Activation of the Signal Transducers and Activators of the Transcription 3 Pathway in Alveolar Epithelial Cells Induces Inflammation and Adenocarcinomas in Mouse Lung

Yuan Li,1,3 Hong Du,2 Yulin Qin,2 Jennifer Roberts,1 Oscar W. Cummings,1 and Cong Yan1,3

1The Center for Immunobiology, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indiana, Indianapolis; 2Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; and 3Department of Pathology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China

Abstract

The lung is an organ for host defense to clear up pathogens through innate and adaptive immunity. This process involves up-regulation of proinflammatory cytokines and chemokines that lead to activation of the signal transducers and activators of the transcription 3 (Stat3) signaling pathway. Overexpression of Stat3C in alveolar type II epithelial cells of CCSP-rTA/(tetO)-Stat3C bitransgenic mice leads to severe pulmonary inflammation, including immune cell infiltration and up-regulation of proinflammatory cytokines and chemokines in the lung. As a consequence, spontaneous lung bronchoalveolar adenocarcinoma was observed in bitransgenic mice. Aberrantly expressed genes in the bitransgenic model were identified and served as biomarkers for human bronchoalveolar adenocarcinoma. During tumorigenesis, genes that are critical to epithelial cell proliferation in lung development were reactivated. Therefore, Stat3 is a potent proinflammatory molecule that directly causes spontaneous lung cancer in vivo. [Cancer Res 2007;67(18):8494–503]

Introduction

Mature alveoli have the largest epithelial surface area of the body to facilitate air exchange. During respiratory cycles, respiratory epithelium is repeatedly exposed to airborne particles and pathogens from the external environment. In coordination with the innate and adaptive immune systems, the respiratory epithelium protects the lung from microorganisms in part mediated by production of proinflammatory cytokines, chemokines, and growth factors. The magnitude of inflammatory responses is precisely regulated by proinflammatory and anti-inflammatory molecules. However, exuberant inflammation can cause severe consequences and lead to pathogenesis of acute and chronic pulmonary disorders, including lung cancer. Lung cancer causes more deaths than the next three most common cancers combined (colon, breast, and prostate). According to American Lung Association,4 an estimated 1 million people worldwide die from lung cancer annually. Over 3 million people have lung cancer, the majority residing in developed countries.

Signal transducers and activators of the transcription 3 (Stat3), which was originally identified as the acute phase response factor (1, 2), is a major intracellular signaling molecule that mediates the proinflammatory interleukin 6 (IL-6) family cytokines that share the common Gp130 receptor subunit (3, 4). On activation by cytokines or growth factors, phosphorylation at Y705 by Janus-activated kinases (JAK) causes Stat3 dimerization to form a heterodimer with Stat1 or to form a homodimer with itself. Stat3 dimers translocate into the nucleus and activate downstream target genes (5). As we showed previously, Stat3 is expressed in alveolar type II epithelial cells along with IL-6 receptors and JAKs in the lung. Treatment of IL-6 or lipopolysaccharides activates the Stat3 pathway in these cells in vivo (6, 7). Stat3 is required for maintaining alveolar structure and function. Overexpression of dominant-negative Stat3 to subvert endogenous Stat3 activity in respiratory epithelial cells causes alveolar destruction (7). Stat3 is an oncogene (8). Extensive surveys of primary tumors and cell lines derived from tumors indicate that inappropriate activation of Stat3 occurs with surprisingly high frequency (50–90%) in a wide variety of human cancers, including the lung (9, 10–15). It has been reported that ablating Stat3 in hematopoietic cells triggers inhibition of tumor growth and metastasis (16). However, no animal model has been generated to prove spontaneous tumor formation as a direct result of activation of the Stat3 pathway.

We hypothesize that persistent activation of the Stat3 pathway leads to pulmonary inflammation and tumorigenesis. An oncogenic Stat3C form has been designed to mimic the action of activated Stat3 (8). In this molecule, substitution of two cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3 produces a molecule that dimerizes spontaneously. Here, we report that Stat3C overexpression caused a microenvironment change in the lung by using flow cytometry, Affymetrix GeneChip microarray, real-time PCR, and immunohistochemistry analyses. These changes include infiltration of T lymphocytes, B lymphocytes, and macrophages in the lung of bitransgenic mice in association with up-regulation of proinflammatory cytokines, chemokines, oncogenes, apoptosis genes, and developmental genes. Persistent inflammation in this animal model leads to bronchoalveolar adenocarcinoma after 9 months of Stat3C induction. This animal model shows that persistent activation of the Stat3 pathway directly causes spontaneous tumor formation in vivo. In a clinical study, many aberrantly expressed genes in the CCSP-rTA/(tetO)-Stat3C bitransgenic mouse model were proven to be useful for diagnosis of human adenocarcinoma.
Materials and Methods

Animal care. All scientific protocols involving the use of animals and humans have been approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana University School of Medicine and Cincinnati Children's Hospital and followed the guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association. Protocols involving the use of recombinant DNA or biohazardous materials have been reviewed by the Institutional Biosafety Committee and followed guidelines established by the NIH. Animals were housed under IACUC-approved conditions in a secured animal facility. Animals were regularly screened for common pathogens. Experiments involving animal sacrifice use CO2 narcosis to minimize animal discomfort.

Human tissue. Normal and patient human lung tissues were from Indiana University Cancer Center Tissue Procurement Lab and Indiana University/Lilly Clinically Annotated Tissue Databank.

Histology and immunohistochemistry. Lung inflation and embedding were done as described previously (17). Multiple sections from each lung were stained with H&E. Tumor incidence and multiplicity in each section were counted. For immunohistochemistry, lung sections were incubated with Flag (1:500; Sigma), Mac3 (1:500; Santa Cruz Biotechnology), B220 (1:300; eBioscience), CD3 (1:100; DAKO), and thyroid transcription factor-1 (TTF-1;1:1,000; a kind gift from Dr. R. Di Lauro, Department of Cellular and Molecular Biology and Pathology, University of Naples “Federico II”, Naples, Italy) primary antibodies at 4°C overnight. The next day, tissue sections were washed and treated with biotinylated secondary antibodies. Signals were visualized with the Vectastain Elite ABC kit following the procedure recommended by the manufacturer.

Immunofluorescence staining. Alveolar type II epithelial cells were purified from 9-month doxycycline-treated or not treated Stat3C bitrans-...
of 95°C for 15 s, and 60°C for 1 min. Statistic differences among different animal groups or human samples were measured by ANOVA. P < 0.05 was considered significant.

**Results**

**Expression of Stat3C in alveolar type II cells of Stat3C bitransgenic mice.** A CCSP-rTA/((teto)γ)-Stat3C bitransgenic mouse system has been generated as reported previously, in which Stat3C overexpression can be induced by doxycycline treatment (20). To identify where Stat3C is expressed in the lung, a Flag sequence was attached to the COOH terminus of the Stat3C molecule to distinguish it from the endogenous Stat3 molecule. At 1 month old, bitransgenic mouse littermates were treated with doxycycline. After 1 month of doxycycline treatment, expression of the Stat3C-Flag fusion protein was readily detected and highly restricted in alveolar type II epithelial cells using an antibody against the Flag sequence (Fig. 1). This is consistent with our previous observations using reverse transcription-PCR, in which Stat3C mRNA and its downstream target surfactant protein B mRNA were induced by doxycycline treatment (20).

**Overexpression of Stat3C induced bronchoalveolar adenocarcinoma in the lung of Stat3C bitransgenic mice.** To determine if continuous Stat3C overexpression causes lung tumors, Stat3C bitransgenic mice were treated with doxycycline for 3, 6, and 9 months. Untreated animals were used as control.

No tumors were observed in 3- and 6-month treated animals. In 9-month treated animals, tumors resembling bronchoalveolar adenocarcinoma were observed in multiple bitransgenic mice, although the sizes of tumors in different bitransgenic mice varied (representing different stages of tumorigenesis; Fig. 2A–C). No tumors were observed in doxycycline untreated bitransgenic mice. None of wild-type (WT) and single transgenic mice (n = 10) developed tumors regardless of doxycycline treatment. Therefore, the tumor formation is an event associated with Stat3C overexpression in alveolar type II epithelial cells. To see if Stat3C-Flag fusion protein was still expressed in alveolar type II epithelial cells at this stage of tumorigenesis, alveolar type II epithelial cells were purified from 9-month doxycycline-treated Stat3C bitransgenic lung. Expression of Stat3C-Flag protein was detected in alveolar type II epithelial cells of doxycycline-treated bitransgenic mice, but not in untreated mice by immunofluorescence staining with anti-Flag antibody (Fig. 2D). These positively stained alveolar type II epithelial cells also showed Stat3 phosphorylation at Y705 in doxycycline-treated bitransgenic mice, indicating a highly inflammatory environment in the lung. This agrees with Affymetrix GeneChip microarray analysis (Supplementary Table S3), in which expression of IL-6 family members (upstream stimuli of Stat3) was highly increased in Stat3C bitransgenic lung. No Stat3 phosphorylation was observed in the noninflammatory lung of doxycycline untreated mice. In our previous observation, unstimulated WT mouse lungs by IL-6 showed very few Stat3 phosphorylation (6).

**Inflammatory cell infiltration in Stat3C induced bronchoalveolar adenocarcinoma.** In Stat3C bitransgenic mice, one striking feature is that inflammatory cells were highly associated with tissue remodeling in tumor areas, including massive plasma protein leakage (Fig. 3A, 1) with hemosiderin-laden macrophages (RBC engulfed by macrophages; Fig. 3A, 2 and 5) and massive lymphocyte infiltration (Fig. 3A, 3 and 4 arrow). At this stage of tumor formation, immunohistochemical staining using Mac3, B220, and CD3 antibodies confirmed dramatically increased macrophages, B lymphocytes, and T lymphocytes in the lung of this animal model (Fig. 3B). In doxycycline untreated control animals, no inflammatory cell infiltration was observed.

**Identification of aberrant gene expression in the lungs of Stat3C bitransgenic mice by Affymetrix GeneChip microarray analysis.** Lung cancer is a complex disease that is caused by multiple factors. To define molecules and molecular pathways that mediate bronchoalveolar adenocarcinoma formation in Stat3C bitransgenic mice, Affymetrix GeneChip microarray analysis was done using total RNA that was isolated from the lungs of bitransgenic mice after 9 months of doxycycline treatment. Approximately 800 genes were either up-regulated or down-regulated (2-fold change; P < 0.05). Supplementary Tables S1 and S2 show the most up-regulated and down-regulated genes in response to activation of the Stat3 pathway in the lung of bitransgenic mice. They represent an overall level of gene expression that is contributed by both residential (e.g., alveolar type II epithelial cells) and migrating cells (e.g., immune cells) in the lung. Expression of these genes forms a microenvironment in favor of tumor formation. These genes may potentially serve as biomarkers for clinical diagnosis. In an initial attempt, 11 up-regulated genes on the most up-regulated gene list (Supplementary Table S1) and two down-regulated genes on the most down-regulated gene list (Supplementary Table S2) were selected and tested in lung biopsies of 5 normal human subjects and 10 bronchoalveolar adenocarcinoma patients by real-time PCR using sequence-specific primers. As shown in Table 1, 10 of 11 genes...
that were up-regulated in bitransgenic mice showed up-regulation and 2 down-regulated genes in the bitransgenic mice showed down-regulation in human bronchoalveolar adenocarcinoma compared with normal human subjects. The only exception was the Scc2a3 gene, which was up-regulated in bitransgenic mice but down-regulated in the human. As a foot note, a few overlapping genes were identified between our list and a previously reported Stat3C microarray gene list (12). Two reasons may account for this. (a) The previous study used a homogeneous in vitro lung epithelial cell line. We used in vivo whole lung tumor tissues. (b) The previous study used human GeneChips. We used mouse GeneChips. Our result is more close to the real situation of in vivo lung microenvironment in Stat3C bitransgenic mice.

Increase of inflammatory molecules and cells in the lungs of Stat3C bitransgenic mice. Gene grouping by Affymetrix GeneChip microarray analysis identified a set of cytokines and chemokines that are up-regulated in the lung of bitransgenic mice (Supplementary Table S3). Most of these results were confirmed by real-time PCR using sequence-specific primers for each gene (data not shown). A group of oncogenes and apoptosis genes was also induced in Stat3C bitransgenic lung to contribute to tumor formation (Supplementary Table S3). Therefore, Stat3C-induced pulmonary inflammation is an important step for initiation and progression of lung tumor. It is crucial to identify which proinflammatory cytokine and chemokine genes are turned on early in the process of Stat3C induction in alveolar type II epithelial cells. These genes are potentially important for triggering and recruiting immune cells into lung tissue to initiate inflammation and tumorigenesis. Because inflammatory cell infiltration was not observed until 3 months after Stat3C induction, we decided to use 1-month doxycycline-treated sample as an early time point to study early-induced proinflammatory cytokines and chemokines. As shown in Fig. 1, the lung structure of Stat3C bitransgenic mice seemed normal. After 1 month of doxycycline treatment, total RNAs were purified from alveolar type II epithelial cells of Stat3C bitransgenic lungs. Using Supplementary Table S3 as a guide, expression levels of multiple cytokines and chemokines were quantitatively determined by real-time PCR. Gpl30 (78.7-fold), Lif (5.6-fold), IL-6 (22.6-fold), Csf2 (22.0-fold), Tnfsf9 (28.1-fold), Ccl5 (3,258.5-fold), Ccl8 (3.48-fold), Cxcl2 (39.3-fold), and VEGF (1,438.3-fold) genes were highly induced in alveolar type II cells at this early stage of lung inflammation (Fig. 4A). Other cytokine/chemokine genes listed in Supplementary Table S3 showed no change in alveolar type II cells at this early stage (data not shown), suggesting that they are involved in tumorigenesis at later stages and probably come from other infiltrated immune cells. In a similar study, we previously showed significant Stat3C elevation in Stat3C bitransgenic lungs after 2 weeks of doxycycline treatment (20). None of these molecules were induced in WT and single transgenic mice regardless of doxycycline treatment (Fig. 4A).

Following up-regulation and secretion of these cytokines and chemokines, inflammatory cell infiltration was observed. By flow cytometry analysis, T lymphocytes (CD3 staining), B lymphocytes (B220 staining), and macrophages (CD11b staining) were all increased significantly 3 months after Stat3C induction by doxycycline treatment in gated myeloid or lymphoid areas of bitransgenic mice (n = 5 per group; Fig. 4B). Removal of doxycycline only partially reduced inflammatory cell infiltration in the lung (Fig. 4B). This study shows that Stat3C is a proinflammatory molecule in the lung.

Reactivation of lung developmental genes in the lungs of Stat3C bitransgenic mice. During examination of gene changes by Affymetrix GeneChip microarray analysis, we noticed that some genes critical to epithelial growth during lung development were reactivated, including Shh gene (7.5-fold) and TTF-1 gene (2-fold). Surprisingly, HNF4α gene (41.76-fold) and Foxa3 gene (4.28-fold) were also up-regulated. This observation has been confirmed by real-time PCR analysis (Fig. 5A). Expression of Shh is required for lung epithelial cell proliferation and branching morphogenesis during embryogenesis. TTF-1 is a homeodomain-containing tissue-specific transcription factor of Nkx2.1 family members (also termed TEBP and Nkx2.1). During lung development, expression of TTF-1 is required for lung epithelial cell proliferation and branching.

Figure 1. Stat3C expression in alveolar type II epithelial cells of Stat3C bitransgenic mice. Expression of Stat3C-Flag fusion protein in the CCSP-rtTA/(tetO)-Stat3C bitransgenic lung by immunostaining with Flag antibody 1 mo after doxycycline treatment. The Stat3C-Flag protein was detected in alveolar type II epithelial cells (arrows). −Dox, doxycycline untreated; +Dox, doxycycline treated.
morphogenesis (17). Figure 5B (2) shows that TTF-1 expression was detected in tumor epithelial cells of Stat3C bitransgenic mice by immunohistochemical staining assay using anti-TTF-1 antibody. This relationship existed even at the onset of tumorigenesis (Fig. 5B, 3, arrows). TTF-1 and Stat3C-Flag fusion protein were colocalized in alveolar type II epithelial cells of doxycycline-treated bitransgenic mice (Fig. 5C).

**Discussion**

In this report, we showed for the first time that persistent activation of the Stat3 signaling pathway by overexpression of Stat3C directly leads to inflammation and spontaneous tumor formation in the lung. Therefore, persistent activation of the Stat3 signaling by upstream proinflammatory cytokines (e.g., IL-6 family members) and growth factors (e.g., epithelial growth factor) in various disease conditions (bacterial infection, chronic inflammation, etc.) has the potential to induce cancer in the body. Cancer formation is a complex process that is influenced by both genetic arrangement and surrounding microenvironment. Based on our studies, Stat3C induces lung tumor formation by two steps in Stat3C bitransgenic mice. The first step involves aberrant expression of cytokines/chemokines and abnormal inflammatory cell infiltration, which hijack the immune system to inhibit the immune surveillance and promote neoplastic process. The second step involves reactivation of developmental genes that stimulate epithelial cell growth.

The lung has more than 40 cell types. Alveolar type II cells are well known for its function of synthesizing and secreting pulmonary surfactant to maintain the alveolar structure from collapse during respiratory cycles. They also serve as terminal progenitor cells for alveolar type I epithelial cells during lung injury and repair. Alveolar type II epithelial cells actively participate in pulmonary inflammation and host defense by secreting various cytokines and chemokines in response to various pathogenic environmental assaults. As we showed previously, alveolar type II epithelial cells express receptors for IL-6 family members and Stat3 protein (6, 7). Endogenous Stat3 can be activated by treatment of IL-6 family members to stimulate downstream gene expression (6, 7). Therefore, alveolar type II cells are sensitive and respond to the surge of Stat3 upstream cytokines and growth factors. It seems that overexpression of Stat3C in alveolar type II epithelial cells is sufficient to trigger pulmonary inflammation that eventually leads to lung tumor. The event is initiated by increased expression of a subset of cytokines, chemokines, and their receptors (Fig. 4A). These molecules are known for their ability to recruit inflammatory cells from the bone marrow and immune systems into inflammatory tissue sites. It is worthy to note that the IL-6 family cytokines and receptor unit (Lif and IL-6, and Gp130) were highly induced by Stat3C overexpression. It is conceivable that up-regulation of these molecules further stimulates activation of endogenous Stat3B by phosphorylation to exacerbate inflammation. This has been confirmed in Fig. 2D. It has been reported that IL-6 trans-signaling via Stat3 directs T-cell infiltration in inflammation (21). IL-6–induced proliferation of T cells was severely impaired in Stat3-deficient T cells. Stat3 activation is responsible for IL-6–dependent T-cell proliferation by preventing apoptosis (22).
may provide a mechanism to explain massive T-cell accumulation as a result of Stat3C overexpression in the Stat3C bitransgenic lung (Figs. 3 and 4). Granulocyte macrophage colony-stimulating factor (csf2) plays a crucial role in growth and differentiation of hematopoietic cells. It stimulates proliferation and differentiation of myeloid progenitors. GM-CSF has been shown to play a role in tumor progression (23). Ccl8 encodes for monocyte chemotactic protein 2, which is a tumor-derived monocyte chemotactic chemokine that drives the recruitment of monocytes from the bloodstream to tissues and differentiate them into tumor-associated macrophages (24). CxcR2 is a chemokine receptor for ELR+ CXC chemokines. Depletion of CxcR2 inhibits lung tumor growth and angiogenesis in the mouse model (25). Identification of CxcR2 up-regulation in alveolar type II epithelial cells after Stat3C induction suggests a new role for this molecule in lung tumorigenesis. Ccl5 (RANTES) plays an active role in recruiting leukocytes into inflammatory sites and increases the adherence of monocytes to endothelial cells. It supports the migration of monocytes and T lymphocytes (26). VEGF is a highly specific mitogen for vascular endothelial cells that significantly influences vascular permeability. Ccl5, IL-6, and VEGF are known downstream target genes of Stat3 (27, 28).

In Stat3C bitransgenic lungs, inflammatory cells including macrophages and lymphocytes are readily detected in the alveolar region before (3 months of doxycycline treatment) and after (9 months of doxycycline treatment) tumor formation with
doxycycline treatment. Macrophages are highly versatile cells that regulate tumor growth, angiogenesis, invasion, and metastasis (29). In the later stage of tumor formation, macrophages were present in the tumor area in association with massive plasma protein leakage in doxycycline-treated Stat3C bitransgenic lung (Fig. 3). Infiltration of innate and adaptive immune cells into lung changed the local microenvironment and hijacked the immune surveillance system to favor tumor growth. This has been further supported by Affymetrix GeneChip microarray analysis (Supplementary Tables S1–S3) and real-time PCR (Fig. 4), in which the expression of multiple genes was altered. The functional roles of many of these genes are poorly understood in tumorigenesis. This unique gene profile defines a genetic network and a set of biomarkers for bronchoalveolar adenocarcinoma caused by persistent activation of the Stat3 pathway in the mouse. This offers novel insight into mechanisms that regulate premalignant and malignant states of inflammation-induced tumorigenesis. Many of these genes can be used for tumor diagnosis in the human as shown in Table 1.

It has been suggested that lung tumor cell ontogeny is determined by consequences of gene expression that recapitulate events important in embryonic lung development (30). In Stat3C bitransgenic mice, TTF-1 and Shh genes that are critical for epithelial cell growth during lung development were reactivated. Expression of Shh is required for normal development of the lung during embryogenesis (31–33). During pulmonary development, Shh contributes to branching morphogenesis and epithelial cell proliferation. Shh is produced and secreted by epithelial cells in embryonic lung buds. Lack of Shh signaling, mediated by the Gli proteins, leads to severe pulmonary hypoplasia (34), inhibits branching morphogenesis, and disrupts pulmonary vascular development (35, 36–37). TTF-1 is expressed in the lung, thyroid, and part of the forebrain (17, 38, 39). In the lung, TTF-1 is expressed in epithelial tubules during lung development and restricted to alveolar type II and conducting airway epithelial cells in the adult lungs (17). Gene-targeted deletion of the mouse TTF-1 gene causes severe pulmonary hypoplasia (38). It is noteworthy as well that HNF4α and Foxa3 were also up-regulated in the CCSP-rtTA/(tetO)−Stat3C bitransgenic lung. Foxa3 and HNF4α were originally identified in the liver and regulate liver-specific gene regulation (40, 41). Because HNF4α and Fox3α are potent transcription factors, misexpression of these molecules in lung tissue may perturb the local genetic setting and promote unwanted cell growth that induces lung tumors. Importantly, up-regulation of these genes was observed in human bronchoalveolar adenocarcinoma (Table 1). Stat3 is a developmental gene required for normal embryonic development and tissue formation. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality (42).

In summary, the CCSP-rtTA/(tetO)−Stat3C bitransgenic mouse model provides a unique and valuable tool to broadly study functional roles of the Stat3 pathway in lung biology and pathology. Previously, we showed that overexpression of Stat3C protects lung tissue from acute lung inflammation and injury caused by hyperoxia (20). Therefore, Stat3C has both anti-inflammatory and proinflammatory functions in the lung. Genes identified by Affymetrix GeneChip microarray analysis in the

| Table 1. Aberrant expression of biomarkers in human bronchoalveolar adenocarcinomas |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Gene            | Cbln1            | Cldn2            | Dlk1             | Fgf1             | Foxa3            | Gjb1             | HNF4x            | Shh              | Slc2a3           |
| Normal (ΔCt)    |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| 1               | 6.27             | 4.42             | 6.79             | 5.26             | 5.20             | 7.90             | 4.36             | 9.20             | 5.37             |
| 2               | 6.34             | 6.06             | 6.53             | 6.48             | 5.87             | 7.96             | 4.34             | 8.89             | 1.23             |
| 3               | 5.02             | 4.85             | 5.27             | 8.15             | 4.33             | 7.18             | 2.81             | 7.98             | 1.17             |
| 4               | 5.85             | 5.46             | 7.37             | 5.76             | 4.83             | 8.10             | 3.69             | 8.59             | 1.42             |
| 5               | 7.40             | 6.41             | 8.39             | 7.76             | 6.69             | 8.87             | 5.12             | 11.26            | 2.86             |
| Average         | 6.18             | 5.44             | 6.87             | 6.68             | 5.38             | 8.00             | 4.06             | 9.14             | 2.01             |
| Patient (ΔCt)   |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| 1               | 2.55             | 0.82             | 3.88             | 4.92             | 1.15             | 3.81             | −0.012           | 5.89             | 5.56             |
| 2               | 4.56             | 2.30             | 4.79             | 8.57             | 2.79             | 5.50             | 2.16             | 7.29             | 4.72             |
| 3               | 6.99             | −0.35            | 6.54             | 4.2              | 3.86             | 3.74             | 3.89             | 8.99             | 6.85             |
| 5               | 0.83             | 0.14             | 3.13             | 1.26             | −0.33            | 2.08             | −1.43            | 4.74             | 5.88             |
| 6               | 0.86             | 0.45             | 2.34             | 5.34             | −0.05            | 3.29             | −1.26            | 5.07             | 6.02             |
| 7               | 2.00             | 1.00             | 3.00             | 0.32             | 1.00             | 3.64             | 0.00             | 6.00             | 6.00             |
| 8               | 4.81             | 2.26             | 4.77             | 2.58             | 2.94             | 6.32             | 2.51             | 8.41             | 5.13             |
| 9               | 4.55             | −2.88            | 4.88             | −1.19            | 3.06             | 4.93             | 1.41             | 8.09             | 5.26             |
| 10              | 5.95             | 5.89             | 1.80             | 0.06             | 5.88             | 7.89             | 4.46             | 8.59             | 4.88             |
| Average patient | 3.95             | 1.35             | 4.21             | 2.74             | 2.52             | 4.91             | 1.59             | 7.28             | 5.62             |
| ΔΔCt            | 2.23             | 4.09             | 2.66             | 3.94             | 2.86             | 3.09             | 2.48             | 1.86             | −3.61            |
| Fold change     | 4.68             | 16.98            | 6.31             | 15.39            | 7.28             | 8.55             | 5.57             | 3.63             | 0.08             |

NOTE: Genes that were up-regulated or down-regulated in Stat3C bitransgenic mice (Supplementary Tables S1 and S2) were examined in the lungs of 5 normal human subjects and in bronchoalveolar adenocarcinoma of 10 human patients. Real-time PCR was normalized by GAPDH mRNA expression. Fold changes were determined by $2^{ΔΔCt}$, in which ΔΔCt = ΔCt (normal) − ΔCt (patients). Normal: lung tissues from human subjects without bronchoalveolar adenocarcinoma. Patient: lung tissues from human subjects with bronchoalveolar adenocarcinoma.
Stat3C bitransgenic mouse model can potentially serve as molecular markers for human cancer patient diagnosis. Further studies of interrelationships between these molecules will provide significant insight into initiation and progression of chronic inflammation-induced lung cancer. The Stat3C bitransgenic mouse model can help define the intrinsic immune surveillance system in tumor immunology. It can also help elucidate mechanisms for epithelial cell growth and differentiation.

Figure 4. Up-regulation of inflammatory molecules and inflammatory cell infiltration in Stat3C bitransgenic lung. A, total RNA was purified from alveolar type II epithelial cells of Stat3C bitransgenic mice, WT mice, and CCSP/rtTA single transgenic mice that were treated or not treated with doxycycline for 1 mo. Real-time PCR was used to quantify mRNA expression levels of cytokines and chemokines as normalized by GAPDH mRNA expression. Relative expression was determined by \( \frac{\Delta \Delta CT}{DCT} \), in which \( \Delta CT = CT_{testing\ molecule} - CT_{GAPDH} \). Therefore, \( DCT = CT_{testing\ molecule} - CT_{GAPDH} \). \( +Dox \), mRNA expression levels from doxycycline-treated samples; \( -Dox \), mRNA expression levels from samples not treated with doxycycline. B, Stat3C bitransgenic mice were treated with \( (+Dox) \) or without doxycycline \( (-Dox) \) for 3 mo. In a separate experiment, Stat3C bitransgenic mice were treated with doxycycline for 2 mo and followed by doxycycline removal for 1 mo \( (+-Dox) \). Lung mononuclear cells were stained with fluorochrome-conjugated antimouse antibodies. B lymphocytes (B220 antibody staining), T lymphocytes (CD3 antibody staining), and macrophages (CD11b staining) were analyzed by flow cytometry in gated myeloid or lymphoid areas.
Figure 5. Reactivation of lung developmental genes in bitransgenic mice. A, up-regulation of developmental genes in the lung of Stat3C bitransgenic mice. Total RNA was purified from whole lungs of Stat3C bitransgenic mice that were treated (+Dox) or untreated (−Dox) with doxycycline for 9 mo. Real-time PCR was used to quantify mRNA expression levels of developmental genes and normalized by GAPDH mRNA expression. B, lung sections from the Stat3C bitransgenic mice after 9 mo of doxycycline treatment were immunostained with TTF-1 antibody. TTF-1–positive signals were detected in epithelial cells at both early (3, arrows) and late (2) stages of tumors. Control staining of the tumor area without primary antibody addition (1). Original magnification, ×20. C, immunofluorescence double staining of Stat3C-Flag and TTF-1 in alveolar type II epithelial cells of Stat3C bitransgenic mice. DAPI, nucleus staining; Flag, Stat3C-Flag fusion protein staining; TTF-1, staining of TTF-1; Overlay, colocalization of Flag and TTF-1. −Dox, doxycycline untreated; +Dox, doxycycline treated for 9 mo.
during lung development. New approaches and pharmacologic drugs can be designed to combat lung cancer by using this animal system.

Acknowledgments


References


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