SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production

Gang Chen,1 Thomas R. Korfhagen,1 Yan Xu,1 Joseph Kitzmiller,1 Susan E. Wert,1 Yutaka Maeda,1 Alexander Gregorieff,2 Hans Clevers,2 and Jeffrey A. Whitsett1

1The Perinatal Institute, Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center and the University of Cincinnati School of Medicine, Cincinnati, Ohio, USA. 2Netherlands Institute of Developmental Biology, Utrecht, The Netherlands.

Various acute and chronic inflammatory stimuli increase the number and activity of pulmonary mucus-producing goblet cells, and goblet cell hyperplasia and excess mucus production are central to the pathogenesis of chronic pulmonary diseases. However, little is known about the transcriptional programs that regulate goblet cell differentiation. Here, we show that SAM-pointed domain–containing Ets-like factor (SPDEF) controls a transcriptional program critical for pulmonary goblet cell differentiation in mice. Initial cell-lineage–tracing analysis identified nonciliated secretory epithelial cells, known as Clara cells, as the progenitors of goblet cells induced by pulmonary allergen exposure in vivo. Furthermore, in vivo expression of SPDEF in Clara cells caused rapid and reversible goblet cell differentiation in the absence of cell proliferation. This was associated with enhanced expression of genes regulating goblet cell differentiation and protein glycosylation, including forkhead box A3 (Foxa3), anterior gradient 2 (Agr2), and glucosaminyl (N-acetyl) transferase 3, mucin type (Gcnt3). Consistent with these findings, levels of SPDEF and FOXA3 were increased in mouse goblet cells after sensitization with pulmonary allergen, and the proteins were colocalized in goblet cells lining the airways of patients with chronic lung diseases. Deletion of the mouse Spdef gene resulted in the absence of goblet cells in tracheal/laryngeal submucosal glands and in the conducting airway epithelium after pulmonary allergen exposure in vivo. These data show that SPDEF plays a critical role in regulating a transcriptional network mediating the goblet cell differentiation and mucus hyperproduction associated with chronic pulmonary disorders.

Introduction

Goblet cell “hyperplasia” and mucus hypersecretion contribute to the pathogenesis of common pulmonary disorders, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis (CF). Allergens, cigarette smoke, inhaled toxicants, and chronic infections induce mucous hyperproduction in conducting airways, causing airway obstruction and tissue remodeling. While initially derived from shared endodermal progenitors during lung morphogenesis (1), the conducting regions of the respiratory tract are lined by a diversity of epithelial cell types, including Clara, serous, basal, goblet (mucous), neuroepithelial, and ciliated cells, that together mediate innate host defense and mucociliary clearance to maintain sterility of the lung. While goblet cells are generally not abundant in the normal lung, goblet cell hyperplasia is induced by acute and chronic inflammation, influencing mucociliary clearance and innate host defense in the lung (2, 3). The differentiation of various pulmonary epithelial cell types is determined by both genetic and environmental factors that, in turn, regulate transcriptional programs controlling epithelial cell differentiation and behavior. During development, alveolar type II and Clara cell differentiation are dependent upon interactions of a number of transcription factors that regulate a group of genes mediating host defense and other aspects of lung function (4). In contrast, there is a paucity of knowledge regarding transcriptional programs regulating goblet cell differentiation. Goblet cells are found in many epithelial-enriched tissues where they synthesize, store, and secrete large mucopolysaccharide-rich mucins that influence mucociliary clearance and innate defense of the lung (5, 6). In the gastrointestinal tract, goblet cells are relatively abundant, and their differentiation is regulated by the Notch signaling pathway (7, 8). In the lung, goblet cells (mucous cells) are present in submucosal glands but are not abundant in conducting airways in the absence of inflammation. Numbers and activity of goblet cells are induced by a variety of acute and chronic inflammatory stimuli. Goblet cells are observed following pulmonary allergen sensitization, mediated primarily by the TH2-associated cytokines IL-4 and IL-13, which activate the IL-4 receptor, STAT6 phosphorylation, and subsequent gene expression (9–12). At the transcriptional level, pulmonary goblet cell genes are increased by allergens, dust mite, or IL-13 exposure and are associated with the decrease of forkhead box a2 (Foxa2) mRNA in bronchial and bronchiolar epithelial cells (13). In the mouse lung, deletion of the Foxa2 gene in respiratory epithelial cells is sufficient to induce goblet cell differentiation in vivo (14). The potential role of SAM-pointed domain–containing Ets-like factor (SPDEF), a member of the Ets family of transcription factors (15), in goblet cell differentiation is supported by the finding that SPDEF is induced following pulmonary allergen and IL-13 exposure (16). Chronic expression of SPDEF in epithelial cells of the mouse lung is associated with extensive goblet cell differentiation in the airways of transgenic mice (16).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 119:2914–2924 (2009). doi:10.1172/JCI39731.
The present study was undertaken to identify the role and mechanisms by which SPDEF regulates goblet cell differentiation in conducting airways of the lung. SPDEF was required for normal goblet cell differentiation in laryngeal-tracheal submucosal glands and in the bronchial-bronchiolar epithelium during experimental pulmonary allergen sensitization. SPDEF regulates a transcriptional network inhibiting Clara cell differentiation and inducing goblet cell differentiation and mucus production.

Results
Cellular origins of goblet cells induced by allergen exposure. To define the cellular precursors of goblet cells in the respiratory epithelium, lineage tracing was performed by conditionally labeling Clara cells using Cre recombinase to recombine the ROSA26 loxP/stop locus, thereby permanently expressing β-gal in Clara cells or their derivatives (Figure 1A). Administration of doxycycline to Scgb1a1-rtTA/Otet-Cre/R26R (ROSA26 Cre reporter) transgenic mice caused extensive recombination in Clara cells within 5 days, at which time β-gal staining was confined to Clara cells and not detected in ciliated cells. Goblet cell differentiation, including increased MUC5AC staining (Figure 1C), occurred without evidence of cellular proliferation, as assessed by BrdU and phospho-histone H3 labeling (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI39731DS1). Consistent with these findings, conditional expression of SPDEF

![Figure 1](https://doi.org/10.1172/JCI39731)

SPDEF caused differentiation of goblet cells from Clara cells. (A) Adult Scgb1a1-rtTA/Otet-Cre mice were mated to R26R mice. (B) To permanently label Clara cells, doxycycline (Dox) was administered from days 12 to 17 during i.p. sensitization with OVA. Mice were sacrificed either before receiving the first i.n. sensitization (day 24) to assess Clara cell labeling with β-gal or after the second i.n. sensitization (day 29) with OVA to induce goblet cell hyperplasia. (C) Before pulmonary OVA sensitization, β-gal was expressed in nonciliated epithelial cells, as shown by its exclusion from FOXJ1-expressing cells and colocalization with CCSP. After sensitization, β-gal was detected (white pseudocolor) in most goblet cells, identified by MUC5AC, indicating their derivation from Clara cells. MUC5AC, β-gal, and FOXJ1 were not colocalized. (D) Scgb1a1-rtTA/TRE2-Spdef mice were treated 3 days with or without doxycycline. SPDEF induced goblet cell differentiation as detected by Alcian blue (AB) and by changes in cell morphology. SPDEF staining decreased 3 and 8 days after withdrawal of doxycycline, at which time goblet cell differentiation was substantially resolved. CCSP was decreased in the conducting airway epithelium 3 days after induction of SPDEF. 4 to 8 days after withdrawal of doxycycline, CCSP staining was restored. Inserts show higher magnifications of the regions indicated by arrows. Scale bars: 25 μm.
research article

**Figure 2**

mRNA microarray analysis of bronchiolar epithelial cells: heat map and partial list of SPDEF-regulated genes. Bronchiolar cells were isolated by LCM and mRNAs isolated and subjected to mRNA microarray analysis after treating Scgb1a1-rtTA/TRE2-Spdef mice for 3 days with or without doxycycline. (A) Heat map of the mRNAs. Red indicates mRNAs increased by SPDEF; green indicates those decreased. A number of mRNAs that were previously associated with pulmonary allergen exposure, including Foxa3, Gcnt3, Clica1, Agr2, Ptger3, and Muc16 were induced by SPDEF. SPDEF inhibited genes selectively expressed in normal airway epithelial cells, including Abca3, Slfpa1, Sftpb, Slfcd, and Foxa2 (A). Quantitative RT-PCR was performed in triplicate using cDNAs obtained from bronchiolar cells by LCM. Spdef mRNA was induced by doxycycline treatment (B). SPDEF induced Muc16 (C), Gcnt3 (D), Clica1 (E), and Ptger3 (F) mRNAs. Results were expressed as mean ± SD of 3 independent mice for each treatment. *P < 0.05 versus off-doxycycline control littermates (Hsu’s MCB test).

using the Clara cell–specific promoter (Scgb1a1-rtTA/TRE2-Spdef) induced marked goblet cell differentiation without causing proliferation in the conducting airways within 3 days. Neither BrdU labeling, phosphohistone H3, nor the number of cells per unit length of the bronchioles (latter data not shown) were increased during allergen sensitization or after expression of SPDEF in vivo. This process was rapidly reversible and associated with the restoration of Clara cell morphology and Clara cell secretory protein (CCSP) expression (Figure 1D). Thus, goblet cell differentiation in this model occurs without proliferation, a finding that does not support the term “hyperplasia.”

SPDEF regulates gene expression in the respiratory epithelium. To identify mRNAs regulated by SPDEF during goblet cell differentiation, laser capture microdissection (LCM) was used to obtain proximal bronchiolar epithelial cells before and 3 days after treatment with doxycycline to induce SPDEF expression in Clara cells (Supplemental Figure 2). SPDEF influenced the expression of 306 unique mRNAs (P < 0.01 level, using differences of 2-fold; Figure 2). SPDEF induced expression of a number of genes involved in the regulation of many aspects of mucus production, including protein glycosylation and mucin secretion, as well as genes that are highly represented in experiments in which mice were exposed to pulmonary allergens or IL-13. For example, mucin 16 (Muc16) (17), anterior gradient 2 (Ag2) (Xenopus laevis) (18, 19), chloride channel calcium activated 1 (Clica1) (20), prostaglandin E receptor 3 (subtype EP3) prostaglandin E receptor 4 (subtype EP4) (Ptger3/4) (21, 22), glucosaminyln (N-acetyl) transferase 3, mucin type (Gcnt3) (23), Foxa3 (13), serine (or cysteine) peptidase inhibitor, clade B (ovalbumin) member 11 (Serpinb11) (24), lumican, and versican (25) were markedly induced by SPDEF. Although Muc5ac mRNA was increased 2-fold by SPDEF, it was prefiltered out because of the low hybridization signals (close to background signal). In contrast, SPDEF inhibited the expression of groups of genes associated with Clara and type II alveolar cell differentiation, including Foxa2, Titf1, and a number of genes known to be directly regulated by FOXA2 and thyroid transcription factor 1 (TTF-1, also termed Nkx2-1), including ATP-binding cassette, subfamily A (ABC1), member 3 (Abca3), surfactant-associated protein A (SftpA), SftpB, SftpD, P450 (cytochrome) oxidoreductase (Por), aquaporin 5 (Aqp5), and secretoglobin, family 1A, member 1 (uteroglobin) (Scgb1a1) (26, 27) (Figure 2A). SPDEF inhibited Scnn1b and Scnn1g, consistent with a role for SPDEF in the regulation of fluid and electrolyte transport, which is important for mucociliary clearance in the lung. Quantitative RT-PCR was used to confirm the SPDEF-related changes in a number of these mRNAs (Figure 2, B–F). Of note, mouse Clica1 mRNA was significantly induced by SPDEF in vivo. Human CLCA1, a calcium-activated chloride channel associated with mucus production (28), is the closest ortholog of mouse CLCA1 (29) (Swiss-Prot accession number Q9D7Z6). The finding that SPDEF inhibited Titf1 and
Foxa2 mRNAs was confirmed by immunohistochemistry. Staining for FOXA2 and TTF-1 was markedly inhibited in goblet cells induced by SPDEF or after pulmonary OVA sensitization, while FOXA3 and AGR2 were induced (Figure 3, A–D). Consistent with the role of SPDEF in promoting mucin biosynthesis, protein glycosylation in the goblet cells induced by SPDEF or pulmonary OVA sensitization was detected by staining of the lectin Ulex euopeaus agglutinin I (UEA-I), which recognized the L-fucose moiety of glycoproteins (30) (Figure 3E). The induction of AGR2 was of particular interest, since Agr2 encodes a potential chaperone required for mucin packaging in goblet cells in vivo (31). SPDEF was colocalized with FOXA3 and AGR2 in goblet cells in vivo, indicating that SPDEF and FOXA3 may cooperate in the regulation of gene expression (Figure 4, A and B). SPDEF and FOXA3 synergistically activated the Agr2-luciferase promoter and endogenous Agr2 mRNA expression in vitro (Figure 4, C and D). We conclude that SPDEF induces goblet cell differentiation, increasing the expression of genes associated with mucin biosynthesis, glycosylation, and packaging, while inhibiting genes characteristic of Clara cells in the normal bronchiolar epithelium, including genes regulating fluid and electrolyte transport as well as innate host defense. Since TTF-1 and FOXA2 are important transcriptional regulators of genes expressed selectively in Clara cells, their inhibition by SPDEF likely accounts for changes of gene expression associated with Clara cells.

Coexpression of SPDEF, FOXA3, and AGR2 in pulmonary diseases. Immunohistochemical staining for SPDEF, FOXA3, and AGR2 was assessed in bronchial tissues from patients with pulmonary diseases caused by CF or cigarette smoking, disorders in which goblet cell hyperplasia and mucus hypersecretion are prominent. Alcian blue, SPDEF, FOXA3, and AGR2 staining were markedly increased at sites of goblet cell hyperplasia and were not readily detected in normal airway epithelium (Figure 5). In contrast, SPDEF, FOXA3, and AGR2 were expressed in goblet cells in the normal submucosal glands in both humans and mice (Figure 5F and data not shown).

SPDEF is required for allergen-induced goblet cell differentiation. Spdef–/– mice breed and survive normally in the vivarium. Spdef–/–, Spdef+/–, and Spdef+/+ mice were sensitized by repeated systemic injection followed by intrapulmonary administration of OVA. Lung histology was unaltered in Spdef–/– mice as assessed by light microscopy; however, goblet cells were absent in the tracheal and laryngeal submucosal glands of Spdef–/– mice prior to allergen exposure (Figure 6). As in wild-type mice, OVA sensitization induced goblet cell differentiation in Spdef+/+ mice as indicated by Alcian blue staining of acidic mucopolysaccharides and increased staining of SPDEF, FOXA3, MUC5AC, and UEA-I lectin (Figure 7). In contrast, neither goblet cell morphology nor the goblet cell–associated transcription factor FOXA3 was detected in the bronchial-bronchiolar epithelial cells in Spdef–/– mice before or after allergen sensitization. MUC5AC and staining for mucus-associated fucosyl glycoconjugates with UEA-I lectin were markedly inhibited but not absent in the Spdef–/– mice after allergen exposure (Figure 7). Pulmonary inflammation and eosinophilic and lymphocytic infiltration associated with allergen exposure were similar in Spdef+/+ and Spdef+/– mice (Supplemental Figure 3). To assess whether expression of SPDEF in airway epithelial cells was sufficient to induce MUC5AC expression in vitro, NCI-H292 cells and HBEC 3KT (human bronchial epithelial cells) (32) were infected with lentivirus expressing the Spdef cDNA. SPDEF induced endogenous MUC5AC expression in both cell lines (Figure 8). SPDEF was induced by IL-13 in primary mouse tracheal epithelial cells grown at air-liquid interface (Supplemental Figure 4), consistent with the role for TH2 cytokine signaling in the regulation of SPDEF.

Discussion

The respiratory tract is continuously exposed to inhaled irritants, particles, and pathogens, necessitating the evolution of robust innate and acquired host defense systems to maintain pulmonary...
sterility and homeostasis. Mucociliary clearance, the production of innate defense proteins, and the activity of the immune system all serve to maintain lung function after birth. The respiratory epithelium adapts to inflammatory stimuli, often dramatically increasing the number and activity of goblet cells. Goblet cell differentiation and excessive production of mucus are common features of many chronic pulmonary diseases, including asthma, CF, and chronic obstructive pulmonary disease. The present study demonstrates that goblet cells induced by allergens or by the expression of SPDEF are derived by differentiation of existing Clara cells and not by proliferation. Thus, the findings support the concept that goblet cells formed in the present model occur by metaplasia rather than by “hyperplasia.” SPDEF is required for goblet cell differentiation in normal tracheal-laryngeal submucosal glands and is required for goblet cell differentiation following allergen exposure. SPDEF regulates a group of genes associated with goblet cell differentiation, mucin biosynthesis, and secretion. SPDEF plays a central role in the regulation of goblet cell differentiation in epithelial cells in the respiratory tract, interacting with a transcriptional network that includes suppression of TTF-1 and FOXA2 and induction of FOXA3.

SPDEF was initially identified in prostate epithelium, where it interacts with NKX-3.1 in the regulation of prostate-specific antigen (PSA) (33). Spdef mRNA and protein are expressed in a number of organs that are enriched in epithelial cells of mucus and other secretory lineages, including breast, pancreas, and both reproductive and gastrointestinal tracts, supporting its potential role in goblet cell differentiation. In the intestine, secretory cell (goblet and Paneth cells) differentiation is regulated by the Notch signaling pathway (7, 8) and transcription factors including MATH1 (also termed atonal homolog 1 [ATOH1], Drosophila) (34) and growth factor independent 1 (GFI1) (35), while in the colon, KLF4 regulates goblet cell differentiation (36). Loss of MATH1/ATOH1 caused loss of GFI1, a zinc-finger transcription factor required for intestinal secretory lineage specification, which in turn decreased Spdef mRNA and reduced goblet cell number (35). Thus, expression of Spdef in postmitotic intestinal epithelial cells is regulated by the Notch signaling pathway and its downstream transcription factors MATH1/ATOH1 and GFI1, which are required for secretory lineage specification. However, it is still unclear that expression of SPDEF is regulated by Notch in the mature respiratory epithelium. Notch signaling pathway has been recently reported to promote airway goblet cell differentiation in a STAT6-independent manner in fetal tracheal explants in vitro (37). In the adult respiratory epithelium, SPDEF expression and goblet cell differentiation were induced by Th2 cytokines in a STAT6-dependent manner in vivo (10, 16). In the lung, GFI1 expression is confined to pulmonary neuroendocrine cells (38), and KLF4 mRNA was not induced in association with goblet cell differentiation in the pulmonary allergy and IL-13 microarray databases presently analyzed, suggesting that at least some aspects of the transcriptional regulation of goblet cell differentiation may be tissue specific. Nevertheless, SPDEF is expressed in many tissues containing goblet cells, including the respiratory and gastrointestinal epithelium (16, 39).

The present data support potential roles of FOXA2, TTF-1, and FOXA3 in a transcriptional network associated with goblet cell differentiation or function (Figure 9). SPDEF inhibited FOXA2 and TTF-1 and induced FOXA3, the latter a transcription factor that is not abundantly expressed in the normal lung (40). We observed reduced expression of FOXA2 and increased expression of SPDEF and FOXA3 during goblet cell differentiation caused by OVA sensitization and in lung tissue from patients with CF and chronic smoking. FOXA2 and FOXA3 staining were mutually exclusive following expression of SPDEF or its induction by allergens. Their colocalization in goblet cells and the finding that SPDEF and FOXA3 synergistically induced Agr2 indicate their potential roles in goblet cell differentiation.

SPDEF regulates expression of genes regulating mucin biosynthesis. The mRNA microarray analysis of SPDEF-induced genes in the conducting airways indicated that the expression of genes associated
Mucin biosynthesis begins with the attachment of N-acetylgalactosamine (GalNAc) to a serine or threonine residue, a process catalyzed by various UDP-GalNAc:polypeptide (where UDP indicates uridine diphosphate) GalNAc transferases (pGalNAc-T subfamily) (42). The synthesis of more complex, functional mucin carbohydrate requires the formation of core 2 β1,6 branched structure in the mucin glycan chain by the β1,6-N-acetylglucosaminyltransferase family of enzymes (also known as the C2GnT family) (43). mRNAs of all 3 members in the C2GnT family that mediate production of functional mucins (Gent1, -2, and -3) were significantly induced by SPDEF (increased 3.4-, 1.8-, and 9.3-fold respectively), strongly supporting its regulatory role in mucin biosynthesis and secretion. Among these enzymes, GCNT3 was induced the most by the expression of SPDEF in the airway epithelium (Figure 3E). GCNT3 mediates both core 2 and core 4 O-glycan branching, 2 important steps in mucin-type biosynthesis (44). Gent3 mRNA is expressed primarily in mucus-secreting tissues, including the gastrointestinal tract and trachea, and is induced by retinoic acid, TNF-α, and IL-13 (45, 46). Previous studies demonstrated that GCNT1 and 3 enzyme activities and mRNA levels were induced in airway epithelial cells following exposure to Th2 cytokines (IL-4 and IL-13) in a process mediated by the JAK-STAT signaling pathway (23, 45). Consistent with the important role of SPDEF in the regulation of mucin glycosylation, staining for UEA-I lectin was induced by SPDEF.

In the present study, goblet cell differentiation was associated with the increased expression of SPDEF and FOXA3 in bronchiolar epithelial cells, consistent with the microarray results showing the induction of many asthma-related and IL-13–responsive genes. Muc5ac, encoding a major mucin type secreted from airway goblet cells, was induced by SPDEF expression in vitro and in vivo. Expression of SPDEF was sufficient to induce MUC5AC in H292 cells, was induced by SPDEF expression in vitro and in vivo. MUC5AC expression in tracheal epithelium, submucosal glands, and cultured bronchial epithelial cells was recently
reported (17). These findings indicate that SPDEF regulates a number of genes involved in mucin synthesis, its glycosylation, and packaging. It remains unclear whether the effects of SPDEF represent direct cell autonomous effects on target gene expression or more complex effects on other regulators of goblet cell differentiation and function.

Expression of SPDEF in Clara cells caused their differentiation into goblet cells and inhibited a number of genes regulating host defense as well as fluid and electrolyte transport that are normally expressed in Clara cells and are known transcriptional targets of FOXA2 and TTF-1. This inhibitory effect is mediated in part by loss of FOXA2 and TTF-1 expression caused by induction of SPDEF in the airway epithelium. It is presently unclear whether this inhibition is mediated by direct or indirect effects of SPDEF on these transcriptional targets (Figure 9).

SPDEF, FOXA3, and AGR2 were increased in both the mouse models and in human lung tissue wherein their expression was associated with the presence of goblet cells, supporting the conservation of the proposed regulatory network. Since the cellular composition of the mouse airway differs from that of the human, the cellular origin of goblet cells in the human airway in which basal rather than Clara cells are more prominent, remains unclear.

Pulmonary allergen exposure and chronic inflammatory diseases of the lung are associated with infiltration by many cell types and the expression of numerous cytokines, chemokines, and other inflammatory mediators. The present findings indicate that these complex signals influence goblet cell differentiation in the respiratory epithelium via the transcription factor SPDEF and its influence on an extended transcriptional network (Figure 9). Changes in gene expression, cell differentiation, and morphology caused by SPDEF occur rapidly and reversibly without the activation of cell proliferation. Goblet cell differentiation and mucin secretion also occur following acute inflammation. Thus, SPDEF plays a central role in the regulation of a gene network that responds to pathogens or toxicants, in turn changing epithelial cell differentiation and mucociliary clearance that together play a role in innate host defense of the lung. Such changes in cell differentiation and function may represent an adaptive change in the epithelium that occurs without cell death, minimizing the need to activate cell proliferation. Since mucus hyperproduction contributes to the pathogenesis of acute and chronic pulmonary disorders, knowledge regarding the regulation and function of SPDEF in the respiratory tract provides a framework for the development of new strategies for diagnosis and therapy for chronic lung diseases.

Methods

Mouse models, OVA sensitization, and BrdU administration. Mouse strains included in this study were Spdef–/– mice produced in the laboratory of Hans Clevers, Netherlands Institute of Developmental Biology. Young adults of approximately 6 to 8 weeks of age were utilized in most studies. Scgb1a1-rtTA/TRE2-Spdef (line 2) (47)/TRE2-Spdef (16) mice in the FVB/N strain were treated with doxycycline (625 mg/kg of food). R26R mice, kindly provided by P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA) (48), were bred to Scgb1a1-rtTA (line 2) (Oetl)-CMV-Cre mice for lineage tracing (Figure 1A). The OVA sensitization protocol was described previously (14). Wild-type FVB/N mice were obtained from The Jackson Laboratory. To induce pulmonary goblet cell hyperplasia, mice received i.p. injections with 100 μg OVA (grade V; Sigma-Aldrich) and 1 mg Imject Alum (Thermo Scientific) as adjuvant, followed by 2 i.n. instillations of 50 μg of OVA or saline 3 days apart, starting at least 10 days after the second sensitization. BrdU (BS002; Sigma-Aldrich) was administered daily by i.p. injection (50 μg/g body weight at a concentration of 10 mg/ml in sterile saline) during treatment with doxycycline (over 3 days) and nasal sensitization with OVA (day 24 through day 29; Figure 1B). Animal protocols were approved by the Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committee in accordance with NIH guidelines.

Laser capture, RNA purification, and amplification. LCM was performed as described (49). To induce Spdef expression, 8-week-old adult male Scgb1a1-rtTA/TRE2-Spdef transgenic mice were treated with doxycycline for 3 days (n = 3). The control mice were the same age, sex, and genotype but were not treated with doxycycline (n = 3). Mice were anesthetized and exsanguinated, and the lungs were inflated with OCT (Fisher Scientific)/DEPC-PBS (where DEPC indicates diethylpyrocarbonate) with 10% sucrose (50% v/v) (sucrose, S0389; Sigma-Aldrich). After inflation, lungs were dissected and lobes were separated, embedded in OCT, and snap-frozen in isopentane, and stored at −80°C. Tissue was sectioned at −20°C in the cryostat. Thin sections (10 μm) were collected on 1:20 poly-l-lysine–coated (P8920; Sigma-Aldrich) slides and stored at −80°C. Prior to laser capture microscopy, slides were fixed in icec DEPC 70% ethanol, washed in DEPC-H2O, dehydrated in 95% and 100% ethanol, xylene, and air dried. Bronchiolar cells were captured by Veritas Microdissection Instrument (Model 704; Molecular Devices) with a laser set at 15 μm. Total RNAs were purified by Arcturus PicoPure RNA Isolation Kit (Molecular Devices). RNAs were then subjected to 2 rounds of amplifica-
Gene Ontology Analysis was performed using a random variance test, as its variance estimates are made on all genes with the same treatment without assuming that all genes have the same variance (52). Gene expression changes were considered statistically significant if their P-values were less than 0.01 and fold changes were greater than 1.5. We also performed a permutation test to provide a 90% confidence level that the false discovery rate was less than 10%. The false discovery rate is the proportion of the list of genes claimed to be differentially expressed that are false positives. In addition, Affymetrix Present Call in at least 2 of 3 replicates and coefficient of variation among replicates of 50% or less were set as a requirement for gene selection.

Functional classification and pathway analysis. Gene Ontology Analysis was performed using the publicly available web-based tool DAVID (database for annotation, visualization, and integrated discovery). Gene sets associated with known pathways and disease states from Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/), GenMAPP (http://www.genmapp.org), and GEArrays (http://www.sabiosciences.com/) were identified by comparing the overlap of pathway genes with the differentially expressed genes and all genes in the MOE430 mouse genome. A gene ontology term is considered to be overrepresented when a Fisher’s exact test P-value is less than or equal to 0.01 and gene hits are equal to 10 or more. Potential protein/protein or protein/DNA interactions were identified using Ingenuity Pathway Analysis (Ingenuity). Genetic networks preferentially enriched for input genes were generated based on their connectivity. Statistical scores were calculated to rank the resulting networks and pathways using Fisher’s right-tailed exact test. The score indicates the degree of relevance of a network to the input gene set, which takes into account the number of network-eligible genes and the size of the network.

Human specimens. Anonymous, deidentified, human adult and pediatric lung samples were obtained through the Department of Pathology, University of Cincinnati College of Medicine, and the Division of Pathology, Cincinnati Children’s Hospital Medical Center, in accordance with institutional guidelines for use of human tissue for research purposes (courtesy of Gail Deutsch, Kathryn Wikenheiser-Brokamp, and Robert Baughman).

Immunohistochemistry, immunofluorescence, X-gal, and Alcian blue staining. Adult mouse lung was inflation fixed, embedded, sectioned, and immunostained. Alcian blue and immunohistochemical staining for SPDEF, CCSP, and phosphohistone H3 followed previously described methods (16). TTF-1 was detected by a mouse monoclonal TTF-1 antibody (8G7G3/1) as previously described (53). AGR2, MUC5AC (ab47044 and ab3649, respectively; Abcam), FOXA3 (SC-5361; Santa Cruz Biotechnology Inc.), BrdU (Zymed BrdU staining Kit; Invitrogen), and UEA-1 (L9006; Sigma-Aldrich) staining followed standard procedures, as recommended by the manufacturers. Rat anti-mouse CD68 antibody (AbD Serotec) was used to detect monocytes and macrophages on frozen sections. Human SPDEF antibody (S4) used on all human specimens was kindly provided by Dennis Watson (Medical University of South Carolina, Charleston, South Carolina, USA). Immunohistochemistry was performed using human SPDEF antibody at a dilution of 1:500 after antigen retrieval with citrate buffer and heat. Major basic protein (MBP) antibody was kindly provided by Jamie and Nancy Lee (Mayo Clinic, Scottsdale, Arizona, USA).
To detect β-gal expression, lungs were inflation fixed with 2% PFA fixative for 10 hours at 4°C and then processed for preparation of frozen sections. X-gal enzymatic reaction was performed by incubating the lung sections with 5 mM K₄Fe(CN)₆, 5 mM K₃(CN)₆, and 1 mg/ml X-gal in PBS (pH 7.2) at 30°C for 4–8 hours. After X-gal staining, the same slides were subjected to immunofluorescence staining for CCSP and FOXJ1 following previously described methods (16). To identify goblet cells, Alcian blue staining was performed after immunohistochemical staining, using a 3-minute incubation in 3% acetic acid and a 10-minute incubation in 1% Alcian blue (Poly Scientific). Then slides were rinsed with running water for 5 minutes followed by 2 minutes in nuclear fast red, dehydration, and coverslipping with mounting media.

**Plasmids and cell lines.** Promoter regions were selected based on the sequence similarity and the conservation of the predicted transcription factor binding sites. The network indicates a regulatory relationship but does not imply direct transcriptional control of each gene by SPDEF or other transcription factors.
ing sites present in human, mouse, and rat genomes. All the PCR reactions were performed using GC-Rich PCR system (Roche) and then cloned into TA cloning vector pSC-A (StrataClone PCR cloning kit; Stratagene) for sequencing. The primer sets for cloning human FOXA3 3-kb promoter were as follows: 5′-GCCTGAGCCTGAGAAGCTATGTTTATGC-3′ and 5′-ATTCGAGGTCTCAGACGCCCAGG-3′. The PCR product was cloned into pSC-A vector, isolated by cutting with HindIII and SpeI, and cloned into HindIII and Nhel sites of the pGKL basic vector (Promega). The primer sets for cloning the mouse Agr2 1.2 kb promoter were as follows: 5′-TTCTCAGAATGTTGTTATTCGCGT-3′ and 5′-ATTCGAGGTCTCAGACGCCCAGG-3′. The PCR fragments were then cloned into the Xhol site of pGKL basic vector. The FOXA3 vector was made by amplifying the mouse 1-kb Foxa3 cDNA using the primer sets 5′-TGGATCACATGCTGGCTCAGTGAAGATG-3′ and 5′-TGGATCACATGCTGGCTCAGTGAAGATG-3′ and subcloned into the BamHI site of pCDN5/TO (Invitrogen). SPDEF expression vector was made by cloning 1-kb Spdef cDNA from TRE-Spdef (16) plasmid using the primer sets 5′-AATCTCTAGATGGATGCATTAAGCAGAGAGCG-3′ and 5′-ATTCTCTAGATGGATGCATTAAGCAGAGAGCG-3′. Those for mouse Muc16 2.6 kb promoter were as follows: 5′-TTCTCAGTACTCTCCTATATAAGAGTCC-3′ and 5′-TTCTCAGAATGTTGTTATTCGCGT-3′. The PCR fragments were then cloned into the XbaI site of pGKL basic vector. The FOXA3 expression vector was then used to transfect primary sheep tracheal epithelial cells (aSTEpC) with CMV–\textit{FLAG}–\textit{myc}–\textit{CMV} expression vector (16) plasmid (Clontech) and/or transcription factor expression plasmid using Lipofectamine 2000 (Invitrogen) as previously described (16). Growth medium was changed into a differentiation medium (MTEC/Nu, mouse tracheal epithelial cells culture medium, 2% Nu serum) (56) after transfection. Cell lysates were collected for luciferase activity assay 24 hours after transfection. All transfection assays were performed with primary sheep adult tracheal epithelial cells at passage 3 or 4. Relative promoter activities were normalized to internal control, the β-gal activity, and shown as mean ± SD.

Quantitative RT-PCR and statistics. Total RNAs obtained from LCM of bronchiolar tissue were reverse transcribed to cDNA by Verso cDNA Kit (Thermo Scientific). Quantitative RT-PCR was performed using Taqman probes and primer sets (Applied Biosystems) specific for \textit{Spdef} (assay ID Mm00600221_m1), \textit{Muc16} (assay ID Mm0177119_g1), \textit{Pgr3} (assay ID Mm01316856_m1), \textit{Clcat} (assay ID Mm00777368_m1), \textit{Agr2} (assay ID Mm00507853_m1), \textit{Gnt3} (assay ID Mm00512133_m1), and \textit{Muc5ac} (assay ID Hs03163661_m1). A probe and primer set for ribosomal 18S (part number 432930) was used as normalization control. PCR reactions were performed by using 25 ng cDNA per reaction in a 7300 real-time PCR System (Applied Biosystems). The quantification RT-PCR data to confirm microarray results were analyzed by Hsu’s MCB (best) test (59). Other quantification data were analyzed by 2-tailed, equal t Student’s t test in this study, P < 0.05 was considered significant.

Acknowledgments

The authors acknowledge support from Ann Maher for preparation of the manuscript; the Morphology Core in the Division of Pulmonary Biology at Cincinnati Children’s Hospital Medical Center; Michael Mucenski, Michael Bruno, and Angela Keiser for technical support; and Johannes C.M. van der Loo, Punam Malik, and Anusha Sridharan for assistance with lentiviral vectors. Grant support was provided by the NIH to J.A. Whitsett (HL090156 and HL095580) and T.R. Korfhagen (HL095580).

Received for publication May 4, 2009, and accepted in revised form July 15, 2009.

Address correspondence to: Jeffrey A. Whitsett, Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, MLC 7029, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039, USA. Phone: (513) 803-2790; Fax: (513) 636-7868; E-mail: jwhitsett@ccccmc.org.

3. Rose, M.C., and Voynow, J.A. 2006. Respiratory epithelial cells at passage 3 or 4. Relative promoter activities were normalized to internal control, the β-gal activity, and shown as mean ± SD.