FGF signaling is required for myofibroblast differentiation during alveolar regeneration

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Perl AK, Gale E. FGF signaling is required for myofibroblast differentiation during alveolar regeneration. Am J Physiol Lung Cell Mol Physiol 297: L299–L308, 2009. First published June 5, 2009; doi:10.1152/ajplung.00008.2009.—Normal alveolarization has been studied in rodents using detailed morphometric techniques and loss of function approaches for growth factors and their receptors. However, it remains unclear how these growth factors direct the formation of secondary septae. We have previously developed a transgenic mouse model in which expression of a soluble dominant-negative FGF receptor (dnFGFR) in the prenatal period results in reduced alveolar septae formation and subsequent alveolar simplification. Retinoic acid (RA), a biologically active derivative of vitamin A, can induce regeneration of alveoli in adult rodents. In this study, we demonstrate that RA induces alveolar reseptation in this transgenic mouse model and that realveolarization in adult mice is FGF dependent. Proliferation in the lung parenchyma, an essential prerequisite for lung regeneration, was enhanced after 14 days of RA treatment and was not influenced by dnFGFR expression. During normal lung development, formation of secondary septae is associated with the transient presence of α-smooth muscle actin (αSMA)-positive interstitial myofibroblasts. One week after completion of RA treatment, αSMA expression was detected in interstitial fibroblasts, supporting the concept that RA-initiated realveolarization recapitulates aspects of septation that occur during normal lung development. Expression of dnFGFR blocked realveolarization with increased PDGFR receptor-α (PDGFRα)-positive cells and decreased αSMA-positive cells. Taken together, our data demonstrate that FGF signaling is required for the induction of αSMA in the PDGFRα-positive myofibroblast progenitor and the progression of alveolar regeneration.

transgenic mouse model; interstitial myofibroblast; emphysema; platelet derived growth factor receptor

SECONDARY SEPTATION IN NORMAL lung development: The parenchyma of the newborn mouse and rat lung is still in the saccular stage of development. There are no alveoli, and the primary septae are thick and contain a double capillary network (7). At the beginning of alveolarization, small ridges appear along the primary septae. These ridges increase in height to become the secondary septae that subdivide the air spaces into smaller units, the alveoli. Immature primary and secondary septae contain two capillary layers, one on each side of a central layer of interstitium, which consists of elastin deposits and the interstitial myofibroblast. Although the supporting septum is immature, successive rounds of alveolar expansion and subdivision occur, resulting in the large increase in alveolar number that occurs between birth and 21 days of age. In the adult lung, septae are thin, containing only a single capillary layer, and the interstitial myofibroblast has disappeared (7).

Retinoic acid (RA)-synthesizing enzymes, receptors, and cytoplasmic proteins are abundant during alveolar septation (30). Mice with deletions in RA receptors fail to form alveoli normally (34, 49). Retinol-rich lipofibroblasts are present in the alveolar wall during the period of normal septation and diminish after septation has been completed (55). Interstitial lipofibroblasts, which “traffic” the lipids and store retinoids (44), are positioned in close apposition to alveolar type II cells (17). Lipofibroblasts express PDGFR receptor-α (PDGFRα), and lack of these lipofibroblasts results in failed septation in PDGFRα knockout mice (5, 24). Boström, Lindahl, and others (5, 24, 54) hypothesized that lipofibroblasts are the progenitors of the interstitial myofibroblasts, the cellular core of the newly forming alveolar septum. Recent confocal microscopy by McGowan et al. (35) revealed that lipofibroblasts with high lipid content are located at the base of the alveolar septum and express low levels of PDGFRα. The same studies show that cells expressing high levels of PDGFRα have characteristics of myofibroblasts and are located at the alveolar entry ring (35). Interstitial myofibroblasts have the morphology of fibroblasts (21) but contain contractile elements as shown by ultrastructural analysis (1). They also express α-smooth muscle actin (αSMA) (23). It remains unclear how the formation of new septae is regulated and whether the same processes are used during reseptation in repair of the postnatal lung.

RA and lung regeneration. RA, the biologically active derivative of vitamin A, is known to be involved in lung development and maintenance of alveolar structures in the adult (3, 11, 13, 30, 33, 36). Many clinical studies and animal models support the importance of dietary retinoids in lung morphogenesis and repair (43, 46–48). It has been known for many decades that vitamin A deprivation in rats causes squamous metaplasia of the conducting airway epithelium that can be reversed by vitamin A restoration (3, 61). As seen in vitamin A-depleted animals, infants who develop bronchopulmonary dysplasia (BPD) show squamous metaplasia in conducting airways and arrested alveologenesis. In a multicenter clinical trial, vitamin A supplementation caused a small but significant reduction in the incidence of BPD (40). RA treatment of adult rats, previously made emphysematous by the intratracheal instillation of elastase or perinatal dexamethasone treatment, reinitiated septation and realveolarization (19, 31). The effects of RA treatment on alveolar regeneration have been subject to numerous studies with mixed success (4, 16, 19, 25, 26, 28, 29, 32, 38, 53). Differences in the pharmacokinetics of retinoid metabolism between strains may be a partial explanation for the negative studies (51). The molecular mechanisms involved in normal alveolar septation and new septum formation during
alveolar repair remain unclear. In this study, we used RA to induce realveolarization and to determine the role of FGF signaling in the formation of secondary septa.

**FGF signaling during alveolarization, lung injury, and lung repair.** Growth factors are key regulators of alveolar formation and have also been implicated in repair mechanisms to restore lung integrity after acute lung injury. In mice, diffuse alveolar damage induced by hyperoxia is associated with increased lung levels of FGF7 and FGF2 that promote type II cell proliferation and facilitate repair of the damaged epithelium (6, 9). Moreover, intratracheal instillation or intravenous injection of FGF7 induces alveolar type II cell proliferation and reduced mortality after exposure to hyperoxia in mice and rats (2, 39). FGF receptor (FGFR) signaling is important for normal alveolar septation, since mice deficient in both FGFR3 and FGFR4 do not form secondary septae (59). Moreover, using a conditional transgenic mouse model to express a soluble dominant-negative FGFR (dnFGFR), we demonstrated that FGFR signaling is critical before the initiation of alveolar septation but not important during normal alveolarization, thus implicating a role of FGF signaling in lung progenitor cell expansion and/or commitment (20).

In the present study, we used a conditional transgenic mouse model to study the role of FGF signaling in realveolarization induced by RA treatment. We demonstrate that a-SMA expression is induced in PDGFRα-positive interstitial fibroblasts during realveolarization, thus recapitulating the developmental program. Concurrent expression of a secreted dnFGFR had no effect on proliferation but blocked induction of a-SMA expression in the secondary crescents resulting in an accumulation of PDGFRα and inhibition of realveolarization.

**MATERIALS AND METHODS**

**Transgenic animals used.** The conditional system consists of two transgenes: the activator transgene and the operator transgene. In the activator line, the 3.7-kb human surfactant protein C (SFTPC) promoter [FVB.Cg-Tg(SFTPC-rTA)5Jaw/J; The Jackson Laboratory] drives expression of the reverse tet activator to the epithelial cells in the lung (41). The operator line has the transgene of interest, a soluble dominant-negative FGFR (dnFGFR), under the control of the tet operator (tetOdnFGFR-Hfc) (20). In the presence of doxycycline (dox), the reverse tet activator can bind the operator sequence and activate expression of dnFGFR (20). Gene activation is detected as early as 16 h after dox treatment in this activator line (41). Wild-type and single transgenic littermates showed no alveolar simplification after dox treatment and served as controls along with untreated double transgenic (DbTg) mice. All animal studies were conducted on FVB/N mice unless otherwise mentioned. For each treatment group, experiments were performed on at least three to five animals of each genotype to meet statistical requirements (Fig. 1).

**PDGFRαGFP/WT mice.** B6.129S4-Pdgfratm11(EGFP)Sor/J mice were provided by Dr. Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA). This mouse line carries a green fluorescent protein (GFP) allele fused to a histone 2B (H2B) moiety introduced into the PDGFRα endogenous locus. The H2B domain targets nuclear localization of GFP in cells that express endogenous PDGFRα. Genotyping of all alleles was done by polymerase chain reaction (18, 20).

**Animal care.** All transgenic mice were housed in a barrier facility with purified air and water and were provided with either autoclaved or irradiated food containing dox in accordance with federal and institutional guidelines. Mice were supplied with food and water ad libitum and were exposed to a 12:12-h light-dark cycle. Mice were routinely screened for viral and bacterial infections by placing sentinel in their cages. The animal experiments were approved by the Cincinnati Children’s Hospital institutional animal care and use committee (IACUC), 7B11078, accreditation number A3108-01 (11/13/07). Euthanizing of mice with ketamine, xylazine, and acepromazine has been approved by and is in accordance with guidelines specified by the American Veterinary Medical Association.

**RA treatment.** All trans-RA (Sigma) was dissolved in DMSO and peanut oil (Sigma) at a dose of 2 μg/g body wt, administered daily in a 50-μl intraperitoneal injection for a period of 10 days from postnatal day 35 (PN35) to PN46 with a 2-day break on PN40 and PN41 (27, 60). As control, DbTg mice received DMSO/peanut oil only [DbTg, dox treatment from embryonic day 14.5 (E14.5) to E18.5 (E-Dox), and DMSO]. Lungs were harvested 7, 14, 21, 28, and 35 days after initiation of RA treatment (RA7, RA14, RA21, RA28, and RA35). Please note that RA was given the first 12 days of the

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**Fig. 1.** A: schematic drawing of the mouse breeding to generate double transgenic (DbTg) mice. Heterozygote mice of the activator line, SP-C-rTA line 1, are crossed to heterozygote mice of the operator line, tetOdnFGFR-Hfc. According to Mendelian inheritance, only 25% of the offspring carry both transgenes, DbTg mice. Only in DbTg progenies doxycycline (dox) treatment activates dominant-negative FGF receptor (dnFGFR) expression specifically in the lung epithelium (20). B: timeline of dox and retinoic acid (RA) treatment to induce alveolar simplification and alveolar repair, respectively, in DbTg mice. Dox treatment from embryonic day 14.5 (E14.5) to E18.5 (E-Dox) induces expression of dnFGFR and inhibits postnatal alveolarization. RA treatment from postnatal day 35 (PN35) to PN48 induces alveolar repair. Postnatal dox treatment from PN35 throughout RA treatment (PN-Dox) induces dnFGFR expression and inhibits alveolar repair. Alveolar repair in the presence and absence of FGF signaling was assessed in DbTg mice after embryonic inhibition of FGF signaling 7, 14, 21, 28, and 35 days after initiation of RA treatment (RA7, RA14, RA21, RA28, and RA35). Wild-type and single transgenic littermates showed no alveolar simplification after dox treatment and served as controls along with untreated double transgenic (DbTg) mice. All animal studies were conducted on FVB/N mice unless otherwise mentioned. For each treatment group, experiments were performed on at least three to five animals of each genotype to meet statistical requirements (Fig. 1).
treatment regimen (RA7 and RA14), and progression of lung regeneration was assessed 1, 2, and 3 wk (RA21, RA28, and RA35) thereafter.

**Time line of dox and RA treatment.** Pregnant dams from the breeding scheme in Fig. 1 were treated with dox in the food (625 mg/kg; Harlan Teklad, Madison, WI) from gestational age E14.5 to E18.5 (41). Activation of the dnFGFR from E14.5 to E18.5 results in reproducible emphysema in the adult lung. These mice are referred to as DbTg, E-Dox (20). A subset of these mice was subsequently treated with RA starting on PN35. These mice are referred to as DbTg, E-Dox, RA. Postnatal dox treatment from PN35 throughout RA treatment (PN-Dox) induces dnFGFR expression in most alveolar type II cells in the adult lung. These mice are referred to as DbTg, E-Dox, RA, PN-Dox. After embryonic dnFGFR expression, RA-mediated alveolar repair was assessed on RA7, RA14, RA21, RA28, and RA35 (Fig. 1).

**Tissue harvest.** Before euthanasia, mice were killed by lethal injection of ketamine, xylazine, and acepromazine. For optimal lung histology, lungs were inflation-fixed with 4% paraformaldehyde in PBS at 25 cmH2O pressure via a tracheal cannula. All lungs were inflated with closed chests to ensure the same inflation volume and subsequent comparable histology of fixed tissue. Lungs were fixed overnight at 4°C. All lungs were washed with PBS, dehydrated through a graded series of ethanol solutions, and processed for paraffin embedding. Sections (5 µm) were loaded onto polysine slides for analysis.

**Morphometric point and intersection counting analysis.** To quantify alveolar simplification, fractional air space area by morphometric point intersection analysis was determined on histological sections of inflation-fixed adult lungs 3 wk after completion of RA treatment (RA35). For each animal, three to five sections from different parts of the lung, showing all five lobes, were analyzed. A 120-point grid was overlaid over five to seven random pictures of each lobe. The alveolar space was calculated as percentage of grid intersections over alveolar space vs. alveolar tissue. Each symbol represents the average percentage of air space of one animal. Values for each individual animal were averaged per experimental group (58).

**Immunohistochemistry.** Fluorescent immunohistochemistry was performed on paraffin sections as previously described (64). The following antibodies have been used. Phospho-histone H3: 1:500, polyclonal rabbit anti-phospho-histone H3, H5110-14B, from United States Biological, Swampscott, MA, histone H3: 1:500, polyclonal rabbit anti-phospho-histone H3, (64). The following antibodies have been used. Phospho-histone H3 was performed on paraffin sections as previously described. Individual animal were averaged per experimental group (58).

**Morphometric analysis of αSMA and PDGFRα expression 1 wk after completion of RA treatment.** To quantify induction of interstitial myofibroblasts by morphometric analysis, immunohistochemistry for αSMA (red) and PDGFRα (green) was performed on histological sections of inflation-fixed adult lungs 1 wk after completion of RA treatment (RA21). Three to seven animals were evaluated from each treatment group from control mice (single and DbTg mice with no embryonic dox treatment), DbTg mice after embryonic dox treatment (DbTg, E-Dox), and DbTg mice with embryonic and adult dox treatment (DbTg, E-Dox, PN-Dox). For each animal, we triple-labeled (αSMA, PDGFRα, and DAPI) histological sections and analyzed 10–12 random, nonoverlapping ×10 magnification fields from each slide. αSMA, PDGFRα staining and DAPI-positive nuclei of large airways, bronchioles, and larger vessels were excluded. The three-dimensional nature of the lung sections cause regions of overlapping nuclei, which were excluded from the counts. αSMA- and PDGFRα-positive staining was expressed as percentage of all DAPI-positive nuclei. ANOVA was used for comparison of percentages, and differences (means ± SE) were assessed by Student’s t-test and the Student-Newman-Keuls test of the means was used for pairwise comparison of significance.

**RESULTS**

**Realveolarization is dependent on FGF signaling.** We (8, 20) previously developed a mouse model in which prenatal inhibition of FGF signaling caused by conditional expression of a soluble dnFGFR results in alveolar simplification, and emphysema in the adult mouse. To test whether RA induces realveolarization in the emphysema model, histological and morphometric analysis were performed on lungs after prenatal dnFGFR expression and postnatal RA treatment. The RA treatment protocol and morphometric analyzes were previously performed on histological sections of inflation-fixed adult lungs 1 wk after completion of RA treatment (RA21). Three to seven animals were evaluated from each treatment group from control mice (single and DbTg mice with no embryonic dox treatment), DbTg mice after embryonic dox treatment (DbTg, E-Dox), and DbTg mice with embryonic and adult dox treatment (DbTg, E-Dox, PN-Dox). For each animal, we triple-labeled (αSMA, PDGFRα, and DAPI) histological sections and analyzed 10–12 random, nonoverlapping ×10 magnification picture frames from each slide. αSMA, PDGFRα staining and DAPI-positive nuclei of large airways, bronchioles, and larger vessels were excluded. The three-dimensional nature of the lung sections cause regions of overlapping nuclei, which were excluded from the counts. αSMA- and PDGFRα-positive staining was expressed as percentage of all DAPI-positive nuclei. ANOVA was used for comparison of percentages, and differences (means ± SE) were assessed by Student’s t-test and the Student-Newman-Keuls test of the means was used for pairwise comparison of significance.

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described by Hind and Maden (19). Mice were first made emphysematous by embryonic dox treatment. At 35 days of age, mice were subjected to 10 days of subcutaneous RA injections (DbTg, E-Dox, RA; see timeline in Fig. 1). Three weeks after RA treatment (RA35), effects on realveolarization were assessed by hematoxylin-eosin (H&E) staining (Fig. 2) and by morphometric point intersection analysis of fractional air space area (Fig. 3). In DbTg control mice (no dox, no RA), the normal fractional air space was 65%. Air space was increased to 75% in mice with emphysema. RA treatment induced alveolar regeneration, and air space returned to 65% (Figs. 2C, 2F, and 3). Expression of dnFGFR during and after RA treatment (RA1 to RA35) inhibited alveolar regeneration, and alveolar air space remained at 75%. Morphometric analysis revealed that RA treatment induced realveolarization and that concurrent dnFGFR expression inhibited realveolarization. Expression of dnFGFR in the postnatal lung did not affect alveolar air space in adult mice (20).

Elastin fibers are an important component of the parenchymal architecture of lungs and are increased in FGFR3 and FGFR4 double knockout mice (59). To determine whether FGF signaling affected elastin expression during realveolarization, elastin protein concentrations were assessed at completion of RA treatment (RA15) and 1 wk thereafter (RA21). Expression of dnFGFR did not change elastin concentration in RA15 lungs (no dox: 21.7 ± 0.6 μg/mg protein vs. on dox: 24.5 ± 6.6 μg/mg; P = 0.998) or in RA21 lungs (no dox: 26.5 ± 2.1 μg/mg vs. on dox: 31.0 ± 6.1 μg/mg; P = 0.217). These data demonstrate that expression of dnFGFR does not significantly increase elastin expression at RA15 or RA21.

FGF does not regulate proliferation after RA-mediated repair. During normal septation, the lung grows rapidly from PN3 to PN10 and continues to grow thereafter, maintaining a constant lungs-to-body weight ratio (Ref. 56; Perl, unpublished data). The mitotic index in adult lungs is extremely low (56).

To test whether dnFGFR expression interfered with proliferation, the mitotic index of the lung parenchyma was determined before and after RA treatment (Fig. 4). We first assessed baseline proliferation in emphysematous lungs in the presence and absence of dnFGFR (Fig. 4A). The mitotic index in the lung parenchyma of normal age-matched control mice was 0.98 ± 0.002. Prenatal inhibition of FGF signaling (E-Dox) caused emphysematous lungs in adult mice, and the mitotic index was increased to 1.87 ± 0.043. Inhibition of FGF signaling by expression of dnFGFR in adult emphysematous lungs significantly reduced the mitotic index to 0.81 ± 0.060 (P = 0.00019). These data demonstrate that adult emphysematous lungs have a higher mitotic index at baseline, which is not sufficient to induce alveolar regeneration and moreover is dependent on FGF signaling.

To further understand the role of cellular proliferation in RA-mediated realveolarization, we determined the mitotic index at RA7, RA14, and 1 wk after completion of RA treatment (RA21) (Fig. 4B). After 7 days of RA treatment, the proliferation index was 1.4 ± 0.037. After 14 days of RA, the mitotic index peaked at 2.48 ± 0.070 and was significantly reduced 1 wk after completion of RA treatment (1.14 ± 0.040). When dnFGFR was expressed during RA treatment, the mitotic index was not significantly different compared with regenerating lung parenchyma (P > 0.1; RA7: 0.94 ± 0.028; RA14: 2.08 ± 0.086; RA21: 1.07 ± 0.021). These data show that the RA-mediated increase in mitotic index was independent of dnFGFR expression and implicate other roles of FGF in alveolar regeneration.

Induced αSMA expression in interstitial fibroblasts during realveolarization is dependent on FGF signaling. αSMA is a cytological marker for interstitial myofibroblasts in the newly forming, immature alveolar septum (7). αSMA and elastic fibers accumulate in the alveolar entry ring, a preferred location of PDGFRα expressing cells (35). To determine whether

Fig. 2. RA improves alveolar simplification in DbTg mice. Lung histology was analyzed by hematoxylin-eosin (H&E) staining of DbTg mice at RA35. A and D show normal lung, no prenatal dox and RA treatment from PN35 to PN48. B and E show that prenatal dox induced dnFGFR expression and caused emphysema. C and F show that RA treatment resolved emphysema. Arrows indicate average alveolar size. Scale bar = 100 μm.
RA treatment induces myofibroblast differentiation during reseption, immunohistochemistry for αSMA was performed on lung sections of adult RA-treated mice (Fig. 5). αSMA expression in fibroblasts associated with airways and arteries was unchanged in any of the treatment groups and thus served as an internal control for antibody staining. αSMA expression in the alveolar septae was absent at the end of RA treatment (RA14; Fig. 5A), was increased 1 wk after RA treatment was completed (RA21; Fig. 5B), and was absent again 2 wk after completion of RA treatment (RA28; Fig. 5C). Expression of the dnFGFR receptor during and after RA treatment inhibited αSMA expression in the regenerating alveolar septae (RA21; Fig. 5D). The number of αSMA-expressing interstitial myofibroblasts was determined by morphometric analysis 1 wk after completion of RA treatment (RA21; Fig. 5E). During alveolar regeneration, the percentage of αSMA-positive cells increased significantly from 3.5 to 30.4%. When dnFGFR was expressed, the percentage of αSMA-positive cells did not increase and was comparable with control animals.

Inhibition of FGF signaling increases the number of PDGFRα-positive fibroblasts in the alveolar interstitium. Boström et al. (5), Lindahl et al. (24), and McGowan and Torday (37) hypothesized that the PDGFRα-positive lipofibroblast differentiates into the αSMA-positive interstitial myofibroblast during alveolarization. Recent studies by McGowan et al. (35) show that during normal alveolarization the abundance of PDGFRα-expressing cells increases as the alveolar septae form. Lipofibroblasts expressed low levels of PDGFRα, whereas myofibroblasts expressed high levels of PDGFRα during normal alveolarization (35). To test whether expression of dnFGFR influences PDGFRα expression in interstitial fibroblasts during alveolar regeneration, we performed dual immunohistochemistry for PDGFRα (green) and αSMA (red) expression at completion of RA treatment (RA15) and 3, 7, and 14 days thereafter (RA18, RA21, and RA28) (Fig. 6). During normal regeneration, PDGFRα was detected at all time points (Fig. 6, A, C, E, and G), whereas the number of αSMA-expressing cells was maximal at RA21 (Fig. 6E). In the presence of dnFGFR, expression of PDGFRα was detected at all time points (Fig. 6, B, D, F, and H; dox), but αSMA staining was not detected (Fig. 6F).

The number of PDGFRα-positive cells in the lung interstitium was quantified by morphometrics. One week after RA treatment was completed, the percentage of PDGFRα-positive cells in the lung interstitium was similar to untreated normal lungs (Fig. 6, E and I). Expression of dnFGFR during lung regeneration significantly increased the percentage of PDGFRα-positive cells (Fig. 6, F and I). To determine whether myofibroblasts in regenerating septae express PDGFRα, immunohistochemistry for αSMA (red) and PDGFRα (green) was performed on RA21 lungs. Colocalization of PDGFRα and αSMA expression in the same cells was found 1 wk after RA treatment was completed (RA21; Fig. 7A). No αSMA staining was detected in PDGFRα-positive cells.
when dnFGFR was expressed. To identify PDGFRα-positive cells by nuclear GFP signal, PDGFRα<sup>GW</sup> mice were crossed into our emphysema model and subjected to RA-mediated regeneration (18). The nuclear GFP signal corresponds with PDGFRα gene transcription and not PDGFRα protein expression and was expected to appear earlier in alveolar regeneration, and therefore confocal microscopy was performed on triple transgenic lungs at RA18. Nuclear GFP and cytosolic αSMA (red) were colocalized in interstitial fibroblasts as early as 4 days after completion of RA treatment (Fig. 7B), which confirmed the presence of dual expressing cells in the regenerating alveolar septum. No colocalization could be found in the presence of dnFGFR expression. These data demonstrate that dnFGFR inhibits the induction of αSMA in PDGFRα-positive cells during alveolar repair.

**DISCUSSION**

RA enhances alveolar regeneration in some but not all animal models of lung injury (4, 16, 19, 25, 26, 28, 29, 32, 38, 50, 53). In this study, we show that RA reinitiates formation of new alveolar septae in emphysema caused by prenatal inhibition of FGF signaling. We demonstrated that lungs with increased alveolar air space, due to prenatal block of FGF signaling, have increased proliferation but do not repair unless treated with RA. Analysis of the temporal course of alveolar regeneration revealed induction of αSMA, a marker for interstitial myofibroblasts, 1

Fig. 5. Alveolar α-smooth muscle actin (αSMA) expression is induced 1 wk after completion of RA treatment. Immunohistochemistry for αSMA (red) was performed on lung sections of adult DbTg mice after prenatal inhibition of FGF signaling and postnatal RA treatment. No αSMA expression in the alveolar septum was found at the end of RA treatment (A; RA14). One week after completion of RA treatment, αSMA expression was detected in the alveolar interstitium (B; RA21). Expression thereafter decreased and was not detected 2 wk after completion of RA treatment (C; RA28). dnFGFR expression (PN-Dox) suppressed induction of αSMA (D). Peribronchiolar and perivascular αSMA expression was not affected at any time point and served as an internal control of the immunohistochemistry staining. Scale bar = 100 μm and 20 μm in the inset. V, vessel. Red: αSMA. Blue: DAPI.

E: morphometric analysis of septal αSMA expression in RA21 lungs. White column: control: DbTg mice, no dox, RA: 3.46% (± 0.64%) of the cells expressed αSMA (n = 4). Black column: DbTg, E-Dox: 30.40% (± 4.04%) of the cells expressed αSMA (*P < 0.001; n = 7). Gray column: DbTg, E-Dox, PN-Dox: expression of dnFGFR suppressed expression of αSMA. Compared with DbTg, E-Dox animals, only 5.13% (±5.2%) of the cells expressed αSMA (P = 0.38; n = 3).
wk after completion of RA treatment. These data support the concept that RA-induced alveolar regeneration recapitulates some aspects of alveolar development. Using immunohistochemistry and confocal microscopy, we confirmed that PDGFRα was induced in PDGFRα-expressing fibroblasts during alveolar regeneration. Expression of dnFGFR had no effect on Fig. 6. αSMA expression but not PDGFRα expression is suppressed by dnFGFR. Immunohistochemistry is shown for αSMA (red), PDGFRα (green), and DAPI (blue) DbTg mice after prenatal inhibition of FGF signaling and 3, 7, and 14 days after RA treatment. Expression of PDGFRα (arrows) on the cell membrane of some alveolar fibroblasts can be found at all time points after RA treatment is finished (A, C, E, and G). In the presence of FGF signaling, αSMA (arrowheads) expression was induced 7 days after RA treatment (E). dnFGFR was expressed during RA treatment and alveolar repair (B, D, F, and H). Expression of dnFGFR suppressed αSMA after RA treatment (F and H). More cells expressed PDGFRα (arrows) 1 wk after completion of RA treatment (compare D, F, and H with C, E, and G). Scale bar = 5 μm. Autofluorescence of red blood cells is found in the red and green channels and results in a yellow signal. I: morphometric analysis of PDGFRα expression in RA21 lungs revealed a significant increase in PDGFRα-expressing cells when dnFGFR was expressed. White column: control; 5.21% (±1.23%) of the cells express PDGFRα (n = 4). Black column: DbTg, E-Dox; 9.43% (±4.35%) of the cells express PDGFRα (P < 0.0068; n = 7). Gray column: DbTg, E-Dox, PN-Dox; dnFGFR expression increases percentage of PDGFRα cells 18.32% (±5.52%). *P < 0.0044; n = 3.

Fig. 7. Dual PDGFRα- and αSMA-positive cells during alveolar regeneration. A: immunohistochemistry for αSMA (red), PDGFRα (green), and DAPI (blue) DbTg lungs 7 days after completion of RA treatment. B: confocal microscopy after immunohistochemistry for αSMA (red) on triple transgenic RA18 lungs [nuclear green fluorescent protein (GFP) in PDGFRα(GFP/WT)] demonstrate existence of double-positive PDGFRα and αSMA cells. Expression of dnFGFR during alveolar repair results in no dual positive. Scale bar = 5 μm.
proliferation during RA repair, blocked αSMA induction, and increased PDGFRA-α-positive fibroblasts, and alveoli did not regenerate. These data suggest that FGF signaling is required for myofibroblast differentiation as a requisite step in alveolar formation. The lack of fibrotic lesions and the obvious plasticity of existing septae and progenitor cell pools in this emphysema model makes it a valuable tool to study molecular processes and signaling pathways in alveolar reseptation.

The dnFGFR used in this study is a truncated version of FGFR2b and has been demonstrated to inhibit signaling of FGF1, FGF3, FGF7, and FGF10 but not FGF2, FGF4, epidermal growth factor, hepatocyte growth factor, or insulin-like growth factor (8). Dox-mediated expression of dnFGFR by epithelial cells creates an extracellular trap that binds and inactivates a specific subset of FGFs (FGF1, FGF3, FGF7, and FGF10) that are bound and inactivated before they can interact with the native FGFs on both the epithelium and the mesenchyme. FGF1, FGF2, FGF7, FGF9, FGF10, and FGF18 are all expressed in the developing lung (45). FGF1, FGF2, and FGF7 are expressed in the adult lung, but little is known about the expression of other FGFs or whether FGFs play a role in alveolar regeneration (12, 62).

Since FGFs are known for their mitogenic role in development and repair, the impact of FGF signaling on cellular proliferation in the absence and presence of RA mediated alveolarization was analyzed. The mitotic index in emphysematous DbTg lungs was increased twofold in the absence of RA. Despite the increase in proliferation, these animals do not form new septae without RA treatment. Expression of dnFGFR, which is known to block signaling of FGF1, FGF3, FGF7, and FGF10 (8), significantly reduced proliferation in the absence of RA but not in the presence of RA treatment.

Since the mitotic index was already increased in adult emphysematous lungs and dnFGFR expression did not affect proliferation after RA treatment, we further analyzed molecular changes during alveolar reseptation, which were regulated by FGF. Based on the known role of the interstitial myofibroblast in normal alveolarization, we investigated myofibroblast differentiation during alveolar regeneration. Our data demonstrate that αSMA was induced in the interstitial fibroblast during alveolar reseptation. Since αSMA expression was observed 1 wk after completion of RA, it is unlikely that RA directly induces αSMA expression.

There are at least three different types of myofibroblasts in the adult lung, vascular, bronchiolar, and interstitial. Very little is known about the lineage relationship of the bronchiolar, vascular, or interstitial myofibroblasts and whether different FGFs and FGFRs serve different functions. It was previously shown that FGFR2 influences commitment of smooth muscle cell progenitors (14). We show that expression of a truncated FGFR2b isoform, which is known to block FGF1, FGF3, FGF7, and FGF10 but not FGF9 signaling, inhibited expression of αSMA in interstitial fibroblasts during reseptation. Yi et al. (63) demonstrated an inhibitory role of FGF9 on the differentiation of the peribronchiolar myofibroblast during early lung development. FGF9 is expressed in the epithelium of the developing bronchi and the mesothelial cells and signals to the mesenchyme through the FGFR2IIIc isoform (10, 15, 57). Expression of the dnFGFR in our system had no effect on peribronchiolar or perivascular αSMA expression.

Alveolar staining for αSMA- and PDGFRα-positive cells is missing specifically from lungs lacking PDGFRα (5, 22, 24, 52). Therefore, Lindahl et al. (24) hypothesized that the PDGFRα-positive mesenchymal cells in the perinatal lung may be precursors of alveolar myofibroblasts or provide signals that induce septum formation, matrix deposition, and the differentiation of myofibroblasts. McGowan et al. (35) demonstrated that during alveolar development fibroblasts with high expression levels of PDGFRα contain low levels of lipids, express αSMA, and are preferentially located at the alveolar entry ring. Our data identified cells in the regenerating alveolus that express both αSMA and PDGFRα, suggesting that reseptation recapitulates aspects of normal alveolarization. In this paper, we provide evidence that inhibition of FGF signaling inhibits the induction of αSMA in the PDGFRα-positive cell and results in an increase in PDGFRα-positive cells. Our data show that the peak of proliferation after RA-induced reseptation is at

Fig. 8. Conceptual model of RA-induced reseptation and the role of FGF in the induction of αSMA in a PDGFRα-positive progenitor cell. RA induces proliferation after 14 days, and after 18 days the number of low lipid and PDGFRα-positive cells (green) increases. Based on Lindahl’s and McGowan’s data (24, 35), we propose that the PDGFRα-positive cell, which is low in lipids, is a precursor cell for the interstitial myofibroblast and requires FGF signaling to induce expression of αSMA. Our data show that in the presence of FGF signaling, PDGFRα-positive cells induce αSMA (red) expression and that expression of dnFGFR inhibits induction of αSMA in PDGFRα-positive cells, resulting in a block of myofibroblast differentiation and increase of PDGFRα-positive cells.
RA14, whereas the increase in PDGFRα-positive cells in the absence of FGF signaling is observed at RA18, thus suggesting that the increase of PDGFRα-positive cells is due to a lack in differentiation rather than an increase in proliferation. This finding is supported by data from McGowan et al. (35) that demonstrate that PDGFRα-expressing cells are not proliferating more rapidly than cells that do not express the receptor and that there is an inverse correlation between an age-related increase in PDGFRα-positive cells and decrease in proliferative PDGFRα-expressing cells during normal alveolarization. Based on our data and data published by Lindahl and McGowan (24, 35), we propose the following conceptual model of realveolarization. After reseptation is initiated by RA, downstream mediators induce differentiation of the PDGFRα-positive myofibroblast progenitor to the αSMA-positive myofibroblast, which is FGF dependent (Fig. 8). Because of the lack of molecular or genetic tools to specifically label subsets of lung fibroblasts and their progenitors, it is not possible to perform lineage studies at present. Until new molecular or genetic tools are available to specifically label subsets of fibroblasts that might serve as progenitor cells and to follow their daughter cells in lung development and lung regeneration, the lineage relationship of the interstitial fibroblasts will remain unclear.

In the future, this transgenic mouse model will allow valuable studies on lung regeneration without the initiation of lung fibrosis and facilitate dissection of the molecular regulators of alveolar repair.

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GRANTS

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