ORIGINAL ARTICLE

Foxa3 Induces Goblet Cell Metaplasia and Inhibits Innate Antiviral Immunity

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Abstract

Rationale: Goblet cell metaplasia accompanies common pulmonary disorders that are prone to recurrent viral infections. Mechanisms regulating both goblet cell metaplasia and susceptibility to viral infection associated with chronic lung diseases are incompletely understood.

Objectives: We sought to identify the role of the transcription factor FOXA3 in regulation of goblet cell metaplasia and pulmonary innate immunity.

Methods: FOXA3 was identified in airways from patients with asthma and chronic obstructive pulmonary disease. We produced transgenic mice conditionally expressing Foxa3 in airway epithelial cells and developed human bronchial epithelial cells expressing Foxa3. Foxa3-regulated genes were identified by immunostaining, Western blotting, and RNA analysis. Direct binding of FOXA3 to target genes was identified by chromatin immunoprecipitation sequencing correlated with RNA sequencing.

Measurements and Main Results: FOXA3 was highly expressed in airway goblet cells from patients with asthma and chronic obstructive pulmonary disease. FOXA3 was induced by either IL-13 or rhinovirus. Foxa3 induced goblet cell metaplasia and enhanced expression of a network of genes mediating mucus production. Paradoxically, FOXA3 inhibited rhinovirus-induced IFN production, IRF-3 phosphorylation, and IKKε expression and inhibited viral clearance and expression of genes required for antiviral defenses, including MDA5, RIG-I, TLR3, IRF7/9, and nuclear factor-κB.

Conclusions: FOXA3 induces goblet cell metaplasia in response to infection or Th2 stimulation. Suppression of IFN signaling by FOXA3 provides a plausible mechanism that may serve to limit ongoing Th1 inflammation during the resolution of acute viral infection; however, inhibition of innate immunity by FOXA3 may contribute to susceptibility to viral infections associated with chronic lung disorders accompanied by chronic goblet cell metaplasia.

Keywords: rhinovirus; IFN; transcription factors; mucus

Goblet cell metaplasia, hypersecretion of mucus, and inflammation complicate common acute and chronic pulmonary disorders that contribute to morbidity and mortality from lung diseases throughout the world (reviewed in Reference 1). Mucous metaplasia, as seen in asthma, cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), bronchiectasis, and other

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At a Glance Commentary

Scientific Knowledge on the Subject: Mucous metaplasia in chronic pulmonary disorders is commonly associated with recurrent viral and bacterial infections that contribute to clinical exacerbations. Mechanisms mediating goblet cell differentiation and the increased susceptibility to recurrent infections associated with chronic lung diseases are incompletely understood.

What This Study Adds to the Field: FOXA3, a transcription factor expressed in the goblet cells in the airways of patients with asthma and COPD, regulates a network of genes mediating mucus production and causes goblet cell differentiation. FOXA3 also inhibits rhinovirus-induced IFN production and viral clearance by the lung. The suppression of antiviral responses by FOXA3 provides a plausible mechanism linking goblet cell metaplasia and susceptibility to infection associated with chronic pulmonary diseases.

chronic pulmonary disorders, is commonly associated with recurrent viral and bacterial infections that contribute to clinical exacerbations and declines in pulmonary function (2–4). Paradoxically, in spite of the inflammation associated with chronic respiratory disorders, these patients are susceptible to both viral and bacterial infections and often fail to fully clear microbial pathogens from the airways. Secondary infections are a common occurrence during recovery from pulmonary viral infections (5). The mechanisms underlying the susceptibility to recurrent infection associated with chronic lung diseases are complex and incompletely understood.

Goblet cell metaplasia and hyperplasia are induced by various inflammatory mediators, including Th2 cytokines after pulmonary aeroallergen, viral, and toxicant exposure, and after activation of epidermal growth factor receptor and Notch signaling (6–9). At the transcriptional level, airway goblet cell metaplasia induced by Th2 cytokines is mediated by SPDEF, the activation of which requires STAT6 and the inhibition of NKX2-1 and FOXA2 in airway epithelial cells (10–12). SPDEF is sufficient and necessary for goblet cell metaplasia, and expression of SPDEF is increased in airway epithelial cells of patients with CF and in chronic smokers (13). In transgenic mice, expression of Spdef induces Foxa3 (also termed HNF3γ) in airway goblet cells (13). Likewise, FOXA3 is increased after aeroallergen exposure in mice but is not required for goblet cell metaplasia in that model (14). The role of FOXA3 in the regulation of respiratory epithelial cell mucous metaplasia and innate immune responses is unknown.

Rhinoviruses (RVs) are the pathogen most commonly associated with the respiratory infections termed "the common cold." RV infections are associated with respiratory tract infection and inflammation that also frequently complicate the clinical course of patients with chronic lung diseases (2–4). RV readily infects respiratory epithelial cells, eliciting inflammation and antiviral responses that are dependent on IFN production and expression of IFN-stimulated genes (ISGs). Production of double-stranded RNAs during RV replication leads to signaling through endosomal TLR3 activating IRF3 (15) and the production of IFNs and intracellular RNA helicases RIG-I and MDA-5 that are critical for viral clearance (16, 17). RV induces neutrophilic and lymphocytic inflammation and enhances Muc5ac expression in mouse lungs (18).

The present study was designed to identify the role of FOXA3 in the regulation of goblet cell metaplasia and innate immunity in airway epithelial cells. FOXA3 was induced in airway epithelial cells after exposure to RV *in vivo* and *in vitro*. FOXA3 was sufficient to cause goblet cell metaplasia in transgenic mice and in human bronchial epithelial cells (HBECs) *in vitro* and inhibited IFN responses. Although inhibitory effects of FOXA3 on IFN signaling may serve to dampen inflammatory responses during resolution of acute infections, chronic expression of FOXA3 associated with mucous metaplasia may contribute to susceptibility to infection associated with chronic pulmonary disorders.

Methods

Human Specimens

Human samples were deidentified and studies were performed in accordance with institutional review board (IRB) approval at Cincinnati Children's Hospital (CCHMC ID: 2012-2853). Samples from patients with COPD were obtained from pathological tissues provided by Dr. Andreas Gunther, University of Giessen Lung Center, Giessen, Germany in accordance with IRB approval. HBECs and tissue from patients with asthma were obtained under approved protocols at the University of North Carolina, Chapel Hill. HBECs were produced under Biomedical IRB Protocol #103-1396.

Mouse Models, Ovalbumin, House Dust Mite, and RV1B Sensitization

Mouse strains included in this study were C57/B6 *Foxa3*$^{-/-}$ mice and FVB/N *Scgb1a1-rtTA* (line 2) bred to *Otet*$_7$*-Foxa3-IRES-EGFP* mice. Ovalbumin, house dust mite (HDM), and RV1B sensitization protocols are provided in the online supplement.

Immunohistochemistry, Alcian Blue Staining, and Confocal Microscopy

Adult mouse lungs sections were stained with Alcian blue and/or immunohistochemical staining as previously described (12, 13, 19). For confocal microscopy, BEAS2B cells that were stably transfected with lentiviral constructs were dual stained with antibodies for FOXA3 and MUC5AC (13) (online supplement).

RV Culture, Infection, IFN-β, and IL-13 Administration

Amplification of RV1B followed the standard protocols as previously described (18). Infection with RV and treatment of primary HBECs with IFN-β or IL-13 were previously described (20–23) (online supplement).

Chromatin Immunoprecipitation Sequence

Chromatin immunoprecipitation (ChIP) assays were conducted as described previously (24). BEAS2B-GFP and BEAS2B-Foxa3 transfected cells were fixed with 1% formaldehyde for 10 minutes at room temperature. Chromatin was sonicated and immunoprecipitated

using a FOXA3 antibody (Santa Cruz Biotechnology, Dallas, TX) overnight. ChIP–polymerase chain reaction analysis was conducted using real-time polymerase chain reaction. ChIP-sequence (ChIP-Seq) libraries were generated and sequenced using standard Illumina protocols. Reads (approximately 50 bp per fragment) were mapped to the human genome (UCSC hg19) using the Bowtie2 algorithm (3′ trimmed 40 bp reads and three mismatches). Only sequences that mapped to a single genomic location were selected (online supplement).

Statistics

Student *t* test (two-tailed, unpaired) and nonparametric Mann-Whitney test (two-tailed, unpaired) (Prism 6; Graphpad, La Jolla, CA) were used for comparison of statistical differences between two groups. Nonpairing one-way analysis of variance (Prism 6) was used for comparison of statistical differences between three or more groups; *P* values of less than 0.05 were considered significant difference.

Results

FOXA3 Is Highly Expressed in Airway Goblet Cells from Patients with Asthma and COPD

Intense nuclear staining of FOXA3 was detected in airway goblet cells in tissue from patients with COPD and asthma. FOXA3 staining was restricted to epithelial cells and closely associated with Alcian blue and SPDEF, both characteristic of airway goblet cells. FOXA3 was much less abundant in airway epithelial cells in tissues from healthy individuals (Figure 1). Th2 cytokines, including IL-13, cause goblet cell metaplasia in airway epithelial cells *in vivo* (25). We therefore assessed the effects of IL-13 on expression of FOXA3 and goblet cell–related genes in primary HBECs. Recombinant human IL-13 caused mucous metaplasia and increased expression of FOXA3, SPDEF, and MUC5AC in well-differentiated primary HBECs cultured at air–liquid interface (Figure 2A).

FOXA3 Is Sufficient to Induce Goblet Cell Metaplasia *In Vitro* and *In Vivo*

Lentiviral-mediated expression of the mouse *Foxa3* cDNA in BEAS2B cells, a transformed HBEC line that does not express endogenous FOXA3, increased expression of SPDEF, MUC5AC, MUC5B, AGR2, and ITLN1 (Figures 2B–2D). Because both SPDEF and AGR2 play critical roles in the regulation of mucus production (13, 26), we tested the effects of Foxa3 on their transcriptional activity. Foxa3 activated both *Agr2* and *Spdef* gene promoters *in vitro* (*see* Figure E1 in the online supplement). To test whether Foxa3 was sufficient to induce goblet cell differentiation *in vivo*, transgenic mice were generated in which the mouse *Foxa3* cDNA was conditionally expressed in nonciliated airway epithelial cells using *Scgb1a1*-rtTA (line 2) (27) transgenic mice (Figure 3A). Doxycycline induced Foxa3 and caused marked goblet cell metaplasia (Figures 3B and 3C). Conditional expression of Foxa3 in nonciliated secretory cells inhibited Foxa2 in goblet cells (Figure 3B), the latter

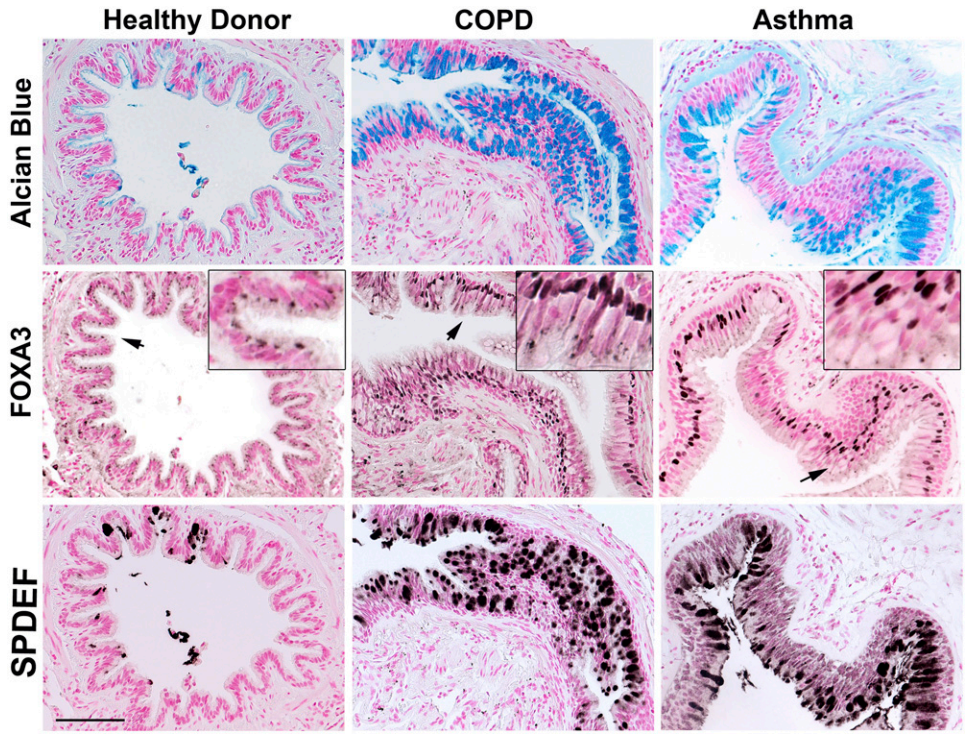

Figure 1. FOXA3 and SPDEF in airway goblet cells from individuals with asthma and chronic obstructive pulmonary disease (COPD). FOXA3 and SPDEF were detected by immunohistochemistry in lung tissue from the human patients with COPD and asthma. FOXA3 was present in nuclei and SPDEF in both nuclei and cytoplasm of goblet cells in airways from patients with COPD (n = 5) and asthma (n = 3). Alcian blue staining of adjacent slides illustrates airway mucus. FOXA3, SPDEF, and Alcian blue staining was less abundant in healthy lung tissue (n = 5) in the absence of goblet cells. Scale bar: 100 μm. *Insets* show higher magnification of regions indicated by *arrows*.

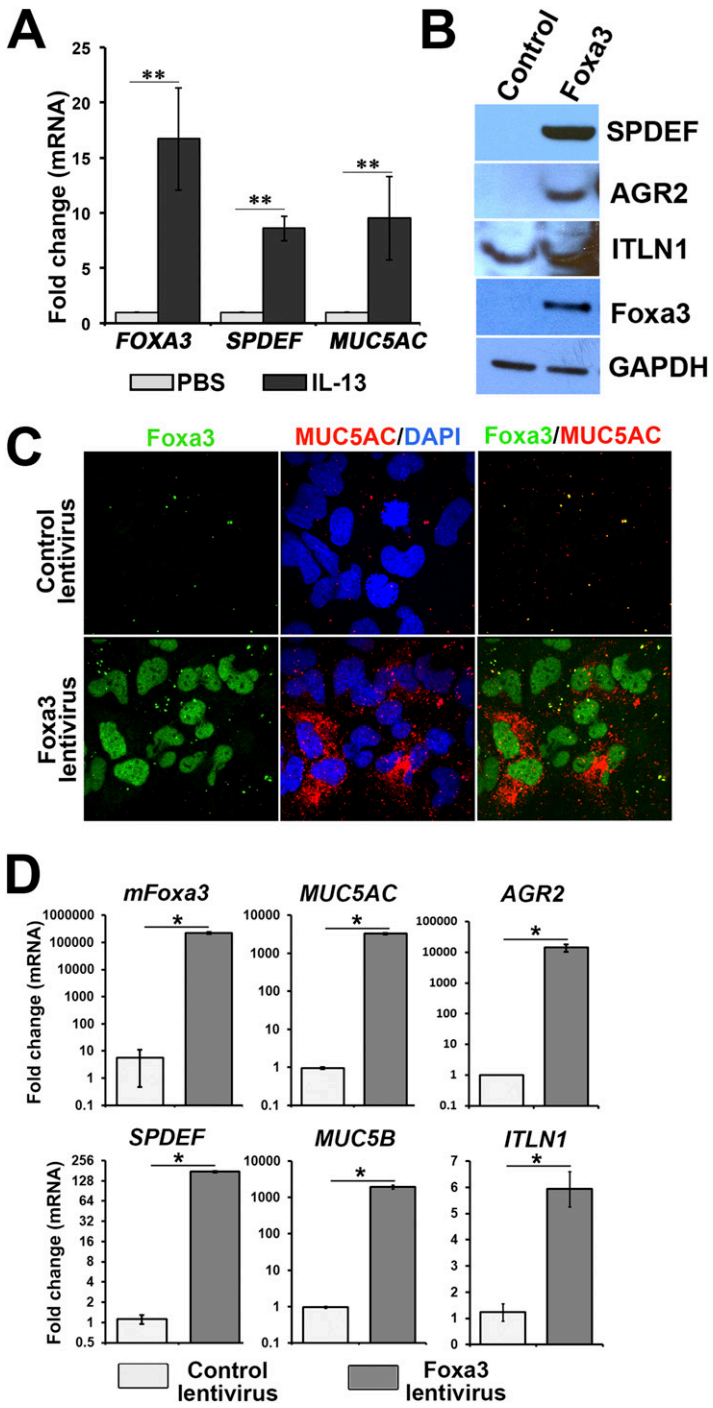

Figure 2. Foxa3 induced goblet cell differentiation *in vitro*. (*A*) *FOXA3*, *SPDEF*, and *MUC5AC* mRNAs were induced by IL-13 (10 ng/ml) in primary human bronchial epithelial cells during air–liquid interface culture for 5 days. Graph is representative of n = 5 independent experiments, mean ± SE. (*B–D*) BEAS2B cells were stably transduced with a lentivirus expressing green fluorescent protein (GFP, control) or Foxa3-IRES-GFP for 2 weeks. (*B*) Foxa3 increased SPDEF, AGR2, and ITLN1 in BEAS2B cells. Glyceraldehyde 3-phosphate dehydrogenase was used as loading control for Western blotting. (*C*) MUC5AC was induced by Foxa3 in BEAS2B cells shown by immunofluorescence staining. (*D*) Expression of Foxa3 in BEAS2B cells increased endogenous *SPDEF*, *MUC5AC*, *MUC5B*, *AGR2*, and *ITLN1* mRNAs, detected by quantitative reverse transcriptase–polymerase chain reaction after normalization to 18S rRNA. Data are expressed as mean ± SD of three independent experiments, $^{*}P < 0.001$ and $^{**}P < 0.05$ versus controls (Student *t* test, two-tailed, unpaired).

a forkhead transcription factor family member known to suppress goblet cell differentiation and Th2-mediated inflammation in the developing lung (12). Transient expression of Foxa3 in transgenic mice did not cause pulmonary inflammation or alter epithelial cell morphology in the alveoli but induced dramatic goblet cell metaplasia in conducting airways.

FOXA3 Is Required for SPDEF and Mucin Gene Expression in Human Airway Epithelial Cells

Unlike Spdef, Foxa3 was not required for differentiation of mucous cells in submucosal glands or in airway goblet cells after sensitization of mice to ovalbumin (14) (Figure E2). In contrast to these findings, inhibition of endogenous FOXA3 by lentiviral shRNA in primary HBECs and Calu-3 cells, the latter a human cell line with characteristics of submucosal gland epithelial cells, strongly inhibited IL-13–induced *SPDEF*, *MUC5AC*, *MUC5B*, *AGR2*, and *ITLN1* mRNAs (Figures 4A–4D). In primary HBECs, IL-13 induced FOXA3, SPDEF, and mucus-related RNAs; furthermore, inhibition of FOXA3 by shRNAs blocked IL-13–induced mucus-related RNAs (Figures 4C and 4D).

Goblet Cell Metaplasia Induced by FOXA3 Is Not Dependent on SPDEF

Spdef is both necessary and sufficient for airway goblet cell metaplasia in mice (13). Because Foxa3 induced SPDEF, we used siRNA to test whether the effects of FOXA3 on mucus production were dependent on SPDEF. In BEAS2B cells expressing exogenous Foxa3, expression of ITLN1 and AGR2 persisted in spite of the inhibition of SPDEF. Similarly, the increase in MUC5AC induced by Foxa3 was not dependent on SPDEF (Figures 4E and 4F).

Rhinoviral Infection Induces FOXA3 and Causes Goblet Cell Metaplasia *In Vivo* and *In Vitro*

RVs, pathogens associated with the common cold and clinical exacerbations in chronic pulmonary diseases (e.g., asthma, CF, and COPD), readily infect airway epithelial cells causing airway inflammation and mucus production. RV1B (a mouse-adapted human RV, the minor group serotype of RV) induced Spdef, Foxa3, and Muc5ac, and goblet cell metaplasia in conducting airways 3 days after pulmonary

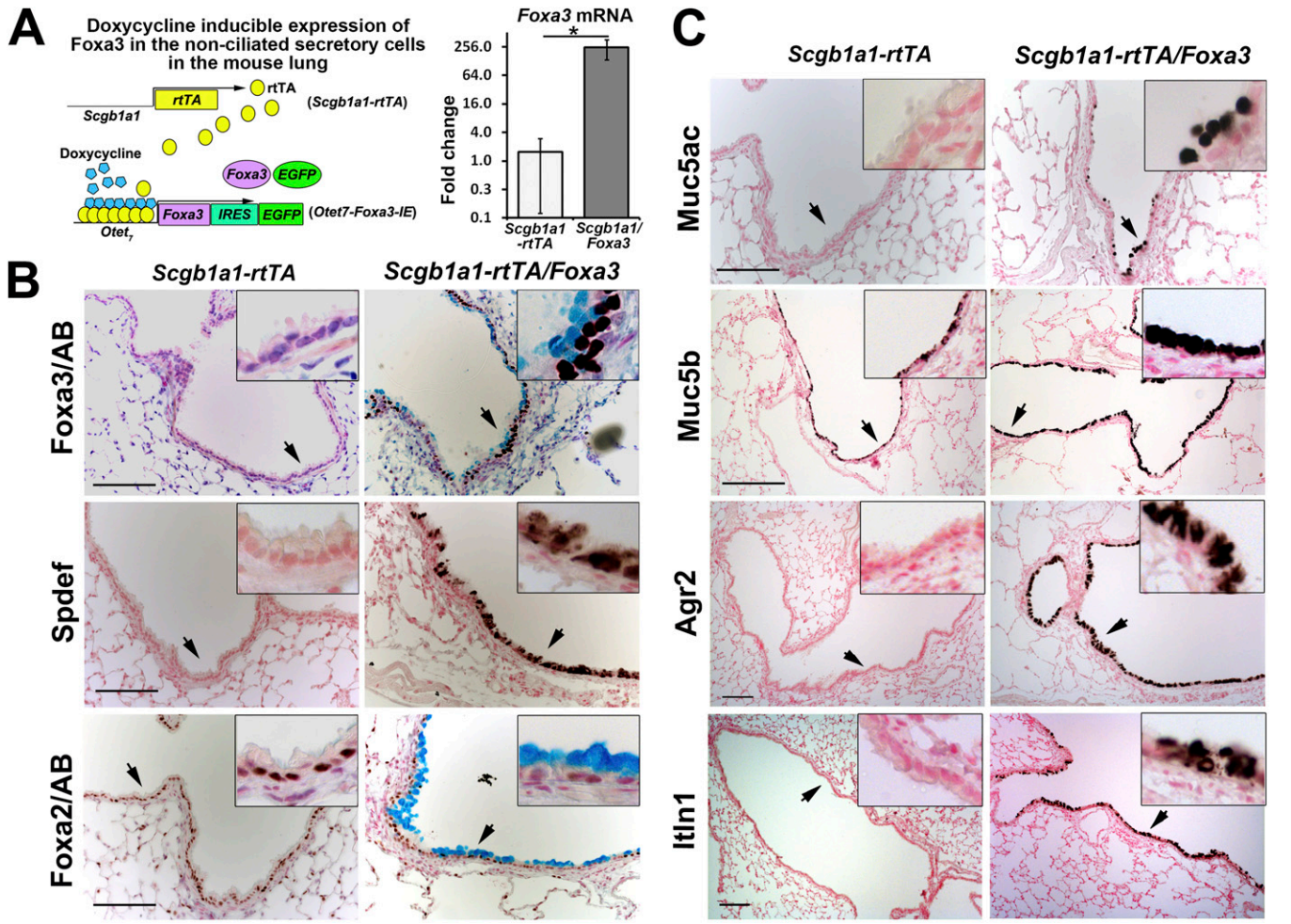

Figure 3. Foxa3 caused goblet cell metaplasia *in vivo*. (*A*) Transgenic mice were produced in which Foxa3 was conditionally induced in airway epithelial cells under control of the *Scgb1a1* gene promoter. Mice were treated with doxycycline for 3 days. (*A*, *B*) *Foxa3* mRNA and protein were increased. Foxa3 caused goblet cell metaplasia as indicated by increased Alcian blue (AB), Spdef, and inhibition of Foxa2 staining. (*C*) Foxa3 increased Muc5ac, Muc5b, Agr2, and Itln1 staining in airway epithelial cells. Scale bar: 100 μm. *Insets* show regions indicated by *arrows*. The graph represents mean ± SD of five independent transgenic mice of each genotype, $^*P < 0.01$ versus control mice (Student *t* test, two-tailed, unpaired).

infection (Figure 5A). Likewise, exposure of primary HBECs to RVs (both RV16, a major group serotype, and RV1B) *in vitro* increased *FOXA3*, *SPDEF*, and *MUC5AC* mRNAs 48 hours after infection (Figure 5B). Because RV infection activates IFN to mediate antiviral innate host defenses in airway epithelial cells, we assessed whether IFN-β regulated *FOXA3* and goblet cell–related gene expression. IFN-β increased *FOXA3*, *SPDEF*, and *MUC5AC* mRNAs in primary HBECs 24 hours after exposure (Figure 5C).

Foxa3 Inhibits RV Clearance *In Vivo*

Because Foxa3 is not normally widely expressed in airway epithelial cells in the adult mouse lung, we used HDM sensitization to enhance Foxa3 before RV instillation. To assess the role of Foxa3 in viral clearance, wild-type and *Foxa3*$^{-/-}$ mice were sensitized with HDM lysates, followed by RV1B infection. As expected, FOXA3 was expressed in *Foxa3*$^{+/+}$ control mice and was absent in the *Foxa3*$^{-/-}$ mice after sensitization with HDM and exposure to RV1B (Figure 6A). Although lack of Foxa3 did not block expression of goblet cell–related mRNAs (e.g., *Spdef*, *Muc5ac*, and *Muc5b*) (Figure E3), *Ifnb1* mRNA and clearance of RV1B vRNA 24 hours after infection were significantly increased in *Foxa3*$^{-/-}$ mice (Figure 6B). To test whether Foxa3 influenced viral clearance from the lung, Foxa3 was induced by treatment of *Scgb1a1-rtTA*, *Otet*$_7$*-Foxa3-IRES-EGFP* mice with doxycycline for 2 weeks before RV1B infection. Conditional expression of Foxa3 in airway epithelial cells significantly inhibited RV clearance in the mouse lung (Figure 6C).

FOXA3 Inhibits ISGs, Viral Clearance, and Apoptosis after RV Infection *In Vitro*

In BEAS2B cells, Foxa3 strongly inhibited type I (*IFNβ1*) and type III (*IFNλ1*, *IFNλ2/3*) IFN mRNAs from 8 to 48 hours after RV1B infection. mRNAs encoding critical regulators of IFN responses in airway epithelial cells, including *TLR3*, *MDA5* (*IFIH1*) (16, 17), and *IKKε* (*IKBKE*) (28), were suppressed by Foxa3. Foxa3 strongly inhibited ISGs, including *MX1*, *IFIT1*,

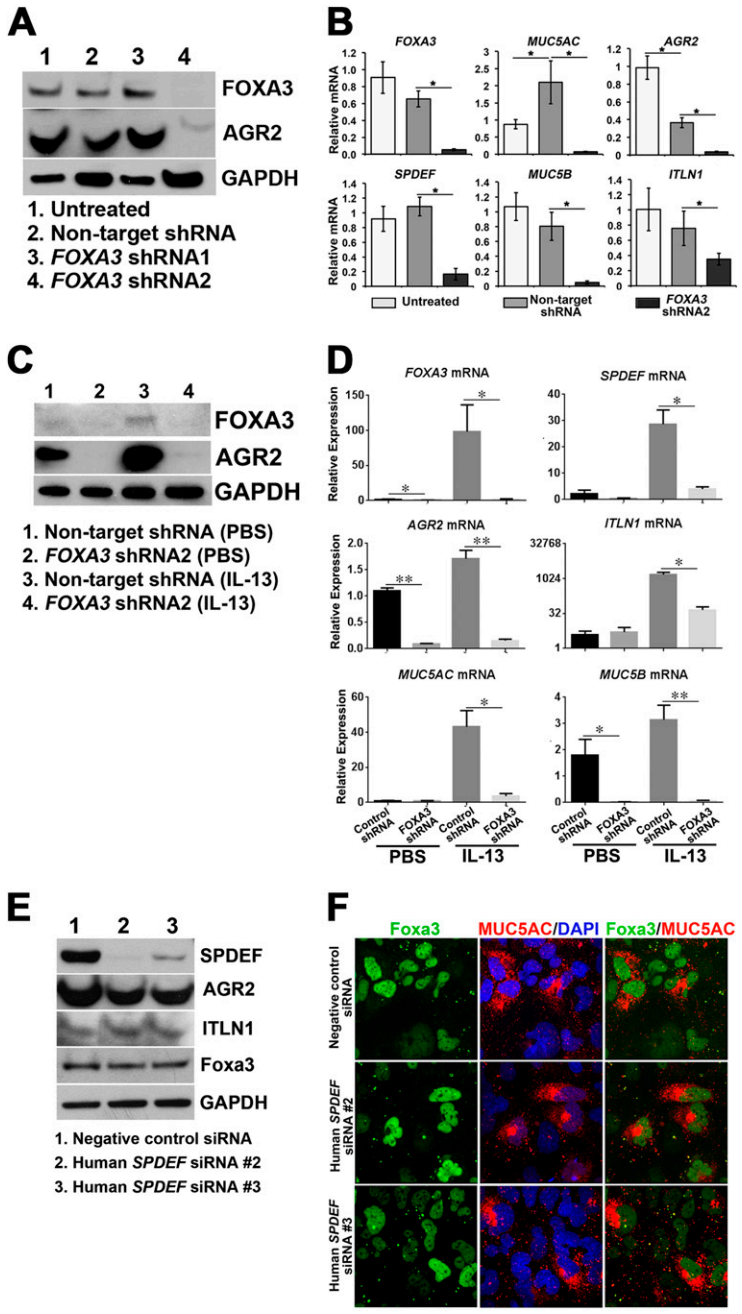

Figure 4. Mucous metaplasia–associated gene expression was dependent on FOXA3 in human respiratory epithelial cells. (*A*, *B*) FOXA3 was inhibited by lentiviral shRNA-mediated knockdown in Calu-3 cells resulting in suppression of *SPDEF*, *MUC5AC*, *MUC5B*, *AGR2*, and *ITLN1* mRNAs and decreased AGR2 as assessed by Western blot. FOXA3 shRNA1 was not effective. (*B*) Graphs are representative of three independent experiments, means ± SD, $^{*}P < 0.01$ versus controls, Student *t* test, two-tailed, unpaired; mRNA normalized to endogenous 18S rRNA. (*C*, *D*) FOXA3 shRNA2 lentivirus-mediated knockdown inhibited endogenous *FOXA3* mRNA and protein in primary human bronchial epithelial cells (HBECs). Primary HBECs were transduced with either nontarget shRNA or FOXA3 shRNA2 lentivirus and cultured under air–liquid interface for 10 days with puromycin (1 μg/ml). Cells were then treated with either phosphate-buffered saline (PBS) or recombinant human IL-13 (10 ng/ml) for 5 days. Loss of FOXA3 suppressed IL-13–induced AGR2 protein and *SPDEF*, *ITLN1*, *MUC5AC*, and *MUC5B* mRNAs. Graphs represent means ± SE of three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ versus controls, Student *t* test, two-tailed, unpaired; mRNA normalized to endogenous 18S rRNA. (*E*) siRNA-mediated inhibition of endogenous SPDEF did not inhibit lenti-Foxa3–induced AGR2 or INTL1 or MUC5AC expression as shown by immunofluorescence staining (*F*) in BEAS2B cells.

OAS2, *ISG15*, *RIG-I* (*DDX58*), and VIPERIN (*RSAD2*) mRNAs (Figure 7A and E4A). Consistent with these findings, rhinoviral clearance was suppressed (Figure 7A). Inhibition of IFNs and ISGs by Foxa3 after RV1B infection was not due to decreased expression of the RV1B receptor, *LDLR*, or the IFN receptors, *IFNAR1* and *IFNAR2* in BEAS2B cells (Figure E4B). Type I IFNs induce ISG factor-3 (ISGF3), a complex composed of activated (phosphorylated) STAT1, STAT2, and IRF9. Activated ISGF3 is translocated to nucleus and binds to IFN-Stimulated Response Elements (ISRE) present on promoters of ISGs (29). Foxa3 inhibited activation of ISGF3 downstream of IFN signaling, inhibiting STAT1 and STAT2 phosphorylation, RV-induced MDA5, and cleaved caspase-3 in BEAS2B cells (Figure 7B). Transcription of IFNs is regulated by phosphorylation of IFN Regulatory Factor 3 (IRF3) (30) by IKKε and TBK1 (28). It is likely that FOXA3 inhibits IRF-3 phosphorylation by suppressing expression of IKKε but not TBK1 in BEAS2B cells (Figure 7C). Inhibition of IFN responses by Foxa3 was demonstrated *in vitro* using reporter constructs consisting of the *IFN-β* (*pRD I/III-luc*) and ISGs promoters (*pISRE-luc*) in luciferase assays. Foxa3 inhibited TRIF-induced activation of *pRD I/III-luc*, *pISRE-luc* promoters, and mRNAs of *IFNs* and *ISGs* in HEK293-T cells (Figure E5) and inhibited poly I:C and RV1B-induced IFN production in H358 cells (Figure E6).

Inhibitory Effects of FOXA3 on Antiviral Gene Expression and Viral Clearance Are Reversed by IFN-β

IFN production, viral clearance, and apoptotic responses to RV infection are deficient in airway cells from patients with asthma and CF (20, 22). Treatment of Foxa3-expressing BEAS2B cells with IFN-β restored STAT1 and STAT2 phosphorylation and expression of ISGs. IFN-β rescued viral clearance and induced apoptosis after RV infection in Foxa3-expressing BEAS2B cells (Figure E7 and E8). Inhibition of endogenous *IFNβ1*, *IFNλ1*, *IFNλ2/3*, *TLR3*, IKKε, and phosphorylation of IRF3 were not reversed by IFN-β (Figures E7A and E7C). Taken together, inhibition of IFN production by Foxa3 is likely mediated, at least in part, by inhibition of IRF-3 phosphorylation and IKKε expression.

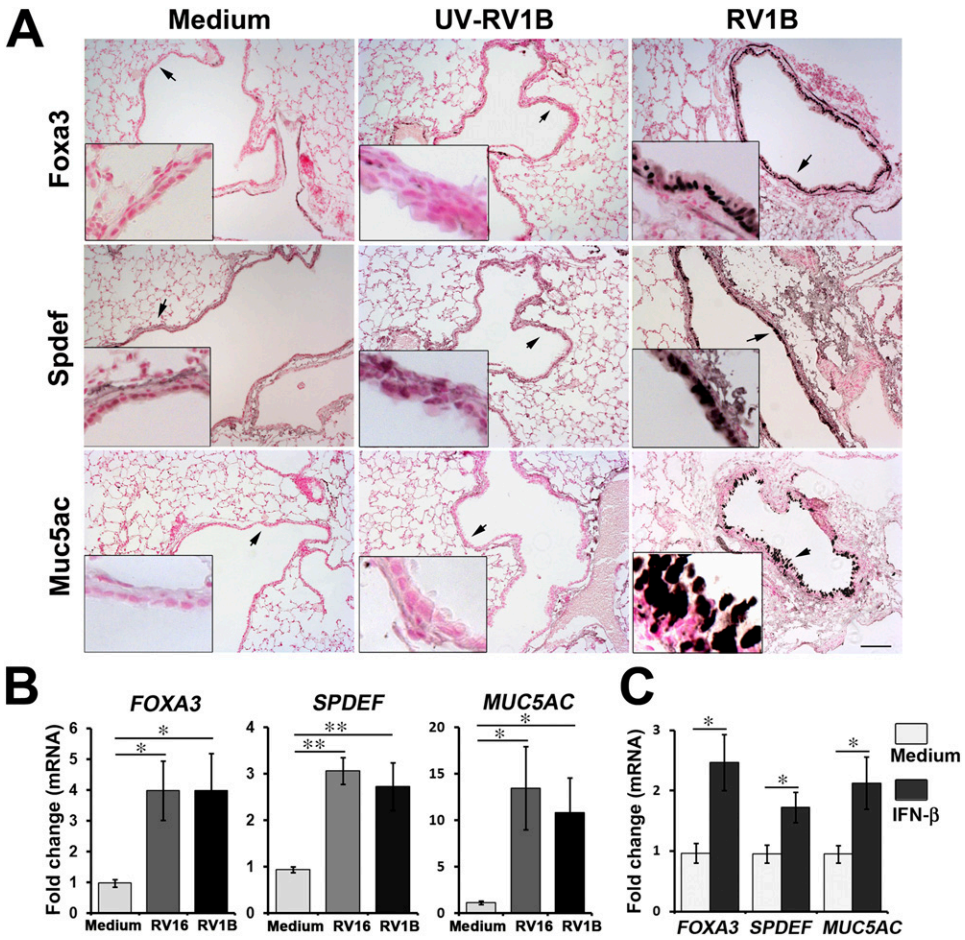

Figure 5. Rhinovirus and IFN-β caused goblet cell metaplasia. (*A*) Foxa3 staining and goblet cell differentiation were assessed *in vivo*. Adult FVB/N mice were treated with 10^7 pfu (100 μl of volume) of rhinovirus (RV1B), ultraviolet (UV)-irradiated RV1B, or HeLa cell conditioned medium by intratracheal injection. Foxa3, Spdef, and Muc5ac were increased in airways 3 days after RV1B infection. Sections are representative of n = 3–5 mice in each treatment group. Scale bar: 100 μm. (*B*, *C*) Exposure of primary human bronchial epithelial cells (HBECs) to RV16, RV1B, and IFN-β–induced *FOXA3*, *SPDEF*, and *MUC5AC* mRNAs. Data are expressed as mean ± SE of five independent experiments, $^*P < 0.05$ and $^{**}P < 0.01$ versus controls (Student *t* test, two-tailed, unpaired). mRNA was normalized to endogenous 18S rRNA.

mRNA and ChIP-Sequence Analysis Identify FOXA3-regulated Genes

mRNA microarray, RNA sequence (RNA-seq), and ChIP-Seq analysis were used to identify potential transcriptional targets of Foxa3. BEAS2B cells were engineered to express mouse *Foxa3* cDNA with a lentiviral vector. A heat map of mRNAs altered by Foxa3 is shown in Figure 8A. In the absence of RV, Foxa3 induced the expression of groups of genes associated with goblet cell metaplasia, including *SPDEF*, *MUC5AC*, *MUC5B*, and *AGR2* mRNAs. Foxa3 suppressed genes involved in antiviral responses, cell survival, and programmed cell death, including *TLR3*, *NFKBIA* (31), *CASP1,4,8* (32, 33), and *FAS* (34). In the presence of RV, Foxa3 inhibited groups of genes involved in "response to virus," "virus RNA replication," and "IFN signaling" (Figure 8B). As shown in Figure E9, network analysis supports a model by which FOXA3 inhibits IFN signaling through the interactions of regulatory hubs including IRF, STAT, and nuclear factor (NF)-κB. RNA-Seq analyses of BEAS2B cells infected with FOXA3 showed a high degree of correlation with microarray data (83% overlap, $r = 0.64$, Figure E10).

ChIP-Seq analyses were used to identify potential sites by which FOXA3 regulated goblet cell and innate immune response–related gene expression. Foxa3 lentivirus-transduced BEAS2B cells were subjected to Foxa3 ChIP-Seq, and two biological replicate libraries were generated. Approximately 28 million reads were sequenced, of which ∼4.1 million clustered into regions that were enriched with FOXA3 binding compared with genomic background (false discovery rate < 0.01). Irreproducible discovery rate analysis of the two ChIP-Seq experiments identified ∼14,000 replicated regions of FOXA3 occupancy (irreproducible discovery rate < 0.1). FOXA3 occupancy was enriched at transcriptional start sites (Wilcoxon rank sum, $P < 1 \times 10^{-11}$) and highly correlated with mRNAs induced by Foxa3 in the lentivirus-transduced cells (Figure 9A–9D). FOXA3 ChIP-Seq peaks were enriched at the promoters of genes activated and repressed by FOXA3 (Wilcoxon rank sum, $P < 1 \times 10^{-6}$; Figures 9A–9C). To further assess the specificity of the ChIP-Seq data, we performed *de novo* motif analyses of the

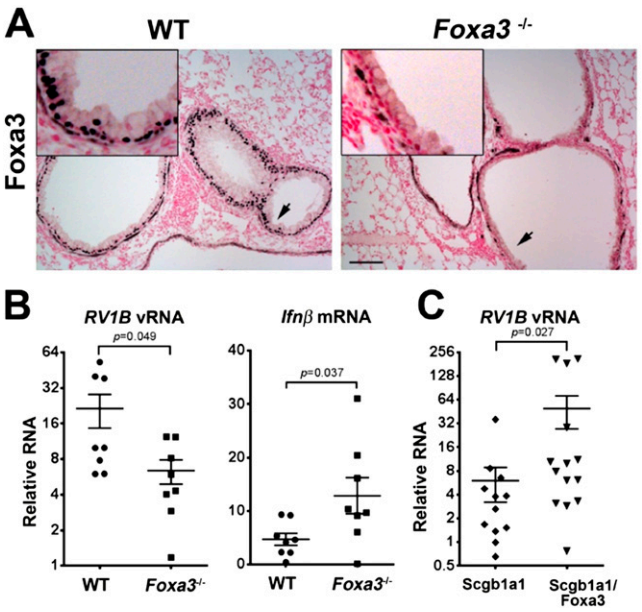

Figure 6. Foxa3 inhibited rhinovirus clearance *in vivo*. Wild-type (WT) and Foxa3$^{-/-}$ mice were sensitized with house dust mite (HDM) extract to induce endogenous Foxa3 in the airway epithelium before rhinovirus (RV1B) infection. (*A*) Nuclear staining of Foxa3 was detected in the airway epithelium of HDM- and RV1B-sensitized WT mice (n = 3) and was absent in *Foxa3*$^{-/-}$ mice (n = 3). Scale bar: 100 μm. (*B*, *C*) RV1B viral RNA (vRNA) was assessed in lung homogenates from *Foxa3*$^{-/-}$ and *Scgb1a1/Foxa3*-expressing mice after viral infection. Increased viral clearance, indicated by decreased *RV1B* vRNA remaining, and increased *Ifnβ* mRNA in the lung were observed in the *Foxa3*$^{-/-}$ mice (n = 8) compared with WT mice (n = 8) (*B*). (*C*) Conditional expression of Foxa3 in the airway epithelium impaired viral clearance from the lung. Foxa3 was induced in the airway epithelium of *Scgb1a1-rtTA/Otet*$_7$*-Foxa3-IRES-EGFP (Scgb1a1/Foxa3)* transgenic mice by treatment with doxycycline for 2 weeks before RV1B exposure. Viral clearance was decreased in mice expressing Foxa3 (*Scgb1a1/Foxa3*, n = 14) compared with control mice (*Scgb1a1*, n = 12). Data were presented as means ± SE, analyzed by a two-tailed, unpaired, nonparametric Mann-Whitney *t* test. vRNA and mRNA were normalized to endogenous 18S rRNA.

top 500 ChIP-Seq peaks. Weeder and Meme2 algorithms (35, 36) identified a motif similar to the previously described FOXA3 consensus binding site TGTTTAC, consistent with previous findings in HepG2 cells (Figure 9E) (37). Importantly, accumulation of this motif at FOXA3 ChIP-Seq peaks was highly significant (Wilcoxon rank sum, $P < 1 \times 10^{-6}$) and highly correlated with peak rank (as expected for a Poisson distribution) (Figure 9F). There was no significant enrichment for this motif at randomized regions of the genome, including regions offset from FOXA3 peaks, or at transcriptional start sites that lacked adjacent FOXA3 peaks. Moreover, FOXA3 ChIP-Seq peaks adjacent to the *SPDEF*, *AGR2*, *MUC5AC*, and *IKKε* gene transcriptional start sites correlated well with the presence of predicted FOXA3 binding sites (Figures 9G, 9H, and E11). Taken together, FOXA3 binds to and activates *SPDEF*, *MUC5AC*, and *AGR2* genes that play important roles in goblet cell metaplasia (13, 26, 38). We also linked FOXA3 occupancy to the repression of genes, such as *IKKε* (*IKBKE*) and *NFKBIA*, which may contribute to suppression of innate antiviral immunity and programmed cell death.

Foxa3 Induced *TSLP* and *CCL-26* in Airway Epithelial Cells

Because FOXA3 was induced by IL-13 (Figures 2A and 4), we tested whether FOXA3 regulated factors expressed by epithelial cells that influence Th2-mediated immunity. Th2 cytokines and RV infection are known to enhance TSLP and CCL-26 (eotaxin-3) expression in primary human airway epithelial cells (39, 40). Foxa3 markedly stimulated *TSLP* and *CCL-26* mRNAs in BEAS2B cells (Figure E10, E12A, and E12B). *CCL-26* mRNA was further enhanced when Foxa3-expressing BEAS2B cells were exposed to RV1B and IL-13 (Figure E12C), supporting the concept that expression of Foxa3 in respiratory epithelial cells may influence Th2-mediated inflammation. Likewise, *FOXA3* shRNA blocked IL-13 induced *CCL26* in primary HBECs in air–liquid interface cultures (Figure E12D). Taken together, FOXA3 induces goblet cell differentiation, inhibits Th1 antiviral responses, and enhances expression of *TSLP* and CCL-26 that may serve to enhance Th2 immunity.

Discussion

The present study demonstrates that FOXA3 plays an unexpected, epithelial cell–autonomous role integrating goblet cell metaplasia/mucus production and suppression of antiviral innate immunity. FOXA3 was sufficient to induce goblet cell metaplasia *in vivo* and *in vitro*. We propose a model that supports a counterregulatory role for FOXA3 in suppressing ongoing inflammation, perhaps mediating resolution of acute infections in the normal airway. In turn, persistent expression of FOXA3 and accompanying goblet cell metaplasia may inhibit antiviral responses that may render individuals with chronic pulmonary disorders susceptible to recurrent pulmonary infection.

In both *in vitro* and *in vivo* studies, we found that FOXA3 was sufficient to cause goblet cell metaplasia in airway epithelial cells. Because FOXA3 bound to and induced *SPDEF*, a gene required for goblet cell differentiation in the airway epithelium, the observed effects of FOXA3 on mucus-related gene expression are likely mediated, at least in part, by its ability to induce SPDEF. However, FOXA3 directly bound to and induced *AGR2* and *MUC5AC* that are critical for mucus production/goblet cell metaplasia in the airways (1, 26, 38), functioning independently of SPDEF to regulate these genes in human airway epithelial cells (Figures 4, 9, and E11). Present findings that FOXA3 mediated goblet cell–associated genes in human respiratory epithelial cells, including HBECs, Calu3 cells, and BEAS2B cells, may contrast with findings in the allergen-exposed mouse models wherein *Spdef*, but not *Foxa3*, was required for goblet cell metaplasia (13, 14), differences that may be related to epithelial cell types, species

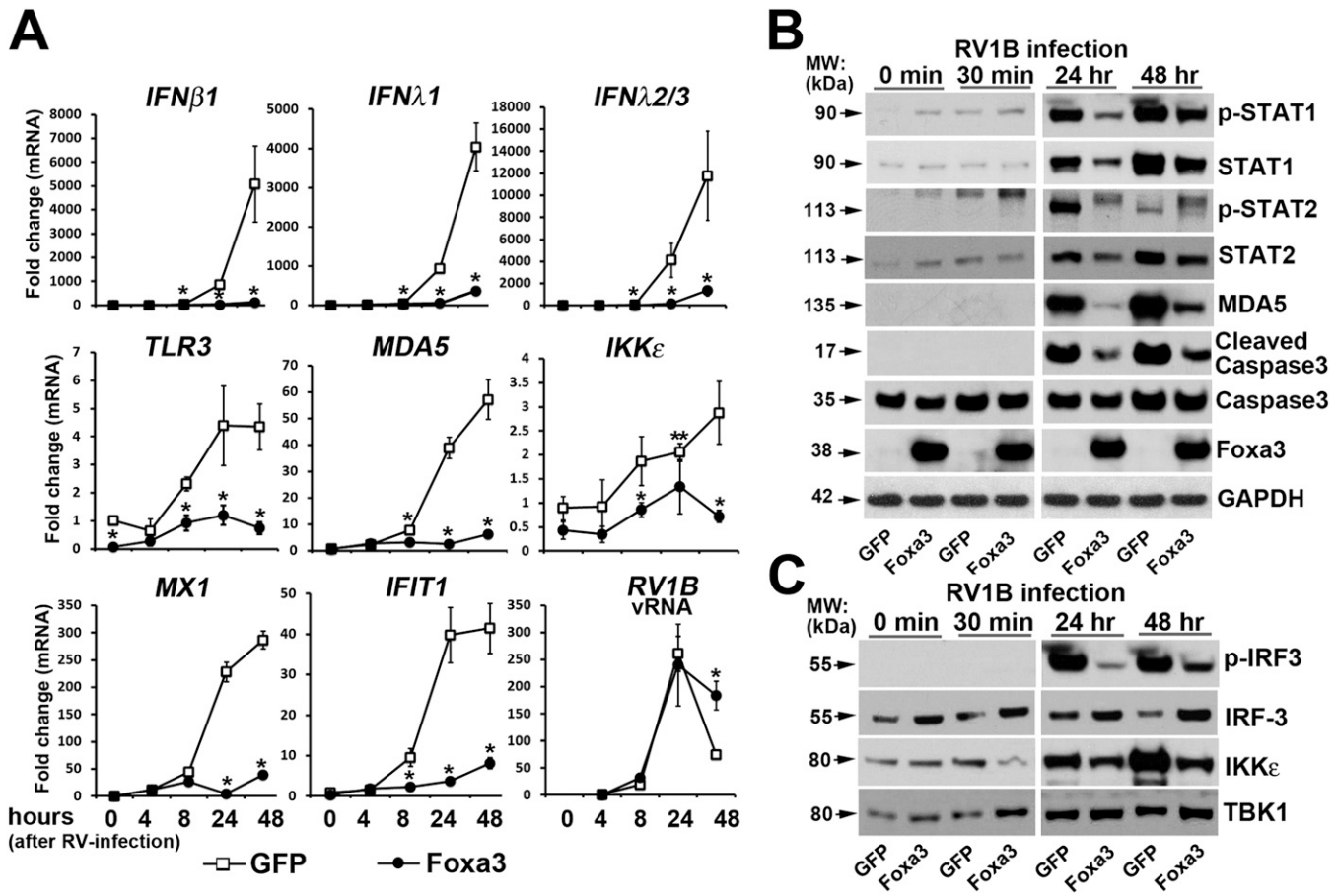

Figure 7. FOXA3 inhibited IFNs, IFN-stimulated genes, and viral replication *in vitro*. BEAS2B cells expressing lenti-GFP or lenti-Foxa3 were exposed to rhinovirus (RV1B) (multiplicity of infection of 2) for 30 minutes to 48 hours (panels *A–C*). (*A*) Foxa3 inhibited *IFNβ1*, *IFNλ1*, *IFNλ2/3*, *TLR3*, *MDA5*, *MX1*, *IFIT1*, and *IKKε* mRNAs. *RV1B* viral RNA (vRNA) was increased by Foxa3, indicating inhibition of viral clearance at 48 hours. vRNA and mRNA were normalized to endogenous 18S rRNA. Data represent means ± SD of three independent experiments, $^{*}P < 0.001$, $^{**}P < 0.05$ versus control, Student *t* test, two-tailed, unpaired. (*B*) Western blot analysis demonstrated that Foxa3 inhibited STAT1, STAT2 phosphorylation, and MDA5. Foxa3 inhibited RV1B-induced apoptosis shown by decreased cleaved-caspase 3. (*C*) Western blot demonstrated that IKKε and phosphorylation of IRF3 were inhibited by Foxa3. TBK1 expression was induced in the presence of Foxa3 at 30 minutes, and only slightly increased by Foxa3 24 hours and 48 hours after RV infection. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control for Western Blotting. GFP = green fluorescent protein; MW = molecular weight.

differences, and the nature and duration of inducing stimuli *in vivo* and *in vitro*.

Mucous metaplasia associated with chronic lung diseases is often accompanied by increased sensitivity to respiratory viral infection. Bronchial epithelial cells from patients with asthma lack normal IFN responses to RV and fail to clear the virus normally (20). The relationship between virus, FOXA3, and IFN response are complex and are likely dose dependent. For example, IFN-α/β has paradoxical effects on hepatitis B virus replication. IFN-α/β enhanced viral replication, Foxa3, and activating STAT3, which together stimulated hepatitis B virus gene expression and replication (41, 42). In the current study, we found that FOXA3 was induced by both RV and IFN-β and strongly suppressed IFN production and an entire network of genes regulated by IFNs in airway epithelial cells, including those critical for viral recognition and viral replication. FOXA3 inhibited *STAT-2*, *TLR3*, *IRF-1*, *IRF-7*, and *IRF-9* mRNAs, supporting its broad role in the regulation of ISGs. Because IRF-3 phosphorylation by IKKε is known to be a critical transcriptional regulator of IFN production (28), the finding that FOXA3 inhibited RV-induced IKKε, and directly bound to the *IKKε* gene promoter in ChIP-Seq experiments (Figures E11B and E11D) is consistent with a direct transcriptional role for FOXA3 in the inhibition of IFN pathways that is mediated, at least in part, via its inhibitory effects on IRF-3 phosphorylation. Some of the antiinflammatory effects of FOXA3 may be influenced by its ability to enhance SPDEF, the latter recently shown to inhibit NF-κB by binding to and inhibiting the adaptor proteins MyD88 and TRIF in the cell cytoplasm (23). Thus, as observed in the regulation of mucus-associated gene expression, FOXA3 and SPDEF may serve both overlapping and complementary roles in the regulation of innate immunity and viral host defenses in airway epithelial cells. Paradoxically, although RV and IFN initiate strong antiviral innate immune responses in respiratory epithelial cells, we speculate that the induction of Foxa3 may serve a counterregulatory role that may be useful in dampening inflammation during the recovery from acute infections but may inhibit antiviral responses in the setting of

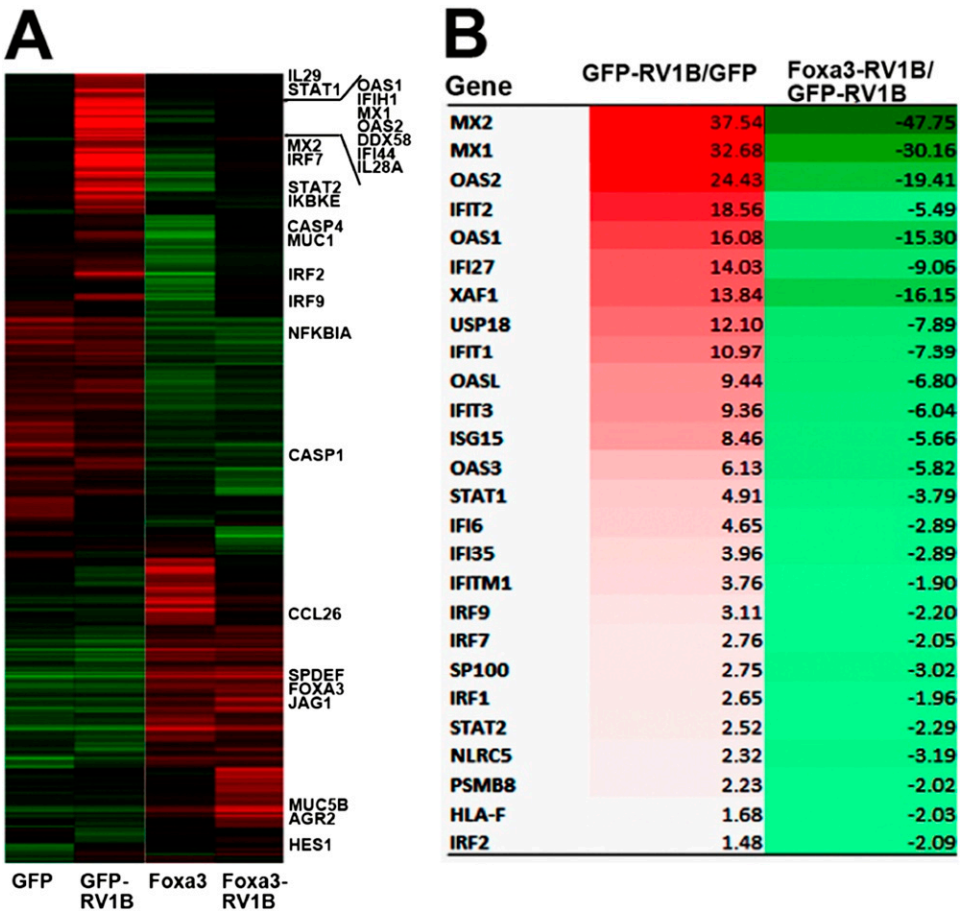

Figure 8. FOXA3 induced mucus-related gene expression and suppressed genes involved in response to virus. (*A*) mRNAs were isolated from lentiviral Foxa3-expressing and control BEAS2B cells and subjected to mRNA microarray analysis. A heat map of mRNAs altered by Foxa3 in presence or absence of rhinovirus (RV1B) is shown. *Green* and *red* denote mRNAs decreased and increased, respectively, by Foxa3 with or without RV1B infection. (*B*) Type I interferon-mediated signaling pathway (green fluorescent protein [GFP]-RV1B vs. GFP) was the most suppressed bioprocess when comparing Foxa3-RV1B versus GFP-RV1B. A list of mRNAs involved in type I IFN-mediated signaling pathway influenced by Foxa3 in the presence of virus is shown.

chronic mucous metaplasia. The present findings are consistent with recent studies wherein IFN was required for normal viral clearance during acute infection but suppressed viral responses in the setting of chronic infection (43, 44).

Treatment with IFN enhanced RV clearance and restored apoptotic responses in airway epithelial cells derived from patients with asthma and CF in which these responses were impaired (20–22, 45, 46). We presently observed that Foxa3 inhibited RV-induced caspase-3 cleavage, as well as *caspase-1* and *caspase-4* mRNAs, inhibition of caspase-3 cleavage being reversed by treatment with IFN-β. The inhibitory effects of Foxa3 on apoptotic pathways in airway epithelial cells may contribute to its inhibitory effects on RV clearance, findings consistent with previous studies that associated goblet cell metaplasia with poor apoptotic response (47, 48) and increased susceptibility to RV infection (49).

Respiratory epithelial cells produce cytokines and chemokines that serve as proximal events in initiating and amplifying inflammatory and immune responses in bone marrow–derived cells (e.g., dendritic cells, macrophages, neutrophils, lymphocytes, eosinophils, and mast cells) (50). Previous studies indicate that the *TSLP*, the epithelial cell–derived cytokine that activates maturation of dendritic cells, is induced by NF-κB (51) and by RV (39), the latter in a process mediated by IRF-3 (52). In the present study, we demonstrated that FOXA3 induced expression of *TSLP* and *CCL26*, providing a potential mechanism linking goblet cell metaplasia to the expression of Th2 polarizing mediators by the respiratory epithelium.

The present studies identify a novel role of FOXA3 in the regulation of goblet cell differentiation and suppression of antiviral responses in airway epithelial cells. FOXA3 plays a role in a transcriptional network with SPDEF that induces goblet cell metaplasia and modulates Th1 and Th2 innate immune responses. The current study defines a novel molecular mechanism that may contribute to mucus production, impairment of IFN responses, and deficient viral host defenses characteristic of individuals with asthma, COPD, and CF. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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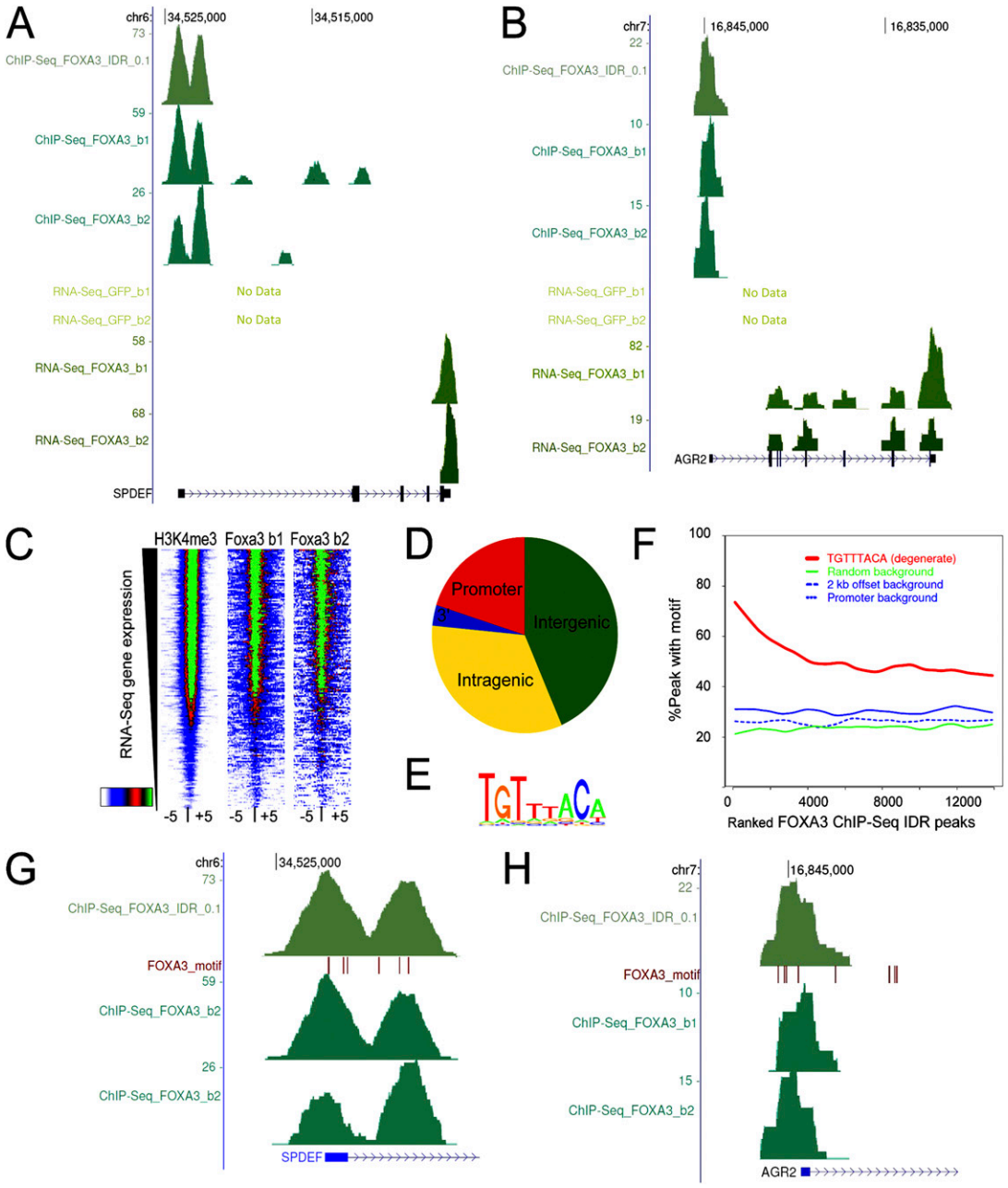

Figure 9. Chromatin immunoprecipitation sequencing (ChIP-Seq) and RNA sequencing (RNA-Seq) identify transcriptional targets of FOXA3 involved in goblet cell metaplasia. ChIP-Seq was performed on BEAS2B cells after infection with lenti-Foxa3 or control lentivirus as described in the online supplement. (*A*, *B*) UCSC Genome browser tracks depict FOXA3 ChIP-Seq peaks and irreproducible discovery rate (IDR)-selected peaks (IDR is analogous to the false discovery rate) from two separate experiments (b1 and b2) demonstrating FOXA3 binding to the promoter and first intronic region of the *SPDEF* and *AGR2* genes. The ChIP-Seq peaks are shown at the top three tracks of the figure. RNA-Seq reads density are shown at the bottom two tracks of the figure. (*C*) Heat maps depict BEAS2B ChIP-Seq sequence density (500-bp bins) of all RefSeq genes rank-ordered by levels of gene expression from RNA-Seq data that show the correlation of H3K4me3 (activation mark) with the density of FOXA3 binding sites in two independent experiments (b1 and b2). The color ramp shows maximum–minimum–scaled expression as follows: *green* > *red* > *blue* > *white*. Regions 5 kb up- and downstream of transcriptional start sites (TSS) are depicted with the TSS located in the middle. Regions surrounding TSS displayed highest ChIP-Seq tag density as well as highest level of gene expression. Expression levels were determined by sorting RNA-Seq data by normalized tag counts annotated to RefSeq gene exons. All RefSeq gene lengths were scaled to 1. (*D*) Pie chart depicts the genomic annotation of FOXA3 binding sites with respect to known RefSeq genes in UCSC genome browser. Percentage distribution of FOXA3 binding sites are shown by color: promoter, *red*; 3′, intergenic, *green*; intragenic, *yellow*. (*E*) *De novo* motif algorithm identifies the FOXA3 consensus motif overrepresented in the FOXA3 ChIP-Seq peak regions. (*F*) An accumulation plot depicts the percentage of FOXA3 consensus binding motif relative to all FOXA3 IDR-selected ChIP-Seq peaks. Offset denotes a region 2 kb upstream of the ChIP-Seq locus used to assess nonspecific binding. Random indicates randomized motif positions. "Promoter" indicates gene start sites without adjacent ChIP-Seq peaks. (*G*, *H*) UCSC genome browser tracks highlight FOXA3 motifs relative to the promoter-proximal *SPDEF* and *AGR2* ChIP-Seq peaks.

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