Promotion of Lung Carcinogenesis by Chronic Obstructive Pulmonary Disease–Like Airway Inflammation in a K-ras–Induced Mouse Model

Seyed Javad Moghaddam^{1*}, Huaiguang Li^{2*}, Sung-Nam Cho², Megan K. Dishop³, Ignacio I. Wistuba^{4,5}, Lin Ji⁵, Jonathan M. Kurie⁶, Burton F. Dickey¹, and Francesco J. DeMayo²

Departments of ¹Pulmonary Medicine, ⁴Pathology, ⁵Thoracic and Cardiovascular Surgery, and ⁶Thoracic/Head and Neck Medical Oncology, the University of Texas M. D. Anderson Cancer Center, Houston, Texas; ²Department of Molecular and Cellular Biology, and ³Department of Pathology, Baylor College of Medicine, Houston, Texas

Lung cancer is the leading cause of cancer deaths in the United States. In addition to genetic abnormalities induced by cigarette smoke, several epidemiologic studies have found that smokers with chronic obstructive pulmonary disease (COPD), an inflammatory disease of the lungs, have an increased risk of lung cancer (1.3- to 4.9-fold) compared to smokers without COPD. This suggests a link between chronic airway inflammation and lung carcinogenesis, independent of tobacco smoke exposure. We studied this association by assaying the inflammatory impact of products of nontypeable *Haemophilus influenzae*, which colonizes the airways of patients with COPD, on lung cancer promotion in mice with an activated K-ras mutation in their airway epithelium. Two new mouse models of lung cancer were generated by crossing mice harboring the LSL-K-ras^{G12D} allele with mice containing Cre recombinase inserted into the Clara cell secretory protein (CCSP) locus, with or without the neomycin cassette excised (CCSP^{Cre} and CCSP^{Cre-Neo}, respectively). Lung lesions in CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice appeared at 4 and 1 month of age, respectively, and were classified as epithelial hyperplasia of the bronchioles, adenoma, and adenocarcinoma. Weekly exposure of CCSP^{Cre}/LSL-K-ras^{G12D} mice to aerosolized nontypeable *Haemophilus influenzae* lysate from age 6–14 weeks resulted in neutrophil/macrophage/CD8 T-cell-associated COPD-like airway inflammation, a 3.2-fold increase in lung surface tumor number (156 ± 9 versus 45 ± 7), and an increase in total lung tumor burden. We conclude that COPD-like airway inflammation promotes lung carcinogenesis in a background of a G12D-activated K-ras allele in airway secretory cells.

Keywords: K-ras; lung cancer; inflammation

Lung cancer is the leading cause of cancer deaths in the world, and accounts for 29% of all cancer deaths in the United States (1). Cigarette smoking causes 90% of all lung cancers, and is thought to do so primarily by inducing DNA mutations (2). In addition, epidemiologic data indicate that chronic inflammation also plays a role in lung epithelial carcinogenesis (3). Multiple studies have found that smokers with chronic obstructive pulmonary disease (COPD), an inflammatory disease of the airways and alveoli, have a 1.3- to 4.9-fold-increased risk of lung cancer compared with smokers without COPD (3–5). Despite the fact that smoking causes most cases of COPD, only 25% of smokers develop COPD. This variable susceptibility to COPD most likely reflects genetic variations in the inflammatory

CLINICAL RELEVANCE

An association between chronic obstructive pulmonary disease (COPD) and lung cancer has been recognized clinically but not mechanistically. Our findings demonstrate a causal role for a COPD-like airway inflammation in lung cancer promotion, and provide a baseline for future clinical research.

response to inhaled smoke and to microorganisms colonizing the injured airways of smokers (6, 7). The most common colonizing bacterium is nontypeable (i.e., unencapsulated) *Haemophilus influenzae* (NTHi) (8, 9). This organism is found in the lower respiratory tract of roughly 30% of individuals with COPD at any time, and the acquisition of new serotypes is associated with exacerbations of COPD (8, 10–12). On the basis of existing *in vitro* studies showing that NTHi activates proliferative and antiapoptotic signaling pathways (13–15), colonization with this bacterium may also promote carcinogenesis by stimulating growth and inhibiting apoptosis. Here, we report the impact of NTHi products on the progression of lung cancer in a newly developed mutant K-ras mouse model of lung cancer.

The K-ras protein, which belongs to a larger family of small GTP-binding proteins, acquires transforming activity when amino acids are substituted at one of a few specific sites (16). The K-ras gene is the most frequently mutated member of the Ras family in human tumors, and approximately 30% of all the lung adenocarcinomas from smokers carry point mutations in codon 12 of the K-ras protooncogene (17). Lung tumorigenesis in murine models has been achieved by expression of this mutant K-ras allele using several different strategies (18–22). In the present study, we used mice in which the Cre recombinase gene had been inserted into the mouse Clara cell secretory protein (CCSP) gene (CCSP^{Cre}) (23). The insertion of Cre into the CCSP locus ensured Clara cell-specific expression of the Cre recombinase. These mice were crossed with the LSL-K-ras^{G12D} mice to restrict K-ras^{G12D} expression to Clara cells of the conducting airways, and the developmental progression of lung cancer was characterized in the resulting CCSP^{Cre}/LSL-K-ras^{G12D} mice. We then applied our previously established COPD-like model of chronic airway inflammation induced by repetitive exposure to aerosolized killed NTHi lysate (24) to test the role of chronic airway inflammation on lung cancer progression in CCSP^{Cre}/LSL-K-ras^{G12D} mice.

MATERIALS AND METHODS

Animal Models

Homologous recombination in embryonic stem cells was used to generate mice in which Cre recombinase and a PGK-neo cassette

(Received in original form May 30, 2008 and in final form September 25, 2008)

* These authors contributed equally to this manuscript.

This work was supported by National Cancer Institute grant UO1 CA105352 (F.J.D.).

Correspondence and requests for reprints should be addressed to Seyed Javad Moghaddam, M.D., Department of Pulmonary Medicine, the University of Texas M. D. Anderson Cancer Center, 2121 W. Holcombe Boulevard, Suite 703F, Houston, TX 77030. E-mail: smoghadd@mdanderson.org

Am J Respir Cell Mol Biol Vol 40, pp 443–453, 2009

Originally Published in Press as DOI: 10.1165/rcmb.2008-0198OC on October 16, 2008

Internet address: www.atsjournals.org

flanked with Frt sites was inserted into exon 1 of the mouse CCSP gene. The mice generated were termed CCSP^{Cre-Neo} mice. CCSP^{Cre-Neo} mice were crossed to FLP^{er} (R26^{fl}) mice (25) for *in vivo* Flp-mediated excision of the PGK-neo cassette to generate CCSP^{Cre} mice (23). The CCSP^{Cre-Neo} and CCSP^{Cre} mice were bred to LSL-K-ras^{G12D} mice, generously provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA; [20]), to obtain double mutant CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice. CCSP^{Cre}/LSL-K-ras^{G12D} mice were also crossed with ROSA26 reporter mice (R26R) (26) for further characterization of cells undergoing Cre-mediated recombination. CCSP-TAG-transgenic mice were previously characterized (27).

The genetic background of the CCSP^{Cre-Neo}, CCSP^{Cre}, and LSL-K-ras^{G12D} mice was 129SvJ-C57BL/6. The CCSP-TAG mice were on a C57BL/6/J background, and wild-type (WT) C57BL/6/J mice (Jackson Laboratory, Bar Harbor, ME) served as controls. All mice were housed at the Baylor College of Medicine pathogen-free animal facility or the M. D. Anderson Cancer Center biohazard facility, and studied with the approval of the respective institutional review boards. Mice were monitored daily for evidence of disease or death.

Histologic Analysis

Tissues were taken from mice with the following genotypes: CCSP^{Cre-Neo}/LSL-K-ras^{G12D}; CCSP^{Cre}/LSL-K-ras^{G12D}; LSL-K-ras^{G12D}; CCSP^{Cre-Neo}; CCSP^{Cre}; and WT. The latter four genotypes served as negative controls. Mice were killed by lethal injection of avertin (Sigma, St. Louis, MO). Trachea were cannulated with PE-50 tubing (Becton Dickinson, Franklin Lakes, NJ) and sutured into place. The right lungs were frozen in liquid nitrogen, and the left lungs were infused with 10% buffered formalin (Sigma), removed, and placed in 10% buffered formalin for 18 hours. At the same time, brain, liver, kidney, spleen, intestine, and muscle were also removed and placed in 10% buffered formalin for 18 hours. Tissues then were transferred to 75% ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Serial midsagittal sections were prepared for immunohistochemical study. Lungs from CCSP^{Cre}/LSL-K-ras^{G12D}/R26R and CCSP^{Cre}/R26R mice were also stained for β -galactosidase, as previously described (23). Briefly, lungs were rinsed with 1 $\times$ PBS and fixed in 4% paraformaldehyde/PBS at 4°C for 2 to 3 hours, then incubated with 1 mg/ml X-gal (15520-034; Invitrogen, Carlsbad, CA) in tissue stain base solution (BG-8-C; Chemicon, Temecula, CA) in a dark, humid chamber at room temperature overnight. For histology, lungs were embedded in paraffin and sectioned at 5-mm thickness and counterstained with Nuclear Fast Red (H-3403; Vector, Burlingame, CA).

Immunohistochemical and Dual Immunofluorescence Staining

Sections were dried at 60°C for 15 minutes and then deparaffinized. The sections were treated with citrate for antigen retrieval for 25 minutes at 100°C, then cooled for 10 minutes. Blocking was achieved with 3% H₂O₂/methanol for 15 minutes, followed by rinsing in water and then Tris-buffered saline tween-20 (TBST) (Dako, Glostrup, Denmark). The sections were incubated with primary antibody against CCSP at 1:2,000 (28), or against surfactant protein C (SPC) at 1:500 (Seven Hills Bioreagents, Cincinnati, OH), followed by rinsing in TBST. Subsequently, sections were stained with a multilink detection kit containing biotinylated antibody and horseradish peroxidase conjugated streptavidin (BioGenex, San Ramon, CA) for 10 minutes at room temperature, followed by rinsing in TBST, application of aminoethyl carbazole (Dako), then rinsing in TBST and then water. The sections were counterstained in hematoxylin, dipped into Bluing Reagent (Anapath, Cheyenne, WY), then mounted. In addition, six complete sets of slides from each mouse were labeled separately with antibodies against macrophages (rat α -F4/80; Abcam, Cambridge, MA), T-cell subsets (goat α -CD4 and rabbit α -CD-8; Santa Cruz Biotechnology, Santa Cruz, CA), B cells (goat α -CD-20, Santa Cruz), and neutrophils (rat α -p40; SeroTec, Raleigh, NC) to assess inflammatory cell populations. NF- κ B activation was assessed immunohistochemically by nuclear translocation of p65 (rabbit α -p65; Abcam), as previously described (24). For dual immunofluorescence staining, after citrate antigen retrieval, slides were quenched in 3% H₂O₂-MeOH, avidin/biotin solution (Zymed, South San Francisco, CA), then blocked with tyramide signal amplification (TSA) buffer (Perkin Elmer, Boston, MA) for 1 hour at room temperature. The primary antibodies, α -CCSP from rabbit (28), α -SPC antibody from

rabbit (WRAB-SPC, 1:1,500; Seven Hills Bioreagents), and anti- β -galactosidase from goat (4,600–1,409, 1:1,000; Biogenesis, Kingston, NH), were incubated at 4°C overnight. After washing with PBS containing 0.2% Triton X-100, the sections were incubated for 30 minutes with secondary antibodies using biotinylated anti-rabbit (BA-1000, 1:200; Vector) or anti-goat (BA-5000, 1:200; Vector) antibodies. The immunofluorescence was developed using TSA kits (Perkin Elmer, Boston, MA) based on the manufacturer's instructions.

NTHi Lysate Aerosol Exposure

A lysate of NTHi strain 12 (14) was prepared as previously described (24), the protein concentration was adjusted to 2.5 mg/ml in PBS, and the lysate was frozen in 10-ml aliquots at -80°C. To deliver the lysate to mice, a thawed aliquot was placed in an AeroMist CA-209 nebulizer (CIS-US, Bedford, MA) driven by 10 liters/minute of room air supplemented with 5% CO₂ for 20 minutes. Mice were exposed to the lysate weekly for 8 weeks. CCSP^{Cre}/LSL-K-ras^{G12D} and LSL-K-ras^{G12D} mice were exposed starting at 6 weeks of age, and CCSP-TAG and C57BL/6 mice were exposed starting at 14 weeks of age, with the different time frames based upon pilot experiments, to result in similar maximal tumor densities to avoid confounding effects, such as hypoxemia from excessive lung replacement by tumor.

Assessment of Lung Tumor Burden and Inflammation in Mice Exposed to the NTHi Aerosol

On the first day after the fourth and eighth NTHi exposure, animals were killed by intraperitoneal injection of a lethal dose of avertin. In some mice, lung surface tumor numbers were counted, then the lungs were prepared for histologic analysis. In other mice, bronchoalveolar lavage fluid (BALF) was obtained by sequentially instilling and collecting two aliquots of 1 ml PBS through a tracheostomy cannula. Total leukocyte count was determined using a hemacytometer, and cell populations were determined by cyto centrifugation of 300 μ l of BALF followed by Wright-Giemsa staining (24). The remaining BALF (~1,400 μ l) was centrifuged at 1,250 $\times$ g for 10 minutes, and supernatants were collected and stored at -70°C. Cytokine concentrations were measured in duplicate by multiplexed sandwich ELISA using SearchLight Proteome Arrays (Pierce, Rockford, IL).

RT-PCR Analysis of K-ras^{G12D} and CCSP Expression

Total RNA was isolated from lung and lung tumors according to the TRIzol reagent protocol (Invitrogen). The cDNA was generated from RNA samples by SuperScript II reverse transcriptase (Invitrogen), followed by PCR amplification. The expression of the K-ras^{G12D} allele was identified by RT-PCR, as previously described (20). The expression of WT K-ras and K-ras^{G12D} expression was differentiated by HindIII digestion of the amplified product (29). Quantitative RT-PCR was used to assay the expression of the CCSP mRNA. Reverse-transcribed cDNA from total RNA isolated from whole lung tissue was added to PCR master mix consisting of 1 $\times$ PCR buffer (Invitrogen), 5 mM MgCl₂, 0.3 mM deoxyribonucleotide triphosphate, 0.4 μ M forward and reverse primers, 0.1 μ M probe, and 0.026 U Taq polymerase (Invitrogen). CCSP forward (CCT TTC AAC CCT GGC TCA GA) and reverse (AGG GTA TCC ACC AGT CTC TTC AG) primers and the TaqMan probe (5' FAM-CCA AAT GCG GGC ACC CAG-BHQ1 3') were designed using Primer Express software, and ribosomal 18S RNA was measured for reference using a predesigned assay, all from ABI Systems (Foster City, CA). PCR was performed according to a standard protocol of 2 minutes at 95°C, 40 cycles of 30 seconds each at 95°C, and 60 seconds at 60°C, and products measured on an ABI Prism 7,000 sequence detector. Absolute quantitation was accomplished using ABI analysis software with standard curves created from synthesized oligonucleotides corresponding to the amplified sequences.

Western Blot Analysis of CCSP Expression

CCSP protein in BALF was measured by Western blotting as previously described (30), using rabbit anti-CCSP antibodies at 1:10,000 dilution (28), horseradish peroxidase-labeled goat anti-rabbit IgG antibodies at 1:20,000 dilution, and chemiluminescent signal detection (Supersignal West Pico; Pierce) with densitometry.

RESULTS

Activation of K-ras^{G12D} in CCSP^{Cre-Neo}/LSL-K-ras^{G12D}, CCSP^{Cre}/LSL-K-ras^{G12D}, CCSP^{Cre-Neo}/LSL-K-ras^{G12D}, and CCSP^{Cre}/LSL-K-ras^{G12D} mice were generated by crossing LSL-K-ras^{G12D} with CCSP^{Cre-Neo} and CCSP^{Cre} mice in which Cre recombinase expression is under control of the endogenous DNA regulatory elements of the airway epithelial cell-specific CCSP gene. Activation by Cre recombinase induces the excision of the "stop" cassette and expression of Lox-K-ras^{G12D} (20) (Figures 1A and 1B). To determine whether the K-ras^{G12D} allele was activated in the lungs of CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice, PCR and RT-PCR analyses were performed to show the excision of the floxed stop cassette by Cre recombinase. The PCR primers were outside the floxed stop cassette in the WT K-ras sequence. Thus, the presence of the stop cassette in the mutant LSL-K-ras^{G12D} allele makes the PCR product too large to be amplified, and the presence of the remaining loxP site in the activated mutant Lox-K-ras^{G12D} allele makes the PCR product (315 bp) longer than that in the WT K-ras allele (285 bp) (20). The 315-bp product was found in CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice, and the 285-bp product was found in LSL-K-ras^{G12D}, CCSP^{Cre-Neo}, CCSP^{Cre}, and WT mice, indicating that the floxed stop cassette in the LSL-K-ras^{G12D} allele was excised after Cre recombination in lung tumors from CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice (Figures 1C and 1D).

The mRNA expression of K-ras^{G12D} was examined by RT-PCR. A HindIII restriction site allele was engineered in the K-ras^{G12D} allele, but not in the WT K-ras allele. After obtaining the 448-bp RT-PCR product, digestion with HindIII generates 300- and 148-bp fragments in the activated Lox-K-ras^{G12D} allele, but not in the WT K-ras allele (29). Using this analysis, the 300- and 148-bp fragments were found in lung tumors from CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice, but only the 448-bp fragment was found in LSL-K-

ras^{G12D}, CCSP^{Cre-Neo}, CCSP^{Cre}, and WT mice, indicating that the K-ras^{G12D} allele was transcribed after Cre recombination in lung tumors from CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice (Figures 1E and 1F).

Developmental Course of Lung Tumor Progression

The CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice were viable at birth and fertile. To determine the developmental time course of lung lesions, four CCSP^{Cre-Neo}/LSL-K-ras^{G12D} mice were killed per month from ages 1 to 12 months, and four CCSP^{Cre}/LSL-K-ras^{G12D} mice were killed from ages 1 mo to 6 months. Littermate-genotyped LSL-K-ras^{G12D}, CCSP^{Cre-Neo}, CCSP^{Cre}, and WT mice were killed at the same time points as control animals, and the lungs of these control mice appeared normal. In the gross pathological examination, isolated small lesions in the lungs were observed in 6-month-old CCSP^{Cre-Neo}/LSL-K-ras^{G12D} mice (Figure 2A) and 2-month-old CCSP^{Cre}/LSL-K-ras^{G12D} mice (Figure 2B). Microscopic examination of the lungs showed small, isolated lesions at 4 months of age in CCSP^{Cre-Neo}/LSL-K-ras^{G12D} mice (Figure 2A), and at 1 month of age in CCSP^{Cre}/LSL-K-ras^{G12D} mice (Figure 2B). Increasing size of the lung lesions correlated with the age of the mice. No lesions were observed in other organs, including brain, liver, kidney, intestine, and muscle.

The CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice displayed a shortened life span when they were compared with LSL-K-ras^{G12D} littermate control animals. The median survival time was 10 months in CCSP^{Cre-Neo}/LSL-K-ras^{G12D} mice and 6 months in CCSP^{Cre}/LSL-K-ras^{G12D} mice (Figure 2C). The difference of survival time was most likely due to differences in the level of Cre activity, resulting in fewer recombination events occurring in the CCSP^{Cre-Neo} model (23). Together, these results show that CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} are two different lung tumor models with respect to onset and progression of tumorigenesis.

Figure 1. Cre-mediated activation and expression of K-ras^{G12D} in lung tumors. (A) Schematic of wild-type (WT) (top), floxed (middle), LSL-K-ras^{G12D}, and Lox-K-ras^{G12D} (bottom) alleles. K-ras exons 0 and 1 are indicated in boxes. P₁ and P₂ represent the location of the primers used to detect excision of the stop cassette after Cre recombination. (B) Schematic of Clara cell secretory protein (CCSP) targeting event (CCSP with the neomycin cassette excised [CCSP^{Cre}] or without the neomycin cassette excised [CCSP^{Cre-Neo}]). (C and D) Identification of Cre editing of the LSL-K-ras^{G12D} allele in DNA isolated from WT, CCSP^{Cre-Neo}, LSL-K-ras^{G12D}, and CCSP^{Cre-Neo}/LSL-K-ras^{G12D} (lanes 1–4). The WT, CCSP^{Cre-Neo}, and LSL-K-ras^{G12D} allele (lanes 1–3) generates a 285-bp PCR product, whereas the activated mutant allele, Lox-K-ras^{G12D}, from CCSP^{Cre-Neo}/LSL-K-ras^{G12D} (lane 4), generates a product of 315 bp as a result of the presence of the remaining loxP site. (C) CCSP^{Cre-Neo}. (D) CCSP^{Cre}. (E and F) RT-PCR analysis of mRNA expression in the lungs of WT, CCSP^{Cre-Neo}, LSL-K-ras^{G12D}, and CCSP^{Cre-Neo}/LSL-K-ras^{G12D}

(lanes 1–4). Only in the PCR product from CCSP^{Cre-Neo}/LSL-K-ras^{G12D} mouse (lane 4), the WT K-ras band is seen at 448 bp. The mutant K-ras^{G12D} is identified by the 300- and 148-bp products after HindIII digestion (lane 4). (E) CCSP^{Cre-Neo}. (F) CCSP^{Cre}.

A CCSP^{Cre-Neo}/LSL-K-ras^{G12D} **B** CCSP^{Cre}/LSL-K-ras^{G12D} **C**

Figure 2. Tumor growth and survival. (A and B) Developmental time course of lung lesions after expression of oncogenic K-ras. The first isolated small lung lesions were observed in 4- and 1-month-old mice in CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice, respectively, and progress after that (arrows) (10× magnification; scale bars = 200 μm). (C) Survival curves. The CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice displayed a shortened life span compared with LSL-K-ras^{G12D} littermate controls.

Histologic Analysis of Lung Tumor Progression

All tissues taken from the mice were examined by a pathologist blinded to the genotypes for histologic analysis, and a variety of histopathologies were observed (Figure 3A). The lesions were classified according to the recommendations of the Mouse Models of Lung Cancer Consortium for proliferative pulmonary lesions (31); however, lesions not specifically described in that classification, such as adenomas with atypical cytological features, were also found and recorded. The lesions observed were atypical papillary bronchiolar hyperplasia (Figure 3A, panels 1 and 2), solid (Figure 3A, panels 3 and 4) and papillary adenomas (Figure 3A, panels 5 and 6), adenomas (solid and papillary types) with atypical cytologic features (Figure 3A, panels 5–8), atypical papillary bronchiolar hyperplasia adjacent to adenomas (Figure 3A, panels 9 and 10), and adenocarcinoma (Figure 3A, panels 11 and 12). In both mouse models, the main lesions were epithelial hyperplasia in the early stage, and adenomas in the middle and late stages. The numbers of adenomas in CCSP^{Cre}/LSL-K-ras^{G12D} mice were greater than in CCSP^{Cre-Neo}/LSL-K-ras^{G12D} mice, but the mean size of adenomas in CCSP^{Cre}/LSL-K-ras^{G12D} mice was smaller than in CCSP^{Cre-Neo}/LSL-K-ras^{G12D} mice. The adenomas in both mouse models were predominantly of the papillary subtype. Lesions that showed atypical cytological features

and invasive histologic features in CCSP^{Cre}/LSL-K-ras^{G12D} mice were identified as adenocarcinomas. From focal to extensive acidophilic pneumonia was observed surrounding adenomas and adenocarcinomas in 30% of the total lung tumors in both mouse models. Adenocarcinomas were only observed in the very late stage of CCSP^{Cre-Neo}/LSL-K-ras^{G12D}. By gross examination of autopsies, there were no metastases in either of these two mouse models.

In the early stages, epithelial hyperplasia was only found in bronchioles, not in the alveolar sac area. This is in agreement with the activation of the LSL-K-ras^{G12D} oncogene in Clara cells of the airways using Cre inserted into the CCSP locus. Observation of later pathologies made it difficult to determine whether an individual tumor had arisen from bronchioles or alveolar sacs. These data suggest that the lesions come from Clara cells of conducting airways, and that they progress from epithelial hyperplasia to adenocarcinoma. We next confirmed this immunohistochemically.

Immunohistochemical Analysis of Cell Types Involved In Lung Tumor Progression

To determine the pulmonary epithelial cell types undergoing hyperplasia and transformation, an immunohistochemical anal-

Figure 3. Pathology and immunostaining of lung lesions. (A) Pathological diagnosis of lung lesions (panels 1 and 2). Atypical papillary bronchiolar hyperplasia (panels 3 and 4), solid adenoma (panels 5 and 6), papillary adenomas (panels 7 and 8), solid adenoma with atypical cytological features (panels 9 and 10), papillary adenoma with atypical cytological features, and adenocarcinoma (panels 11 and 12). Panels 1, 3, 5, 7, 9, and 11 are 10× magnification; scale bars = 100 μm; panels 2, 4, 6, 8, 10, and 12 are 40× magnification; scale bars = 40 μm. (B) Immunohistochemistry of lungs from CCSP^{Cre}/LSL-K-ras^{G12D} using anti-CCSP, and anti-SPC in regions with atypical hyperplasia, adenoma and adenocarcinoma (panels 1, 2, 4, and 5 are 20× magnification; scale bars = 50 μm; panels 3 and 6 are 40× magnification; scale bars = 20 μm). (C) LacZ staining of CCSP^{Cre}/R26R mouse lung shows activity (blue staining) in the airways (panels 1 and 2), whereas CCSP^{Cre}/LSL-K-ras^{G12D}/R26R mouse lung shows staining in airways and alveoli (panels 3 and 4). Dual immunofluorescence staining of CCSP^{Cre}/R26R mouse lung for CCSP (green) and β-galactosidase (red) (panel 5), and SPC (green) and β-galactosidase (red) (panel 6) shows colocalization for CCSP and β-galactosidase (arrows), whereas CCSP^{Cre}/LSL-K-ras^{G12D}/R26R mouse lung shows colocalization for CCSP and β-galactosidase (arrows) (panel 7) and SPC and β-galactosidase (arrows) (panel 8) (panels 1 and 3: 4× magnification; panels 5 and 6: 40× magnification; panels 2, 4, 7, and 8: 20× magnification; scale bars = 50 μm).

ysis was conducted. Airway cells were identified using anti-CCSP antibodies (Clara cell marker), and alveolar cells were identified using anti-pro-SPC antibodies (alveolar epithelial type II cell marker). Normal bronchial cells and cells exhibiting epithelial hyperplasia consistently stained strongly positive for CCSP (Figure 3B, panel 1), whereas strong SPC staining was observed in alveolar sacs, but was only weak and patchy in areas

of epithelial hyperplasia (Figure 3B, panel 4). In contrast, regions showing adenomas and adenocarcinomas in the lung periphery stained positive for SPC (Figure 3B, panels 5 and 6), whereas only normal airway cells stained positive for CCSP (Figure 3B, panels 2 and 3). These data indicate that the early lung lesions exhibited a Clara cell phenotype, with only a small population demonstrating an alveolar type II cell phenotype.

However, the more advanced pathologies demonstrated an alveolar type II cell phenotype.

Lineage Analysis of Tumors in CCSP^{Cre}/LSL-K-ras^{G12D} Mice

The appearance of tumors expressing both the Clara cell marker, CCSP, and the alveolar type II cell marker, SPC, raises a question regarding the lineage of the cells giving rise to these tumors. To address this question, CCSP^{Cre}/LSL-K-ras^{G12D} mice were crossed with R26R mice (26). The R26R mice express the LacZ reporter gene in cell lineages in which Cre recombinase has been expressed. The results of this analysis are shown in Figure 3C. CCSP^{Cre}/R26R mice showed expression of LacZ only in the airways (Figure 3C, *panels 1 and 2*), and the expression colocalized in CCSP-expressing cells (Figure 3C, *panels 5 and 6*). The Clara cell specificity was similar to that previously reported for these mice (23). Analysis of the CCSP^{Cre}/LSL-K-ras^{G12D}/R26R mice showed expression in airways and alveoli (Figure 3C, *panels 3 and 4*), and lacZ colocalized with cells expressing both CCSP and SPC (Figure 3C, *panels 7 and 8*). This indicates that activation of the K-ras^{G12D} oncogene promotes development of an alveolar type II cell phenotype in cells expressing CCSP^{Cre}.

NTHi-Induced Airway Inflammation

To test the role of airway inflammation in promotion of lung carcinogenesis, we exposed WT, CCSP^{Cre}/LSL-K-ras^{G12D}, and LSL-K-ras^{G12D} littermate control mice to the aerosolized NTHi lysate once weekly for 8 weeks. In WT mice, this exposure results in a rise in neutrophil numbers as early as 1 hour after exposure, increasing from less than 1% of total BALF leukocytes before exposure to 81% 24 hours after exposure, with total leukocyte numbers increasing 10-fold (24). The increase in neutrophil numbers resolves over the ensuing 3–7 days, followed by a more modest increase in lymphocytes and macrophages, with all leukocyte numbers returning nearly to baseline after 7 days (24). Analysis of the BALF from LSL-K-ras^{G12D} littermate control and CCSP^{Cre}/LSL-K-ras^{G12D} mice exposed to aerosolized NTHi lysate also showed an increase in total white blood cell and neutrophil numbers (Figure 4A). However, CCSP^{Cre}/LSL-K-ras^{G12D} mice showed elevated macrophages even in the absence of exposure to NTHi (Figure 4A), similar to what has been reported in other models that induce expression of activated K-ras in the airways (22, 32). Paradoxically, the rise in neutrophil numbers in CCSP^{Cre}/LSL-K-ras^{G12D} mice after exposure to the NTHi lysate was less than in WT mice (Figure 4A). This is reminiscent of the declining neutrophil numbers with repetitive exposure of WT mice to the NTHi lysate, and suggests the development of immune tolerance (24). Leukocyte recruitment was accompanied by an increase in cytokines and chemokines in BALF 24 hours after 8 weekly exposures (Table 1). There were increased levels of proinflammatory cytokines (IL-6, TNF- α , and IL-1 β), and neutrophil (keratinocyte-derived chemokine [KC], macrophage inflammatory protein [MIP]-2), monocyte (IFN- γ -inducible protein of 10 kD and MIP-1 α), and T helper cell (Th) 1 (IFN- γ -inducible protein of 10 kD), particularly CD8 cell (MIP-1 α) chemokines. Consistent with the changes in the levels of inflammatory cytokines, the p65 subunit of NF- κ B had already translocated from the cytoplasm to the nucleus of most airway epithelial, tumoral, and inflammatory cells upon completion of exposure to the aerosolized NTHi lysate (Figure 5A), whereas it was translocated only in some tumoral cells and surrounding macrophages in the absence of NTHi exposure.

Histopathologically, there was severe inflammatory cell infiltration surrounding airways and blood vessels in the lungs of

10- and 14-week-old control mice after four and eight weekly NTHi lysate aerosol exposures (Figure 4B, *panel 2*), and peribronchial lymphoid aggregates were also occasionally observed (data not shown). Smaller numbers of leukocytes were also seen in alveolar airspaces (data not shown). The identities of infiltrating leukocytes were determined by immunohistochemical labeling with lineage-specific antibodies (Figure 5). The most abundant leukocyte population was that of the macrophages, and this was followed by B cells, cytolytic T cells, neutrophils, and Th cells, as previously described in WT mice (24). The lungs of 14-week-old CCSP^{Cre}/LSL-K-ras^{G12D} mice not exposed to the lysate showed multiple foci of atypical adenomatous hyperplasia and papillary proliferation in peripheral bronchial structures, and multiple alveolar adenomas (Figure 4B, *panel 3*). This was associated with focal infiltration of macrophages around hyperplastic and adenomatous lesions detected by immunohistochemical (IHC) staining (not shown). In 10- and 14-week-old CCSP^{Cre}/LSL-K-ras^{G12D} mice exposed to the NTHi lysate four and eight times weekly, there was dense infiltration of the lung parenchyma with macrophages, neutrophils, and lymphocytes, and multiple foci of atypical adenomatous hyperplasia, papillary bronchial proliferation, and alveolar adenomas. The inflammatory infiltrate was centered around airways and blood vessels, but extended widely into alveolar spaces (Figure 4B, *panel 4*). IHC staining of lungs from 14-week-old CCSP^{Cre}/LSL-K-ras^{G12D} mice exposed to the NTHi lysate eight times weekly showed a diffuse infiltration of neutrophils (Figure 5B) around and into the adenomatous lesions, surrounded by large numbers of macrophages (Figure 5C), considerable numbers of B lymphocytes (Figure 5D), and cytolytic T cells (Figure 5F). B cells were usually clustered next to a peritumoral vessel (Figure 5D), and small numbers of Th cells were seen around the lesions (Figure 5E).

Effect of NTHi-induced Airway Inflammation on Lung Tumor Progression and Transgene Expression

The effect of airway inflammation induced by the NTHi lysate on lung tumor progression was analyzed by determining the number of tumors present on the pleural surface of the lungs in CCSP^{Cre}/LSL-K-ras^{G12D} mice. As shown in Figure 6A, eight weekly NTHi exposures increased lung surface tumors 3.2-fold (156 ± 9 in NTHi-exposed versus 45 ± 7 in control mice). No tumors were observed in control mice exposed to the NTHi lysate for a similar duration (data not shown). To exclude the possibility that increased lung surface tumor number in CCSP^{Cre}/LSL-K-ras^{G12D} mice exposed to the aerosolized NTHi lysate was caused by an increased rate of LSL-K-ras^{G12D} recombination induced by activation of the promoter of the CCSP^{Cre} transgene, we measured CCSP transcripts in the lungs of WT mice exposed to the aerosolized NTHi lysate. In contrast to allergic inflammation (30), CCSP transcript levels fell dramatically (65%) by 24 hours after the first exposure to the NTHi lysate, remained low (75%) throughout the 8 weeks of exposure, and then slowly rose over a 2-week period after the last exposure (Figure 6B). CCSP protein levels were similarly suppressed by exposure to the NTHi lysate, though there was a delay in the fall of protein levels consistent with the reduction in protein being due primarily to reduced gene expression, rather than increased protein degradation (Figure 6C). Collectively, these data show that the endogenous CCSP promoter is sensitive to inflammation induced by the NTHi lysate. However, the effect is a negative one, suggesting that increased Cre expression does not explain the increase in tumorigenesis in CCSP^{Cre}/LSL-K-ras^{G12D} after chronic NTHi exposure.

To determine whether the effects of NTHi lysate-induced airway inflammation on CCSP promoter-driven transgene

LSL-K-ras^{G12D} with NTHi exposure versus CCSP^{Cre}/LSL-K-ras^{G12D} without NTHi exposure). (B) hematoxylin and eosin-stained sections of lungs after the eighth NTHi aerosol exposure are shown (scale bar = 20 μm).

expression are functionally significant, we analyzed tumor progression in the CCSP-T antigen (TAG) mouse. This mouse is a transgenic mouse model in which Clara cells are transformed by the expression of the SV40 large TAG under control of the mouse CCSP promoter. In this model, the TAG is expressed exclusively in Clara cells, and lung tumors develop and advance rapidly (27). Four and eight weekly exposures to the NTHi lysate from 14 to 22 weeks of age almost completely suppressed the appearance of tumors on the lung surface (7.3 ± 6.8 in NTHi-exposed versus 184 ± 21.1 in control mice) (Figure 6D). Histopathologically, the lungs of control CCSP-TAG mice at 22 weeks of age showed airway lumens partially occluded by hyperplastic/dysplastic epithelial cells, and widespread infiltration of the lung parenchyma surrounding airways by the same dysplastic epithelial cells, with minimal inflammatory cell infiltration, as previously described (27, 33). In contrast, the lungs of 22-weeks-old CCSP-TAG mice exposed weekly for 8 weeks to the aerosolized NTHi lysate showed similar inflammatory cell infiltration to that seen in WT mice (data not shown); however, many fewer hyperplastic/dysplastic epithelial cells were present in the airways and lung parenchyma. Thus, we conclude that NTHi lysate-induced airway inflammation has a strong negative effect on CCSP promoter-driven transgene expression. The increase in lung surface tumor number in CCSP^{Cre}/LSL-K-ras^{G12D} mice, therefore, represents a lower limit of the effect of

NTHi-induced airway inflammation on tumor promotion, because it may have reduced the number of LSL-K-ras^{G12D} recombination events.

TABLE 1. CHEMOKINE AND CYTOKINE LEVELS IN BRONCHOALVEOLAR LAVAGE FLUID AFTER NONTYPEABLE HAEMOPHILUS INFLUENZA EXPOSURE

Mediators	Control* (pg/ml)		K-ras ^{G12D} * (pg/ml)	
	-NTHi	+NTHi†	-NTHi	+NTHi†
Chemokines				
KC	19.9 ± 1.6	78 ± 48.5	29.7 ± 8.9	60.5 ± 25.4
IP-10	1.1 ± 0.3	73.6 ± 23.3	0.9 ± 0.3	15 ± 1.8
MIP-1α	1.4 ± 0.2	25.9 ± 2.6	1.5 ± 0.1	12.4 ± 1.1
MIP-2	13.1 ± 0.5	93 ± 9.1	9.9 ± 1.2	47.5 ± 15.7
Cytokines				
IL-6	11.3 ± 1.2	76.4 ± 18.9	15.2 ± 3.4	260.1 ± 92.9
TNF-α	2.7 ± 0.1	19.5 ± 2.7	3.8 ± 0.3	20.2 ± 2.1
IL-1β	8.3 ± 0.7	15.7 ± 3.4	5.3 ± 0.6	10.6 ± 1.5

Definition of abbreviations: BALF, bronchoalveolar lavage fluid; IP-10, IFN-γ-inducible protein of 10 kD; KC, keratinocyte-derived chemokine; MIP, macrophage inflammatory protein 1α; NTHi, nontypeable *Haemophilus influenza*.
* All data are means (±SEM).
† BALFs were collected first day after eighth NTHi exposure.

Figure 4. Analysis of lung inflammation after repetitive exposure to the aerosolized NTHi lysate. Control and CCSP^{Cre}/LSL-K-ras^{G12D} (K-ras^{G12D}) mice were exposed weekly for 8 weeks to an NTHi aerosol, and then killed the first day after the last exposure for analysis. (A) Total and lineage-specific leukocyte numbers in bronchoalveolar lavage fluid (BALF) after the eighth NTHi aerosol exposure are shown (mean ± SE; *P < 0.05 for control with NTHi exposure or CCSP^{Cre}/LSL-K-ras^{G12D} without NTHi exposure, or CCSP^{Cre}/LSL-K-ras^{G12D} with NTHi exposure versus WT without NTHi exposure; #P < 0.05 for CCSP^{Cre}/

Figure 5. Immunohistochemical identification of NF-κB activation and leukocytes infiltrating the lungs after repetitive NTHi lysate aerosol exposure. CCSP^{Cre}/LSL-K-ras^{G12D} mice were exposed weekly for 8 weeks to the aerosolized NTHi lysate, then killed 1 day after the last exposure and their lungs processed for light microscopy by immunohistochemical labeling with specific antibodies and hematoxylin counterstaining. (A) Labeling with α-p65 (antibody against the p65 subunit of NF-κB) showing intense nuclear staining of tumoral, and inflammatory cells. (B) Labeling with α-p40 to detect neutrophils. (C) Labeling with α-F4/80 to detect macrophages. (D) Labeling with α-CD20 to detect B cells. (E) Labeling with α-CD4 to detect helper T cells. (F) Labeling with α-CD8 to detect cytolytic T cells. Scale bar = 50 μm (applicable to all panels).

Figure 6. Lung tumor burden and CCSP expression after repetitive exposure to the aerosolized NTHi lysate. WT, CCSP^{Cre}/LSL-K-ras^{G12D} (K-ras^{G12D}), and CCSP-T antigen (Tag) mice were exposed weekly for 8 weeks to an NTHi aerosol, and killed at various time points to assess lung surface tumor number or CCSP expression. (A) Lung surface tumor numbers in CCSP^{Cre}/LSL-K-ras^{G12D} mice before aerosol exposure (Week 6), after four and eight weekly exposures to the aerosolized NTHi lysate (Weeks 10 and 14, open circles), or after 4 and 8 weeks without aerosol exposure (Weeks 10 and 14, closed circles) are shown (mean $\pm$ SE; **P* < 0.05 for NTHi exposed versus unexposed). (B) Quantitative RT-PCR analysis of CCSP transcripts in whole-lung lysate from WT C57BL/6 mice 1 day after a single exposure to the aerosolized NTHi lysate (W1-D1) and at multiple time points after eight weekly exposures are shown (mean $\pm$ SE; **P* < 0.05 for NTHi exposed versus unexposed). (C) Western blot analysis of CCSP protein in BALF from WT C57BL/6 mice 1 day after a single exposure to the aerosolized NTHi lysate (W1-D1) and at multiple time points after eight weekly exposures are shown (mean $\pm$ SE; **P* < 0.05 for NTHi exposed versus unexposed). (D) Lung surface tumor number in CCSP-TAG mice before aerosol exposure (Week 14) after four and eight weekly exposures to the aerosolized NTHi lysate (Weeks 18 and 22, open circles), or after 4 and 8 weeks without aerosol exposure (Weeks 18 and 22, closed circles) are shown (mean $\pm$ SE; **P* < 0.05 for NTHi exposed versus unexposed).

DISCUSSION

Two New Genetic Models of Lung Cancer with Airway-Specific Expression of Activated K-ras

Three different strategies have been previously used to express a mutant K-ras allele for inducing lung tumors in mice. First, expression of a doxycycline-regulated K-ras^{G12D} transgene was achieved using transgenic mice expressing the reverse tetracycline transactivator transgene under the control of the rat CCSP promoter, CCSP-rtTA, and the mutant K-ras^{G12D} gene under the control of a rtTA responsive promoter, Tet-op-K-Ras^{G12D} (18). This model resulted in doxycycline-dependent pulmonary tumor growth. Second, a “hit and run” strategy that relied on somatic recombination was employed to achieve expression of the engineered K-ras^{G12D} allele. Although expression and tumors were observed in several tissues, the lungs were the predominant site (19). Finally, the expression of an engineered K-ras^{G12D} allele was accomplished by Cre-mediated removal of a stop codon sequence flanked by loxP sites inserted into the K-ras^{G12D} allele, LSL-K-ras^{G12D}. In this last case, Cre activity has been achieved either by lung infection with adenoviral vectors carrying a Cre-expressing transgene (20, 21), or by crossing to a transgenic mouse in which the Cre recombinase gene was placed under the rat CCSP promoter, which also induces expression in alveolar type II cells (22). All three models resulted in lung tumors, with differences among them resulting from differences in the specific pulmonary epithelial cell types that expressed the Cre recombinase and in the strength of transgene expression.

In our study, the insertion of the Cre recombinase into the mouse CCSP locus places it under the transcriptional control of the native CCSP regulatory elements, ensuring expression in conducting airways, but not alveoli (23). The use of two models, CCSP^{Cre-Neo} and CCSP^{Cre}, when crossed with the LSL-K-ras^{G12D} mouse, allowed for Clara cell-specific mutant K-ras^{G12D} expression (23). Animals developed lung cancer with different latency periods due to the difference in the number of Cre-mediated recombination events, as CCSP^{Cre-Neo} mice express Cre recombinase at levels significantly lower than CCSP^{Cre} mice

(23). Despite this difference, the pathology of CCSP^{Cre-Neo}/LSL-K-ras^{G12D} and CCSP^{Cre}/LSL-K-ras^{G12D} mice is similar. Differences in the longevity of these two models allow versatility for the investigation of lung cancer. The faster-progressing tumor model, CCSP^{Cre}/LSL-K-ras^{G12D}, is well suited to investigate cancer prevention, whereas the more indolent model, CCSP^{Cre-Neo}/LSL-K-ras^{G12D}, can be used to study the effects of cancer promoting factors, such as oncogenes, genomic instability, or inflammatory mediators.

Phenotypes and Lineages of Tumors

Clara cells are the predominant apical secretory cells of human distal airways, and of mouse airways at all levels proximal to the alveoli. As would be expected for recombination driven by the CCSP promoter, all of the lesions appeared to have a secretory (adenomatous) phenotype. During the progression of tumorigenesis in both mouse models, the main lesions were epithelial hyperplasia in the early stages, and adenomas in the middle and late stages, with a small number of adenocarcinomas at late stages. Previous observations using adenoviral-expressed Cre in combination with LSL-K-ras^{G12D} showed that the tumors arose from transformation of bronchioalveolar stem cells (BASCs), which are dual positive for CCSP and SPC (34). We have previously shown that CCSP^{Cre}/LSL-K-ras^{G12D} mouse lungs show an increase in BASCs (35, 36). In this model, LSL-K-ras^{G12D} could be activated in any cell expressing the CCSP gene, which includes differentiated Clara cells as well as BASCs. The fact that early hyperplastic lesions are strongly immunohistochemically positive for CCSP, but negative or only weakly positive for SPC, suggests a Clara cell origin. However at more advanced stages, tumors stained predominantly for the alveolar type II cell marker. This raises the possibility that the pathology results from a non-BASC Clara cell lineage in which cells begin to express alveolar type II cell markers as tumors progress. Alternatively, the primary cellular target for transformation could be a subpopulation of the BASCs that normally develops to the Clara cell phenotype, but the activated K-ras oncogene shifts development of

this cell lineage toward a type II phenotype. The lineage analysis of the CCSP^{Cre}/LSL-K-ras^{G12D}/R26R mice (Figure 3C) supports this hypothesis. An important advantage of the models presented in this manuscript is that, because Cre recombinase is knocked into the endogenous CCSP locus, all of its native 5' regulatory elements are preserved, so the LSL-K-ras^{G12D} allele is activated in a cell-specific manner.

Role of Inflammation in Lung Cancer Promotion

Recent studies have demonstrated that tumor neutrophils and macrophages can be intrinsic components of tumorigenesis. Specifically, they may influence tumor behavior by activation or degradation of angiogenic factors, growth factors, and cytokines, as well as their ability to remodel the extracellular matrix using matrix metalloproteinases and serine proteinases (37, 38). For example, CXCL-8 induction is necessary for RasV12-induced tumor growth, and ablation of CXCL-8 function in RasV12-expressing tumors leads to a substantial decrease in tumor vasculature and extensive tissue necrosis (39). In this report, we similarly show the presence of an underlying inflammatory condition in the activated K-ras model by demonstrating high number of macrophages in BALF, even in the absence of exposure to NTHi. Others have also found that expression of K-ras^{G12D} within the bronchiolar epithelium of mice induces the production of chemokines that leads to the accumulation of macrophages and neutrophils within the lung, which contribute to the expansion of early alveolar neoplastic lesions induced by oncogenic K-ras (22, 32). Inhibition of this intrinsic inflammation has been shown to suppress the progression of premalignant alveolar lesions and induce apoptosis of vascular endothelial cells within alveolar lesions (40). However, there are no data indicating whether extrinsic inflammation can further promote tumorigenesis in K-ras-induced mouse lung tumors.

Promotion of Lung Cancer by Exogenous COPD-Like Airway Inflammation

An association between COPD and lung cancer has been recognized clinically for a long time. The existing epidemiologic data suggest that chronic airway inflammation caused by microbial infection or noninfective irritants, such as tobacco smoke, promote lung carcinogenesis (41, 42), and that lung cancer risk is positively associated with the severity and duration of inflammation (43). However, although these data underline the relationships, they do not establish causal links. Here, we have demonstrated a causal role for extrinsic inflammation in the promotion of lung cancer in a mouse model by showing a 3.2-fold increase in lung surface tumor number after establishment of NTHi-induced COPD-like airway inflammation. This effect of NTHi challenge on tumorigenesis was not due to an effect on CCSP promoter-driven Cre expression (Figures 6B and 6C). In fact, NTHi lysate repressed CCSP promoter activity and inhibited tumorigenesis in an SV40 large TAg model of carcinogenesis in the CCSP-TAg model (Figure 6D), so the 3.2-fold increase in lung surface tumor number is a lower limit of tumor promotion by NTHi-induced COPD-like airway inflammation in CCSP^{Cre}/LSL-K-ras^{G12D} mice.

NTHi lysate challenge resulted in a shift from macrophage-predominant to neutrophilic airway inflammation in the CCSP^{Cre}/LSL-K-ras^{G12D} mouse associated with significant tumor promotion (Figure 5). Consistent with our findings, several studies have shown an association between poor cancer prognosis, neutrophil infiltration, and neutrophil elastase expression (44, 45). They have also been able to completely inhibit the

growth and metastasis of human lung cancer cell lines transplanted into severe combined immunodeficiency mice using a specific neutrophil elastase inhibitor (46, 47). Our study indicates that, even though K-ras activation in airway epithelial cells elicits inflammation that supports tumor progression, this intrinsic inflammation is insufficient for full tumor promotion, and extrinsic (particularly neutrophilic) inflammation further promotes tumorigenesis.

Role of the NF-κB Pathway in Lung Cancer Promotion

We have previously reported rapid activation of NF-κB in airway epithelial cells after exposure to NTHi in our COPD-like model of airway inflammation (24). We have also shown its activation in tumoral and surrounding inflammatory cells in CCSP^{Cre}/LSL-K-ras^{G12D} mice (Figure 5A). NF-κB inhibits apoptosis, induces proliferation, and is found to be constitutively active in many cancers (48). Recently, several groups have shown that activation of NF-κB is essential for promoting inflammation-associated cancers in other organs (49, 50). Conversely, inactivation of the NF-κB pathway decreases tumor multiplicity and delays cancer progression (49–51). In the lungs, NF-κB inhibition suppresses airway inflammation (52–55) and urethane-induced lung cancer (56). Based upon the demonstrated role of NF-κB in inflammation-associated cancer in other tissues, and the activation of NF-κB in patients with COPD (57), and in our present and previous studies (24), NF-κB likely plays a role in promotion of airway epithelial carcinogenesis by inflammation in COPD. Therefore, NF-κB and its downstream mediators may be targets for future preventive and therapeutic studies.

Conclusions

In summary, we have developed two new mouse models of lung adenocarcinoma that offer airway-specific activation of K-ras with differing efficiencies. These should be useful in further dissecting the mechanism of lung cancer progression and in testing preventive and therapeutic strategies. We also demonstrate that COPD-like airway inflammation promotes lung carcinogenesis, in good agreement with epidemiologic data. In view of the high incidence and mortality of lung cancer (58), and the high prevalence of COPD (59), preventive strategies that stop the progression from the premalignant to the malignant phase are attractive. Further mechanistic *in vivo* analysis of the role that inflammation plays in tumor progression will provide a basis for preclinical testing of the efficacy of antiinflammatory agents.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Ms. Meirong Gu, Jinghua Li, Jie Wang, Janet DeMayo, and Drs. Michael Tuvim and Christopher Evans for their technical assistance in the completion of this project, and Dr. Tyler Jacks, Massachusetts Institute of Technology, for kindly providing the LSL-K-ras^{G12D} mouse.

References

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. Cancer statistics, 2005. *CA Cancer J Clin* 2005;55:10–30.
2. Stellman SD, Takezaki T, Wang L, Chen Y, Citron ML, Djordjevic MV, Harlap S, Muscat JE, Neugut AI, Wynder EL, et al. Smoking and lung cancer risk in American and Japanese men: an international case-control study. *Cancer Epidemiol Biomarkers Prev* 2001;10:1193–1199.
3. Shacter E, Weitzman SA. Chronic inflammation and cancer. *Oncology (Williston Park)* 2002;16:217–226, 229; discussion 230–232.
4. Tockman MS, Anthonisen NR, Wright EC, Donithan MG. Airways obstruction and the risk for lung cancer. *Ann Intern Med* 1987;106:512–518.
5. Skillrud DM, Offord KP, Miller RD. Higher risk of lung cancer in chronic obstructive pulmonary disease: a prospective, matched, controlled study. *Ann Intern Med* 1986;105:503–507.

6. Rennard SI. COPD: overview of definitions, epidemiology, and factors influencing its development. *Chest* 1998;113:235S–241S.
7. Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;163:1304–1309.
8. King PT, Hutchinson PE, Johnson PD, Holmes PW, Freezer NJ, Holdsworth SR. Adaptive immunity to nontypeable *Haemophilus influenzae*. *Am J Respir Crit Care Med* 2003;167:587–592.
9. Murphy TF. *Haemophilus influenzae* in chronic bronchitis. *Semin Respir Infect* 2000;15:41–51.
10. Bandi V, Apicella MA, Mason E, Murphy TF, Siddiqi A, Atmar RL, Greenberg SB. Nontypeable *Haemophilus influenzae* in the lower respiratory tract of patients with chronic bronchitis. *Am J Respir Crit Care Med* 2001;164:2114–2119.
11. Soler N, Ewig S, Torres A, Filella X, Gonzalez J, Zaubet A. Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *Eur Respir J* 1999;14:1015–1022.
12. Monso E, Ruiz J, Rosell A, Manterola J, Fiz J, Morera J, Ausina V. Bacterial infection in chronic obstructive pulmonary disease: a study of stable and exacerbated outpatients using the protected specimen brush. *Am J Respir Crit Care Med* 1995;152:1316–1320.
13. Jono H, Shuto T, Xu H, Kai H, Lim DJ, Gum JR Jr, Kim YS, Yamaoka S, Feng XH, Li JD. Transforming growth factor-beta-Smad signaling pathway cooperates with NF-kappa B to mediate nontypeable *Haemophilus influenzae*-induced MUC2 mucin transcription. *J Biol Chem* 2002;277:45547–45557.
14. Wang B, Lim DJ, Han J, Kim YS, Basbaum CB, Li JD. Novel cytoplasmic proteins of nontypeable *Haemophilus influenzae* up-regulate human MUC5AC mucin transcription via a positive p38 mitogen-activated protein kinase pathway and a negative phosphoinositide 3-kinase-Akt pathway. *J Biol Chem* 2002;277:949–957.
15. Shuto T, Xu H, Wang B, Han J, Kai H, Gu XX, Murphy TF, Lim DJ, Li JD. Activation of NF-kappa B by nontypeable *Haemophilus influenzae* is mediated by Toll-like receptor 2-TAK1-dependent NIK-IKK alpha/beta-I kappa B alpha and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. *Proc Natl Acad Sci USA* 2001;98:8774–8779.
16. Slebos RJ, Rodenhuis S. The ras gene family in human non-small-cell lung cancer. *J Natl Cancer Inst Monogr* 1992;13:23–29.
17. Westra WH, Slebos RJ, Offerhaus GJ, Goodman SN, Evers SG, Kensler TW, Askin FB, Rodenhuis S, Hruban RH. K-ras oncogene activation in lung adenocarcinomas from former smokers: evidence that K-ras mutations are an early and irreversible event in the development of adenocarcinoma of the lung. *Cancer* 1993;72:432–438.
18. Fisher GH, Wellen SL, Klimstra D, Lenczowski JM, Tichelaar JW, Lizak MJ, Whitsett JA, Koretsky A, Varmus HE. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. *Genes Dev* 2001;15:3249–3262.
19. Johnson L, Mercer K, Greenbaum D, Bronson RT, Crowley D, Tuveson DA, Jacks T. Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. *Nature* 2001;410:1111–1116.
20. Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, Jacks T, Tuveson DA. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev* 2001;15:3243–3248.
21. Meuwissen R, Linn SC, van der Valk M, Mooi WJ, Berns A. Mouse model for lung tumorigenesis through Cre/lox controlled sporadic activation of the K-Ras oncogene. *Oncogene* 2001;20:6551–6558.
22. Ji H, Houghton AM, Mariani TJ, Perera S, Kim CB, Padera R, Tonon G, McNamara K, Marconcini LA, Hezel A, et al. K-ras activation generates an inflammatory response in lung tumors. *Oncogene* 2006;25:2105–2112.
23. Li H, Cho SN, Evans CM, Dickey BF, Jeong JW, DeMayo FJ. Cre-mediated recombination in mouse Clara cells. *Genesis* 2008;46:300–307.
24. Moghaddam SJ, Clement CG, De la Garza MM, Zou X, Travis EL, Young HW, Evans CM, Tuvim MJ, Dickey BF. *Haemophilus influenzae* lysate induces aspects of the chronic obstructive pulmonary disease phenotype. *Am J Respir Cell Mol Biol* 2008;38:629–638.
25. Farley FW, Soriano P, Steffen LS, Dymecki SM. Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* 2000;28:106–110.
26. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999;21:70–71.
27. Magdaleno SM, Wang G, Mireles VL, Ray MK, Finegold MJ, DeMayo FJ. Cyclin-dependent kinase inhibitor expression in pulmonary Clara cells transformed with SV40 large T antigen in transgenic mice. *Cell Growth Differ* 1997;8:145–155.
28. Ray MK, Wang G, Barrish J, Finegold MJ, DeMayo FJ. Immunohistochemical localization of mouse Clara cell 10-KD protein using antibodies raised against the recombinant protein. *J Histochem Cytochem* 1996;44:919–927.
29. Caulin C, Nguyen T, Longley MA, Zhou Z, Wang XJ, Roop DR. Inducible activation of oncogenic K-ras results in tumor formation in the oral cavity. *Cancer Res* 2004;64:5054–5058.
30. Evans CM, Williams OW, Tuvim MJ, Nigam R, Mixides GP, Blackburn MR, DeMayo FJ, Burns AR, Smith C, Reynolds SD, et al. Mucin is produced by clara cells in the proximal airways of antigen-challenged mice. *Am J Respir Cell Mol Biol* 2004;31:382–394.
31. Nikitin AY, Alcaraz A, Anver MR, Bronson RT, Cardiff RD, Dixon D, Fraire AE, Gabrielson EW, Gunning WT, Haines DC, et al. Classification of proliferative pulmonary lesions of the mouse: recommendations of the mouse models of human cancers consortium. *Cancer Res* 2004;64:2307–2316.
32. Wislez M, Spencer ML, Izzo JG, Juroske DM, Balhara K, Cody DD, Price RE, Hittelman WN, Wistuba II, Kurie JM. Inhibition of mammalian target of rapamycin reverses alveolar epithelial neoplasia induced by oncogenic K-ras. *Cancer Res* 2005;65:3226–3235.
33. DeMayo FJ, Finegold MJ, Hansen TN, Stanley LA, Smith B, Bullock DW. Expression of SV40 T antigen under control of rabbit uteroglobin promoter in transgenic mice. *Am J Physiol* 1991;261:L70–L76.
34. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005;121:823–835.
35. Iwanaga K, Yang Y, Raso MG, Ma L, Hanna AE, Thilaganathan N, Moghaddam S, Evans CM, Li H, Cai WW, et al. Pten inactivation accelerates oncogenic K-ras-initiated tumorigenesis in a mouse model of lung cancer. *Cancer Res* 2008;68:1119–1127.
36. Yang Y, Iwanaga K, Raso MG, Wislez M, Hanna AE, Wieder ED, Molldrem JJ, Wistuba II, Powis G, DeMayo FJ, et al. Phosphatidylinositol 3-kinase mediates bronchioalveolar stem cell expansion in mouse models of oncogenic K-ras-induced lung cancer. *PLoS One* 2008;3:e2220.
37. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860–867.
38. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. *Mol Cancer Res* 2006;4:221–233.
39. Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 2004;6:447–458.
40. Wislez M, Fujimoto N, Izzo JG, Hanna AE, Cody DD, Langley RR, Tang H, Burdick MD, Sato M, Minna JD, et al. High expression of ligands for chemokine receptor CXCR2 in alveolar epithelial neoplasia induced by oncogenic kras. *Cancer Res* 2006;66:4198–4207.
41. Dhala A, Pinsker K, Prezant DJ. Respiratory health consequences of environmental tobacco smoke. *Med Clin North Am* 2004;88:1535–1552, xi. Review.
42. Philip M, Rowley DA, Schreiber H. Inflammation as a tumor promoter in cancer induction. *Semin Cancer Biol* 2004;14:433–439.
43. Borm PJ, Driscoll K. Particles, inflammation and respiratory tract carcinogenesis. *Toxicol Lett* 1996;88:109–113.
44. Yamashita J, Ogawa M, Shirakusa T. Free-form neutrophil elastase is an independent marker predicting recurrence in primary breast cancer. *J Leukoc Biol* 1995;57:375–378.
45. Yamashita J, Ogawa M, Abe M, Hayashi N, Kurusu Y, Kawahara K, Shirakusa T. Tumor neutrophil elastase is closely associated with the direct extension of non-small cell lung cancer into the aorta. *Chest* 1997;111:885–890.
46. Inada M, Yamashita J, Ogawa M. Neutrophil elastase inhibitor (ONO-5046-Na) inhibits the growth of human lung cancer cell lines transplanted into severe combined immunodeficiency (scid) mice. *Res Commun Mol Pathol Pharmacol* 1997;97:229–232.
47. Inada M, Yamashita J, Nakano S, Ogawa M. Complete inhibition of spontaneous pulmonary metastasis of human lung carcinoma cell line EBC-1 by a neutrophil elastase inhibitor (ONO-5046-Na). *Anticancer Res* 1998;18:885–890.
48. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002;2:301–310.
49. Luo JL, Maeda S, Hsu LC, Yagita H, Karin M. Inhibition of NF-kappaB in cancer cells converts inflammation-induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. *Cancer Cell* 2004;6:297–305.

50. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004;431:461–466.
51. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004;118:285–296.
52. Newton R, Holden NS, Catley MC, Oyelusi W, Leigh R, Proud D, Barnes PJ. Repression of inflammatory gene expression in human pulmonary epithelial cells by small-molecule IkappaB kinase inhibitors. *J Pharmacol Exp Ther* 2007;321:734–742.
53. Inayama M, Nishioka Y, Azuma M, Muto S, Aono Y, Makino H, Tani K, Uehara H, Izumi K, Itai A, et al. A novel IkappaB kinase-beta inhibitor ameliorates bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Crit Care Med* 2006;173:1016–1022.
54. Broide DH, Lawrence T, Doherty T, Cho JY, Miller M, McElwain K, McElwain S, Karin M. Allergen-induced peribronchial fibrosis and mucus production mediated by IkappaB kinase beta-dependent genes in airway epithelium. *Proc Natl Acad Sci USA* 2005;102:17723–17728.
55. Yang L, Cohn L, Zhang DH, Homer R, Ray A, Ray P. Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *J Exp Med* 1998;188:1739–1750.
56. Stathopoulos GT, Sherrill TP, Cheng DS, Scoggins RM, Han W, Polosukhin VV, Connelly L, Yull FE, Fingleton B, Blackwell TS. Epithelial NF-kappaB activation promotes urethane-induced lung carcinogenesis. *Proc Natl Acad Sci USA* 2007;104:18514–18519.
57. Di Stefano A, Caramori G, Oates T, Capelli A, Lusuardi M, Gnemmi I, Ioli F, Chung KF, Donner CF, Barnes PJ, et al. Increased expression of nuclear factor-kappaB in bronchial biopsies from smokers and patients with COPD. *Eur Respir J* 2002;20:556–563.
58. Guessous I, Cornuz J, Paccaud F. Lung cancer screening: current situation and perspective. *Swiss Med Wkly* 2007;137:304–311.
59. Halbert RJ, Natoli JL, Gano A, Badamgarav E, Buist AS, Mannino DM. Global burden of COPD: systematic review and meta-analysis. *Eur Respir J* 2006;28:523–532.