Rapid Induction of Lung Adenocarcinoma by Fibroblast Growth Factor 9 Signaling through FGF Receptor 3

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Abstract

Fibroblast growth factors (FGF) are expressed in many non–small cell lung carcinoma (NSCLC) primary tumors and derived cell lines, and mutations in FGF receptor 3 (FGFR3) have been identified in human lung adenocarcinoma. FGF9 has been implicated in the pathogenesis of NSCLC by synergizing with EGFR pathways or by providing an escape pathway mediating resistance to EGFR inhibition. To model pathogenic mechanisms mediated by FGF signals, we have established a mouse model in which FGF9 expression can be induced in adult lung epithelium. Here, we show that induced expression of FGF9 in adult lung leads to the rapid proliferation of distal airway epithelial cells that express the stem cell marker, Sca-1, and the proximal and distal epithelial markers, Sftpc and CC10, the rapid formation of Sftpc-positive adenocarcinomas, and eventual metastasis in some mice. Furthermore, we have identified FGFR3 as the obligate receptor mediating the FGF9 oncogenic signal. These results identify an FGF9–FGFR3 signal as a primary oncogenic pathway for lung adenocarcinoma and suggest that this pathway could be exploited for customized therapeutic applications for both primary tumors and those that have acquired resistance to inhibition of other signaling pathways. Cancer Res; 73(18): 5730–41. ©2013 AACR.

Introduction

Non–small cell lung carcinoma (NSCLC) is a disease with considerable molecular heterogeneity (1–3), and thus, pathway-specific inhibitors have become attractive as candidates for customized therapy. Inhibitors of the EGF receptor (EGFR) tyrosine kinase have proven effective in patients with activating mutations in the EGFR tyrosine kinase domain (4, 5). However, EGFR mutations account for only 10% of patients with NSCLC in North America and 20% to 50% of patients with NSCLC in Asia (6), and patients inevitably acquire resistance to EGFR inhibition (7). These observations highlight the need to identify additional signaling pathways that are activated in NSCLC as potential targets for new molecular therapies. One pathway of great interest in NSCLC is the fibroblast growth factor (FGF) signaling pathway (8).

FGF signaling has been implicated in the etiology and pathogenesis of many cancers, including myeloma, bladder, prostate, cervical, ovarian endometrioid, endometrial, gastric, breast, melanoma, and lung (9). A subset of NSCLC tumor cells expresses FGF2 and its receptor, FGFR1 (7, 14, 20). Treatment of gefitinib-resistant cell lines, microRNA-mediated downregulation of FGF receptor 3 (FGFR3) correlated with decreased plating efficiency (17). In addition, the FGFR1 gene is amplified in 22% of human squamous cell lung cancers and 3% of human lung adenocarcinomas (18, 19). Thus, the FGF signaling pathway may be a clinically relevant target for a subset of lung cancers.

Several NSCLC cell lines that acquired resistance to gefitinib (EGFR kinase inhibitor) were found to increase expression of FGFR2, FGFR3, FGF9, FGFR1, and/or FGFR3 (7, 14, 20). Treatment of gefitinib-resistant cell lines that coexpressed either FGF2 or FGF9 and FGFR1 with a small-molecule FGF tyrosine kinase inhibitor, RO483596, resulted in decreased expression of phosphorylated extracellular signal–regulated kinase (p-ERK) and inhibition of cell growth. Cell lines that did not coexpress both ligand and receptor did not show growth inhibition. These findings suggest that FGF-mediated autocrine signaling may play an important role in the development and progression of NSCLC. In other studies, FGF9 expression
was observed in 86 of 146 primary human NSCLC specimens, 77% of which were adenocarcinomas (21). FGF9 expression correlated with the expression of aryl hydrocarbon receptors and activation of aryl hydrocarbon receptors by benzopyrenes (known lung carcinogens) increased expression of FGF9 (21–23). FGF9 expression in an adenocarcinoma cell line increased its invasive properties in vitro and high FGF9 expression was associated with increased tumor stage and lymph node metastasis (23). Although, all these data point to the fact that alterations in FGF9 may play an important role in NSCLC, so far, no data exist supporting a role for FGF9 in cancer initiation.

Here, we show that induction of FGF9 in adult lung results in the rapid formation of epithelial tumors that resemble papillary adenocarcinomas. We further show that early targets of FGF9 include cells in the bronchioalveolar duct junction (BADJ) that coexpress surfactant protein C (Sftpc), Clara cell antigen 10 (CC10, Sgb1a1), and Sca-1, suggesting that a cell with progenitor properties may be particularly sensitive to FGF9. Finally, we identify FGF9R3 as an essential mediator of FGF9-induced tumorigenicity. This mouse model thus identifies FGF9–FGF9R3 as an oncogenic signal that should be studied further in human lung cancer as a potential target for therapeutic intervention. Furthermore, this model should serve as a valuable tool to screen and evaluate pathway-specific pharmacologic therapies.

Materials and Methods

Mice

All mouse strains, including Sftp-Cre, Tre-Fgf9-ires-eGfp, TetO-Cre, Fgf9–/+, and Rosa26 reporter (R26R), have been previously described (24–28). Sftp-Cre and Tre-Fgf9-ires-eGfp mice were maintained on the FVB genetic background. Other mice were maintained on a mixed FVB/129SvJ/C57B6J background. Doxycycline diet was purchased from Bio-Serv Inc. (200 mg/kg green pellets, S3888). All mice were housed in a pathogen-free animal facility under the veterinary care of the Department of Comparative Medicine at Washington University School of Medicine (Saint Louis, MO), and used at the age of 6 to 12 weeks. All protocols were approved by the Washington University Animal Studies Committee and were conducted in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

Whole mount, histology analysis, and immunohistochemistry

Mice were anesthetized with Avertin (2,2,2 tribromoethanol, 2-methyl-2-butanol) or KXA (31 mg/kg ketamine, 6 mg/kg xylazine, and 1 mg/kg acepromazine) and transcardially perfused with a vascular rinse of 0.9% NaCl followed by 10% phosphate-buffered formalin (1:444:9; Fisher Scientific). Lung and other organs were dissected, whole mount enhanced GFP (eGFP) fluorescence was viewed with a fluorescence dissecting microscope (SZX12-ILLD100; Olympus Optical Co. Ltd.). Tissues were postfixed in 10% phosphate-buffered formalin overnight at 4°C. Frozen or paraffin sections were prepared using standard procedures. For histology, slides were stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were dehydrated and treated with 0.3% hydrogen peroxide in methanol for 15 minutes to suppress the endogenous peroxidase activity. Antigen retrieval was achieved by boiling at 121°C for 15 minutes in 10 mmol/L citrate buffer followed by gradual cooling to room temperature. Sections were incubated overnight with the primary antibodies at 4°C. Secondary antibodies were visualized using Broad Spectrum (AEC) kit (95–9743; Zymed Laboratories Inc.) or Vectastain Elite ABC (AEC) kit (PK–4005; Vector Laboratories). Sections were photographed on an Axioplan2 microscope (Zeiss). For immunofluorescence staining, 0.3% hydrogen peroxide treatment was omitted, and after incubation with the primary antibodies, appropriate Alexa Flour–coupled secondary antibodies (Molecular Probes) were applied at a 1:200 dilution. Sections were photographed on a ApoTome fluorescence microscope (Zeiss). The following primary antibodies were used for staining: FGF9 (AF-273-NA; R&D Systems; 1:100), Nkx2.1 (TTF-1, M3575; DacoCytomation; 1:100), CC10 (Sgb1a1, sc-9772; Santa Cruz Biotechnology Inc.; 1:200), pro-SP-C (SftpC, AB3786; Millipore; 1:2,000), PCNA (sc-56; Santa Cruz Biotechnology Inc.; 1:100), Sca-1 (557403; BD Pharmingen; 1:200), p-Erk (4370S; Cell Signaling Technology; 1:200), E-cadherin (610181; BD Pharmingen; 1:200); p-Erk (4370S; Cell Signaling Technology; 1:200), E-cadherin (610181; BD Pharmingen; 1:200), FGF2 (1005; BD Pharmingen; 1:500), FGF9 (rabbit polyclonal), and GFP (NeuroMab N86/38, 1:500).

For quantification of cell number, multiple optical sections were scored manually to distinguish cell boundaries. Three different whole-lung longitudinal sections containing the main axial bronchi were scored for each mouse at each time point.

RNA isolation, cDNA synthesis, and RT-PCR analysis

Mouse lung total RNA was prepared, using the TRIzol LS Reagent (Life Technologies) following the manufacturer’s instructions. One microgram of RNA was used for cDNA synthesis using M-MLV reverse transcriptase and oligo dT primers (Promega). Fgf9 transcripts were amplified, using Klen Taq polymerase (Department of Biochemistry, Washington University, Saint Louis, MO). Amplification: 95°C for 3 minutes followed by 24 to 30 cycles of 95°C for 1 minute, 55°C to 65°C for 2 minutes and 72°C 2 minutes, followed by a 5-minute extension at 72°C. PCR primers were as follows: Fgf9, forward 5′-CAAGCTTTGATATACCTC-3′, reverse, 5′-CAGCGGAATCTCACCC-3′ (30 cycles); L19, forward 5′-CTGAAGGTGAAGGGGAATGTG-3′, reverse 5′-GGATAAATGTCGTTGAC-3′ (24 cycles). The PCR products were electrophoresed on a 1% agarose–tris-acetate/EDTA (TAE) gel and visualized by ethidium bromide staining.

Quantitative RT-PCR

Residual exonuclease–based fluorogenic PCR was conducted using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). TaqMan Gene Expression Assay probes (Applied Biosystems) were used for mouse β2-microglobulin (Mm00437764_m1) and mouse Fgf9 (Mm01319105_m1). The relative amounts of each gene mRNA in the samples were assessed by interpolation of their threshold cycles from a standard curve, which were then normalized against β2-microglobulin mRNA.
**In situ hybridization**

RNA probes were transcribed and labeled with SP6 RNA polymerase (for antisense orientation) or T7 RNA polymerase (for sense orientation) using DIG-UTP labeling mix (Roche Applied Science).

**Whole-mount β-galactosidase staining**

Lungs were dissected in ice-cold PBS and fixed with 0.5% glutaraldehyde in PBT (PBS, 0.1% Tween-20) overnight at 4°C. Tissues were washed in PBT twice for 10 minutes before incubation with β-Gal staining solution (2 mmol/L MgCl₂, 35 mmol/L potassium ferrocyanide, 35 mmol/L potassium ferricyanide, 1 mg/mL X-Gal in PBT) at room temperature in the dark. Following adequate color reaction, tissues were washed twice in PBT for 10 minutes each to stop the reaction. Paraffin sections were prepared with standard procedures and slides were counterstained with Fast Red. All staining patterns are representative of at least 3 mice.

**Lung cell transplantation**

*Sftpc-rtTA* and *Tre-Fgf9-ires-eGfp* mice were induced with doxycycline for 12 days. Lung cells were then isolated as described previously (29) with minor modifications. Mice were anesthetized and exsanguinated. The thoracic cavity was opened, and the lungs were exposed and perfused with 10 mL of PBS, 0.1 mmol/L EDTA. The trachea was cannulated and 3
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mL of dispase (BD) with 8U elastase solution (Worthington Biochemical) was instilled into the airways and incubated for 15 minutes at 37°C. Lung lobes were removed following dispase/elastase digestion, minced, and incubated with 7 mL of collagenase type II (3.6 U/mL; Worthington Biochemical) plus 1 mL of DNase I (2 U/mL; Invitrogen) for 40 minutes at 37°C with rotation. Cells were passed through a 70-μm cell strainer (BD Biosciences), and red blood cells were lysed using RBC lysing buffer (eBioscience Inc.).

The digested tissue was then dispersed with an 18-gauge needle, washed, and cells were resuspended in PBS. A total of 5.5 × 10⁶ cells/100 μL were injected subcutaneously (n = 4), or 2.25 × 10⁶ cells/100 μL were injected intravenously (n = 2) through the tail vein of female wild type FVB mice.

MRI

Respiratory-gated, spin-echo magnetic resonance images were collected using a small-animal magnetic resonance scanner built around an Oxford Instruments 4.7 T, 40-cm bore magnet interfaced to an Agilent/Varian DirectDrive console. The magnet is equipped with Agilent/Magnex actively shielded, high-performance (21 cm inner diameter; 32 G/cm²) gradient power supplies. All data were collected using a Stark contrast 2.5-cm, birdcage-style, quadrature radiofrequency (rf) coil. Before the imaging experiments, mice were anesthetized with isoflurane and were maintained on isoflurane/O₂ (1% v/v) throughout data collection. Animal core-body temperature was maintained at 37°C ± 1°C by circulation of warm air through the bore of the magnet. During the imaging experiments, the respiratory rates for all mice were regular and approximately 2 per second. Synchronization of magnetic resonance data collection with animal respiration was achieved with a respiratory-gating unit (30) and all images were collected during postexpiratory periods. Twenty-four to 30 contiguous coronal slices, ventral to dorsal, were collected for each mouse. Imaging parameters were repetition time (TR), ~3 seconds; echo time (TE), 30 milliseconds; field of view (FOV), 4.0 cm × 4.0 cm²; slice thickness, 0.5 mm; 128 × 128 data matrix, four averages.

Results

FGF9 overexpression in adult lung leads to adenocarcinoma

To investigate the immediate and long-term consequences of FGF9 expression in adult lung, we used a doxycycline-regulated inducible genetic system comprising the surfactant protein C–reverse tetracycline activator (Sftpc-rtTA) and tetracycline responsive, Tre-Fgf9-ires-eGfp transgenes (26, 31). In the absence of doxycycline, adult Sftpc-rtTA and Tre-Fgf9-ires-eGfp double-transgenic mice were phenotypically normal and healthy, and their lung tissue did not express GFP and was histologically normal (Fig. 1A–C and N). Real-time PCR (RT-PCR), eGFP fluorescence, in situ hybridization, and immunostaining of lung tissue in the absence of doxycycline showed very low or undetectable Fgf9 expression (Fig. 2A–E and Supplementary Fig. S1). However, in response to doxycycline (provided in chow), Fgf9 and eGFP expression were robustly induced (Fig. 2A and F–H and Supplementary Fig. S1).
Examination of whole lungs, 1 day following doxycycline administration, revealed the rapid formation of small eGFP positive nodules visible on the lung surface (Fig. 1D and E). Histologic examination revealed small epithelial clusters, located in distal airways and BADJ regions with benign appearing nuclei (Fig. 1F and F). One day after induction, Fgf9 expression was readily detected by RT-PCR, and 4 days after induction, in situ histologic analysis showed high Fgf9 and eGFP expression in tumor nodules (Fig. 2A and Supplementary Fig. S1). Consistent with the Sftpc-rtTA driver, induced FGF9 colocalized with Sftpc in lung tissue following 16 hours of doxycycline induction (Fig. 2F–H). At 4 and 8 days of induction with doxycycline, the number and size of nodules continued to increase and the lung surface took on a cobblestone appearance with visible nodules corresponding to high eGFP fluorescence (Fig. 1G, H, J and K). Histologic examination after 4 days of doxycycline induction was consistent with papillary adenoma architecture (Fig. II). After eight days of induction, the cells forming papillary structures became atypical with larger and hyperchromatic nuclei, consistent with the diagnosis of papillary adenocarcinoma (Fig. I and L). Other tissues examined (brain, heart, liver, spleen, and kidney) did not express Fgf9 or eGFP and were histologically normal (Fig. 2B and data not shown).

MRI was used to assess tumor progression (Fig. 1M). Because of low water content and the effects of magnetic susceptibility, healthy lung parenchyma was not visible on hydrogen MRI, and lungs from uninduced mice appeared clear of lesions. Consistent with the gross pathology, MRI showed progressive formation of multiple high-density nodules following doxycycline induction. Consistent with widespread lung tumorigenesis, the primary cause of death of doxycycline-induced mice seemed to be respiratory failure. The median survival of mice induced with doxycycline (n = 25) was 89 days (Fig. 1N). Mice not exposed to doxycycline remained disease free for more than 700 days (n = 20).

FGF9 targets the adult lung epithelium

During embryonic development, FGF9 primarily signals to mesenchyme, where it promotes proliferation and suppresses smooth muscle differentiation. In addition, FGF9 directly and indirectly induces proliferation of developing lung epithelum, resulting in epithelial dilation (26, 32, 33). In contrast to the mesenchymal growth and epithelial dilation seen in response
to FGF9 in embryos. FGF9 induction in adult lung resulted in epithelial proliferation with minimal stromal response. Immunostaining for Nkx2.1 (TTF-1) showed that the tumor nodules are derived from lung epithelium (Fig. 3A and B). To characterize the type of lung epithelial cells, lung tissue was also stained for Sftpc and CC10. Interestingly, all tumor nodules were positive for Sftpc and negative for CC10 (Fig. 3C–F), suggesting that the tumors arise from progenitor-like type II pneumocytes or progenitor cells that lose expression of CC10 as they progress toward malignancy.

We next assessed proliferation, differentiation, and FGFR signaling. Eight days after doxycycline induction, levels of proliferating cell nuclear antigen (PCNA) and p-Erk were markedly elevated within tumor nodules (Fig. 3G–J). In addition, E-cadherin, an adhesion molecule expressed in differentiated lung epithelial cells, was initially expressed in tumor nodules after 2 days of induction, but then lost from larger tumors after 8 days of induction (Fig. 3K, L, and L'). Decreased E-cadherin expression is associated with increased risk for metastasis of NSCLC (34). Consistent with the potential for FGF9-induced lung cancer to progress to malignancy, animals surviving 4 months of doxycycline induction developed ascites. Of 5 mice with ascites, 2 contained cells with malignant cytology that were positive for eGFP expression (Fig. 4A and B). No evidence of metastatic seeding was identified in other tissues including brain and liver (data not shown).

To further assess malignant potential, syngeneic mice (FVB background), injected intravenously (n = 4) or transplanted subcutaneously (n = 2) with dissociated cells from FGF9-induced lung tissue and maintained on a doxycycline-containing diet for 2 to 5 months, developed small lung nodules that were often associated with blood vessels (Fig. 4C). Immunostaining revealed that many of these nodules continued to express eGFP (Fig. 4D). In addition, in one intravenously injected mouse, a peribronchiolar lymph node was also found to contain eGFP-positive cells (Fig. 4E and F).

Persistence of lung tumors after withdrawal of FGF9 induction

To evaluate the reversibility of tumor formation, Sftpc-rtTA and Tet-Fgf9-ires-eGfp double-transgenic mice were induced with doxycycline and then placed on a doxycycline-free diet. Two experimental designs were used. After 2 to 4 days of induction, followed by 1 month of doxycycline withdrawal (3 mice), small tumor nodules persisted throughout the lungs. However, immunostaining for FGF9 showed that small clusters of cells within each nodule remained FGF9-positive after 4 weeks withdrawal (Fig. 5A–C, arrow), whereas in surrounding cells, expression of FGF9 and eGFP (not shown) returned to baseline levels. Because some cells remained FGF9-positive, we induced 2 more mice (for 2 weeks) and then withdrew doxycycline for 18 weeks. In this second experiment, MRI was used to confirm that lungs were tumor-free before induction and to follow large tumor nodules during the withdrawal period (Fig. 5D). Following doxycycline withdrawal, imaging at 9 and 18 weeks showed that large tumor nodules persisted with no apparent change in size (red arrow). Endpoint histologic evaluation confirmed the persistence of tumors even after doxycycline withdrawal (Fig. 5E and F). Immunostaining for FGF9 showed only background levels of FGF9 expression in most tumors (Fig. 5G). These data indicate that once tumors are induced, they persist and become partially independent of FGF9. This is in contrast to lung tumors induced by expression of FGF3, FGF7, or FGF10 (targeted with Sftpc regulatory sequences), which showed rapid regression following doxycycline withdrawal (31, 35, 36).

FGF9 promotes proliferation of Sftpc, CC10-expressing cells in the BADJ

Initial studies suggest a very rapid response of adult lung tissues to FGF9, with small epithelial nodules visible within 24 hours after FGF9 induction. To determine which cells are potentially targeted for FGF9 expression, Sftpc-rtTA mice were crossed to Tet0-Cre and ROSA26 reporter alleles. Following induction with doxycycline for 7 days, β-galactosidase (β-Gal) activity was localized to type II pneumocytes (arrows in Supplementary Fig. S2B) and a subset of bronchiolar epithelial...
cells (arrowheads in Supplementary Fig. S2B). It should be noted that not only type II pneumocytes, but also distal airway epithelial cells, are potentially targeted for FGF9 induction with the Sftpc-rtTA allele.

To identify which cells initially proliferate in response to FGF9, lung sections were stained for PCNA after 16 hours of doxycycline induction. Interestingly, the majority of PCNA-positive cells were localized to distal bronchiolar epithelium (Supplementary Fig. S2C and S2D). A small population of cells in the distal bronchiolar epithelium, referred to as bronchio-alveolar stem cells (BASC), coexpress Sftpc and CC10 and have been reported to have progenitor-like properties. Although BASCs can contribute to alveolar and bronchiolar lineages in vitro, it is controversial as to whether they can contribute to all lung epithelial lineages in vivo (37–40). To determine whether these proliferating cells expressed markers of putative progenitor cells, lung tissue was immunostained for Sftpc and CC10 (Fig. 6). In uninduced lung, 2 ± 1.7 Sftpc, CC10-positive cells (double-positive cells) could be identified within 25 cells of the BADJ (defined as the terminal bud; Fig. 6W; ref. 40). Of these, none were positive for PCNA (Fig. 6A–F and X). Following induction of FGF9, 22 ± 8.3 cells within 25 cells of the BADJ were Sftpc, CC10-positive, and 94% ± 0.1% of these were also positive for PCNA (Fig. 6G–K, W and X). In contrast, only 17% ± 0.1% of peripheral Sftpc-positive cells were also positive for PCNA (Fig. 6F, L and X).

Sca-1 is a marker of a broad population of bronchiolar progenitor cells, including BASCs (29, 37). Lungs induced to express FGF9 showed a high level of Sca-1 immunoreactivity in cells that were also positive for Sftpc and CC10 (Fig. 6R–V) compared with very low Sca-1 expression in uninduced lung tissue (Fig. 6M–Q). Together, these data identify a putative population of epithelial cells located in distal bronchiolar epithelium as the primary cell type that proliferates in response to FGF9. However, within the time frame of 16 hours, it is unlikely that the small population of preexisting BASCs could proliferate in response to FGF9 to an extent that would displace preexisting CC10-positive cells in the distal bronchiolar epithelium.

FGFR3 is essential for FGF9-induced lung adenocarcinoma

Epithelial FGFR2 is required for lung branching morphogenesis (41). FGFR3 and FGFR4, together, regulate alveolar

Figure 5. Persistence of lung tumors after withdrawal of FGF9 induction. A–C, example of lung histology from mice (n = 3 mice) that were induced with doxycycline (Dox) for 2 to 4 days followed by withdrawal of doxycycline for 1 to 2 months. Low magnification view of an H&E-stained section (A) and high-magnification view of the boxed region (B) showing focal tumor masses in otherwise normal lung tissue. C, adjacent section to A and B immunostained with an anti-FGF9 antibody showing small clusters of cells within the tumor that continued to express FGF9 (arrow). D, example of MRI (n = 2 mice) showing absence of lung masses before induction, clear lung masses following doxycycline induction for 2 weeks, and persistence of lung masses for 9 and 18 weeks after withdrawal of doxycycline (red arrows). E–G, endpoint histology from the mouse imaged in D. Low- (E) and high-magnification (F) views of an H&E-stained section. G, FGF9 immunostaining of an adjacent section showing only background immunoreactivity (bright green spots are autofluorescent red blood cells). Scale bars: E and G, 100 μm.
Figure 6. FGF9 induces Sftpc and Sca-1 expression in CC10-positive cells and promotes proliferation of bronchiolar epithelial cells. A–L, immunofluorescence showing Sftpc, CC10, and PCNA expression in lungs from Sftpc-rtTA and Tre-Fgf9-ires-eGfp control (A–F; –Dox) and 16-hour induced (G–L; +Dox) mice. A–E and G–K show distal bronchiolar epithelial regions and F and L show alveolar regions. Arrows indicate cells expressing multiple markers as indicated. M–V, immunofluorescence showing Sftpc, CC10, and Sca-1 expression in lungs from Sftpc-rtTA and Tre-Fgf9-ires-eGfp control (M–Q; –Dox) and 16-hour induced (R–V; +Dox) mice. All of the Sftpc, CC10 double-positive cells (DPCs) in induced mice were also Sca-1-positive. Arrows indicate examples of Sftpc, CC10 double-positive cells. W, quantification of Sftpc, CC10 double-positive cells in the terminal bud (TB) region (defined as within 25 cells of the BADJ) after doxycycline (Dox) induction for 16 hours. X, quantification of the ratio of PCNA-positive (PCNA+) cells to Sftpc, CC10 double-positive cells compared with the ratio of PCNA-positive cells to distal Sftpc-positive cells showing that Sftpc, CC10 double-positive cells have a much higher proliferation rate compared with distal Sftpc-positive cells. Data plotted as mean ± SD; *, P < 0.001. 4',6-Diamidino-2-phenylindole (DAPI; blue). Scale bar, 50 μm.
Figure 7. FGFR3 is essential for FGF9-induced lung adenocarcinoma. A–D, immunohistochemistry staining for FGFR2 (A and B) and FGFR3 (C and D) showing that both FGFRs are expressed in lung epithelial cells and tumors from Sftpc-rtTA and Tre-Fgf9-ires-eGfp control mice and mice induced with doxycycline (Dox) for 4 days, respectively. E, I, and M, images of whole lungs from Sftpc-rtTA and Tre-Fgf9-ires-eGfp mice lacking either one allele (Fgfr3+/−/C0) or both alleles (Fgfr3−/−/C0) of Fgfr3, as indicated. Mice were either not induced (I) or induced with doxycycline for 1 month (E and M). F, J, and N, corresponding fluorescent images showing eGFP expression. Lungs that are heterozygous for Fgfr3 show surface nodules, whereas lungs homozygous for the Fgfr3 null allele have a smooth surface appearance. G, K, and O, representative histology (H&E) of the lungs shown in E, I, and M. Note that SftpC-rtTA, Tre-Fgf9-ires-eGfp, and Fgfr3−/− mice induced with doxycycline for 1 month showed normal histology, whereas mice heterozygous for Fgfr3 developed adenocarcinoma. (Continued on the following page.)
septation in the neonatal lung (42). In adult lung, immunostaining showed that FGFR2 and FGFR3 are expressed in lung epithelium and in FGFR9-induced adenocarcinoma (Fig. 7A–D). Furthermore, following induction of FGFR9, FGFR3-expressing cells in the BADJ also coexpress Sca-1 (Supplementary Fig. S3). Because more than 90% of human NSCLC cell lines were found to express FGFR3b (43) and FGFR3c is a unique target for the FGFR3 sub-family (44), we hypothesized that FGFR3 could mediate the in vivo epithelial response to FGFR9.

To evaluate the contribution of FGFR3 to FGFR9-induced tumorigenesis, the Sftpc-rtTA and Tre-Fgf9-ires-eGfp alleles were mated with mice harboring a null allele of Fgfr3 (Fgf3<sup>–/–</sup>) to generate double-transgenic mice that are heterozygous or null for Fgfr3. Adult mice were then induced (or not induced) with doxycycline for variable lengths of time (16 hours to 6 months) and their lungs were examined for eGFP expression and for any histopathology (Fig. 7I–P). Induction of FGF9 expression in Fgfr3<sup>+/–</sup> mice (n = 20) resulted in the robust formation of tumor nodules after 1 week (not shown) or 1 month (Fig. 7E–H) on doxycycline. In contrast, the induction of FGF9 expression in Fgfr3<sup>–/–</sup> mice (total n = 26) did not result in the formation of lung tumors, and lung histology remained normal after induction for 1 (Fig. 7I–P; n = 4) to 6 months (data not shown; n = 4). Expression of the Tre-Fgf9-ires-eGfp transgene was monitored by GFP fluorescence and immunostaining for FGF9. After 1 month induction, Sftpc-rtTA, Tre-Fgf9-ires-eGfp, and Fgfr3<sup>+/–</sup> lungs were positive for eGFP fluorescence and expressed immuno-reactive FGF9 in airway epithelium even though they were histologically normal (Fig. 7I, L, N, and P).

We next evaluated the early response of distal bronchiolar epithelial cells to FGF9 expression. After 16 hours of induction, lungs were examined for expression of Sftpc and CC10 and for any increased proliferation. Fgfr3<sup>+/–</sup> mice responded to FGF9 induction with increased PCNA labeling and increased Sftpc, CC10, colabeling of cells in the distal bronchiolar epithelium (Fig. 7Q–T). In contrast, Fgfr3<sup>–/–</sup> mice showed no increased cell proliferation and few double-positive cells in the distal bronchiolar epithelium (Fig. 7U–X). These data show that FGFR3 is required for FGF9-induced activation of preexisting CC10 positive cells in the distal bronchiolar epithelium and ensuing oncogenesis in adult lungs.

**Discussion**

Studies on primary human lung adenocarcinomas and derived cell lines revealed an association between FGFR9 expression and tumorigenesis; however, little is known about the ability of FGF9 to directly induce lung tumors in vivo. To evaluate the oncogenic potential of FGF9 in adult lung, Fgf9 mRNA was selectively and conditionally expressed in distal lung epithelial cells and type II pneumocytes. Forced induction of Fgf9 rapidly caused multifocal papillary adenomas, which progressed to adenocarcinoma with the ability to metastasize. Although the cell of origin of these tumors cannot be unequivocally identified, we show that cells in the distal bronchiolar epithelium express FGFR3, rapidly enter the cell cycle in response to FGF9, and express markers (Sftpc, CC10, and Sca-1) that are associated with progenitor-like cells. We also show that FGFR3 is absolutely essential for the response of distal bronchiolar epithelial cells to FGF9 and the formation of lung tumors. Importantly, after induction of FGF9 is stopped, the tumors persist, suggesting that they rapidly become independent of the initiating FGF9 stimulus. These data show that unlike other FGF ligands, FGF9 may uniquely target the expansion of lung epithelial progenitor-like cells and induce the formation of papillary adenomas that progress to adenocarcinoma.

Identification of the cell of origin of FGF9-induced lung lesions remains an important goal. Tumors may derive from alveolar type II pneumocytes, BASCs, or Clara cells. Examination of cell proliferation at early time points following FGF9 induction showed that the highest density of proliferating cells are located near the BADJ and 94% of these also express SftpC and CC10. Nevertheless, 17% of type II pneumocytes were also PCNA-positive. Between 1 and 2 days following FGF9 induction, papillary adenomas were associated with the BADJ and often occupied the distal bronchiole, again suggesting that some expanding epithelial tumors arise from distal bronchiolar epithelial cells. Supporting this model, clusters of proliferating type II pneumocytes peripheral to the BADJ were rarely observed. Careful sequential examination at early time points suggests the conversion of ‘facultative’ Clara cells into Sftpc, CC10 double-positive cells following induction of FGF9. Ensuing papillary adenoma formation seemed to be contiguous with expansion of columnar distal bronchiolar epithelial cells that subsequently lose expression of CC10.

Similar to what is observed in this FGF9 induction model, increased numbers of Sftpc, CC10 double-positive cells have often been observed in mice with reduced expression of proteins that normally function to suppress cell proliferation, such as PTEN (45), MapK14 (i.e., p38<sup>∥</sup>; ref. 46), p27<sup>Kip1</sup> (47), and GATA-6 (48). Increased numbers of proliferating Sftpc, CC10 double-positive cells have been observed in response to K-ras activation (37, 40). However, Xu and colleagues concluded that Sftpc, CC10 double-positive cells only underwent hyperplasia and that tumors were most likely to arise from alveolar type II cells. Another possibility is that Sftpc, CC10 double-positive cells could progressively lose CC10 expression. Loss of CC10 expression has been associated with increased tumorigenicity in human NSCLC (49) and in mouse models of lung cancer (50).

Several mouse models for the role of FGFs in lung tumorigenesis have been described in which epithelial hyperplasia and/or adenoma were observed (31, 35, 36). In these models, expression of members of the Fgf7 subfamily (Fgf3, Fgf7, and Fgf10) was induced in mouse lung using the Sftpc-rtTA transgene. Further characterization of these models may provide insights into the role of FGFs in human lung tumorigenesis.
regulatory system. However, in all these models, invasive cancer was not observed and the adenomatous hyperplasia was reversible upon withdrawal of doxycycline. In contrast, tumors arising from FGFR9 induction do not seem to regress upon withdrawal of doxycycline. FGFRs 3, 7, and 10 activate epithelial splice variants of FGFR1 and FGFR2, but do not have known activity toward FGFR3 (44). In contrast, FGFR9 has the unique ability to activate both β and c splice variants of FGFR3 (44). We showed that FGFR3 is predominantly expressed in bronchiolar epithelial cells, which is consistent with published data showing high-level expression of Fgf3 in adult rat lung (51). Collectively, these data suggest that FGFR9 is activating an FGFR3-expressing lung epithelial progenitor-like cell that can then progress through oncogenic stages in the absence of the inducing ligand. This is in sharp contrast to FGFRs 3, 7, and 10, which activate FGFR2-expressing lung epithelial cells that only undergo hyperplasia. Differences in FGF responsiveness may be due to the unique expression of FGFR3 in progenitor-like cells or differences in the signaling properties of FGFR2 and FGFR3 in progenitor-like cells or Clara cells. In support of a potential pathogenic role for FGFR3 in lung cancer, whole-genome sequencing identified FGFR3 among 25 significantly mutated genes in human lung adenocarcinoma (2).

Induction of FGFR9 expression in embryonic lung in vivo or addition of FGFR9 to lung explant cultures in vitro results in mesenchymal proliferation and epithelial dilatation, but not epithelial tumors (26, 33). However, these embryonic epithelial and mesenchymal responses to FGFR9 are not mediated by FGFR3 because both control and Fgf3−/− lung explant cultures showed a similar response to FGFR9 in vitro (data not shown).

In summary, lung tumors robustly arise in mice with lung epithelial-specific induction of FGFR9. The morphologic features in this mouse model are similar to human adenocarcinoma, and also closely reflect the rapid progression in a subset of human lung adenocarcinomas, providing a model that can be experimentally manipulated to resolve questions concerning the role of FGFR9/FGFR3 in lung adenocarcinoma development. Furthermore, the FGFR-TK pathway has become increasingly recognized as an attractive target for cancer therapy (52), and the ability to induce FGFR9 in a mouse model thus provides a powerful tool for assessing the short- and long-term efficacy of anti-FGF pathway molecules for cancer treatment and perhaps prevention (53).

Disclosure of Potential Conflicts of Interest

Y. Yin has a commercial research grant from ImClone Systems. R. Govindan has commercial research grants from Merck, Boehringer Ingelheim, and Pfizer. D.M. Ornitz has a commercial research grant from ImClone Systems. No potential conflicts of interest were disclosed by the other authors.

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