Airway Epithelial Transcription Factor NK2 Homeobox 1 Inhibits Mucous Cell Metaplasia and Th2 Inflammation

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***Rationale*: Airway mucous cell metaplasia and chronic inflammation are pathophysiological features that influence morbidity and mortality associated with asthma and other chronic pulmonary disorders. Elucidation of the molecular mechanisms regulating mucous metaplasia and hypersecretion provides the scientific basis for diagnostic and therapeutic opportunities to improve the care of chronic pulmonary diseases.**

***Objectives*: To determine the role of the airway epithelial–specific transcription factor NK2 homeobox 1 (NKX2-1, also known as thyroid transcription factor-1 [TTF-1]) in mucous cell metaplasia and lung inflammation.**

***Methods*: Expression of NKX2-1 in airway epithelial cells from patients with asthma was analyzed. *NKX2-1*$^{+/-}$ gene targeted or transgenic mice expressing NKX2-1 in conducting airway epithelial cells were sensitized to the aeroallergen ovalbumin. *In vitro* studies were used to identify mechanisms by which NKX2-1 regulates mucous cell metaplasia and inflammation.**

***Measurements and Main Results:* NKX2-1 was suppressed in airway epithelial cells from patients with asthma. Reduced expression of *NKX2-1* in heterozygous *NKX2-1*$^{+/-}$ gene targeted mice increased mucous metaplasia in the small airways after pulmonary sensitization to ovalbumin. Conversely, mucous cell metaplasia induced by aeroallergen was inhibited by expression of NKX2-1 in the respiratory epithelium *in vivo*. Genome-wide mRNA analysis of lung tissue from ovalbumin-treated mice demonstrated that NKX2-1 inhibited mRNAs associated with mucous metaplasia and Th2-regulated inflammation, including *Spdef*, *Ccl17*, and *Il13*. *In vitro*, NKX2-1 inhibited *SPDEF*, a critical regulator of airway mucous cell metaplasia, and the Th2 chemokine *CCL26*.**

***Conclusions:* The present data demonstrate a novel function for NKX2-1 in a gene network regulating mucous cell metaplasia and allergic inflammation in the respiratory epithelium.**

Keywords: asthma; goblet cell; respiratory epithelium; NK2 homeobox 1

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Elucidation of the cellular and molecular mechanisms by which respiratory epithelial cells interact with the innate immune system to modify lung inflammation are providing insights into the pathogenesis of common pulmonary disorders. The present work identifies the role of the respiratory epithelial-specific transcription factors regulating mucous metaplasia and lung inflammation.

What This Study Adds to the Field

We provide *in vivo* and *in vitro* evidence for the airway epithelial-specific transcription factor NK2 homeobox 1 (NKX2-1)/thyroid transcription factor-1 (TTF-1) role in the inhibition of aeroallergen-induced mucous metaplasia and lung inflammation. NKX2-1 inhibited Sam Pointed Domain Ets-like Factor (SPDEF), a transcription factor critical for mucous cell differentiation and mucous production in the lung. Although NKX2-1 is known to play an important role in lung morphogenesis before birth, the present work demonstrates its novel function as a regulator of allergen-induced mucous metaplasia and inflammation in the adult lung.

In the conducting airways, the epithelium is pseudostratified and consists primarily of basal, ciliated, secretory, and relatively lesser numbers of mucous cells (1). These cells form a physical barrier to the external environment and protect the airways surface through secretion of mucus that neutralizes noxious agents and traps inhaled particles, which are moved out of the airways via the action of the mucociliary escalator (2). The gel-like properties of this mucus are dictated by the mucin glycoproteins MUC5AC and MUC5B, derived from goblet cells and submucosal glands (3). Repeated exposure of the airways to toxicants and allergens, as well as viral infections, induces mucous cell metaplasia and increases the production of mucus (4). Overproduction of mucus impairs mucociliary clearance, causing airway obstruction that underlies the recurrent infections and ongoing inflammation associated with pulmonary morbidities in chronic airway diseases, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis (4–6).

Mucous hyperproduction is regulated by a number of signaling pathways, including EGF, Notch, and IL-4 receptors, that influence transcription of genes associated with mucous cell metaplasia in respiratory epithelial cells (4, 7). In mouse models, airway mucous metaplasia was dependent on the expression of Sam Pointed Domain Ets-like Factor (SPDEF)

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that was necessary and sufficient for mucous metaplasia *in vivo* (8, 9). Likewise, expression of SPDEF was induced by exposure of mice to aeroallergens or IL-13, where it was associated with mucous cell metaplasia and the loss of NKX2-1 (NK2 homeobox 1; also known as TTF-1, thyroid transcription factor-1) (9). NKX2-1 plays a central role in the regulation of lung morphogenesis before birth and is required for differentiation and gene regulation of diverse subsets of respiratory epithelial cells (10–12). In the normal human lung, NKX2-1 is selectively expressed in subsets of epithelial cells lining conducting airways and in alveolar type II cells in the lung periphery (13). After aeroallergen exposure in the mouse, epithelial cells (Clara cells) lining the bronchiolar epithelium undergo mucous metaplasia in association with increased expression of genes associated with the synthesis and packaging of pulmonary mucins and the loss of expression of genes expressed in nongoblet secretory cells.

Because SPDEF and NKX2-1 were not coexpressed in epithelial cells after pulmonary allergen sensitization in mouse (9) (*see* Figure E1 in the online supplement), we tested whether NKX2-1 played an important inhibitory role in the regulation of mucous cell metaplasia. NKX2-1 inhibited both mucous cell metaplasia and Th2-mediated inflammation, indicating its important role in the regulation of respiratory epithelial cell differentiation and innate immune responses in the adult lung.

METHODS

Full methodological details are available in the online supplement.

Human Specimens

Lung samples from anonymous patients were obtained at autopsy from S. H. Randell (University of North Carolina at Chapel Hill, NC) and A. Günther (University of Vienna, Austria, and University of Giessen Lung Center, Germany). Bronchial brushings and biopsies from anonymous patients with asthma and healthy control subjects were obtained by fiberoptic bronchoscopy. All of human specimens were obtained in accordance with institutional guidelines for use of human tissue for research purposes.

Transgenic Mice and Animal Husbandry

NKX2-1 heterozygous mice (+/−) were kindly provided by S. Kimura (National Institutes of Health, Bethesda, MD) (11). Transgenic mice bearing the transgene $(tetO)_7$CMV-*Flag-NKX2-1*-IRES-EGFP were generated and mated with *Scgb1a1*-rtTA (line 2) transgenic mice (14).

Pulmonary Sensitization to Ovalbumin

Mice were sensitized to ovalbumin by sequential systemic and pulmonary administration using a protocol previously described (9). Expression of Flag-tagged NKX2-1 in the *Scgb1a1*-rtTA/$(tetO)_7$CMV-*Flag-NKX2-1*-IRES-EGFP mice was induced at 6 to 8 weeks of age by provision of doxycycline 72 hours before mice were sensitized by intratracheal aspiration of ovalbumin. Single transgenic littermates that received the same doxycycline and ovalbumin sensitization were used as controls. Doxycycline was continued until the time of death.

Immunoblot Assays, Immunohistochemistry, and Morphometric Analysis

Immunoblot assays were performed as previously described (15). Staining was performed using 5-μm paraffin-embedded lung sections as previously described (9). Morphometric analysis was performed on lung sections from control and NKX2-$1^{+/-}$ mice (n = 3–4 per group).

RNA Isolation, Quantitative Reverse Transcriptase–Polymerase Chain Reaction Assays, and RNA Microarray Data Analysis

Total RNA was isolated using standard procedures. Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed on a 7300 real-time PCR system (Applied Biosystems, Foster City, CA) with the TaqMan probes. RNA microarray analysis was performed on whole lung RNA in the CCHMC Affymetrix Core using standard procedures. For functional classification and pathway analysis, gene ontology analysis was performed using the publically available web-based tool David (Database for Annotation, Visualization, and Integrated Discovery) (16).

In Vitro Studies in Human A549 and H441 Lung Epithelial Cells

Human lung epithelial carcinoma A549 cells were infected with either control virus or NKX2-1–expressing virus. Five days after infection, cells were harvested for qRT-PCR assay for *SPDEF* mRNA and for immunoblot assay for NKX2-1 and actin. In separate experiments, control virus or NKX2-1–expressing virus-infected A549 cells were treated with IL-13 (catalog # 213-IL-005; R&D Systems, Minneapolis, MN) at a final concentration of 10 ng/ml. Twenty-four hours after treatment, cells were harvested for qRT-PCR assay for *CCL26* mRNA.

The siRNAs targeting *NKX2-1* #1 (ID: 14152), *NKX2-1* #2 (ID: 224731), and negative control siRNA (cat# 4390843) were purchased from Ambion/Applied Biosystems (Austin, TX). Transfection of human lung papillary adenocarcinoma NCI-H441 cells with siRNAs was performed according to a protocol using Lipofectamine RNAiMAX (catalog # 13778–150; Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, total RNA was extracted and qRT-PCR assays for *NKX2-1*, *SPDEF*, *CCL26*, and *MUC5AC* were performed.

Statistics

Statistical differences were determined using Student *t* test (two-tailed and unpaired). The difference between two groups was considered significant when the *P* value was less than 0.05 for all tests of human, mouse, and *in vitro* experiments.

RESULTS

Reduction of NKX2-1 in Human Asthmatic Airways

Decreased expression of NKX2-1 was associated with Th2-mediated inflammation after aeroallergen exposure in the mouse (9) (Figure E1). We determined whether mucous cell metaplasia accompanying human asthma was associated with loss of NKX2-1. NKX2-1 was readily detected in the nuclei of subsets of epithelial cells in conducting airways and in alveolar type II epithelial cells in normal human lung tissue consistent with previous findings (13) (Figure 1A, *top left*). NKX2-1 staining was not detected in mucous cells in autopsy tissue from patients with asthma, wherein staining of NKX2-1 and SPDEF was mutually exclusive (Figure 1A). In bronchial brushings obtained by bronchoscopy from patients with asthma, *NKX2-1* mRNA was significantly decreased compared with that in healthy control subjects (Figure 1B and Table 1).

Increased Mucous Cell Metaplasia in *NKX2-1*$^{+/-}$ Mice

To determine whether reduced *NKX2-1* expression induced mucous cell metaplasia, adult *NKX2-1* gene-deleted mice were studied. *NKX2-1*$^{-/-}$ die at birth related to respiratory failure associated with lack of respiratory epithelial differentiation and peripheral lung formation, whereas *NKX2-1*$^{+/-}$ mice survive normally to adulthood (11). *NKX2-1* mRNA in lungs of the *NKX2-1*$^{+/-}$ mice was significantly reduced, consistent with haploinsufficiency at the locus (17) (Figure E2). Mucous cells,

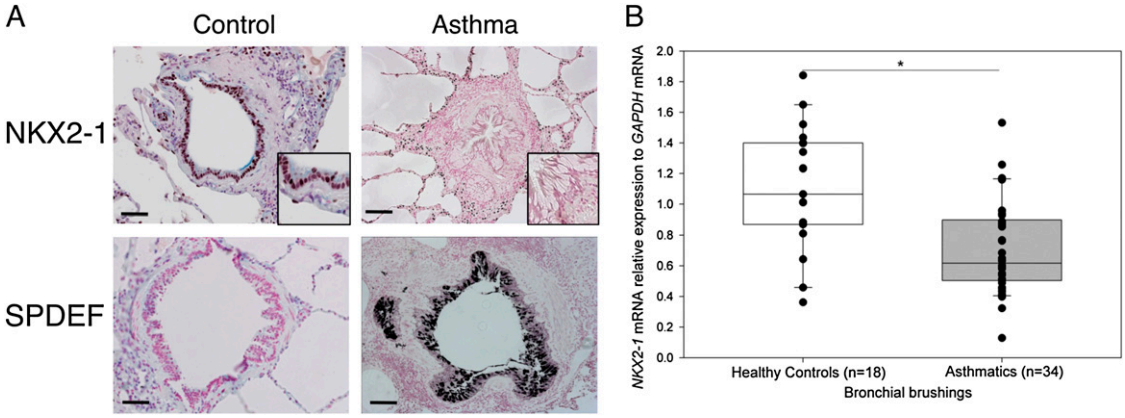


Figure 1. Mucous cells express Sam Pointed Domain Ets-like Factor (SPDEF) and lack NK2 homeobox 1 (NKX2-1). (*A*) Staining of NKX2-1 and SPDEF is shown by immunohistochemistry of normal and asthmatic human autopsy lung specimens. NKX2-1 staining was readily detected in nuclei of subsets of conducting airway cells and type II epithelial cells from normal human lung that lacked mucous cell metaplasia and SPDEF staining (control, *left panels*, counterstained with Alcian Blue). Absence of NKX2-1 and presence of SPDEF in mucous cells are shown in bronchial tissue from a patient who died of acute asthma (*right panels*). Positive-staining cells are seen as dark brown or black. Scale bars: 100 μm. (*B*) *NKX2-1* mRNA in bronchial epithelial brushings by bronchoscopy was significantly decreased in human patients with asthma compared with healthy control subjects (*$P < 0.001$).

detected by Alcian blue, SPDEF, and MUC5AC staining, were sporadically observed in the airways of the *NKX2-1*$^{+/-}$ but not in lungs from *NKX2-1*$^{+/+}$ mice at 5 to 6 weeks of age (Figure 2A). *NKX2-1*$^{+/-}$ mice and littermate control mice were sensitized by pulmonary administration of ovalbumin (Figure 2B). In lungs of *NKX2-1*$^{+/-}$ mice, MUC5AC and SPDEF expression extended into small conducting airways that normally lack mucous cells after aeroallergen exposure (Figure 2B, *right panels*, *arrows*). Morphometric analysis demonstrated that the extent of mucous cell metaplasia induced by ovalbumin sensitization was significantly increased in bronchioles of *NKX2-1*$^{+/-}$ mice compared with littermate control mice (Figure 2C).

Expression of NKX2-1 Inhibited Allergen-induced Mucous Metaplasia

To determine whether NKX2-1 negatively regulates mucous cell metaplasia, transgenic mice were produced in which NKX2-1 was expressed under control of the tet-operator (tetO)$_7$CMV-*Flag-NKX2-1*-IRES-EGFP. These mice were bred to *Scgb1a1*-rtTA transgenic mice (line 2) that express the reverse tetracycline transactivator (rtTA) in nonciliated Clara cells, the predominant secretory cell lining the conducting airways of the mouse (Figure 3A) (14). *Flag-NKX2-1* mRNA and immunostaining were induced when doxycycline was administered in the food (Figures 3B and 3C, *top two panels*). *Scgb1a1*-rtTA/(tetO)$_7$CMV-*Flag-NKX2-1*-IRES-EGFP and single littermate control mice were sensitized to ovalbumin to test whether increased expression of NKX2-1 in airway epithelial cells influenced allergen-induced mucous metaplasia. In control mice, endogenous NKX2-1 was absent and SPDEF was induced in mucous cells by ovalbumin sensitization (Figure 3C, *left panels*, *arrow*; Figure E1). In contrast, when NKX2-1 was induced in respiratory epithelial cells after treatment with doxycycline, mucous cell metaplasia, indicated by Alcian blue, SPDEF, and MUC5AC staining, was markedly inhibited or absent (Figure 3C, *right panels*; Figure 3D).

TABLE 1. SUBJECT CHARACTERISTICS OF BRONCHIAL BRUSHING GROUP

	Healthy Control (*n = 18*)	Mild-Moderate Asthma (*n = 7*)	Severe Asthma (*n = 27*)
Age, yr	26 (19–55)	36 (18–64)	45 (21–69)*
Sex, m/f	10/8	1/6	8/19
Atopy	5	5	16
FEV_1% predicted	97 (83–115)	82 (51–106)	72 (30–101)†
BTS step‡	0	1–3	4–5
SABA	N/A	7	27
ICS	N/A	3	27
LABA	N/A	3	27
OCS	N/A	0	16

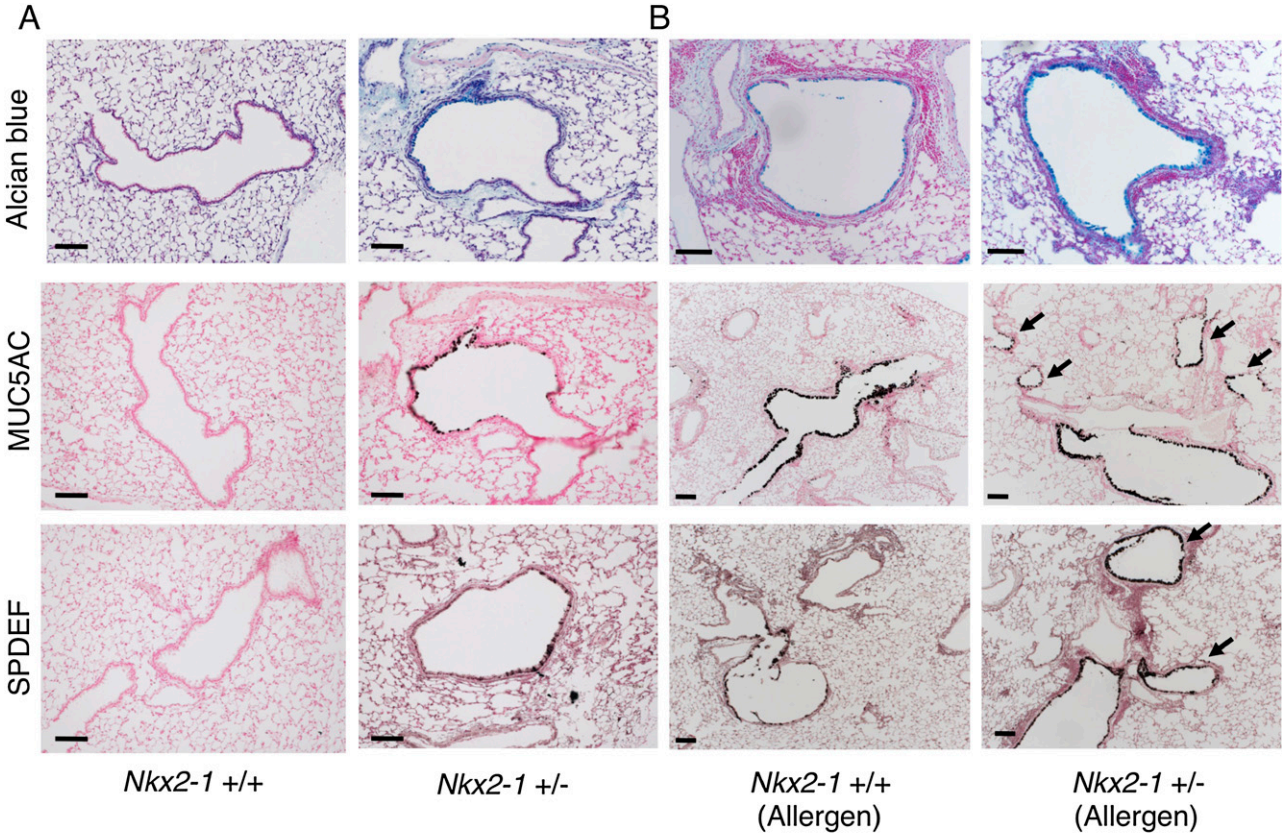
Bronchial brushing samples were obtained from control and patients with asthma for quantification of NKX2-1 and GAPDH mRNAs seen in Figure 1.

Definition of abbreviations: BTS = British Thoracic Society asthma management guidelines; ICS = inhaled corticosteroids; LABA = long-acting beta2-agonists; N/A = not applicable; OCS = oral corticosteroids; SABA = short-acting beta2-agonists.

* One way ANOVA with pairwise multiple comparison and multiple comparisons versus control group (Bonferroni *t* test) ($P < 0.05$).

† Kruskal-Wallis one way ANOVA on ranks with pairwise multiple comparison and multiple comparisons versus control group (Dunn method) ($P < 0.05$).

‡ BTS steps (26): Step1: Inhaled SABA as required. Step2: SABA + ICS 200–800 μg beclomethasone dipropionate or equivalent/d. Step3: SABA + ICS + LABA. Step 4: up to 2,000 μg/d ICS + LABA + others. Step 5: high dose ICS + others + OCS.



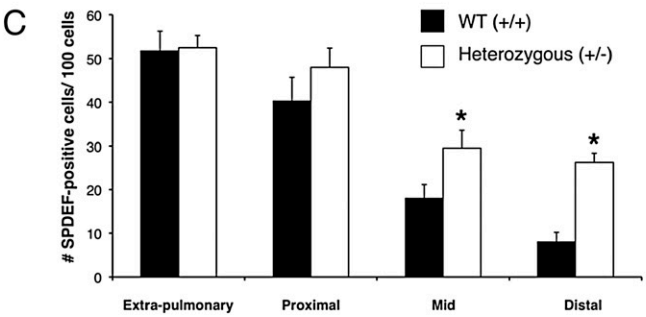


Figure 2. Reduced *NKX2-1* enhances mucous metaplasia in airways. (*A*, *B*), lung sections were stained with Alcian blue or by immunohistochemistry for MUC5AC and Sam Pointed Domain Ets-like Factor (SPDEF). (*A*) In the absence of allergen exposure, mucous cells were sporadically observed in the airways of the *NKX2-1*$^{+/-}$ but not in lungs from *NKX2-1*$^{+/+}$ mice at 5 to 6 weeks of age. Scale bars: 100 μm. (*B*) After ovalbumin sensitization, mucous metaplasia was significantly increased in distal airways of *NKX2-1*$^{+/-}$ mice. Scale bars: 100 μm. (*C*) Morphometric analysis was performed on *NKX2-1*$^{+/+}$ (control) and *NKX2-1*$^{+/-}$ mice after ovalbumin exposure by counting SPDEF-positive cells per total number of cells as described in the METHODS (*$P < 0.05$).

Expression of NKX2-1 Prevented Loss of FOXA2 Triggered by Allergen Sensitization

Although NKX2-1 inhibited the expression of SPDEF and MUC5AC, it prevented the loss of proteins triggered by allergen sensitization, including FOXA2 and cytochrome P450 reductase in Clara cells (Figure 4, *top two panels*; Figure E3). In contrast, FOXJ1, a transcription factor mediating cilia formation, and SOX2, a transcription factor expressed ubiquitously in the conducting airways, were not altered by the expression of NKX2-1. Ki-67, a cell proliferation marker, was not changed in bronchiolar epithelial cells of the NKX2-1–expressing transgenic mice (Figure 4, *bottom three panels*). Staining for CCSP, a Clara cell marker, was reduced in the bronchioles of NKX2-1–expressing transgenic mice at baseline (data not shown); thus, loss of CCSP was not a useful marker for mucous metaplasia in these mice.

NKX2-1 Inhibits mRNAs Associated with Mucous Metaplasia and Th2 Inflammation

mRNA microarray (Figure 5A) and qRT-PCR (Figures 5B and 5C, Figure E4) were used to identify mRNAs influenced by NKX2-1 after pulmonary ovalbumin sensitization. NKX2-1 inhibited mRNAs encoded by genes associated with goblet cell differentiation and mucous production, including *Spdef*, *Muc5ac*, *Muc5b*, *Clca3/1*, *Agr2*, and *Tff2*, consistent with histological data indicating inhibitory effects of NKX2-1 on mucous metaplasia.

To determine whether NKX2-1 inhibits *SPDEF* gene expression in a cell autonomous manner, human lung epithelial A549 cells that express *SPDEF* but not NKX2-1 were infected with a lentiviral vector containing *NKX2-1*. NKX2-1 significantly inhibited the expression of *SPDEF* mRNA in A549 cells (Figure 6A). Conversely, in H441 human lung epithelial cells that express both *NKX2-1* and *SPDEF*, siRNA-mediated inhibition of *NKX2-1* significantly induced the expression of *SPDEF* and *MUC5AC* mRNAs (Figure 6C), whereas siRNA-mediated inhibition of *SPDEF* did not alter *NKX2-1* expression (data not shown). These results indicate that loss of *NKX2-1* in lung epithelium promotes the expression of *SPDEF* and mucous-related genes, including *MUC5AC*.

A number of chemokines, cytokines, and innate immune molecules regulated by ovalbumin sensitization were also counter-

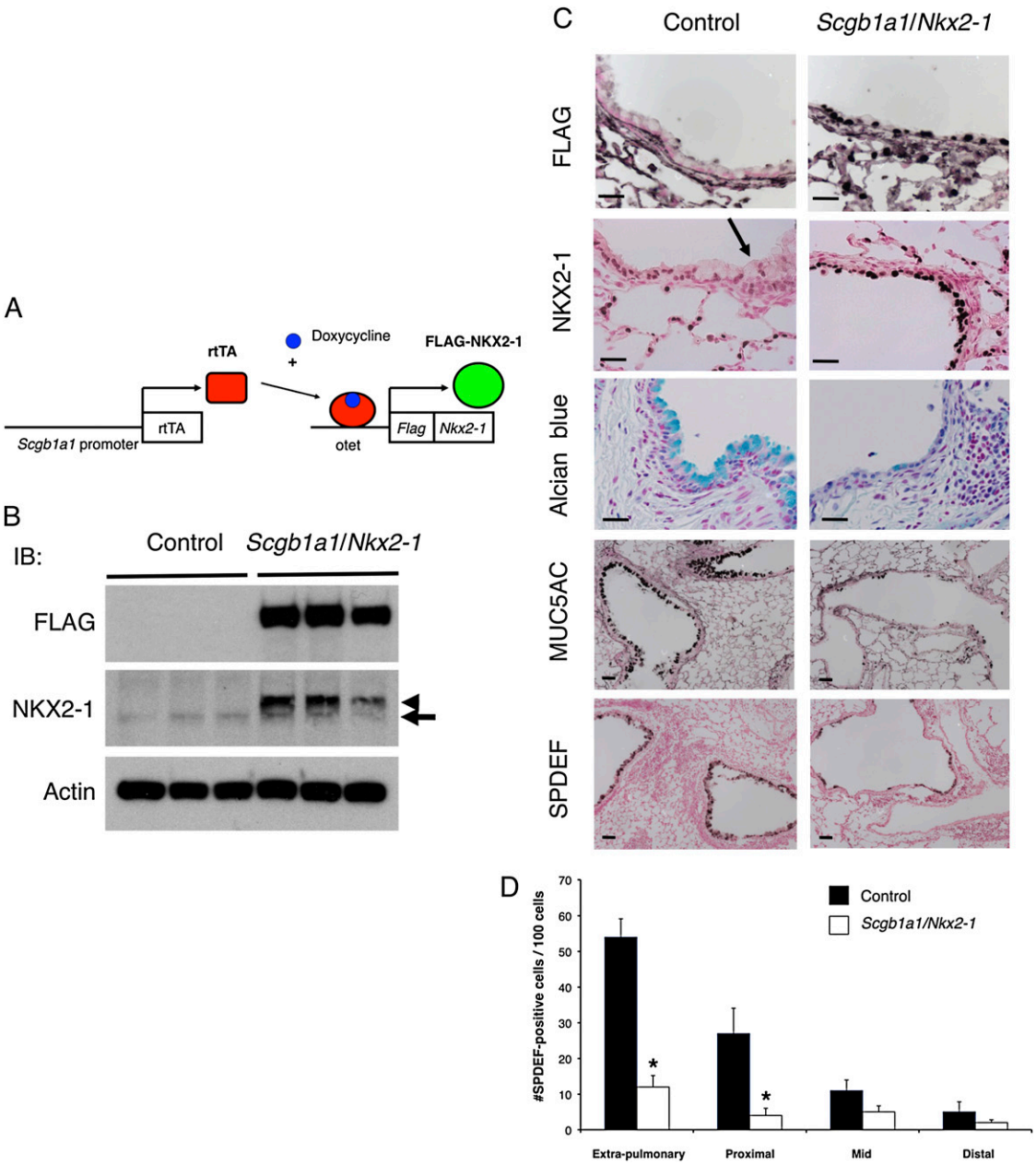


Figure 3. NK2 homeobox 1 (NKX2-1) inhibits allergen-induced mucous metaplasia. (*A*) The construct and strategy used to express Flag-tagged NKX2-1 in Clara cells in conducting airways of the transgenic mice are shown. Mice were sensitized to ovalbumin and Flag-NKX2-1 was induced by administration of doxycycline as described in the METHODS. (*B*) Immunoblotting is shown using FLAG and NKX2-1 antibodies to detect Flag-tagged NKX2-1 increased in lung homogenates from control or the NKX2-1 transgenic mice (*Scgb1a1/NKX2-1*) in the presence of doxycycline after allergen challenge. Actin staining was used for sample control loading. *Arrowhead* is Flag-NKX2-1 and *arrow* is endogenous mouse NKX2-1. (*C*) Lung sections were stained with Alcian blue or by immunohistochemistry for FLAG, NKX2-1, MUC5AC, and Sam Pointed Domain Ets-like Factor (SPDEF). The arrow indicates loss of NKX2-1 in goblet cells increased by the ovalbumin sensitization in control mice. NKX2-1 inhibits allergen-induced mucous cell metaplasia. Data are representative staining of lung sections (n = 5–6 per group). Scale bars: 25 μm. (*D*) Morphometric analysis was performed on control and the NKX2-1 transgenic mice by counting SPDEF-positive cells per total number of cells as described in the METHODS, demonstrating a significant reduction in SPDEF stained cells in mice expressing NKX2-1, $^*P < 0.05$.

regulated by NKX2-1. *Ccl17*, *Ccl22*, *Il13*, and *Il4*, but not *Ifng* mRNAs, were inhibited by expression of NKX2-1 after aeroallergen exposure (Figure 5, Figure E4). CCL17 (also known as TARC) is a Th2-attracting chemokine that is highly induced in the bronchial epithelium of patients with asthma. CCL17 mediates allergic airway inflammation and mucous cell metaplasia by activating the Th2 lymphocytes expressing IL-13 and IL-4 (18, 19). In human lung epithelial A549 cells, IL-13–mediated induction of the Th2 chemokine *CCL26* (also known as eotaxin-3) (20) was significantly inhibited by NKX2-1 (Figure 6B). Conversely, the siRNA-mediated inhibition of *NKX2-1* in H441 human lung epithelial cells significantly induced *CCL26* expression (Figure 6C). CCL26 expression is increased in human bronchial epithelial cells from individuals with asthma (21). In our mouse models, *Ccl26* expression was not altered by NKX2-1; however, the mouse *Ccl26* gene is a pseudogene (22).

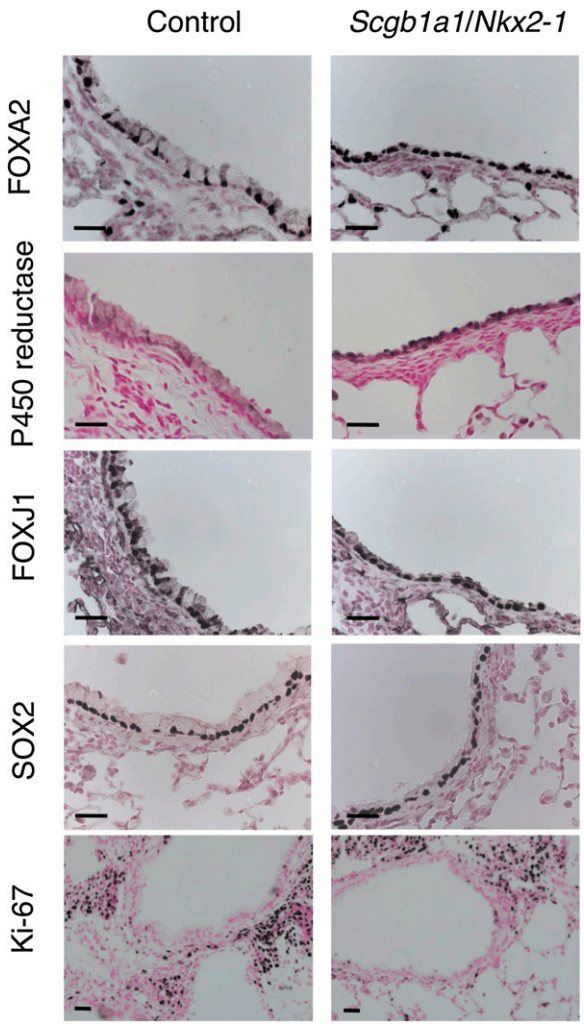


Figure 4. NK2 homeobox 1 (NKX2-1) prevents loss of FOXA2 triggered by allergen sensitization. Lung sections from control or the NKX2-1 transgenic mice (*Scgb1a1/NKX2-1*) in the presence of doxycycline after allergen challenge were stained by immunohistochemistry for FOXA2, P450 reductase, FOXJ1, SOX2, and Ki-67. Scale bars: 25 μm.

DISCUSSION

Airway epithelial cells provide barrier function, mucociliary clearance, and secrete diverse antimicrobials, mucins, chemokines, and cytokines that recruit and instruct inflammatory cells that serve to limit infection. Chronic epithelial cell injury and inflammation cause tissue remodeling and increased susceptibility to infection that together ultimately impair lung function in chronic airway diseases, including asthma, cystic fibrosis, and chronic obstructive pulmonary disease. Although transcriptional networks integrating mucous cell metaplasia and chemokine production by airway epithelial cells are presently poorly understood, the importance of the respiratory epithelium in the instruction of various aspects of innate immune system of the lung is highly relevant to the pathogenesis of common chronic lung diseases. Here, we observed that a transcription factor NKX2-1 in airway epithelial cells was suppressed in patients with asthma. In mouse models and in human epithelial cells, NKX2-1 inhibited genes associated with mucous metaplasia and Th2 inflammation.

To further understand the role of NKX2-1 in airway epithelial cells, we used Ingenuity Pathway Analysis and identified biological relationships of NKX2-1 among gene nodes within a network of corelated genes, the "network" being defined as a group of biologically related genes, proteins, or other molecules. Biological relationships were derived from the combined analyses of the present mRNA microarray data and previously published mRNA arrays obtained from transgenic mice in which *Spdef*, *Foxa2*, or *NKX2-1* expression was selectively induced (9), deleted (23), or inhibited (12); from published and present experimental data that included mRNA, protein analysis, and immunohistochemistry; and from peer-reviewed publications in the Ingenuity knowledge base (Figure 7A). The network analyses revealed important interrelated cellular processes influenced by NKX2-1: (*1*) suppression of mucous cell metaplasia via inhibition of SPDEF-regulated mucous genes (e.g., *Muc5ac*, *Clca3/1*, *Agr2*, and *Tff2*); (*2*) inhibition of mRNAs associated with Th2-mediated inflammation (e.g., *Ccl17*, *Ccl22*, *Ccr8*, *Cd28*, *Mmp12*, *Chia*, and *Icos*); and (*3*) a complementary role of NKX2-1 with FOXA2 in the inhibition of mucous cell metaplasia and Th2-mediated inflammation.

mRNAs decreased by NKX2-1 during allergen exposure partially overlap with those induced by expression of SPDEF in airway epithelial cells (9) as shown schematically in Figure 7A. The finding that NKX2-1 inhibited allergen-induced expression of SPDEF provides a mechanism by which a number of genes associated with mucin production were coordinately regulated (Figures 5 and 7). The concept that NKX2-1 and SPDEF counter-regulate each other and associated target genes is consistent with previous studies in embryonic lungs from transgenic mice bearing phosphorylation sites mutant *NKX2-1* allele (*NKX2-1*^{PM}) (12), in which *Spdef* mRNA was markedly induced in lung before birth (Figure E5). In the present study, SPDEF induced the expression of mucins, including *Muc5ac*, *Muc16*, and a number of genes mediating various aspects of mucin biosynthesis and packaging, (e.g., glycotransferases, *Agr2*, and *Clca3/1*), which were inhibited by NKX2-1. Thus, reciprocal interactions between SPDEF and NKX2-1 likely serve as a switch to control the differentiation of airway epithelial cells to mucous cells.

Th2 chemokines and cytokines induced by allergen sensitization were also inhibited by NKX2-1 *in vivo* and *in vitro*, indicating that NKX2-1 suppresses some aspects of Th2-mediated responses in airway epithelial cells in a cell-autonomous manner. Although a number of mRNAs associated with Th2-mediated inflammation were inhibited by NKX2-1, expression of a number of mRNAs associated with innate immune mediated inflammation, including *Il6*, *Saa3*, *Saa4*, *Orm1*, *and Orm2*, were induced (Figures 5A and 7A). Thus, the inhibitory effects of NKX2-1 on mucous cell metaplasia are likely mediated, at least in part, by inhibition of Th2-associated chemokines/cytokines and increased expression of a subset of mRNAs associated with acute inflammation.

FOXA2 is a member of the forkhead family of transcription factors that is normally expressed in both conducting airway and alveolar respiratory epithelial cells. Conditional deletion of *Foxa2* in the developing respiratory epithelium caused severe eosinophilic lung inflammation, mucous metaplasia, and increased expression of mRNAs associated with mucous cell metaplasia and Th2-mediated inflammation, including *Spdef*, *Muc5ac*, *Il13*, *Ccl17*, and *Ccl22* (23). Expression of FOXA2 in airway epithelial cells blocked allergen-induced mucous cell metaplasia and expression of SPDEF in adult mice (23, 24). In the present study, expression of NKX2-1 prevented the loss of FOXA2 and inhibited various mediators of Th2-mediated inflammation and mucous cell metaplasia after aeroallergen

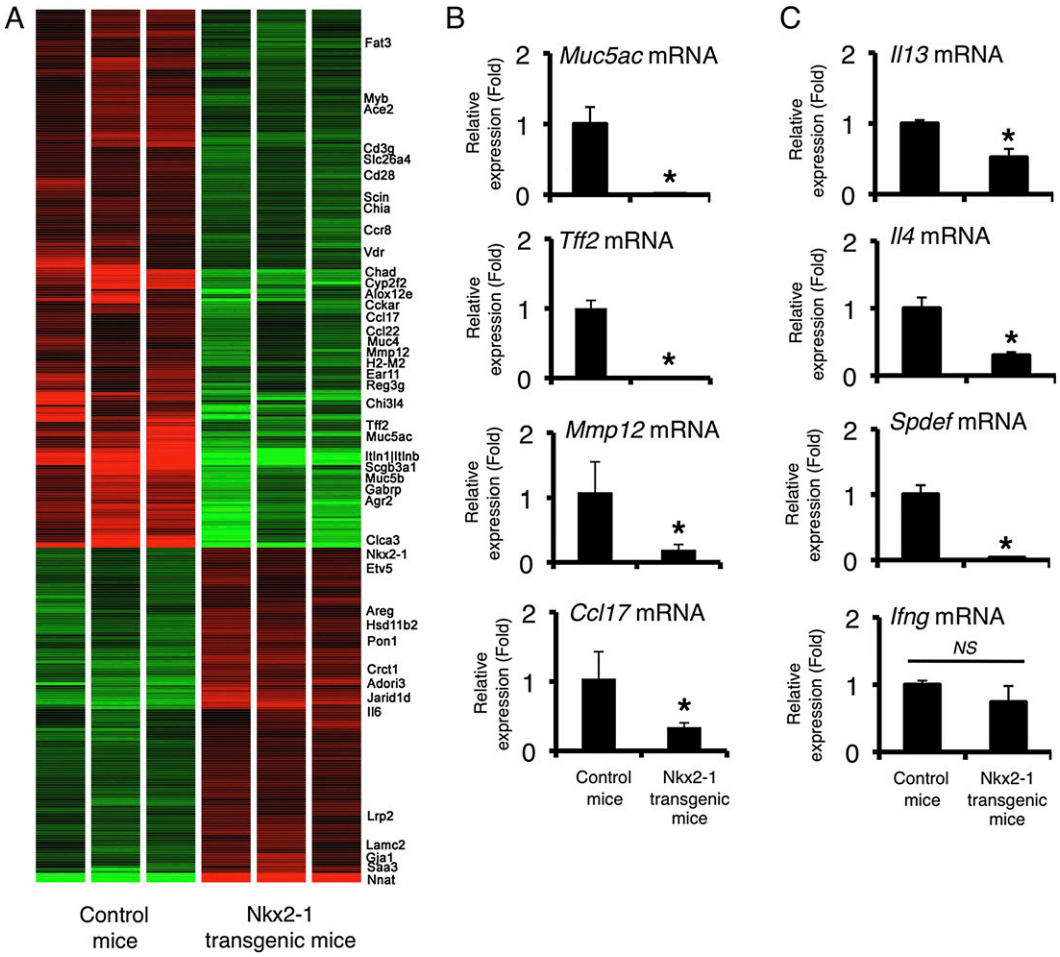


Figure 5. NK2 homeobox 1 (NKX2-1) inhibits the expression of *Spdef* and genes associated with mucous metaplasia and Th2-mediated inflammation. (*A*) Heat map of the mRNAs. Green indicates mRNAs decreased by NKX2-1; red indicates those mRNAs that were increased. A number of mRNAs that were previously associated with pulmonary allergen sensitization, including *Clca3/1*, *Agr2*, *Muc5ac*, *Tff2*, *Mmp12*, and *Ccl17* were suppressed by NKX2-1. (*B*) Validation of microarray results by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed using cDNAs obtained from whole mouse lungs. Results are expressed as mean ± SD of three independent mice for each treatment, $*P < 0.05$ versus control. (*C*) qRT-PCR for Th2 (*Il13*, *Il4*, and *Spdef*) and innate inflammation (*Ifng*)-mediated genes. Results are expressed as described in *B*, $*P < 0.05$. NS = not significant.

sensitization (Figure 4), supporting the concept that NKX2-1 and FOXA2 play complementary roles in the inhibition of mucous metaplasia and lung inflammation (Figure 7A).

Glucocorticoids are often used to treat patients with asthma; thus, the decreased expression of NKX2-1 in patients with asthma might be mediated, in part, by their treatment. Our patient cohort, however, included three patients not treated with glucocorticoids, and the decreased expression of NKX2-1 was also evident in these patients (data not shown). The numbers are small, and to conclusively address this larger numbers of

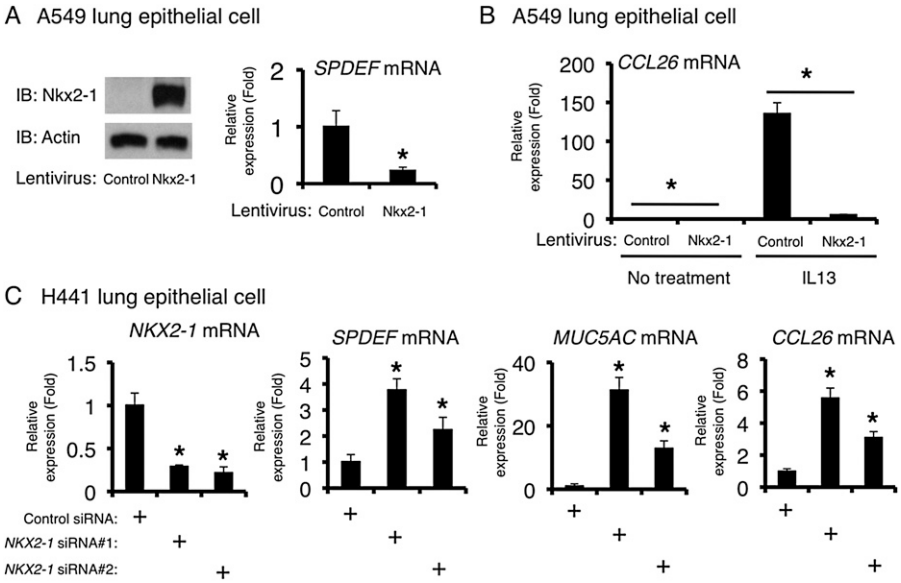


Figure 6. NK2 homeobox 1 (NKX2-1) inhibits *SPDEF* and Th2 chemokine *CCL26* mRNAs in human lung epithelial cells. (*A*) Inhibition of *SPDEF* mRNA by NKX2-1 in A549 human lung epithelial cells. A549 cells were infected with NKX2-1–expressing or control lentivirus and proteins and mRNAs extracted. Protein expression of NKX2-1 was confirmed by immunoblotting (IB) (*left panels*). *SPDEF* mRNA was significantly inhibited by the NKX2-1-expressing lentivirus (*right panel*). Results are expressed as mean ± SD of biological triplicates for each group and are representative of three independent experiments, $*P < 0.05$ versus control virus. (*B*) Inhibition of *CCL26* mRNA by NKX2-1 in A549 human lung epithelial cells. After A549 cells were infected with NKX2-1–expressing or control lentivirus, they were treated with IL-13 for 24 hours and mRNAs extracted. IL-13–mediated induction of *CCL26* mRNA was significantly inhibited by the NKX2-1–expressing lentivirus. Results are expressed as described in *A*, $*P < 0.05$ versus control virus. (*C*) Induction of *SPDEF*, *MUC5AC*, and *CCL26* mRNAs by *NKX2-1*siRNAs. H441 human lung epithelial cells were transfected with control or *NKX2-1*siRNAs (#1 or #2) and mRNAs isolated 48 hours after transfection. Results are expressed as described in *A*, $*P < 0.05$ versus control siRNA.

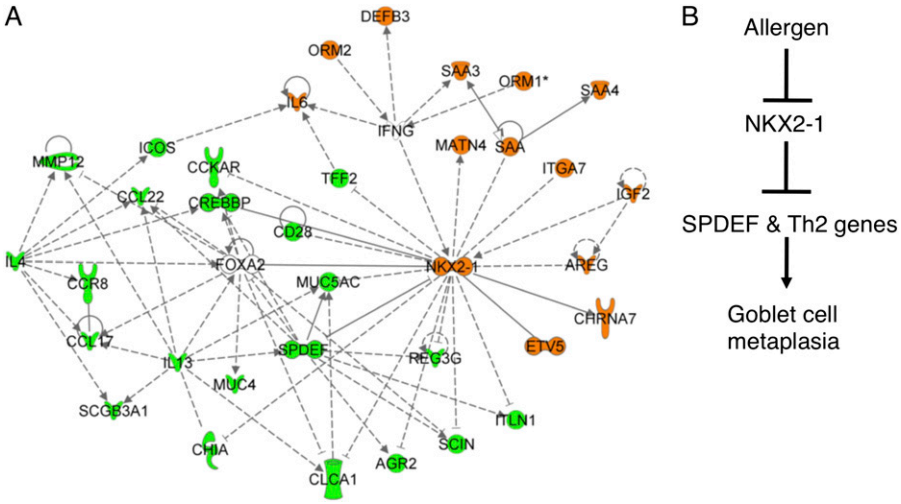


Figure 7. NK2 homeobox 1 (NKX2-1)–Sam Pointed Domain Ets-like Factor (SPDEF)–associated gene network analyzed using Ingenuity Pathway Analysis. (*A*) Genes/proteins are represented as nodes, and the biological relationship between two nodes is represented as a line. Solid lines indicate direct relationships, dashed lines mean indirect relationships, open line is binding, arrow indicates activation, and closed line is inhibition. mRNAs up- or down-regulated in transgenic mice expressing NKX2-1 after ovalbumin sensitization are color coded in orange or green, respectively. Biological relationships are derived from the combined analyses of microarray samples from transgenic mouse models in which *NKX2-1*, *Spdef*, or *Foxa2* genes were selectively perturbed; experimental data (reverse transcriptase–polymerase chain reaction, immunoblot, and immunohistochemistry); and peer-reviewed publications (Ingenuity knowledge base). NKX2-1 expression suppressed genes involved in mucus production, goblet cell differentiation (e.g., *Spdef*, *Muc5ac*, *Muc4*, *Clca1*, *Agr2*, *Scin*), and allergic inflammation (e.g., *Ccl17*, *Ccl22*, *Ccr8*, *Il4*, *Il13*, and *Chia*), while promoting the expression of genes involved in acute inflammatory response (e.g., *Il6*, *Saa1*, *Saa4*, *Adora3*, *Orm1*, and *Orm2*) and epithelial development (e.g., *NKX2-1*, *Etv5*, *Chrna7*, *Areg*). (*B*) Schema of the inhibitory role of NKX2-1 in goblet cell metaplasia.

patients would be needed to assess the potential role of glucocorticoid treatment in the expression of NKX2-1. Nevertheless, in the mouse experiments, *NKX2-1* mRNA was decreased by allergen challenge in the absence of exogenous treatments (Figure E1). Furthermore, NKX2-1 was not reduced by treatment of dexamethasone, a corticosteroid, in H441 human lung epithelial cells (25). These results indicate that the expression of NKX2-1 is influenced by aeroallergen sensitization.

NKX2-1 inhibited aeroallergen-induced mucous cell metaplasia, in part, by inhibiting SPDEF and by maintaining expression of FOXA2. mRNA microarray analysis indicated that NKX2-1 and SPDEF act in an opposing manner within a gene network influencing both respiratory epithelial differentiation and the Th2 immunoregulatory axis. NKX2-1 inhibited allergen-induced *Ccl17*, *Il13*, *CCL26*, and other genes that play important roles in the pathogenesis of asthma and mucous metaplasia (Figure 7B). The present study demonstrates a novel function of NKX2-1 as an inhibitor of aeroallergen-induced airway mucous cell metaplasia in the adult lung and provides further support for the important role of the respiratory epithelium in the regulation of pulmonary inflammation.

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