Dynamic Regulation of Platelet-Derived Growth Factor Receptor α Expression in Alveolar Fibroblasts during Realveolarization

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Although the importance of platelet-derived growth factor receptor (PDGFR)- α signaling during normal alveogenesis is known, it is unclear whether this signaling pathway can regulate realveolarization in the adult lung. During alveolar development, PDGFR- α -expressing cells induce α smooth muscle actin (α -SMA) and differentiate to interstitial myofibroblasts. Fibroblast growth factor (FGF) signaling regulates myofibroblast differentiation during alveolarization, whereas peroxisome proliferator-activated receptor (PPAR)- γ activation antagonizes myofibroblast differentiation in lung fibrosis. Using left lung pneumonectomy, the roles of FGF and PPAR- γ signaling in differentiation of myofibroblasts from PDGFR- α -positive precursors during compensatory lung growth were assessed. FGF receptor (FGFR) signaling was inhibited by conditionally activating a soluble dominant-negative FGFR2 transgene. PPAR- γ signaling was activated by administration of rosiglitazone. Changes in α -SMA and PDGFR- α protein expression were assessed in PDGFR- α -green fluorescent protein (GFP) reporter mice using immunohistochemistry, flow cytometry, and real-time PCR. Immunohistochemistry and flow cytometry demonstrated that the cell ratio and expression levels of PDGFR- α -GFP changed dynamically during alveolar regeneration and that α -SMA expression was induced in a subset of PDGFR- α -GFP cells. Expression of a dominant-negative FGFR2 and administration of rosiglitazone inhibited induction of α -SMA in PDGFR- α -positive fibroblasts and formation of new septae. Changes in gene expression of epithelial and mesenchymal signaling molecules were assessed after left lobe pneumonectomy, and results demonstrated that inhibition of FGFR2 signaling and increase in PPAR- γ signaling altered the expression of *Shh*, *FGF*, *Wnt*, and *Bmp4*, genes that are also important for epithelial-mesenchymal crosstalk during early lung development. Our data demonstrate for the first time that a comparable epithelial-mesenchymal crosstalk regulates fibroblast phenotypes during alveolar septation.

Keywords: progenitor cells; septation; bronchopulmonary dysplasia; chronic obstructive pulmonary disease; *wnt*; *shh*

Arrest of alveolarization in infants with bronchopulmonary dysplasia and loss of alveolar airspace, such as emphysema in patients with chronic obstructive pulmonary disease, poses an enormous

CLINICAL RELEVANCE

This study demonstrates that the number of platelet-derived growth factor receptor (PDGFR)- α -expressing cells and the levels of PDGFR- α per cell dynamically change during compensatory lung growth. We further show that the dim PDGFR- α -expressing cells are the myogenic precursor cells. We provide evidence that dnFGFR and PPAR- γ agonists increase PDGFR- α expression, which inhibits α smooth muscle actin induction and results in a change in fibroblast phenotype. This study provides new insights in epithelial-mesenchymal crosstalk and the differentiation of lung fibroblast phenotypes.

public health burden. Increased knowledge of the cellular and molecular mechanisms regulating alveolar formation may provide therapeutic opportunities to regenerate alveolar surface area. Transgenic mice offer unique opportunities to explore cellular and molecular mechanisms of compensatory lung growth that might be useful to increase the limited potential of adaptive growth in human lungs (1–6).

Unilateral left lobe pneumonectomy (PNX) induces compensatory growth of the remaining right lobes with increased numbers of alveoli (1, 7–9). Molecular mechanisms that regulate the earlier phase of compensatory lung growth have been studied extensively, but little is known about the cellular mechanisms that regulate fibroblast differentiation during the formation of new septa (8, 10–15).

In the developing lung, two populations of interstitial fibroblasts have been described: myofibroblasts and lipofibroblasts (16). Interstitial myofibroblasts contain contractile elements as shown by ultrastructural analysis (17, 18), express α smooth muscle actin (α -SMA) (16, 19), and undergo apoptosis after alveolarization is completed (20). Lipofibroblasts express peroxisome proliferator-activated receptor γ (PPAR- γ) (16), traffic lipids, and store retinoids (21). The transition of the lipofibroblast to the myofibroblast in lung fibrosis can be inhibited by administration of rosiglitazone (RZG), a PPAR- γ agonist (22–30). However, lineage relationships between lipofibroblasts and myofibroblast during lung development and regeneration remain controversial. In this study, we integrated findings from lung development and lung injury to study myofibroblast differentiation during reseptation.

Although lack of PDGFR- α or fibroblast growth factor receptor (FGFR) signaling in mice results in deficient alveolarization and absent or aberrant interstitial myofibroblast differentiation, little is known about how these signaling pathways regulate myofibroblast differentiation (31–36). Previous data demonstrated that dominant-negative FGFR (dnFGFR) expression during retinoic acid-mediated realveolarization inhibited induction of α -SMA and subsequent formation of new septa (37).

It has been shown that PPAR- γ and dnFGFR can change the phenotype of the interstitial fibroblast *in vitro* and *in vivo*. The

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purpose of the current study was to identify whether the common lack of the myofibroblast phenotype was sufficient to block realveolarization and to identify other common or distinct cellular and molecular changes that impair realveolarization. Data from the current study demonstrate that activation of PPAR- γ signaling and inhibition of FGF signaling promote a nonmyogenic phenotype by increasing platelet-derived growth factor receptor (PDGFR)- α expression. However, over time changes in fibroblast populations and expression of retinoic acid signaling molecules between these treatment groups were significantly different, suggesting that these two pathways do not converge. In summary, this study provides new insights in the origin of a population of interstitial myofibroblasts and their dependence on a balanced epithelial-mesenchymal crosstalk.

MATERIALS AND METHODS

Additional details about the methods are provided in the online supplement.

Transgenic Mice

Conditional triple transgenic mice *SFTPC -rtTA/tetOdnFGFR-Hfe/Pdgfra^{tm11(EGFP)Sor}* were subjected to PNx or sham surgery (SHAM) (2, 12, 34, 36–42). To induce dnFGFR, mice were exposed to doxycycline 2 days before surgery and maintained on doxycycline until death (PNx+dnFGFR). To activate PPAR- γ , mice were injected daily with RZG (3 μ g/g body weight) starting the day of surgery (PNx+RZG). The following four groups were obtained for each time point: SHAM, PNx, PNx+dnFGFR, and PNx+RZG. PNx was performed as previously described (41). Three to 10 animals per group and assay were used.

Immunohistochemistry

α -SMA, chondroitin sulfate proteoglycan4 (NG2), and PDGFR- α -green fluorescent protein (GFP) were colocalized by immunofluorescence staining using mouse anti- α -SMA (Sigma-Aldrich, St. Louis, MO) and rabbit anti-NG2 (Milipore, Billerica, MA) with secondary antibodies conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad, CA) and counterstained with DAPI-containing mounting media. GFP signals were detected using a chicken polyclonal antiGFP antibody (Abcam, Cambridge, MA).

Flow Cytometry staining and Flow Cytometry analysis

Freshly isolated lung fibroblasts ($0.5\text{--}1 \times 10^6$) were stained with anti-mouse CD45 eFluor 450 (e-bioscience) with phycoerythrin-anti- α -SMA antibody or PE-mouse IgG2a. Flow cytometry was performed using a two-laser, six-color FACSCanto (BD Bioscience, Sparks, MD) run by FACSDiVa software. Compensation and gating were performed using unstained and single-stained primary lung fibroblasts. Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Quantitative Real-Time PCR

Total RNA from freshly isolated lung fibroblasts and lung epithelial cells was transcribed to cDNA using random primers. qRT-PCR was performed with TaqMan probes (Applied Biosystems, Foster City, CA). RPL32 was used as normalization standard. All changes in gene expression were normalized to one SHAM control mouse. Changes in gene expression are fold changes of the average of three to seven animals per group.

RESULTS

Expression of dnFGFR or Administration of RZG Increased Fractional Airspace

To determine whether inhibition of FGF signaling or activation of PPAR- γ after PNx changed compensatory lung growth, mass specific lung volume and fractional airspace were assessed 21 days after PNx (43, 44). Neither expression of the dnFGFR nor administration of RZG changed mass specific lung volume of

the total lung (Figure 1A) or relative and absolute volume gain of individual lung lobes (see Figures E1A and E1B in the online supplement). Mean fractional airspace of all lobes was not different between SHAM- and PNx-treated mice (Figures 1B and 1C). However, expression of dnFGFR or RZG treatment increased mean fractional airspace of all lobes by 10% of the original value. Individual lung lobes have different capabilities to gain volume after PNx (12). Although the accessory lobe gained the highest absolute and relative volume after PNx (see online supplement), there was no significant difference in alveolar regeneration or inhibition of regeneration between lung lobes (Figure 1D). These data demonstrate that expression of the dnFGFR or administration of RZG during compensatory lung growth did not change expansion and volume gain of the remaining right lung but inhibited alveolar regeneration.

Expression of dnFGFR Altered Lung Mechanics

To determine whether histological changes after pneumonectomy resulted in physiological changes, lung mechanics were assessed 21 days after PNx (45–47) (Figures 1E and 1F). From all groups and parameters analyzed, only PNx+dnFGFR-treated animals compared with PNx-treated animals demonstrated increased tissue damping (PNx: 7.75 ± 0.53 ; PNx+dnFGFR: 9.68 ± 0.44) and tissue elastance (PNx: 7.75 ± 0.53 ; PNx+dnFGFR: 9.68 ± 0.44). These data demonstrate that inhibition of FGF signaling during compensatory lung growth increased the resistance of the lung, resulting in the need for more energy to inflate and deflate the lung.

Induction of an Interstitial Myofibroblast Is Important for Reseption and Contraction

Although an increase in fractional airspace and changes in lung mechanics demonstrate that inhibition of FGFR2 signaling or activation of PPAR- γ impaired realveolarization, the cellular and molecular changes responsible for these functional changes were not clear. Therefore, cellular and molecular changes were assessed at an earlier time point during compensatory lung growth. Because myofibroblast differentiation is important during normal septation, myofibroblast differentiation was assessed after PNx surgery by immunohistochemistry for α -SMA (48) on lung sections of mice (Figure 2). α -SMA protein was induced in PNx lungs 5 days after PNx (Figures 2A and 2B). Expression of the dnFGFR receptor (Figure 2C) or RZG treatment (Figure 2D) inhibited induction of α -SMA expression in interstitial fibroblasts, whereas α -SMA expression in peribronchiolar and perivascular fibroblasts was unchanged. Most mesenchymal progenitor cells in the early mouse embryo express PDGFR- α (49). In the lung, PDGFR- α -GFP-positive cells are exclusively found in the alveolar fibroblasts (Figure 2A). Although it has been demonstrated that interstitial PDGFR- α -expressing cells induce α -SMA (37, 50), it is unclear whether PDGFR- α -expressing cells are pericyte-like cells. NG2 (chondroitin sulfate proteoglycan 4) has been identified as a marker of pericytes and was recently used to identify stromal populations that contribute to pulmonary fibrosis (51–53). Dual-immunofluorescent detection of NG2 and PDGFR- α -GFP revealed that NG2 did not colocalize with PDGFR- α -GFP-expressing cells in adult lungs before and after surgery (Figures 2E–2H). These data suggest that PDGFR- α -expressing cells are not NG2-positive pericytes.

α -SMA is a phenotypic marker of the myofibroblast, and its abundance correlates with the generation of contractile force, which can be measured by gel contraction *in vitro* (54). To test the hypothesis that differentiation of a contractile α -SMA-positive interstitial fibroblast is important for budding and elongation of new septa, primary mouse lung fibroblasts (MLFs) were

Figure 1. Lung volume and lung mechanics 21 days after left lobe pneumonectomy (PNX). The volume of total lungs and single lobes was determined by fluid displacement and corrected for body weight (43). (A) Mass-specific lung volume (ml/g). The right lung (RL) and total lung (RL+ left lung [LL]) volume of double transgenic mice without surgery (RL: 0.013 ± 0.001 ; RL+LL: 0.018 ± 0.002) and with sham surgery (RL: 0.014 ± 0.002 ; RL+LL: 0.019 ± 0.003) was determined (white column). At 21 days after left lung pneumonectomy, the volume of the right lung was determined in PNX animals (PNX: black column: 0.022 ± 0.002) in the absence of fibroblast growth factor (FGF) signaling (PNX + dominant-negative FGF receptor [dnFGFR], light gray column: 0.019 ± 0.004) and after daily injections of rosiglitazone (RZG) (PNX+RZG: dark gray column: 0.019 ± 0.005). *In all groups, mass specific volume of the right lung was significantly increased after surgery and was comparable to right and left lung mass before surgery. (B) Lung histology of SHAM, PNX, PNX+dnFGFR, and PNX+RZG was analyzed by H&E staining 21 days after pneumonectomy. SHAM and PNX animals show normal alveolar tissue. PNX+dnFGFR and PNX+RZG lungs demonstrate alveolar simplification. Scale bar, 50 μ m. (C) Fractional airspace of the right lung was assessed by morphometric point intersection analysis. In SHAM-operated mice, the fractional airspace is $59.08 \pm 0.25\%$ (white column). In PNX, the fractional airspace is $59.03 \pm 0.28\%$ (black column). Compared with PNX lungs, fractional airspace in PNX+dnFGFR lungs is significantly increased to $64.99 \pm 0.34\%$ (light gray column). Compared with PNX lungs, fractional airspace in PNX+RZG lungs is significantly increased to $64.00 \pm 0.24\%$ (dark gray column). (D) Fractional airspace of the accessory lobe was assessed by morphometric point intersection analysis. In SHAM (white) and PNX (black) mice, the mean fractional airspace of the accessory lobe was not significantly changed (SHAM: $59.32 \pm 0.31\%$; PNX: $59.52 \pm 0.48\%$). In the presence of dnFGFR (light gray) or RZG (dark gray), the mean fractional airspace of the accessory lobe was significantly increased (PNX+dnFGFR: $65.85 \pm 0.53\%$; PNX+RZG: $65.13 \pm 0.29\%$). Comparisons among groups were made by one-way ANOVA using the Bonferroni's multiple comparison test. Data are presented as mean $\pm$ SEM, with $P < 0.05$ considered significant ($n \geq 3$ animals per group). (E and F) Lung mechanics were assessed 21 days after surgery. SHAM (white column), PNX (black column), PNX+dnFGFR (light gray column), and PNX+RZG (dark gray column). Lung mechanics in SHAM-, PNX-, and PNX+RZG-treated mice were comparable. Compared with PNX lungs, expression of dnFGFR after PNX surgery significantly increased tissue damping (E) and tissue elastance (F). Statistical differences were analyzed by Student's *t* test ($P < 0.05$; $n = 5$ per group).

Figure 2. Alveolar α smooth muscle action (α -SMA) expression is induced 5 days after PNx. Immunohistochemistry for α -SMA (A–D, red) and NG2 (E–H, red) was performed on lung sections of adult mice 7 days after SHAM (A, E), PNx (B, F), PNx+dnFGFR (C, G), and PNx+RZG (D, H). α -SMA expression was found in peribronchiolar and perivascular fibroblasts in all lungs. Nuclear PDGFR- α -GFP signal could only be found in alveolar fibroblasts (B, arrowhead and insert) and was not found in peribronchiolar or perivascular SMA-positive fibroblasts (A, C, D, arrows). Five days after surgery, α -SMA was induced in PDGFR- α -GFP-positive interstitial fibroblasts (B, arrow). NG2 expression was found in the lung interstitium in all experimental groups and was never colocalized with nuclear PDGFR- α -GFP. Low-magnification inserts in E through H provide overall distribution patterns. Scale bar, 10 μ m. Red in A through D: α -SMA. Red in E through H: NG2. Blue: DAPI. Green: PDGFR- α -GFP. B = bronchus; V = vessel.

subjected to a gel contraction assay in the absence and presence of the FGF inhibitor SU5402 and RZG (Figure 3). After 7 days in culture, MLFs significantly contracted collagen gels to one third of the initial area. In the presence of the FGF inhibitor SU5402 or 100 mM RZG, MLFs did not contract the collagen gels. Immunohistochemical analysis of the collagen gels after 7 days in culture demonstrate that fibroblasts in contracting gels contain α -SMA, whereas fibroblasts in noncontracting SU5402- and RZG-treated gels were negative for α -SMA. These data demonstrate that FGF and PPAR- γ signaling is important for induction of α -SMA and the generation of contractile force in lung fibroblasts.

Figure 3. Primary MLFs generate contractile force in three-dimensional collagen gels. (A) Primary mouse lung fibroblasts (MLFs) were cultured in three-dimensional collagen gels over 7 days in the presence of DMSO (a, b), SU5402 (c), and 100 and 50 mM RZG (d). (B) Gel area size was determined over 7 days of culture. Control pellets significantly contracted over 7 days (Day 0: 1.9 ± 0.01 cm²; Day 3: 1.6 ± 0.08 cm²; Day 5: 0.8 ± 0.15 cm²; Day 7: 0.5 ± 0.09 cm²). In the presence of SU5402, pellets did not contract (Day 0–7: 1.9 ± 0.03 cm²). In the presence of 100 mM RZG pellets did not contract (Days 0–7: 1.9 ± 0.07 cm²). In the presence of 50 mM RZG pellets contracted 10% (Day 0: 1.8 ± 0.02 cm²; Day 3: 1.8 ± 0.02 cm²; Day 5: 1.6 ± 0.11 cm²; Day 7: 1.5 ± 0.13 cm²). (C) Immunohistochemistry for α -SMA on sections of collagen pellets after 7 days in culture. Fibroblasts in control pellets stained positive for α -SMA. α -SMA was not detected in fibroblasts cultured in the presence of SU5402 or RZG.

The Percentages of PDGFR- α -Expressing Cells Dynamically Changed during Compensatory Lung Growth

The PDGFR- α -GFP protein functions as a nuclear reporter for spatial and temporal expression of the endogenous PDGFR- α expression (34, 40). Previous histological data showed that expression

of dnFGFR increased the number of PDGFR- α -expressing fibroblasts during retinoic acid-mediated realveolarization (37). Therefore, the ratio of GFP-positive fibroblasts in primary MLFs was determined by flow cytometry. To focus on nonhematopoietic cells, CD45-positive cells were excluded (Figure 4A), and the percentage of GFP-positive fibroblasts among MLFs was determined and compared over time (3, 5, and 7 d after surgery) and between treatments (SHAM, PNx, PNx+dnFGFR, and PNx+RZG) (Figure 4B). Compared with SHAM, the ratio of GFP-positive cells significantly increased by 3 and 5 days after PNx surgery and was decreased significantly by 7 days (Figure 4B, *black solid line*). These cytometry data demonstrated a rapid change within the fibroblast population during the first week after surgery. Compared with SHAM, the ratio of GFP-positive cells in PNx+dnFGFR significantly increased 5 days after surgery and remained significantly increased after 7 days (Figure 4B, *green dashed line*). These data suggest that expression of dnFGFR does not change induction of PDGFR- α -GFP expression but inhibits the down-regulation of PDGFR- α -GFP expression 7 days after surgery. Compared with SHAM, the ratio of GFP-positive cells in

PNx+RZG did not increase significantly but decreased significantly 7 days after surgery (Figure 4B, *red dotted line*). These data suggest that RZG treatment attenuated the increase of PDGFR- α -GFP-expressing cells and did not change loss of PDGFR- α -GFP-expressing cells 7 days after surgery. Compared with PNx or PNx+RZG, expression of dnFGFR resulted in a significant 3-fold increase of PDGFR- α -GFP-expressing cells 7 days after surgery. These data suggest that dnFGFR expands the populations of PDGFR- α -GFP-expressing cells, whereas RZG attenuates activation of PDGFR- α -GFP-expressing cells.

PDGFR- α Expression Levels Are Dynamically Regulated during Compensatory Lung Growth

Flow cytometry analysis revealed that in adult mice PDGFR- α -GFP-positive cells can be distinguished into two distinct fibroblast populations, GFP^{bright} and GFP^{dim} (Figures 4A and 4C). In SHAM lungs, 16.5% of all GFP-positive cells were GFP^{bright}; during the first 3 days of compensatory lung growth, the percentage of GFP^{bright}-positive cells duplicated and rapidly declined

Line graph of % GFP-positive fibroblasts. Compared with SHAM ($9.2 \pm 0.9\%$), the percentage of GFP-expressing fibroblasts significantly increased 3 and 5 days after PNx and significantly decreased at 7 days (Day 3: $15.6 \pm 1.6\%$; Day 5: $13.2 \pm 2.5\%$; Day 7: $4.9 \pm 0.8\%$). Compared with SHAM the percentage of GFP expressing cells was significantly increased 5 and 7 days after PNx+dnFGFR intervention (Day 3: $11.0 \pm 1.1\%$; Day 5: $15.9 \pm 2.2\%$; Day 7: $16.0 \pm 2.9\%$). Compared with PNx, the expression of dnFGFR delayed the increase of PDGFR- α -expressing cells and significantly blocked the down-regulation of GFP-expressing cells by 7 days. Compared with SHAM, the percentage of GFP-expressing cells was significantly decreased 7 days after PNx+RZG intervention (Day 3: $8.8 \pm 0.9\%$; Day 5: $14.3 \pm 2.0\%$; Day 7: $6.8 \pm 0.6\%$). Compared with PNx, the increase of GFP-expressing cells was delayed to Day 5 but did not interfere with GFP down-regulation by Day 7. (C) Line graph of % GFP^{bright} in all GFP-positive fibroblasts. Compared with SHAM ($16.5 \pm 1.5\%$), the percentage of GFP^{bright}-expressing cells significantly increased after PNx to $34.1 \pm 6.0\%$ on Day 3, decreased significantly to $11.5 \pm 0.8\%$ on Day 5, and reverted to $16.8 \pm 3.1\%$ on Day 7. The same significant increase at Day 3 was seen in PNx+dnFGFR (Day 3: $34.5 \pm 3.2\%$); however, the percentage of GFP^{bright} cells remained significantly increased by 5 days after surgery (Day 5: $31.2 \pm 4.0\%$) but was not significantly changed after 7 days (Day 7: $26.9 \pm 3.1\%$). Compared with SHAM, RZG treatment did not significantly change the percentage of GFP bright cells at any time point (Day 3: $18.3 \pm 2.8\%$; Day 5: $27.0 \pm 1.7\%$; Day 7: $25.2 \pm 4.0\%$). RZG significantly attenuated up-regulation by Day 3 and down-regulation by Day 5 when compared with PNx.

Figure 4. Number and level of PDGFR α expression transiently increases during compensatory lung growth. Flow cytometry analysis of primary lung fibroblast 3, 5, and 7 days after SHAM, PNx, PNx+dnFGFR, and PNx+RZG surgery. (A) Representative FACS histograms of freshly isolated lung fibroblasts 7 days after surgery. Pseudocolor dot plots. Gates identify nonhematopoietic populations as CD45^{neg}. Histograms of CD45^{neg}- and GFP^{pos}-positive cells gated for GFP and divided into GFP^{dim} and GFP^{bright} cells. Line graph of % GFP-positive cells in all CD45^{neg} cells at SHAM (time point 0), 3, 5, and 7 days after surgery in PNx (solid black line), PNx+dnFGFR (dashed green line), and PNx+RZG (dotted red line) animals. *Changes within treatment groups, analyzed by one-way ANOVA and Neuman-Keuls multiple comparison test. #Significant changes between treatments, analyzed by two-way ANOVA and Bonferroni multiple comparison test. Data are presented as means $\pm$ SEM, with $P < 0.05$ considered significant ($n \geq 3$ animals). (B)

by 5 days. Expression of dnFGFR increased the percentage of GFP^{bright} cells 3 and 5 days after surgery, and percentages returned to normal levels by 7 days. RZG treatment did not significantly change the percentages of GFP^{bright} cells at any time point. These data demonstrated that a subpopulation of PDGFR- α -GFP-expressing cells undergoes a transient shift to GFP^{bright} cells after PNx surgery. Expression of dnFGFR increased the percentage of PDGFR- α -GFP-expressing cells among all fibroblasts and prolonged the time of high GFP^{bright} percentages, whereas RZG did not increase percentages of PDGFR- α -GFP-expressing cells or percentages of GFP^{bright} cells. These data suggest that expression of dnFGFR and treatment with RZG have different effects on compensatory lung growth.

Myofibroblasts are PDGFR- α -GFP^{dim} and α -SMA^{bright}

To quantify induction of α -SMA in PDGFR- α -GFP-positive cells, freshly isolated MLFs were fixed, stained for α -SMA, and subjected to flow cytometry analysis (Figure 5). Cells positive for the hematopoietic marker CD45 were excluded. CD45^{neg} and GFP^{pos} fibroblasts were analyzed for α -SMA and PDGFR-

α -GFP expression levels. Pseudocolor plots were subdivided into four quadrants representing subpopulations of PDGFR- α (dim and bright) and α -SMA (dim and bright) (Figure 5A). Five days after surgery, the majority of the myofibroblasts, α -SMA^{bright} cells, were found in the GFP^{dim} cell population. Shifts in population distributions 3, 5, and 7 days after surgery were analyzed and summarized in line graphs for PDGFR- α -GFP^{dim}/ α -SMA^{bright} (Figure 5B), PDGFR- α -GFP^{dim}/ α -SMA^{dim} (Figure 5C), and PDGFR- α -GFP^{bright}/ α -SMA^{dim} (Figure 5D).

Compared with SHAM-operated, PDGFR- α -GFP^{dim}/ α -SMA^{bright} cell percentages increased 2.5-fold 3 days after PNx and 4.5-fold 5 days after PNx and reverted to sham levels by 7 days (Figure 5B). Compared with SHAM-operated, PDGFR- α -GFP^{dim}/ α -SMA^{bright} cell percentages did not change in PNx+dnFGFR lungs. Compared with SHAM-operated, PDGFR- α -GFP^{dim}/ α -SMA^{bright} cell percentages increased 2.5-fold 3 days after PNx+RZG and reverted to normal levels by 5 days.

Compared with SHAM, PDGFR- α -GFP^{dim}/ α -SMA^{dim} cell percentages significantly decreased in all treatment groups after

Figure 5. Expression of dnFGFR and administration of RZG inhibited α -SMA induction in PDGFR- α -GFP^{dim} cells and increased PDGFR- α -GFP expression per cell. α -SMA and PDGFR- α -GFP expression was analyzed by flow cytometry of freshly isolated CD45^{neg} and GFP^{pos} fibroblasts 3, 5, and 7 days after surgery (SHAM, PNx, PNx+dnFGFR, and PNx+RZG). Plots were subdivided into quadrants representing subpopulations of dim and bright cells (y axis: α -SMA-PE; x axis: PDGFR- α -GFP). Average percentages of cell numbers were plotted in line graphs for GFP^{dim}/ α -SMA^{bright} (B), GFP^{dim}/ α -SMA^{dim} (C), and GFP^{bright}/ α -SMA^{dim} (D). After Sham surgery, PDGFR- α -GFP-positive cells were composed of 72.4 $\pm$ 1.6% PDGFR- α -GFP^{dim}/ α -SMA^{dim}, 10.5 $\pm$ 0.8% GFP^{dim}/ α -SMA^{bright}, 16.9 $\pm$ 1.6% GFP^{bright}/ α -SMA^{dim}, and less than 1% GFP^{bright}/ α -SMA^{bright}. Significant shifts of population distributions were observed 3 days after surgery, peaked at 5 days, and reverted to normal population distribution after 7 days. At 5 days after PNx, the percentage of PDGFR- α -GFP^{dim}/ α -SMA^{dim} significantly decreased to 40.4 $\pm$ 1.9%, whereas the percentage of GFP^{dim}/ α -SMA^{bright} increased to 44.3 $\pm$ 1.3%. The percentage of GFP^{bright} cells did not change significantly. Expression of dnFGFR after surgery decreased the percentage of PDGFR- α -GFP^{dim}/ α -SMA^{dim} compared with SHAM-operated mice but significantly less than in PNx-treated mice (52.7 $\pm$ 5.4%). Moreover, in the presence of dnFGFR the percentage of GFP^{dim}/ α -SMA^{bright} (12.2 $\pm$ 0.7%) did not increase, whereas the percentage of GFP^{bright}/ α -SMA^{dim} significantly increased compared with SHAM- and PNx-treated animals (34.3 $\pm$ 5.1%). Treatment with RZG after surgery resulted in a reduction of PDGFR- α -GFP^{dim}/ α -SMA^{dim} cells (57.1 $\pm$ 2.7%) and a shift of cells to GFP^{bright}/ α -SMA^{dim} cells (27.2 $\pm$ 4.6%) and not GFP^{dim}/ α -SMA^{bright} (11.4 $\pm$ 1.0%). Data are presented as means $\pm$ SEM, with $P < 0.05$ considered significant ($n \geq 3$ animals). *Changes within treatment groups, which were analyzed by one-way ANOVA and Neuman-Keuls multiple comparison test. #Significant changes between treatments, which were analyzed by two-way ANOVA and Bonferroni multiple comparison test.

3 and 5 days and revert to almost normal levels by 7 days after surgery (Figure 5C). Compared with PNx, PDGFR- α -GFP^{dim}/ α -SMA^{dim} cell percentages decreased less after expression of dnFGFR or RZG treatment (Figure 5C).

Compared with SHAM, PDGFR- α -GFP^{bright}/ α -SMA^{dim} cell percentages did not change significantly after PNx surgery but increased significantly 3 and 5 days after PNx+dnFGFR and 5 days after PNx+RZG surgery (Figure 5D). All PDGFR- α -GFP^{bright}/ α -SMA^{dim} cells reverted to normal percentages by Day 7 (Figure 5D). Compared with PNx, the percentage of PDGFR- α -GFP^{bright}/ α -SMA^{dim} was 3-fold increased after expression of dnFGFR or treatment of RZG (Figure 5D). No significant changes in cell percentages were found in the PDGFR- α -GFP^{bright}/ α -SMA^{bright} populations.

At 3 and 5 days after PNx surgery, α -SMA was induced in 15 to 30% of GFP^{dim} cells, suggesting that PDGFR- α -GFP^{dim} cells differentiate into myofibroblasts. Although expression of dnFGFR inhibited induction of α -SMA, treatment with RZG transiently induced α -SMA expression in 15% of GFP^{dim} cells for 3 days after surgery. These data suggest that RZG and dnFGFR impair differentiation of the contractile fibroblast in different ways. After 5 days, α -SMA induction in PNx lungs peaked, whereas the percentage of GFP^{bright} cells dipped. In contrast, expression of dnFGFR or treatment with RZG blocked α -SMA expression after 5 days and increased GFP^{bright} cells by 15%. These data suggest that expression of dnFGFR and treatment with RZG promote a PDGFR- α -GFP^{bright} population, which is α -SMA negative.

Changes in mRNA Expression during Compensatory Lung Regrowth

To assess the effects of dnFGFR and RZG on the changes in gene expression involved in the epithelial-mesenchymal cross-talk, changes in mRNA levels of various genes were assessed 3 and 5 days after Sham, PNx, PNx+dnFGFR, and PNx+RZG

by real-time PCR (Figure 6). Compared with PNx lungs, the ratio of bright PDGFR- α -GFP cells increased by 20% in PNx+dnFGFR and in PNx+RZG 5 days after surgery, which was reflected by a doubling of PDGFR- α mRNA in the same treatment group (Figure 6A). qPCR analysis on a mixture of all lung fibroblasts was not sensitive enough to detect induction of *acta2* (α smooth muscle actin), which occurred in only 4.41% of all CD45^{neg} fibroblasts (data not shown). The PPAR- γ agonist RZG has been shown to inhibit profibrotic phenotypes in fibroblasts (27). PPAR- γ expression was decreased in MLFs 3 days after surgery but was not changed in the presence of dnFGFR or RZG (data not shown). PDGFR- α -positive cells have been identified to differentiate into adipocytes in skeletal muscle (55). Because fatty acid binding protein 4 (*Fabp4*) is expressed at high levels in adipocytes, changes in *Fabp4* mRNA expression levels were assessed after PNx. Compared with 3 days after surgery, levels of *Fabp4* were up-regulated (Figure 6A). Tenascin is an extracellular matrix protein secreted by fibroblasts and is associated with injury and remodeling (56). The mRNA levels in fibroblasts were increased 2- to 3-fold in all groups 5 days after surgery (Figure 6A). These data demonstrate that expression of genes characteristic of structural fibroblasts was induced 5 days after PNx and suggest that dnFGFR and RZG promoted the differentiation of structural fibroblasts.

Administration of retinoic acid rescues failed septation in mice, and mRNA expression of RXR- α , RXR- β , and RXR- γ and the retinoic acid-inducible gene *midkine* have been demonstrated to be involved in alveolarization (37, 57–59). At 3 days after surgery, RXR- α and RXR- β expression was reduced in PNx fibroblasts, unchanged in PNx+dnFGFR, and increased in PNx+RZG fibroblasts. RXR- γ and *midkine* expression was unchanged. At 5 days after surgery, RXR- α and RXR- β expression returned to baseline levels, whereas *midkine* expression was increased in PNx+RZG fibroblasts (Figure 6B). These data suggest that dnFGFR represses regulation of RXR- α and RXR- β , whereas RZG treatment increased RXR- α and RXR- β

Figure 6. Dynamic changes in gene expression after PNx. qRT-PCR was performed to determine RNA expression in primary lung fibroblasts or primary lung epithelial cells 3 and 5 days after PNx. (A) Genes expressed in structural fibroblasts: PDGFR- α , *acta2*, *Fabp4*, *tenascin C*. (B) Retinoic acid signaling in fibroblasts: RXR- α , RXR- β , *Midkine*. (C) FGF3 and FGF4 in fibroblasts and epithelial FGF9. (D) Wnt2a in fibroblasts and epithelial β -catenin, Shh, and Bmp4. Gene mRNA levels of SHAM (white bars), PNx (black bars), PNx+dnFGFR (light gray bars), and PNx+RZG (dark gray bars) were compared. Results were normalized to ribosomal L32 mRNA. Results are expressed as the means $\pm$ SEM of three to seven animals per group, and differences were analyzed by Student's *t* test (**P* < 0.05). Only genes and time points with significant changes are shown.

expression, resulting in an up-regulation of midkine after RZG treatment. These data support the finding that dnFGFR and RZG inhibit myofibroblast differentiation in different ways.

Although microarray expression data of postpneumectomy and postnatal lungs (14, 60) did not reveal significant changes in FGF or FGFR gene expression, *in vivo* loss-of-function studies associated FGFR3, FGFR4, and FGF18 with alveolarization (61–63). The dnFGFR receptor used in this study is thought to inhibit FGF7 and FGF10 signaling to the epithelium, which may affect expression of epithelial FGFs (e.g., FGF9). FGF9 and FGF10 have also been associated with lung bud morphogenesis (64, 65). To determine whether differentiation of myofibroblasts is associated with changes in FGF/FGFR mRNA expression, qPCR analysis was performed on primary MLFs and primary lung epithelial cells (Figure 6C). At 3 and 5 days after surgery, FGFR3 expression was reduced in PNx and PNx+dnFGFR fibroblasts and unchanged in PNx+RZG fibroblasts. FGFR4 was up-regulated in PNx+RZG fibroblasts after 5 days. These data suggest that down-regulation of FGFR3 precedes myofibroblast differentiation and that RZG administration inhibits down-regulation of FGFR3 and up-regulates FGFR4. FGF9 expression was reduced 3 days after PNx but was significantly increased after 5 days in PNx+dnFGFR and PNx+RZG fibroblasts. No changes in FGF10 and FGF18 expression were found (data not shown). These data suggest a significant role for FGF9 in the regulation of the fibroblast phenotype during compensatory lung growth.

It is clear from the complexity of epithelial–mesodermal interactions during lung bud morphogenesis that signaling components of Wnt, Shh, FGF, or BMP work together (66). To determine how changes in fibroblast phenotypes and ratios of fibroblast populations affect these major signaling pathways, we performed gene expression analysis on primary lung fibroblasts and epithelial cells (Figure 6D). In MLFs, Wnt2a expression was increased 5 days after surgery in PNx+dnFGFR and PNx+RZG. However, expression of Wnt5a and Wnt7b did not change at any time point

(data not shown). Compared with epithelial cells 3 days after PNx, β -catenin expression was increased in PNx+dnFGFR. Epithelial expression of Shh and Bmp4 increased 5 days after surgery in PNx+dnFGFR and PNx+RZG lungs. β -Catenin and Bmp4 expression did not change in MFLs 5 days after surgery (data not shown).

DISCUSSION

In this study, we have shown that PDGFR- α expression changes dynamically after PNx and that a subpopulation of PDGFR- α -expressing fibroblasts induces α -SMA and differentiate into contractile myofibroblasts. Evidence that FGFR2 and PPAR- γ signaling regulate myofibroblast differentiation was demonstrated *in vivo* and *in vitro*. *In vivo*, α -SMA induction during compensatory regrowth was inhibited by expression of dnFGFR or administration of RZG, whereas treatment with a FGF inhibitor or RZG inhibited contraction of collagen pellets and expression of α -SMA *in vitro*. Our data suggest that RZG delays activation of the PDGFR- α -positive fibroblasts and that dnFGFR2 promotes expansion of PDGFR- α -positive fibroblasts and differentiation into structural fibroblasts with no contractile function (Figure 7). Although the relationship between PDGFR- α expression and the myofibroblast phenotype is not clear, our data demonstrate that FGF and PPAR- γ pathways affect the dynamic changes of the myofibroblast and structural fibroblast phenotypes in the alveolar wall during compensatory lung growth. Changes in these fibroblast phenotypes affect septation, and, as a consequence, fractional airspace was increased 21 days after surgery.

Alveolar septation has been studied during normal development, but most studies on fibroblast differentiation during alveolarization have been descriptive (67, 68). In PDGFA knockout mice, myofibroblast progenitors fail to spread, which demonstrates an important role of the PDGFR- α -positive cell during alveolar septation (31, 32). Other researchers have shown that the number of PDGFR- α -GFP-positive cells increases during

Figure 7. Hypothetical model of molecular and cellular changes during reseptation. (A) PDGFR- α -positive cells increase in numbers during realveolarization. Fibroblasts with elevated PDGFR- α expression are located at the ridge of the newly forming septum (67). Data from this study demonstrate that, in adult lungs, expression of low levels of PDGFR- α is associated with a contractile myofibroblast. Myofibroblasts induce the tension that is necessary for the budding and elongation of a newly forming septum, and a structural fibroblast supports the newly forming alveolar tissue. (B) Inhibition of FGFR2 signaling or administration of RZG increased PDGFR- α expression per cell, which blocks the differentiation of myofibroblasts and new septae fail to bud or elongate. However, the structural function of the interstitial fibroblast remains, resulting in lungs with alveolar simplification that are stiffer, as demonstrated by increased tissue damping and elastance.

postnatal alveolarization and that PDGFR- α -GFP expression levels change as alveolarization progresses (50, 67). In the lung, the lipofibroblast has been implicated in lung development and homeostasis (68). During lung development, lipofibroblasts reside at the base of the secondary septum and are associated with dim levels of PDGFR- α -GFP, whereas non-lipid-containing fibroblasts express bright levels of PDGFR- α -GFP and are located at the alveolar entry ring (67, 69). Based on developmental studies, it is well accepted that PDGFA/PDGFR- α signaling and the interstitial fibroblast are important for elastin and extracellular matrix synthesis (31, 62), which would define a structural phenotype of the PDGFR- α -positive fibroblast. In this study, we demonstrate that PDGFR- α expression was transiently increased after PNX and that expression of dnFGFR further increased PDGFR- α expression and blocked its down-regulation 7 days after surgery. Although α -SMA expression was inhibited by dnFGFR and RZG, expression of Fabp4 and tenascin C was still increased supporting the hypothesis that the structural role of the interstitial fibroblast is independent of the contractile role. Likewise, an increase in tissue damping and elastance suggest changes of extracellular matrix composition and/or contractile elements in the lung parenchyma as a result of the increase in structural fibroblasts.

Based on published data and our own work, we developed a hypothetical model of reseptation (Figure 7). Regeneration is induced by mechanical stretch and results in increased numbers of PDGFR- α -positive fibroblasts with dual function: contraction and synthesis of structural proteins. Low levels of PDGFR- α promote the contractile function, and high levels of PDGFR- α promote the structural function. This study demonstrates that loss of the contractile fibroblast results in failure to bud and elongate new septae and increases the number of PDGFR- α -GFP bright cells. Previous studies demonstrating that RZG reversed perinatal hyperoxia and induced myogenic markers and subsequently alveolar simplification demonstrate that too many contractile fibroblasts result in impaired alveologenesis (30, 70, 71). Recent PNX studies, using 9-month-old mice, demonstrate that although α -SMA protein was transiently increased, fibroblasts had reduced capability for collagen deposition, which also results in partial loss of regenerative capacity (72). Taken together, these studies suggest that a balance of contractile and structural function is necessary for “normal” regeneration and that a shift toward one or the other results in a delay or block of regeneration.

During lung development, α -SMA is transiently expressed in interstitial fibroblasts, and PDGFR- α -positive cells are more likely to express α -SMA during postnatal alveolarization (19, 20, 37, 67). Postnatal alveolar myofibroblasts with higher levels of PDGFR- α expression were more resistant to apoptosis, and cytosolic α -SMA expression was associated with bright PDGFR- α -GFP nuclei in PN12 lung fibroblasts (50, 73). However, in adult lungs, higher levels of PDGFR- α and subsequent decreased apoptosis pose a profibrotic risk. It would therefore be beneficial to maintain low levels of PDGFR- α during regeneration, transiently induce a contractile fibroblast, and then switch to a structural fibroblast. This hypothesis is supported by our findings, which demonstrate that in adult mice α -SMA is rapidly and transiently induced in PDGFR- α -GFP^{dim} fibroblasts (Figure 7).

The retinoic acid receptors, retinoic X receptors (RXRs), and PPAR γ play an important role in postnatal alveolarization, and retinoic acid treatment in adult animals can stimulate reseptation (37, 74–80). Studies have identified binding sites for PPAR- γ /RXR heterodimers in genes involved in fatty acid and lipid metabolism, confirming the role of PPAR γ as the master transcriptional regulator of adipogenesis (81). Real-time PCR analyses demonstrated that RXR- α , RXR- β , and PPAR- γ were down-regulated 3 days after surgery, whereas RZG-treated fibroblasts had increased RXR- α and RXR- β expression. These data suggest that activation

of PPAR- γ by RZG stimulates PPAR/RXR heterodimerization, antagonizing myofibroblast differentiation.

During lung development, constitutive inactivation of FGFR3/FGFR4 in all cells induces interstitial α -SMA expression and blocks septation (61, 62). Real-time PCR analysis on lung fibroblasts after PNX demonstrated that FGFR3 expression was down-regulated before myofibroblast differentiation, suggesting that FGFR3 signaling antagonizes α -SMA, which is consistent with the FGFR3 knock-out data. Treatment with RZG prevented down-regulation of FGFR3 and increased FGFR4 expression, suggesting that FGFR3 and FGFR4 antagonize α -SMA differentiation.

FGF signaling regulates complex epithelial and mesenchymal interactions. During lung development, misexpression of the epithelial FGFR2b isoform in mesenchymal cells established a FGFR2b-FGF10 autocrine feedback loop that inhibited myofibroblast differentiation and reduced fibronectin and elastin deposition (82). In our experiments, conditional expression of a dnFGFR2b by the epithelium using the Sftpc-rtTA also blocked α -SMA expression in fibroblasts, suggesting that disturbed FGFR2b-FGF10 signaling to the epithelium resulted in an indirect effect on fibroblast differentiation (36, 83). When we expressed dnFGFR or administered RZG, we did not detect changes in mesenchymal FGF10 expression but detected doubling of epithelial FGF9 expression, which supports the hypothesis that epithelial FGFR2 signaling effects expression of other epithelial genes, which then directly or indirectly regulate fibroblast phenotypes. This hypothesis is supported by the findings that epithelial Shh and Bmp4 expression and mesenchymal Wnt2a expression are up-regulated. During the early pseudoglandular stages of lung development, an epithelial FGF9-mesenchymal FGFR-WNT signaling pathway regulates mesenchyme development (84, 85). In this study, we find evidence that a FGF9-WNT signaling pathway regulates fibroblast phenotypes during compensatory lung growth. Moreover, we hypothesized that dnFGFR and RZG promote a structural lipofibroblast phenotype over a contractile myofibroblast, which is supported by the findings that inactivation of FGFR2 signaling in mouse adipocytes resulted in increased levels of FGF9 expression and adipocyte hypertrophy (86).

Although this study did not identify the progenitor of the PDGFR- α cell, it suggests that PDGFR- α -expressing cells are the progenitor cells of the contractile and structural fibroblasts and that these cells dynamically change their phenotype during compensatory lung growth. The field of mesenchymal cells in the lung is limited by the use of generic markers for fibroblast subpopulations. Developing additional specific markers will facilitate dissection of different mesenchymal subpopulations and allow us to follow rapid changes in fibroblast phenotypes during lung injury and repair. Identifying the molecular regulation of changes in fibroblast phenotypes will help to understand how the lung repairs itself correctly without inducing fibroproliferation or excessive deposition of extracellular matrix that may result in chronic lung disease.

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