mouse-CD140a (PDGFR-α) (BV421 conjugate) (no. 562774) was from BD Biosciences (San Jose, CA); PoPo3-iodide, mouse anti-bromodeoxyuridine (BrdU) (A-55520) was from 3i (San Diego, CA); recombinant human FGF18 (no. 100-28) was from Peprotech (Rocky Hill, NJ); and recombinant human/mouse FGF8b (no. 423-F8) was from R&D Systems (Minneapolis, MN).

Mice. Mice bearing the PDGFR-α-GFP construct have been described (20). Production and nuclear localization of GFP is under the control of the endogenous pdgfra promoter. GFP expression in the PDGFR-α-GFP mice spatially and temporally recapitulates endogenous pdgfra expression (16). The mice used in this study carried one pdgfra-GFP allele (which does not encode for active PDGFR-α) and one functional pdgfra allele and are genetically identical to wild-type (GFP<sup>−</sup>) mice (16). Mice with a targeted deletion of LoxP-pdgfa have also been described (32). The DNA coding Cre-recombinase was inserted into exon 1 of transgelin (tagln, TG) and mediates Cre recombinase postnatally but not in the embryo (53). Transgelin

![](image.png)

Fig. 1. PDGF receptor (PDGFR)-α green fluorescent protein (GFP)<sup>high</sup> and PDGFR-α-GFP<sup>low</sup> fibroblasts (F) exhibit different kinetics of proliferation. A: proliferating (Ki67<sup>+</sup>), solid portion of bars alveolar cells were enumerated and expressed relative to all cells within a particular population (GFP<sup>high</sup>, GFP<sup>low</sup>, or GFP<sup>neg</sup>) on 3 different postnatal (P) days. The numbers within the bars represent the mean percentages of Ki67<sup>+</sup> cells within the respective parent population (including the Ki67<sup>+</sup> cells, open portion of the bars). Error bars are 1 SE, n = 3 mice at each age. *P < 0.01 P4 vs. P8. B: mean proportions of GFP<sup>high</sup>, GFP<sup>low</sup>, and GFP<sup>neg</sup> cells relative to all alveolar cells at each age. The mean percentages of each population based on PDGFR-α-GFP expression relative to all alveolar cells at a particular age are shown. **P < 0.01 P2 vs. P6. C: thymidine analog bromodeoxyuridine (BrdU) was administered to PDGFR-α-GFP mice during P2 through P4. The same mice received a second thymidine analog, 5-ethyl-2′-deoxyuridine (EdU) on P8, when the lung F were isolated. The F were immediately fixed, permeabilized, and stained for BrdU, EdU, and CD45. Flow cytometry was used to separate the 3 subpopulations based on the intensity of GFP fluorescence and the absence of CD45. Means ± SE, n = 3, 2-way ANOVA, Student-Newman-Keuls post hoc test. *P < 0.01 compared with GFP<sup>neg</sup>, †P < 0.05 comparing GFP<sup>low</sup> and GFP<sup>high</sup>.
is expressed in pulmonary MF, pericytes, and smooth muscle cells. Protocols for animal use were approved by the Iowa City Veterans Affairs Medical Center Animal Use Committee (30).

Isolation of primary mouse F. Lung F were isolated from heterozygous PDGFR-α-GFP mice on P8 or for primary cell culture on P12 using a previously reported method involving digestion with collagenase (30). The dispersed cells were resuspended in Ham’s F-12 medium containing 10% FBS, and F were selected by their adherence to tissue culture dishes for 1 h at 37°C. Approximately one-third of the adherent cells were PDGFRα-GFP+. The purity of the F was assessed by immunostaining for cellular markers specific for epithelial (anti-pan cytokeratin antibody), macrophage (CD 206), and endothelial (CD31) cells (33). Epithelial and endothelial cells comprised ~2.5% and 1.6% respectively, whereas macrophages were only detected in the PDGFR-α-GFPneg population (33).

Quantification of Ki67 as a marker of F proliferation. The lungs from three PDGFR-α-GFP mice at each age (P2, P4, and P8) were perfused to clear the vasculature, fixed while uniformly inflated to 25 cmH2O, embedded in optimal cutting temperature compound, sectioned at 100-μm intervals, and stained overnight at 4°C with anti-Ki67 followed by goat anti rat-IgG A647 (32). Nuclei were stained with PoP3-iodide, and the sections were analyzed using the Zeiss LSM 710, using lungs from one mouse at each age for each staining session. The laser intensities and photodetector gains were optimized within the LSM intervals and converted to TIF format stacks) were acquired at 2.5-

Exposure of mice to BrdU and EdU. Beginning on P2, litters of offspring from breeding pairs of two heterozygotes for PDGFR-α-GFP were treated twice daily through P4 with 100 ng BrdU/g. The pups were genotyped, and those bearing the GFP marker were treated with 100 ng EdU/g 3 h before euthanasia and isolation of lung F. F were isolated and fixed for 20 min at 4°C with 2% paraformaldehyde, washed, permeabilized with Cytperm Plus (BD Biosciences), washed again, and then treated with 25 Kunitz units of DNase1 (D 5025; Sigma Chemical, St. Louis, MO) in 150 mM NaCl, 4.2 mM MgCl2 for 30 min at 37°C. After an additional wash, the Click-It reaction (A647 picolyl azide, Life Technologies) was performed, and the cells were stained with mouse anti-BrdU-A555 and anti-CD45 and then analyzed by flow cytometry (fluorescence-activated cell sorting FACS).

F culture. Primary isolations of mouse F at P8 or P12 were cultured without further subculturing to 60–80% confluence, and serum concentration of the medium was reduced to 4 or 5%. FGF18 was added for 12 h, and, during the last 2 h, the medium was also supplemented with 10 mM EdU (Life Technologies). After 12 h, the cells were released with trypsin, fixed, and reacted with A647-picolyl azide, following the manufacturer’s instructions (Life Technologies) and analyzed by flow cytometry gating on the CD45-, GFP+ cells. Primary mouse lung F cultures were also exposed to 200 ng FGF18/ml for 0, 10, or 60 min, and the cell layers were harvested and subjected to Western immunoblotting and probing for phospho-p44/p42 MAPK (Erk1/2) (Thr202/Tyr204), Erk1/2, and β-tubulin for a loading control (30).

Fig. 2. Abundance and location of FGF receptors (FGFRs) in F subpopulations determined by flow cytometry. F isolated on P8 were fixed immediately after isolation and were either permeabilized (total) or not (surface) before staining for FGFR2 (CD332, n = 5) (A). B: representative dot plots for FGFR3 (CD333) showing isotype controls (b and c) and CD333 (e and f) in permeabilized (b and e) or unpermeabilized (c and f) groups. Combined data for FGFR3 (n = 4) (C) or FGFR4 (CD334, n = 4) (D) are shown. CD45- cells were gated out, and data were expressed relative to the total number of cells in the respective subpopulations, based on the intensity of the PDGFR-α-GFP tag. Comparisons were made among the intracellular pools (open portion of bar) or for the extracellular receptors across (closed portions) the 3 subpopulations. Means ± SE, 2-way ANOVA, Student-Newman-Keuls post hoc test. *P < 0.05 compared with GFPneg, †P < 0.05 comparing GFPneg and GFPneg. FSC, forward scatter; SSC, side scatter.
were as follows: FGFR1 IIIb Fwd AGCATCCGGGAAATATAGCT, Rev CACAGGTCTGGTGACAGT, probe CTAAACAGTCTGGTGACATC; FGFR1 IIIc Fwd AACTTGGCGATATGGCCAGTC, Rev AGAGTCCGATAGGTTACCCG, probe TGAAGCAGCTTCATTCTCTTGTCCG (4). The primer and probe sets corresponded to regions of the transcripts unique to the specific splice products. FGF and FGFR gene expression were normalized to β2-microglobulin using the \(2^{-\Delta\Delta CT}\) relative quantification method (33).

Analytical flow cytometry. LF were isolated, and in most experiments they were fixed before staining. To assess extracellular CD140a (PDGFR-α), F were stained before fixation. Fixed F were permeabil-
ized with 0.1% saponin and stained for intracellular antigens. Intracellular antigen staining was calculated by subtracting surface (unpermeabilized) from total (permeabilized) staining. CD45+ cells, which had adhered to culture plastic after 1 h, were excluded from analysis. Virtually all of the PDGFR-α-expressing F were in the CD45− fraction. Forward and side scatter were used to exclude small (presumably apoptotic) cells and aggregates. Events were captured from 2 × 10^4 cells (except for analysis of pErk and pFGFR3, when 5 × 10^4 events were collected) using a Becton Dickinson LSR2 flow cytometer and analyzed using Cell Quest software (BD Biosciences) (33).

Proximity ligation assay. F were isolated at P8, and cells that adhered to culture plastic after 1 h were removed and replated on glass cover slides (P35G-1.0−14-C; MatTek, Ashland, MA) that had been coated with 10 μg/ml fibronectin peptide (Sigma-Aldrich, F3667). The cells remained on the cover slides overnight, enabling them to spread over the surface but maintain their native level of PDGFR-α expression. The cover slides were fixed for 15 min at 4°C with 2% paraformaldehyde, rinsed, and then stored at 4°C. The Proximity ligation assay (PLA) was carried out using the DuoLink In Situ Fluorescence Kit (Olink Bioscience, Sigma Aldrich), as follows. After hydration in PBS, the cells were permeabilized with 0.3% Triton X-100 and blocked with 2% normal donkey serum, and the cover slides were incubated overnight with rabbit anti-PDGFR3 and goat anti-FGF18 at 4°C. Alternatively, cover slides were incubated overnight with goat-anti-PDGFR3 and rabbit anti-HSP90. After incubation with primary antibodies, the cover slides were washed and incubated with DuoLink anti-rabbit and anti-goat PLA probes following the manufacturer’s instructions. After the washing to remove the unbound probes, the nucleotide ligation reaction was conducted, followed by the amplification reaction to incorporate the DuoLink orange fluorescent probe (excitation 554, emission 579). The actin cytoskeleton was stained with A633 phalloidin. Following a final wash, the cover slides were mounted for analysis using a Zeiss LSM710 confocal microscope and a Zeiss 63 oil-immersion objective. Cells were analyzed separately for the intensity of PDGFR-GFP fluorescence (an index of PDGFR-α transcriptional activity). Uniform segmentation criteria were applied to identify regions with high and low levels of GFP intensity and to demarcate granules from the PLA reaction (IP Laboratory, BD Biosciences) (20).

Intracellular locations of FGFR3. Glass cover slides were prepared as they were for the PLA, and cells were analyzed without exposure to exogenous FGF ligand. After being blocked and permeabilized, F were incubated overnight at 4°C with goat anti-FGFR3 and rabbit primary antibodies against EEA1 (early endosomes), CHM2B (MVB), GM130 (Golgi body), or Rab11 (recycling endosomes), washed, and incubated with donkey anti-goat A568 and donkey anti-rabbit A647. To outline the cell borders, the actin cytoskeleton was stained with A488 phalloidin, which could be distinguished from nuclear GFP. Colocalizing pixels were analyzed using the JACoP plugin for ImageJ2 (FIJI, http://imagej.nih.gov/ij). The plasma membrane boundary was outlined to select regions of interest and exclude the portions of the fields between cells. The same region of interest was applied to the channel detecting FGFR3 or Spry-4 (secondary antibody donkey anti-goat A568) and to the channel for the intracellular organelle marker (secondary antibody donkey anti-rabbit A647).

Statistical methods. Data were expressed as the means ± SE of the number of different mice that were used or the number of different experiments that were performed using cell cultures. ANOVA (either 1-, 2-, or 3-way as indicated in the figure legends) was performed using Systat (Chicago, IL) and Student’s t-test using Microsoft Excel. Post hoc tests are described in the figure legends. Values of P < 0.05 were considered significant.

**Fig. 5.** Sprouty (Spry)-4 mRNA is higher, and more Spry4 colocalizes to the multi-vesicular body in GFP<sup>high</sup> F. A: F were isolated and separated into 3 subpopulations by flow cytometric sorting based on the intensity of GFP and absence of CD45. Spry-4 (n = 3) but not Spry-2 (n = 3) mRNA was higher in GFP<sup>high</sup> F. Means ± SE; 2-way ANOVA, Student-Newman-Keuls post hoc test. *P < 0.05 compared with GFP<sup>low</sup>, †P < 0.05 comparing GFP<sup>low</sup> and GFP<sup>high</sup>. B: cover slides were prepared from 3 separate cell isolations as described in Fig. 4, and colocalization of Spry4 with CHM2B was quantified for all F (total) or separately for the GFP<sup>low</sup> and GFP<sup>high</sup> subpopulations. 88 GFP<sup>high</sup> and 45 GFP<sup>low</sup> F were analyzed; *P < 0.05, 2-way ANOVA comparing GFP<sup>low</sup> and GFP<sup>high</sup>.
RESULTS

Differential FGFR expression accompanies divergent F differentiation. We and others have observed two subpopulations of PDGFR-α/H9251-expressing F (10, 33). The LF exhibit lower PDGFR-α gene expression (GFPlow) and remain at the base of secondary septa (28). The MF subpopulation exhibits higher PDGFR-α gene expression (GFPhigh) and is most abundant at the septal tips. We used two approaches to assess whether the kinetics of proliferation differ between the GFPlow F and the GFPhigh subpopulations. 1) A stereological approach was used to quantify GFPhigh, GFPlow, and GFPneg cells in the alveolar region at different ages, to determine whether the three different populations expanded at different rates. Proliferating (Ki67+/H11001) GFPhigh cells were observed at P2, and the proliferation index (Ki67+/H11001/total GFPhigh) increased, between P2 and P4, whereas proliferation of GFPlow F was delayed until P4. The proportion of GFPhigh F steadily increased, whereas GFPlow F decreased as a proportion of total alveolar cells from P2 to P8. Proliferation of the GFPlow population started later and ended sooner than the GFPhigh population. 2) We also used bitemporal, cell-cycle S-phase labeling with BrdU and then EdU (14, 23). BrdU was administered to PDGFR-α-GFP mice on P2 through P4, and they were allowed to mature to P8, when EdU was administered 3 h before euthanasia. A larger proportion of the GFPlow than GFPhigh F retained the BrdU label at P8, whereas a larger proportion of GFPhigh F incorporated EdU, confirming that the GFPhigh F were more proliferative at P8 (Fig. 1) (20). This indicates that proliferation diminishes sooner in the GFPlow than in the GFPhigh F subpopulation. Kinetically equivalent proliferation would yield similar proportions of BrdU-retaining cells in both the GFPlow and GFPhigh subpopulations. These findings suggest that LF and MF proliferate independently along divergent pathways, which may influence their divergent differentiation.

Others have proposed that FGFs regulate the differentiation of the GFPhigh F subpopulation into MF (39). To learn whether FGF signaling has individualized effects on MF precursors, using FACS (representative plots for FGFR3, CD333, shown in Fig. 2B) we examined how FGFs and FGFRs are differentially expressed and localized in the GFPneg, GFPlow, and GFPhigh LF subpopulations, immediately after isolation. FGFR3-staining cells were more prevalent, and a larger fraction of the receptor was located on the cell surface in the GFPhigh compared with GFP low F (Fig. 2C). A larger proportion of GFPlow F contained FGFR2 (Fig. 2A) and FGFR4 (Fig. 2D), which were predominantly located intracellularly. Consistent with the data for immunoreactive FGFR4 protein, FGFR4 mRNA was more abundant in GFPlow F (Fig. 3A).
However, FGFR3 mRNA was higher in GFP<sup>low</sup> F (Fig. 3A) although a larger proportion of GFP<sup>high</sup> than GFP<sup>low</sup> F contained immunoreactive FGFR3 on the cell surface (Fig. 2C). As expected, FGFR2IIIb was less abundant in the PDGFR GFP<sup>low</sup> and GFP<sup>high</sup> cells than in GFP<sup>neg</sup> cells, which contained a small fraction of contaminating epithelial cells (Fig. 3B).

**Endosomal trafficking influences the abundance of FGFR3.** Observing more immunoreactive FGFR3 but less FGFR3 mRNA in GFP<sup>high</sup> compared with GFP<sup>low</sup> F (Fig. 5A, B), greater colocalization (M1, Mander’s coefficient), was greater in GFP<sup>high</sup> than in GFP<sup>low</sup> F, consistent with a prolonged protein half-life (Fig. 4, A and C) (22). Spry4 but not Spry2 mRNA was more abundant in F that express PDGFR-<sup>α</sup>-<sup>lig</sup> as expected, FGFR2IIIb was less abundant in the PDGFR GFP<sup>low</sup> and GFP<sup>high</sup> cells than in GFP<sup>neg</sup> cells, which contained a small fraction of contaminating epithelial cells (Fig. 3B).

We examined two potential mechanisms for increased FGFR3 protein stability in GFP<sup>high</sup> F. The PLA demonstrated greater association of HSP90 with FGFR3 in GFP<sup>high</sup> than in GFP<sup>low</sup> F, immediately after adding an exogenous FGF ligand (Fig. 6A). The PLA was used to assess interactions between endogenous FGFR18 and FGFRs in F that were isolated at P8 and cultured overnight (to enable adherence and spreading but attempt to preserve the native level of PDGFR-<sup>α</sup> expression while in the lung). Complexes between FGFR18 and FGFR3 or FGFR4 were both observed although ligand-receptor interactions were more abundant for FGFR3 (Fig. 6B). This suggests that FGFR18 may signal in an autocrine fashion in elastin-producing GFP<sup>high</sup> MF (28).

FGFRs and PDGFR-<sup>α</sup> both signal through the PI3K/Akt and Erk pathways. Because the FGFRs are differentially expressed in F with high and low levels of PDGFR-<sup>α</sup> gene expression, we examined pErk and pAkt in F that were fixed immediately after their isolation and were not exposed to FGF or PDGF ligands in culture. pS473Akt was more abundant in F that express PDGFR-<sup>α</sup> (GFP<sup>pos</sup>) than in nonexpressing F (GFP<sup>neg</sup>, Fig. 7A). PDGFR-<sup>α</sup> mRNA was significantly reduced in F isolated from TgCre; PDGFR-<sup>α</sup>/F mice (0.33 ± 0.09) compared with littermate controls (1.00 ± 0.01, means ± SE, n = 4 for each genotype, P = 0.01, Student’s t-test for paired variables). Conditional deletion of PDGFR-<sup>α</sup> reduced pS473Akt, distinctly in F that contain α-SMA (MF, Fig. 7B). In contrast, PDGFR-<sup>α</sup> deletion did not significantly alter pErk1/2, suggesting that complexing with HSP90 and sequestration of ubiquitin E3 ligase by Spry4 would stabilize FGFR3 in GFP<sup>high</sup> F, enabling higher levels of FGFR3 protein despite lower FGFR3 mRNA.

**FGF18 signals through FGFR3 to influence F proliferation and migration.** After observing that GFP<sup>high</sup> MF contain more FGFR3, we examined FGFR18, a known FGFR3 ligand, which stimulates MF differentiation (elastin production) during secondary septation (8). FGFR18 mRNA was more abundant in GFP<sup>high</sup> F immediately following isolation, showing that, compared with GFP<sup>neg</sup> or GFP<sup>low</sup> F, MF are primed to stimulate their own FGFR3 (Fig. 6A). The PLA was used to assess interactions between endogenous FGFR18 and FGFRs in F that were isolated at P8 and cultured overnight (to enable adherence and spreading but attempt to preserve the native level of PDGFR-<sup>α</sup> expression while in the lung). Complexes between FGFR18 and FGFR3 or FGFR4 were both observed although ligand-receptor interactions were more abundant for FGFR3 (Fig. 6B). This suggests that FGFR18 may signal in an autocrine fashion in elastin-producing GFP<sup>high</sup> MF (28).

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### Figure 8

Cell-surface PDGFR-<sup>α</sup> (<sup>CD140a</sup>) correlates with intracellular phospho (p) Erk. Lung F were isolated from PDGFR-<sup>α</sup>-GFP mice at P8, an aliquot of live cells was stained for CD140a before fixation, and the remainder was fixed. After permeabilization, some cells were stained for CD140a and all with anti-pErk and were then subjected to fluorescence-activated cell sorting (FACS). Data are expressed relative to cells within a particular subpopulation. A: larger proportion of GFP<sup>pos</sup> than GFP<sup>neg</sup> F exhibited PDGFR-<sup>α</sup> on their surface although similar proportions contained PDGFR-<sup>α</sup> intracellularly. B: larger proportion of GFP<sup>low</sup> F contained pErk, **P < 0.01, 2-way ANOVA, n = 4. C: larger proportion of GFP<sup>high</sup> than GFP<sup>low</sup> F displayed CD140a on the surface. Means ± SE, n = 4, 2-way ANOVA, Student-Newman-Keuls post-hoc test. *P < 0.05 compared with GFP<sup>neg</sup>, †P < 0.05 comparing GFP<sup>low</sup> and GFP<sup>pos</sup>. In both GFP<sup>neg</sup> and GFP<sup>pos</sup>, cell surface CD140a was more prevalent in pErk + F (**P < 0.05).
PDGFR-α primarily signals through PI3K/Akt (Fig. 7C). However, Erk signaling impacts where PDGFR-α localizes in the cell. Although CD140a was detected in equal proportions of GFPlow and GFPhigh F, CD140a was more abundant on the surface in the GFPhigh subpopulation (Fig. 8A). A larger proportion of pErk1/2-containing GFP-positive (GFPpos, both GFPhigh and GFPhigh) LF displayed PDGFR-α (CD140a) on the cell surface than GFPpos F without pErk1/2 (Fig. 8C). A larger proportion of GFPhigh F contained pErk1/2 (Fig. 8B). Therefore, Erk signaling may influence the availability of PDGFR-α to extracellular ligands.

Because F proliferate during septal elongation, we examined the effects of FGF18 on the proliferation of cultured F (Fig. 9A). We observed a decrease in the incorporation of the thymidine analog EdU after exposure to exogenous FGF18. This suggests that FGF18 decreases F proliferation, which would limit septal thickness. We also observed that FGF18 stimulated transient, but not sustained, phosphorylation of Erk1/2 in cultured F (Fig. 9B), indicating that FGF18 signals through Erk kinase in these cells.

F also reposition as the septa progressively thin. FGF18 gene deletion results in thickened saccular walls with excessive mesenchyme (47). Therefore, FGF18 may be required for mesenchymal repositioning and septal thinning, which occur during alveolar formation. The effects of the FGRF3 ligands FGF18 and FGF8 on migration of F were assessed using time-lapse imaging of microfluidic devices, which maintain ligand concentration gradients (31). Both FGF18 and FGF8b were chemoattractants for F, which moved toward the source well, but only FGF8b significantly increased the speed of migration compared with unexposed control cells (Fig. 9, C–F).

**LF express and bear receptors for FGF10.** Others have demonstrated that FGF10 is expressed by lipid-laden LF (11). We also observed that FGF10 mRNA was markedly higher in the GFPhigh (lipid-storing) compared with the GFPhigh or GFPhigh subpopulations (Fig. 10). FGFR2IIIb, the critical receptor for FGF10 in the pulmonary epithelium, was less abundant in the GFPhigh mesenchymal cells than in the GFPhigh cells, which contain ~3% epithelial cells (Fig. 3B) (33). As expected, the mesenchymal mRNA splice form FGFR1 IIIc was observed in both populations of PDGFR-α-expressing F (Fig. 10C). FGFR1 IIIb also binds FGF10, and this interaction impacts the differentiation of cardiomyocytes and adipocytes (48). The FGFR1 IIIb mRNA splice isoform was present in PDGFR-α-expressing F and was significantly higher in the GFPhigh subpopulation (Fig. 10B). Finding both FGF10 and its receptor suggests potential autocrine regulation in LF.
FIG. 10. GFP<sub>low</sub> F express both FGF10 and the FGFR1 IIIb splice variant, a receptor for FGF10. RNA was isolated from F that were separated by flow cytometric sorting based on their levels of PDGFR-α gene expression (GFP fluorescence intensity). Quantitative RT-PCR was performed for FGF10 using a TaqMan probe (ΔΔCT, n = 7). Student-Newman-Keuls post hoc test compared with GFP<sub>low</sub>; *P < 0.05 comparing GFP<sub>low</sub> and GFP<sub>high</sub>. B and C: custom-designed primer and 5'-nuclease probe sets were used to uniquely detect either the FGFR1 IIIb or FGFR1 IIIc splice variants (n = 5). FGF10 and FGFR1 mRNA abundance was normalized to β2-microglobulin for each sample. (*P < 0.05, 2-way ANOVA).

DISCUSSION

Secondary septation requires sequential mesenchymal proliferation, differentiation, and apoptosis to produce the expansive but thin barrier, which is necessary for optimal gas exchange. We previously showed that MF differentiation is accompanied by increasing nuclear p27<sup>kip1</sup> and FoxO3a along with diminishing PDGFR-α signaling through Akt (32). However, additional factors are required for differentiation, indicating that variation in PDGFR-α signaling is necessary but not sufficient to define the fate of PDGFR-α-expressing lung F (39). FGFs, which regulate the differentiation of adipocytes and myocytes, are also required for pulmonary alveolar formation (39). Blocking the function of FGFR2 (primarily FGFR2 IIb) in alveolar epithelial cells altered differentiation in both the epithelial and mesenchymal compartments and, by diminishing secondary septation, reduced alveolar surface area (17). FGFR2 signaling is also required for the differentiation of PDGFR-α+ progenitors into MF during the compensatory alveolar growth, following unilateral pneumonectomy (10, 39).

In addition, deletion of both FGFR3 and FGFR4, which primarily alters mesenchymal cells, increased the MF products tropoelastin, lysyl oxidase, and fibulin at P28 without altering α-SMA (a marker of MF) (44). When combined with our data, these observations suggest that, although FGFR2IIc is expressed by mesenchymal cells, FGFR3 is the predominant cell surface FGR on PDGFR-α-GFP<sup>high</sup> alveolar MF.

To further clarify the importance of FGFR3 and FGFR4 for alveolar formation, others have deleted genes for two of their ligands, FGF8 and FGF18 (54). FGF8 deletion increased proliferation of both epithelial and mesenchymal cells at embryonic day (E) 16.5, resulting in diminished maturation of type 1 epithelial cells, fewer capillaries, and thickened primary septa (52). Germline deletion of FGF18 produced thickened septa and displacement of capillaries from hypoproliferative epithelia at E18 (47). Therefore, remodeling of primary septal walls requires an FGF8-mediated reduction in epithelial proliferation and a concurrent reduction in the mesenchymal compartment, perhaps in response to FGF18. We found that FGF18 diminished proliferation of cultured F (Fig. 9A). Overexpression of FGF18 during the pseudoglandular phase promoted mesenchymal differentiation during the saccular phase, and it reduced epithelial differentiation with fewer primary septa and dilated saccules (51). Mesenchymal FGF18 peaks postnatally during maximal secondary septation (8). In cultured lung F, FGF18 increased several components of the elastic fiber (elastin, fibulin-5, and lysyl oxidase), indicating that its effects are directed at mesenchymal cells (8). Taken together, these observations suggest that FGF18 and FGF8 likely signal through FGFR3 and FGFR4 to regulate pulmonary mesenchymal differentiation during saccular formation.

Our study refines these observations, applies them to two specific populations of mesenchymal cells, and clarifies their unique importance. We have shown that FGFRs differ between GFP<sub>flow</sub> LF (lower PDGFR-α gene expression) and GFP<sub>high</sub> MF. Both FGFR3 and FGFR4 mRNA were more abundant in GFP<sub>flow</sub> cells compared with either GFP<sub>high</sub> or GFP<sub>neg</sub>. Analysis of immunoreactive FGFR2 and FGFR4 using FACS corroborated the findings for mRNA, as both were present in a higher proportion of the GFP<sub>flow</sub> than GFP<sub>high</sub> subpopulation. However, contrary to what was observed for FGFR3 mRNA, more FGFR3 protein was observed both intracellularly and on the surface of GFP<sub>high</sub> compared with the GFP<sub>flow</sub> population. This suggests that the FGFR3 protein half-life is prolonged in the GFP<sub>high</sub> F.

Our findings suggest that Spry4 and HSP90 preferentially stabilize FGFR3 in the GFP<sub>high</sub> subpopulation. Differences in FGFR stability influence disease states and are controlled by events in the endosomal sorting pathways (26) (2). The MVB is an important intracellular depot for FGFR3, where it can retain kinase activity or undergo ubiquitination and subsequent proteasomal degradation (35). Using CHMP2B as a marker, we identified the MVB as the major intracellular reservoir of FGFR3 in PDGFR-α-expressing F. Spry-2 and Spry-4 modulate FGFR3 ubiquitination by acting as a sink for c-Cbl, which titrates this E3 ligase away from FGFR3, thereby slowing its proteolytic degradation (42). HSP90 is a stabilizing molecular chaperone for FGFR3, which would otherwise be marked for degradation in the MVB (45). Defining how differences in FGFRs influence the divergent functions of GFP<sub>high</sub> and GFP<sub>low</sub> F would require population-specific deletion of FG-
FRs. Therefore, we cannot conclude whether a particular FGF is more essential for a particular PDGFR-α-expressing subpopulation.

Figure 1 shows different kinetics for expansion of the GFPhigh and GFPlow F populations. If the GFPlow cells were precursors for GFPhigh F, then one would expect the BrdU label to dissipate as GFPlow proliferates to produce GFPhigh F. Conversely, if GFPhigh F were precursors for GFPlow F, then one would expect the GFPlow cells to increase relative to total alveolar cells between P4 and P8 rather than remain constant (Fig. 1B). Lineage tracing would more precisely define the origins of the GFPhigh and GFPlow F, but no suitable approach has been identified. GI11 and FGF10 respectively define a precursor population for GFPhigh and GFPlow F (25) (11). To our knowledge, no one has identified a unique marker for a common precursor, which is retained in one F subpopulation and not the other.

Although a smaller proportion of GFPlow F incorporated EdU at P8 (Fig. 1), a larger proportion contained pErk, indicating that Erk signaling and proliferation do not directly correlate (Fig. 8B). However, pErk+ GFPlow MF were more likely to display PDGFR-α on their surface than their GFPhigh counterparts, making them potentially more responsive to PDGF-A, which increases cellular proliferation (32). Furthermore, PDGFR-α gene deletion reduced pS473Akt but not pErk1/2, suggesting that PDGF-A signals primarily through PI3K and Akt (Fig. 7B). Using glioma cells, others demonstrated that Erk activation was accompanied by localization of PDGFR-α on the cell surface (9). Thus one effect of FGFR3, which predominantly signals through the Ras-Raf-MEK-Erk pathway, may be modifying the availability of cell-surface PDGFR-α (12).

Others observed that exogenous FGF18 increases tropoelastin in lung F. Our new findings (Fig. 6B) suggest that FGFR3 and/or FGFR4 bind endogenous FGF18, which could potentially increase tropoelastin production by PDGFR-α-expressing MF. Exogenous FGF18 suppressed proliferation in cultured PDGFR-α-GFP-expressing F (Fig. 6C), which differs from the findings of Chaillie-Hue and associates (8) but is similar to what others have observed in the dermis and in intestinal crypts (1, 24). Coordination of a decrease in proliferation with an increase in differentiation (greater tropoelastin and lysyl oxidase gene expression) is consistent with the paradigm that elastogenic cells proliferate before depositing elastic fibers (43). Finding more FGF18 mRNA in GFPhigh than in GFPlow cells (Fig. 6A) also connects FGF18 with MF differentiation, which is accompanied by production of α-SMA and tropoelastin. This is similar to chondrocytes and myocytes, where FGF18 autonomously promotes differentiation during development of the articular cartilage and limbs (37).

Pulmonary mesenchymal cells produce FGF10, which binds to FGFR2IIIb on adjacent epithelial cells, promoting their proliferation and differentiation (34). However, FGF10 also regulates the differentiation of mesenchymal cells, cardiomyocytes (41), adipocytes (21), and cardiac F (48). FGF10 signaling is critical for development of embryonic right ventricular cardiomyocytes, which express FGFR2IIIb. Cardiac F, which do not express FGFR2IIIb, respond to FGF10 by increasing proliferation and migration, apparently through FGFR1 IIIb (48). We have shown that, like cardiac F precursors, GFPlow F express Tcf21 (33). We observed the FGF10 receptor, FGFR1 IIIb, was highest in the GFPlow LF, which also have the highest levels of FGF10 mRNA (Fig. 10B). Our observations parallel those made in adipocytes, where autocrine FGF10 signaling promotes the lipid storage (21). Further studies are required to delineate the significance of FGF10 signaling within the LF. By analogy to adipocytes, FGF10 may regulate the acquisition of the white fat phenotype (lipid storage and Tcf21) (38, 46, 50).

Srisuma and associates (44) demonstrated that compound deletion of both FGFR3 and FGFR4 is required to disrupt alveolar septation. Because the two receptors have overlapping temporal and spatial patterns of expression in the lung, these authors posited that they have an additive effect on alveolarization. Zhang and associates (54) showed that members of the FGFR8/FGF18 family bind more avidly to FGFR3 and FGFR4 than either FGFR1 or FGFR2, which is consistent with an overlapping additive effect. Our studies indicate that FGFR3 is the dominant receptor in the GFPhigh MF, which are more abundant than their GFPlow counterparts at P8. Conversely, FGFR4 is the dominant receptor in the GFPlow subpopulation (Figs. 2D and 3A). Both GFPhigh and GFPlow F express FGF11/FGF13 mRNA, which, along with the coreceptor β-Klotho, binds FGF21, a metabolic regulator in adipocytes (38). Additional studies are required to determine whether FGFR4 and FGFR1/IIc have unique effects in the GFPlow F.

Our findings may help identify new strategies for alveolar repair and regeneration. FGF10 produced by GFPlow LF maintains epithelial stem cells, which yield more alveolar type 2 or differentiate into type 1 cells (3). Selective modulation of FGF and/or PDGF-A signaling may enable expansion of the PDGFR-α-low LF and promote repair in fibrotic lungs. Alternatively, selective modulation FGF signaling in PDGFR-α-high MF may limit the transforming growth factor-β-dependent expansion of F foci and ameliorate the fibrotic response in pulmonary fibrosis (34).

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: S.E.M. conception and design of research; S.E.M. and D.M.M. performed experiments; S.E.M. analyzed data; S.E.M. interpreted results of experiments; S.E.M. prepared figures; S.E.M. drafted manuscript; S.E.M. and D.M.M. edited and revised manuscript; S.E.M. and D.M.M. approved final version of manuscript.

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