Prenatal, but Not Postnatal, Inhibition of Fibroblast Growth Factor Receptor Signaling Causes Emphysema*

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Although fibroblast growth factor (FGF) signaling is required for the formation of the lung in the embryonic period, it is unclear whether FGF receptor activity influences lung morphogenesis later in development. We generated transgenic mice expressing a soluble FGF receptor (FGFR-HFc) under conditional control of the lung-specific surfactant protein C promoter (SP-C-rtTA), to inhibit FGF activity at various times in late gestation and postnatally. Although expression of FGFR-HFc early in development caused severe fetal lung hypoplasia, activation of the transgene in the postnatal period did not alter alveolarization, lung size, or histology. In contrast, expression of the transgene at post-conception day E14.5 decreased lung tubule formation before birth and caused severe emphysema at maturity. FGFR-HFc caused mild focal emphysema when expressed from E16.5 but did not alter alveolarization when expressed after birth. Although FGF signaling was required for branching morphogenesis early in lung development, postnatal alveolarization was not influenced by FGFR-HFc.

The lung buds evaginate from the foregut endoderm and undergo stereotypic branching to form conducting and peripheral airspaces critical for gas exchange after birth. In humans and mice, the alveoli are formed primarily in the postnatal period, during which the numbers of alveoli increase and the alveolar capillary gas exchange area expands. Lung morphogenesis is dependent upon autocrine-paracrine signaling between the splanchnic mesenchyme and the endodermally derived lung buds that form the developing respiratory epithelium. A number of growth factors and transcription factors, including fibroblast growth factor (FGFs),1 plays important roles during lung morphogenesis, regulating cell migration, proliferation, and differentiation (Refs. 1–3 for review). Fibroblast growth factors (FGFs) comprises a family of low molecular weight polypeptides that are involved in the morphogenesis of many organs, including the lung (4). At least seven FGFs are expressed in the developing lung, including aFGF, bFGF, FGF-7, FGF-9, FGF-10, and FGF-18 (5–12). Likewise, all of the known FGF receptors (FGFR1–3) are expressed in the lung (13–18). The extracellular domains of the FGFR consist of three immunoglobulin-like domains (D1, D2, and D3). Ligand binding is mediated by the C-terminal portion of the D3 domain. Classification of the FGFRs is dependent upon differences in the D3 domain. Three discrete subgroups of D3 domains have been identified, each representing variably spliced isoforms (4). Although all FGFRs are expressed in fetal and postnatal lung, the temporal-spatial distribution of each receptor and ligands varies. FGFR2 is restricted to the epithelial cells of the developing lung buds at the onset of lung organogenesis at E9.5 (19). The FGFR2 becomes increasing restricted to the peripheral lung buds during branching morphogenesis. FGFR2IIb is expressed in respiratory epithelial cells, whereas FGFR2IIc is distributed in mesenchymal cells of the lung (16, 19, 20).

The important role of FGF signaling during formation of the lung was revealed by both in vitro and in vivo experiments. FGF-7 caused cystic dilation of the fetal lung and cell proliferation in the postnatal lung (21, 22). A number of in vitro and in vivo experiments support the concept that FGF-10 production by mesenchyme is critical for early lung morphogenesis. FGF-10 gene-targeted mice failed to develop limbs or lungs (23, 24). Likewise, FGF-10 enhanced cell proliferation and chemotaxis of respiratory epithelial cells of the fetal lung in vitro (7, 25). The finding that FGFR2b gene-targeted mice had a phenotype similar to that seen in the FGF-10 (−/−) mice suggests that FGF-10 binds and activates the FGFR2 receptor (20). Expression of either Sprouty-2 or a fusion protein consisting of the FGFR-D3 and D1 domains and the mouse immunoglobulin Fc fragment blocked lung morphogenesis by inhibiting FGF signaling in vivo (27, 28). Taken together, FGF-10 and FGFR2IIb are required for early lung-brancho-mor-phenesis. In contrast, double gene targeting of FGFR3 and FGFR4 did not alter prenatal lung development but caused defects in alveolarization in the postnatal lung (29).

Because deletion of either FGF-10 or inhibition of FGFR2 activity blocked lung formation, FGF signaling plays a critical role in early lung morphogenesis. However, because lung formation fails to occur in these gene-targeted mice, it is unclear if FGF receptor signaling is required only for the initiation of lung morphogenesis or functions later in development. Because most lung growth and alveolarization occurs postnatally, it is not known whether FGF signaling plays a role after the embryonic period and whether the postnatal emphysema seen in the FGFR3/4 (−/−) mice is mediated by events occurring prenatally. To assess the role and timing of FGF signaling in perinatal and postnatal lung morphogenesis, we generated transgenic mice that express a soluble dominant-negative
FGFR2 (FGFR-HFc) under conditional control of doxycycline using the reverse tetracycline transactivator system. Inhibition of FGFR signaling in the late gestation, but not postnatally, inhibited cell proliferation and caused emphysema that was evident at maturation.

EXPERIMENTAL PROCEDURES

Transgenic Construct—FGFR-HFc consists of the extracellular domain of mouse FGFR2b and the heavy chain hinge and Fc domain of mouse immunoglobulin (28). FGFR-HFc cDNA was inserted between the (tetO)7-CMV minimal promoter and the 3′-untranslated region of the bovine growth hormone gene as previously described (22, 30) (see Fig. 1). Transgenic Mouse Lines—Transgenic mouse lines bearing the (tetO)7-CMV-FGFR-HFc were established in the FVB/N strain after oocyte microinjection of the (tetO)7-CMV-FGFR-HFc construct. Heterozygous (tetO)7-CMV-FGFR-HFc mice were viable and had no malformations. Five independent mouse lines bearing the FGFR-HFc were generated. Mice transmitting the (tetO)7-CMV-FGFR-HFc were mated with SP-C-rtTA mice that express the rtTA activator under control of the human 3.7-kb SP-C promoter, selectively directing expression of transgenes in respiratory epithelial cells of the lung (22, 30). Transgenic mice were identified by using PCR primers specific for each transgene. Severe lung hypoplasia was observed in double transgenic offspring from five independent founder lines when the dam was maintained on doxycycline throughout pregnancy. Transgenic line 5.9 was then chosen for further analysis.

Animal Use and Doxycycline Administration—Transgenic mice were kept in the pathogen-free vivarium according to institutional guidelines. Gestation was estimated by the detection of vaginal plug and correlated with average fetal weight at the time of sacrifice. Doxycycline was administered in the drinking water at a final concentration of 1 mg/ml or in the food pellets at concentration of 25 mg/kg (Harlan Teklar, Madison, WI) for described time periods. Drinking water that contained doxycycline was changed three times per week because of light sensitivity. Activity of the doxycycline in the food pellets was stable for up to 6 months.

Tissue Preparation, Histology, and Immunohistochemistry—Mice were killed by lethal injection of anesthetic reagent (ketamine, xylazine, and acepromazine). Lungs were inflated-fixed with 4% parafomaldehyde in PBS at 25-cm water pressure and immersed in same fixative. Tissue was fixed overnight, washed with PBS, dehydrated through a series of ethanol, and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin, orcein, or trichrome for histology. Immunohistochemistry for FcγRIII (CD163) and PECAM-1 (PECAM-1) were performed as previously described (31).

Lung Explant Cultures—Lung buds were isolated from mouse embryos at E11–13.5 obtained from the breeding of SP-C-rtTA and (tetO)7-CMV-FGFR-HFc mice. Lung buds were cultured with or without doxycycline (1 μg/ml) on 13-mm diameter, 8.0-μm pore size Track-Etch membranes (Whatman 110414). Each well contained 1 ml of Dulbecco’s modified Eagle medium. Human recombinant FGF-7 (Pepro Tech Inc., Rocky Hill, NJ) was diluted in PBS and added to each well at final concentrations of 0.625–20 ng/ml. Lung buds were grown at 37 °C in a humidified 5% CO2/85% air incubator. Photographs were taken with a stereoscopic microscope.

RESULTS

Conditional Expression of FGFR-HFc—Double and single transgenic mice of all genotypes survived normally and no abnormalities in lung histology were observed in the absence of doxycycline. To determine the effects of doxycycline on the expression of FGFR-HFc, adult SP-C-rtTA, (tetO)7-CMV-FGFR-HFc double transgenic and single transgenic mice were treated with doxycycline for 3 days. FGFR-HFc mRNA was detected only in double transgenic mice treated with doxycycline (Fig. 1B). When the dams were maintained on doxycycline from conception, severe lung hypoplasia was observed in all double transgenic pups at E18, demonstrating that the expression of the FGFR-HFc markedly abrogated lung morphogenesis during the embryonic period (Fig. 2).

Prenatal Expression of FGFR-HFc Causes Emphysema—To determine the temporal requirements for FGFR signaling during lung morphogenesis, the dams or pups were treated with doxycycline at various developmental time periods (Fig. 3A). When pups were treated from PN0 to PN25, no abnormalities in lung...
growth or histology were observed (Fig. 3, A and E). Double transgenic mice treated with doxycycline from E14.5 developed severe, diffuse emphysema when assessed at PN25 or at 6 weeks of age (Fig. 3, B and F) and data not shown. In contrast, less extensive, focal emphysema was observed when the mice were exposed to doxycycline from E16.5 to PN25 (Fig. 3, C and G). The severity of emphysema was similar in double transgenic mice treated from E14.5 to PN25 (Fig. 3, D and H) after treatment from E14.5, percent airspace, determined by lung morphometry, was significantly increased (78.2 ± 1.9, mean ± S.E., n = 5, p < 0.006) compared with controls (69.9 ± 0.69, n = 4) and was less affected when the mice were treated from E16.5 (74.5 ± 1.7, p = 0.08, n = 5) by ANOVA when assessed at PN25. Lung pathology consisted of airspace enlargement without evidence of inflammation, cellular infiltrates, or fibrosis (as assessed by trichrome and orcein staining, data not shown), suggesting that the lesions represent abnormalities in morphogenesis rather than inflammatory remodeling. At E18.5, the lung weight to body weight ratio was decreased when emphysema was assessed at PN25 (Table I).}

**Maintenance of Cell Differentiation—**To determine whether FGFR-HFc altered epithelial or vascular differentiation, lung sections from double transgenic mice that were treated from E14.5 to PN25 were stained with antibody against mature SP-B, TTF-1, and/or PECAM, respiratory epithelial and pulmonary vascular cell markers, respectively (Figs. 4 and 5). Although the numbers of alveoli and peripheral vessels were decreased, there were no differences in the intensity of staining for PECAM (Fig. 4, C and F). The proportion of SP-B-positive cells in the peripheral lung were significantly decreased in double transgenic mice (Figs. 4 and 5). Morphometric analysis and pulmonary arteriograms demonstrated decreased pulmonary vessel density, consistent with emphysema. A significant reduction in pulmonary vessels was observed in adult mice that had been treated with doxycycline from E14.5 to 6 weeks of age (Fig. 6, A and B). Despite the loss of pulmonary vascularity, combined and right ventricular weights were not different in these mice at 8 months of age. TTF-1 was detected in the nuclei of respiratory epithelial cells in both transgenic mice and controls (Fig. 4, B and E).

**Inhibition of Lung Morphogenesis by FGFR-HFc before Birth—**To determine the timing of events involved in the perturbation of lung morphogenesis following exposure to doxycycline, dams were maintained on doxycycline from E14.5 and the lung tissue evaluated at E16.5 (Fig. 7) and E18.5 (Fig. 8). On embryonic day 16.5, the numbers of respiratory tubules were reduced in lungs of the double transgenic fetuses (Fig. 7). No differences in the intensity of TTF-1 immunostaining or BrdUrd labeling (Figs. 7 and 8) were observed. At E18.5, numbers of respiratory saccules were significantly decreased in the double transgenic mice (Fig. 8). Airspaces were larger than those seen in control fetuses (Fig. 8, A and D). The proliferation index, as assessed by BrdUrd labeling, was reduced at E18.5 (p < 0.01) (Fig. 9). However, no statistical differences were observed in the proliferation index on E16.5 despite the arrest of saccullation observed histologically (Figs. 7 and 9). There was no evidence of cell necrosis, nuclear fragmentation, or condensation, suggesting that widespread cell injury or apoptosis did not account for the observed emphysema or lung hypoplasia.

**FGFR-HFc Did Not Alter Surfactant Protein and TTF-1 mRNAs—**Surfactant protein mRNAs were quantitated by S1 nuclease assay on E16.5 and E18.5 after treatment with doxycycline from E14.5. At E16.5, SP-B mRNA was decreased in double transgenic mice. No significant differences in SP-B mRNA were detected on E18.5. Quantification of TTF-1 mRNA in PN25-old lungs after treatment with doxycycline also revealed no differences (data not shown).

**FGFR-HFc Inhibits Branching Morphogenesis and Responses to FGF-7 in Vitro—**Lung explants from E11.5 embryos were cultured in the presence and absence of doxycycline (Fig. 10). Doxycycline did not alter lung growth or branching in controls. In contrast, lung branching was not altered in tissue from double transgenic mice in the absence of doxycycline (data not shown). Branching of lung tubules from double transgenic mice was inhibited by doxycycline (1 µg/ml) (Fig. 10). To assess
Table I

| Age     | Genotype | Body weight (g) | Lung weight (g) | Lung/body weight ratio × 100 |
|---------|----------|----------------|----------------|-----------------------------|
| E15.5d  | Control  | 0.44 ± 0.011   | 0.01 ± 0.001   | 2.89 ± 0.121                |
|         | FGFR-HFc | 0.45 ± 0.009   | 0.01 ± 0.001   | 2.54 ± 0.104                |
|         | p value  | 0.7            | 0.1            | 0.1                         |
| E18.5d  | Control  | 1.22 ± 0.025   | 0.05 ± 0.002   | 3.79 ± 0.133                |
|         | FGFR-HFc | 1.26 ± 0.031   | 0.03 ± 0.001   | 2.40 ± 0.104                |
|         | p value  | 0.4            | <0.0001        | <0.0001                     |
| PN25d   | Control  | 16.5 ± 0.8     | 0.11 ± 0.004   | 0.68 ± 0.029                |
|         | FGFR-HFc | 14.4 ± 0.8     | 0.10 ± 0.002   | 0.71 ± 0.029                |
|         | p value  | 0.1            | 0.1            | 0.5                         |

Fig. 4. Immunohistochemistry for SP-B, TTF-1, and PECAM-1 on PN25 lungs. Lung sections were prepared on PN25. FGFR-HFc-expressing (D–F) and controls (A–C) mice were maintained on doxycycline from E14.5 to PN25. Lung sections were stained with an antibody against mature SP-B (A and D), TTF-1 (B and E), or PECAM-1 (C and F). Bar = 100 μm. The figures are representative of at least 4 per group.

Fig. 5. Emphysema and decreased numbers of type II epithelial cells in FGFR-HFc-expressing mice. Mice were treated with doxycycline from E14.5 to PN25. Lungs from control (A) or FGFR-HFc-expressing (B) mice were stained for SP-B. Bars = 100 μm. Numbers of SP-B-staining and total cells were counted (C). Five different areas were randomly selected, comparing n = 5 animals in each group. Differences (mean ± S.E.) were assessed by Student’s t test. The proportion of SP-B-staining cells was significantly decreased.

Discussion

Conditional expression of FGFR-HFc in respiratory epithelial cells of the developing fetal lung caused emphysema in the postnatal period. The effects of FGFR-HFc were time-dependent. Severe, permanent emphysema was observed in mature mice when the transgene was activated from E14.5 to birth. Milder lung abnormalities were noted when expressed from E16.5 to birth and thereafter. The effects of FGFR-HFc during late fetal lung development contrasted with the lack of effects of the transgene when expressed only in the postnatal period. Because most of the lung growth and alveolarization occur postnataally, most pulmonary morphogenesis occurs by pathways that are not influenced by the FGFR-HFc transgene.

Once established, emphysema persisted despite the removal of the mice from doxycycline, demonstrating the irreversibility of the architectural abnormalities. Emphysema in the FGFR-HFc transgenic mice was not associated with inflammation, fibrosis, or cellular infiltrates, consistent with defects in lung morphogenesis rather than inflammation. Likewise, expression of the FGFR-HFc did not cause necrosis or cell injury, and there was no evidence of nuclear fragmentation. Taken together, these findings support the concept that FGFR-HFc inhibited expansion of precursor cells that require FGF signaling in the pseudoglandular-canalicular period of development (E12.5–E16.5). Thereafter, lung morphogenesis proceeded relatively normally despite the expression of the FGFR receptor inhibitor.

The FGFR-HFc is a known inhibitor of FGF signaling, binding various FGF ligands, including FGF-10, FGF-1, and FGF-7. Widespread expression of this chimeric gene under control of the murine mammary tumor virus promoter caused abnormalities in the skeleton, limbs, skin, kidney, endocrine organs, and lung (28). Because FGFR2IIIb and FGF-10 are both required for early lung morphogenesis, the presently observed effects of the FGFR-HFc are consistent with inhibition of FGF signaling in epithelial cells of the peripheral lung tubules during their forma-
tion. The SP-C promoter is highly active in the fetal and postnatal respiratory epithelium, directing expression of transgenes in a temporal-spatial pattern similar to that of the FGFR2 (32). Because inhibition of lung morphogenesis was observed when the SP-C promoter was used to express a membrane-associated FGFR2 mutant, the present findings are consistent with the requirement of FGF signaling in a subset of respiratory epithelial cells that are critical for morphogenesis of the peripheral lung.

Interestingly, in FGFR-HFc, FGFR2 mutant-expressing and FGF-10 gene-targeted mice, some elements of the proximal trachea were formed (23, 28, 33). In contrast, deletion of FGFR2IIIb resulted in failure of formation of trachea and main stem bronchi (20). In the present study, maintenance of trachea and main bronchi may indicate that their development is independent of the FGFR-HFc or that the SP-C promoter was not active in the proximal regions of the forming lung buds. Thus, some aspects of proximal lung tubule formation may be independent of FGF-10 but dependent on other FGF signaling, perhaps mediated by other FGF ligands. Recent cell lineage studies demonstrated that proximal and peripheral airways are distinguished early in embryogenesis and that the members of progenitor cells forming the lung periphery were highly restricted at the tips of main bronchi at E12.5–13.5 (34). The present findings support the concept that FGF signaling to this subset of precursors is required between E12.5 and E14.5 for their survival and expansion during formation of the lung periphery, with their loss causing severe lung hypoplasia. We hypothesize that partial loss of these progenitors later (E14.5–E16.5) results in a partial loss of progenitor cells and resultant emphysema. Thus, FGF activity may be required at precise times for survival and/or expansion of only subsets of progenitor cells that are critical for the formation of the peripheral lung.

In contrast to the severe effects of FGFR-HFc on fetal lung formation, postnatal alveolarization proceeded independently of the expression of the transgene. This result is not
likely related to developmental changes in the expression of the SP-C-dependent transgene, which is known to be expressed at high levels in the postnatal period (32, 35). The SP-C promoter is increasingly active in late gestation and in the postnatal period. SP-C promoter-driven rtTA was expressed throughout the peripheral lung prenatally and perinatally in the SP-C-rtTA line used in the present study (32, 35). Previous in situ hybridization studies utilizing this same SP-C-rtTA transgenic line with several (tetO) 7 target constructs demonstrated that target gene expression was readily detected and is highly inducible, the latter being expressed in subsets of acinar and alveolar regions of the postnatal lung (22, 30, 35). In the present study, FGFR-HFc RNA was readily detected in the adult lung after exposure to doxycycline. Thus the lack of effect of FGFR-HFc on alveologenesis in the postnatal period is not likely related to changes in expression of the transgene but to the lack of a requirement for FGF signaling after birth.

Despite the emphysema, proximal-distal patterning of respiratory epithelial cell morphology and mRNAs were maintained in the FGFR-HFc-expressing mice. Both TTF-1 and SP-B mRNAs were expressed at sites similar to these observed in control mice. The emphysematous lungs of the affected mice contained fewer type II epithelial cells and pulmonary vessels, findings likely related to airspace enlargement. Although the lung-body weight ratio was reduced at E18.5 in FGFR-HFc-expressing mice, the ratio was not different at PN25, despite the presence of severe emphysema. This observation may indicate that compensatory growth has occurred during the postnatal period. The relative content of SP-B and TTF-1 mRNAs was similar at E18.5 and thereafter. Maintenance of SP-B expression is consistent with postnatal survival of the FGFR-HFc-expressing mice, because severe deficiency of SP-B causes lethal respiratory distress at birth (26).

Not surprisingly, abnormalities of alveolar structures were associated with decreased vascularity. Morphometric analysis demonstrated decreased numbers of peripheral blood vessels seen after barium labeling of whole mount preparations. Thus, the paucity of endodermally derived respiratory epithelial cells was correlated with a failure to form vascular tissues, perhaps mediated by decreased production of angiogenic and vasculogenic factors. It is also possible that the FGFR-HFc directly inhibited FGF signaling to alter vascular development.

In summary, these studies demonstrate a precise temporal requirement for FGF signaling for formation of the lung during the pseudoglandular-canalicular period of development. Although inhibition of FGF signaling during this period caused irreversible emphysema, postnatal alveologenesis proceeded normally despite expression of the FGFR inhibitor. In human infants, lung hypoplasia is associated with a number of clinical syndromes, including oligohydramnios, renal agenesis, and diaphragmatic hernia. The severity of lung hypoplasia and respiratory dysfunction in these syndromes is strongly influenced by the timing of the initiating events, the human lung being most vulnerable during the canalicular period of development. These clinical observations and the present transgenic model support the concept that perturbation of progenitor cells at critical times during lung morphogenesis can cause irreversible emphysema in the postnatal period.
FGF Signaling and Emphysema

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