IL-13 and Epidermal Growth Factor Receptor Have Critical but Distinct Roles in Epithelial Cell Mucin Production

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Overproduction of mucus is a central feature of asthma. The cytokine, IL-13, epidermal growth factor receptor (EGFR), and transcription factor, FOXA2, have each been implicated in mucus production, but the mechanistic relationships between these molecules are not yet well understood. To address this, we established a primary normal human bronchial epithelial cell culture system with IL-13–induced mucus production and gene transcript expression changes similar to those seen *in vivo* in mice. IL-13 did not stimulate release of the EGFR ligand, transforming growth factor (TGF)- α . However, there was constitutive release of TGF- α from normal human bronchial epithelial cells, and inhibition of TGF- α or EGFR reduced both constitutive and IL-13–induced mucin production. Microarray analysis revealed that IL-13 and the EGFR pathway appear to have almost completely independent effects on transcript expression. IL-13 induced a relatively small set of transcripts, including several novel transcripts that might play a role in pathogenesis of allergic airway disease. In contrast, EGFR activity had extensive effects, including altered expression of many transcripts associated with cell metabolism, survival, transcription, and differentiation. One of the few common effects of IL-13 and EGFR signaling was decreased expression of FOXA2, which is known to prevent mucus production. We conclude that the IL-13 and EGFR pathways make critical but quite distinct contributions to gene regulation in airway epithelial cells, and that both pathways affect expression of the key transcription factor, FOXA2, a known regulator of mucus production.

Keywords: airway epithelium; IL-13; epidermal growth factor receptor; FOXA2; mucus

Mucus overproduction is a clinically important feature of several major airway diseases, including chronic bronchitis, cystic fibrosis, and asthma (1). In asthma, there are increases in the number of mucus-producing goblet cells in airway epithelium (2) and in the expression of the major mucin glycoprotein, MUC5AC (3). Studies of mouse allergic airway disease models show that IL-13, a cytokine produced by T helper type 2 cells and other cells recruited to the lung during allergic responses, is required for allergen-induced airway inflammation and mucus production (4–6). Previous studies have indicated that allergen- and IL-13–induced mucus production in mouse airways is critically dependent

CLINICAL RELEVANCE

Mucus production is a central feature of asthma. We show the relationship between two key pathways (IL-13 and epidermal growth factor receptor) that contribute to mucus production. We also identify a connection between these pathways and FOXA2, a key transcription factor.

upon the expression of both the IL-13 receptor and the IL-13 signaling molecule signal transducer and activator of transcription 6 (STAT6) in airway epithelial cells (7, 8). These *in vivo* studies demonstrate that direct effects of IL-13 on airway epithelial cells are both necessary and sufficient for allergen-induced mucus production in mice, and suggest that a better understanding of the mechanisms of IL-13 effects on epithelial cells could help guide the development of new asthma therapies.

Recent studies have begun to identify key molecular pathways responsible for mucus production induced by various stimuli. Several stimuli reportedly induce mucus production by increasing metalloproteinase-mediated cleavage of epidermal growth factor receptor (EGFR) proligands on the cell surface (9, 10). The EGFR ligand responsible for mucus production appears to depend upon the nature of the stimulus that induces mucus: phorbol 12-myristate 13-acetate and LPS-induced mucus production depend upon transforming growth factor (TGF)- α (10), whereas the effects of cigarette smoke depend upon amphiregulin (11), and the effects of the bacterial component, lipoteichoic acid, depend upon heparin-binding EGF-like growth factor (HBEGF) (9). Relatively little is known about steps that are downstream of EGFR activation, although a pathway that involves Ras and NF- κ B is important for lipoteichoic acid–induced increases in MUC2 expression in NCI-H292 pulmonary mucoidermoid carcinoma cells (9). A recent report indicated that disruption of the *Foxa2* transcription factor gene in bronchial epithelial cells causes mucus metaplasia and increases in MUC5AC protein expression in mice (12), but the relationship (if any) between the EGFR pathway and changes in FOXA2 expression has not been established.

In this report, we investigate the relationships between IL-13, the EGFR pathway, FOXA2, and mucin gene expression. Previous attempts to use human cell culture systems to investigate the effects of IL-13, or the closely related cytokine, IL-4, on epithelial cells have yielded mixed results, with some studies showing that IL-13 increases expression of mucus and MUC5AC (13–15), but others showing that these cytokines had no effect or even decrease mucin production (14, 16–18). Even in systems where IL-13 did stimulate mucin production, the contribution of the EGFR pathway is not clear, with one study showing that EGFR kinase activity was critical for constitutive mucin production but not for IL-13–stimulated mucin production (14),

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and another showing that inhibitors of metalloproteinases responsible for activation of EGFR ligands blocked mucin production by IL-13-stimulated cells (15). A very recent study concluded that EGFR activity is critical for preventing apoptosis of murine tracheal ciliated cells that have the potential to differentiate into goblet cells in response to IL-13 stimulation *in vitro* (19). The explanation for these varying findings is not clear, but may relate to the use of different cell culture systems with uncertain relationships to *in vivo* systems. To address this, we used an *in vivo* mouse model to obtain a detailed picture of the kinetics of IL-13-induced changes in gene expression, and then established a human primary epithelial cell culture system with many similar features. This system allowed us to develop new insights about how IL-13 and the EGFR pathway each regulate the expression of *FOXA2*, *MUC5AC*, and other genes in human bronchial epithelial cells.

MATERIALS AND METHODS

Mouse Models

The University of California, San Francisco, Committee on Animal Research approved the use of mice for these experiments. Care and use of animals complied with the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions (no. 3400-01). To determine the effects of IL-13 *in vivo*, 6- to 8-wk-old male BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with inhaled isoflurane. Recombinant murine IL-13 (2 μ g in 50 μ l saline; Peprotech, Rocky Hill, NJ) was administered intranasally, and lungs were harvested 2–24 h after treatment. Untreated mice were used as control animals. To determine the effects of allergen sensitization and challenge, we used a protocol that has been described previously (20). The FVB/NJ mice used for the allergic model studies reported here, like other mice used in our earlier studies with this model (8, 20, 21), developed eosinophilic airway inflammation, mucus overproduction, and airway hyperreactivity after allergen challenge (data not shown). In brief, mice were sensitized by intraperitoneal administration of ovalbumin mixed with adjuvant three times at weekly intervals. Control mice received adjuvant alone. Beginning 1 wk after the last injection, mice were challenged three times by intranasal administration of ovalbumin at daily intervals. Control mice were challenged with PBS alone. RNA was isolated from lungs harvested 24 h after the last challenge.

Normal Human Bronchial Epithelial Cell Culture

Normal human bronchial epithelial (NHBE) cells (lot 3F1191; Cambrex Bio Science, Baltimore, MD) were cultured at an air-liquid interface using protocols similar to those reported previously (14). Cells were seeded into plastic T-75 flasks (Corning Inc., Corning, NY) and grown in bronchial epithelial cell growth medium (Cambrex Clonetics, Walkersville, MD) supplemented with bovine pituitary extract, hydrocortisone (0.5 μ g/ml), recombinant human EGF (0.5 ng/ml), epinephrine (0.5 μ g/ml), transferrin (10 μ g/ml), insulin (5 μ g/ml), retinoic acid (0.1 ng/ml), triiodothyronine (6.5 ng/ml), gentamycin sulfate (50 μ g/ml), and amphotericin B (50 ng/ml). Medium was changed every 48 h until cells were 90% confluent. Cells were then seeded at 8.25×10^4 cells/insert onto 12-mm-diameter Corning Costar Transwell-Clear inserts with 0.4 μ m pores. Cells were submerged in differentiation medium containing a 1:1 mixture of Dulbecco's modified Eagle's medium and bronchial epithelial cell growth medium with the same supplements as above, except that gentamycin sulfate, amphotericin B, and triiodothyronine were omitted, and penicillin, streptomycin, and 50 nM all-trans retinoic acid (Sigma Chemical Co., St. Louis, MO) were added. Cells were submerged for the first 7 d in culture, and then the apical medium was removed to establish an air-liquid interface that was maintained for the remainder of the culture period. Medium was refreshed three times weekly. The apical surface of the cells was rinsed with PBS (37°C) once weekly to remove accumulated mucus and debris. Cells were maintained at 37°C in 5% CO₂ in a humidified incubator and protected from light.

IL-13 Stimulation of NHBE Cells

To determine the effects of IL-13, cells were stimulated by addition of recombinant human IL-13 (1–25 ng/ml; Peprotech) to the basal medium for varying periods, as indicated in RESULTS. For prolonged IL-13 stimulation (> 48 h), the IL-13-containing medium was replaced every 2 d. For studies of the effects of antibodies and pharmacologic inhibitors, cells were grown in differentiation medium without EGF beginning 11 d after establishment of the air-liquid interface, and cells were stimulated with IL-13 for 48 h beginning the following day (Days 12–14). Antibodies and inhibitors were added to the medium on the basal side 1 h before IL-13. Neutralizing antibodies against EGFR (clone 225, 4 μ g/ml) and TGF- α (clone 189–2130.1, 10 μ g/ml), and the pharmacologic inhibitors AG1478 (10 μ M), GM6001 (40 μ M), and TAPI-1 (30 μ M), were from Calbiochem (San Diego, CA). Antibodies against amphiregulin (clone 31221, 50 μ g/ml), EGF (clone 10825, 20 μ g/ml), and HBEGF (20 μ g/ml), and control mouse IgG1, were from R&D Systems (Minneapolis, MN). In some experiments, the concentration of TGF- α in the basolateral medium of IL-13-stimulated and control cells was determined by ELISA (Calbiochem) according to the manufacturer's protocol.

Quantitative RT-PCR

Total RNA from mouse lungs was isolated using TRIzol (Invitrogen, Carlsbad, CA) and purified using the RNeasy kit (Qiagen Inc, Valencia, CA). Total RNA from NHBE cells was isolated using the RNeasy kit. RNA integrity was verified using a Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). First-strand cDNA synthesis was performed using the SuperScript First-Strand cDNA Synthesis System (Invitrogen). Taqman primers and probes were designed using Primer Express software (Perkin Elmer, Boston, MA). The human *FOXA2* primers were 5'-TCTTAAGAAGACGACGGCTTCAG-3' and 5'-TTGCTCTCACTTGTCTCGAT-3', and the *FOXA2* probe sequence was 5'-FAM-CCGGCTAACTCTGGCACCCCG-TAMRA-3'. Sybr Green real-time PCR was performed for human *CAII*, *CCL26*, *CDH26*, *DPP4*, *FCGBP*, *FOXA3*, *IL19*, *NOS2A*, *SERPINB10*, *SLC39A8*, *STATH*, and some of their mouse orthologs. The sequences of the primers for Sybr Green real-time PCR were obtained from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>). Other primer and probe sequences have been reported previously (20). Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (Foster City, CA) and AmpliTaq Gold DNA polymerase. The cycle threshold (Ct) of each mouse gene transcript was normalized to the average Ct for the housekeeping gene transcripts glyceraldehyde-3-phosphate dehydrogenase (*Gapd*), peptidylprolyl isomerase A (*Ppia*), and ribosomal protein S9 (*Rps9*). The Ct of each human transcript was normalized to the average Ct for *GAPD*, phosphoglycerate kinase 1 (*PGK1*), and ubiquitin (*UBB*). Fold differences were determined by the $2^{-\Delta\Delta C_t}$ method (22).

Alcian Blue Staining and Immunocytochemistry

After 14 d of air-liquid interface culture, the apical surface of the cells was gently rinsed with 37°C PBS to remove accumulated mucus and debris, and cells were fixed in 10% neutral buffered formalin and embedded in paraffin; 5- μ m-thick sections were stained with 1% Alcian blue (in 3% glacial acetic acid, pH 2.5) for detection of mucus. Antibodies against MUC5AC (45M1; Santa Cruz Biotechnology, Santa Cruz, CA) and FOXA2 (rabbit polyclonal; Upstate Biotechnology, Lake Placid, NY), and matched nonreactive control antibodies, were used for immunocytochemistry. Antibodies were detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as directed by the manufacturer.

Microarray Analysis

RNA was prepared from individual wells of NHBE cells that were maintained at air-liquid interface for 14 d. Triplicate samples were prepared for each of four conditions: control (no IL-13 or antibody); IL-13 (25 ng/ml for the final 48 h of culture); anti-EGFR antibody (added during the final 49 h of culture); or IL-13 and anti-EGFR. Total RNA (1 μ g/sample) was amplified using one round of *in vitro* transcription with incorporation of amino-allyl-modified nucleotides (Message Amp II aRNA kit no. 1753; Ambion, Austin, TX), and the

resulting cRNA was coupled to Cy3 or Cy5 fluorescent dyes (Amersham Biosciences, UK). Fluorescently-labeled cRNAs were fragmented using Ambion RNA Fragmentation Reagents and hybridized to DNA microarrays using Ambion SlideHyb Glass Array Hybridization Buffer #1 (Ambion). For each hybridization, Cy3- or Cy5-labeled cRNA from a single control sample was hybridized along with cRNA from a single sample from IL-13-, anti-EGFR-, or IL-13 and anti-EGFR-treated NHBE cells that was labeled with the other dye. Labeled cRNAs were hybridized to 70-mer oligonucleotides microarrays produced using the Operon Human Genome 70-mer Oligo Set Version 2.0 (Operon Biotechnologies, Huntsville, AL). Additional information about microarray protocols and the complete array data are available from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>, series accession GSE4804).

Statistical Analysis

For microarray data analysis, we used an approach that allowed us to estimate differential gene transcript expression using linear models (23). We used a 2×2 factorial model, with two factors representing the IL-13 and the anti-EGFR effects, and a two-way interaction term for the interaction of IL-13 and anti-EGFR. The interaction term was used to identify transcripts that had a different response to IL-13 when anti-EGFR was present than when anti-EGFR was not present. We calculated estimated log ratios, moderated t statistics, log odds ratios of differential expression, and P values for each contrast of interest using functions in the Bioconductor package limma (24). P values were adjusted for multiple testing using the Bonferroni correction. To identify functionally related groups of genes with altered expression, we used Gostat (25) to compare increased or decreased transcripts to the entire set of transcripts represented on the arrays. We considered gene ontology groups to be significantly overrepresented if the Benjamini false discovery rate was $< 5\%$. For analysis of data from other experiments, values are presented as mean $\pm$ SEM. For comparisons of multiple time points to the 0-h control, and comparisons of multiple concentrations of IL-13 to unstimulated controls, statistical significance was determined using Dunnett's test as implemented in JMP version 6 (SAS Institute, Cary, NC). Other comparisons were performed using the Student's t test.

RESULTS

Administration of IL-13 to the Mouse Airway Induces Both Rapid and Delayed Increases in Transcript Expression

We began by measuring the effects of IL-13 stimulation on lung gene expression *in vivo*. We previously identified a set of 11 gene transcripts that are induced by the direct effects of IL-13 on airway epithelial cells (20). In those studies, a transgene resulted in continuous high-level production of IL-13 throughout the life of the animal. We found that 10 of these 11 gene transcripts were increased 24 h after a single intranasal administration of IL-13 (Figure 1a). To determine the kinetics of gene induction by IL-13, we measured expression of each of the 11 gene transcripts between 2 and 24 h after IL-13 (Figures 1b–1e). Three transcripts, the anion exchanger, pendrin (*Slc26a4*), the chitinase, *Chia*, and the secreted pattern recognition protein, intelectin (*Itln*), were increased to near maximal levels by 2 h, the earliest time point we studied (Figure 1b). The minimal early increase in the lipoxygenase, *Alox15*, seen in this model was not statistically significant. Two other transcripts, the calcium-activated chloride channel, *Clca3*, and the actin filament severing protein, Scinderin (*Scin*), were also increased by 2 h, but then had substantial further increases in expression by 8 h after stimulation (Figure 1c). Transcripts encoding the mucin protein *Muc5ac* were increased at 16 and 24 h after stimulation, but not at earlier time points; there was a similar trend for the related mucin, *Muc5b*, but the increases did not reach statistical significance (Figure 1d). Three other transcripts, encoding the secreted proteins trefoil factors (TFFs) 1 and 2 (*Tff1* and *Tff2*) and the anterior gradient homolog 2 protein (*Agr2*), were also increased at

Figure 1. Mouse lung gene expression changes after intranasal IL-13 administration. Lung RNA was obtained from unstimulated mice (no intranasal administration), mice administered intranasal saline, and mice administered intranasal IL-13 (2 μ g). Gene transcript expression was measured by qRT-PCR. Fold difference was calculated relative to expression in mice that did not receive IL-13 (time 0). Results shown are means ($\pm$ SEM) for four mice at each time point. (a) Gene expression changes 24 h after saline or IL-13 administration. * $P < 0.05$ for IL-13 compared with saline administration; † $P < 0.05$ for saline compared with unstimulated mice by Dunnett's test. (b–e) Kinetics of IL-13-induced gene expression changes at 2–24 h after IL-13 administration. The following increases were significant ($P < 0.05$ as compared with unstimulated mice [time 0] by Dunnett's test): *Slc26a4*, *Itln*, *Chia*, *Clca3*, and *Scin* at all time points; *Agr2* at 8–24 h; and *Muc5ac*, *Tff1*, and *Tff2* at 16 and 24 h. There were no significant decreases.

relatively late time points, but not at earlier time points (Figure 1d). All of the transcript expression increases either preceded or approximately coincided with the appearance of mucus-containing goblet cells, which we found occurs between 6 and 24 h after a single intranasal dose of IL-13 (Ref. 21 and D.J.E., unpublished observations), and others have reported is detectable by 6 h and increased by 18 and 48 h (26).

IL-13 Effects on Gene Expression in NHBE Cells

We sought to develop a suitable cell culture system for further analysis of the effects of IL-13 on airway epithelial cell gene expression. We implemented an air-liquid interface culture system for NHBE cells and examined the effects of IL-13 on expression of the major mucin protein gene, *MUC5AC*. When cells were maintained in EGF-supplemented medium for 14 d, but not stimulated with IL-13, there was Alcian blue staining of the glycocalyx, but little intracellular staining with Alcian blue or *MUC5AC* immunostaining, indicating that very few, if any, goblet cells were present (Figures 2a and 2c). When IL-13 was added to the medium for the entire 14-d period, there were many mucus-containing goblet cells that stained intensely with Alcian blue and a *MUC5AC*-specific antibody (Figures 2b and 2c).

We examined the effects of IL-13 stimulation on expression of transcripts for human orthologs of the 11 IL-13-induced genes previously identified in IL-13-overexpressing mice (20). Using quantitative RT-PCR (qRT-PCR), we found that *ALOX15*,

CLCA1 (human ortholog of *Cla3*), *ITLN*, *MUC5AC*, *SCIN*, *SLC26A4*, *TFF1*, and *TFF2* transcripts were increased significantly after NHBE cells were stimulated by IL-13 (Figure 2d). There was no significant change in *AGR2* or *MUC5B*, and we did not detect *CHIA* expression with any of three PCR primer sets used. We conclude that 8 of the 11 gene transcript expression changes identified in IL-13-overexpressing mice can also be seen after prolonged IL-13 stimulation of cultured primary NHBE cells.

Next we examined the kinetics of IL-13-induced gene transcript expression changes in NHBE cells. We cultured NHBE cells at the air-liquid interface for 14 d and added IL-13 during the final 2–48 h (Figure 3). IL-13 produced a substantial increase in *MUC5AC* expression within 24–48 h. Three orthologs (*SLC26A4*, *ITLN*, and *CLCA1*) of transcripts that were induced rapidly in mice were also induced rapidly in NHBE cells. *ALOX15* was also induced rapidly in NHBE cells. The transcript encoding Scinderin (*SCIN*) was induced more slowly in NHBE cells than in mice. Although *MUC5B*, *TFF1*, and *TFF2* transcripts could be induced by prolonged IL-13 stimulation of NHBE cells during the differentiation period (Figure 2), they were not induced within 48 h (Figures 3c and 3d). The temporal pattern of IL-13-induced changes in transcript expression in the NHBE cell culture model was similar to that seen *in vivo* in mice, although the delayed changes tended to be more markedly delayed in NHBE cells. IL-13-induced increases in *MUC5AC*/*Muc5ac*

Figure 2. IL-13 treatment induces mucus metaplasia and alters gene expression in NHBE cells. (a and b) Alcian blue staining was used to detect mucus in cells cultured at the air-liquid interface for 14 d in the absence of IL-13 (a) or in 25 ng/ml IL-13 (b). (c) *MUC5AC* immunostaining was seen in IL-13-stimulated cells, but not in unstimulated cells. (d) NHBE cells were maintained at the air-liquid interface for 14 d in the presence of 0, 1, 10, or 25 ng/ml of IL-13. Gene transcript expression was measured by qRT-PCR. Fold difference was calculated relative to expression in NHBE cells not exposed to IL-13. Data are means ($\pm$ SEM) for three replicate wells. * $P < 0.05$ compared with unstimulated cells (no IL-13) by Dunnett's test.

Figure 3. Gene expression changes after 2–48 h of IL-13 stimulation of mature NHBE cell cultures. NHBE cells were cultured at the air-liquid interface for 14 d. IL-13 (25 ng/ml) was added to the medium during the last 2–48 h. Gene transcript expression was measured by qRT-PCR. Fold difference was calculated relative to expression in NHBE cells not exposed to IL-13. Data are means ($\pm$ SEM) for three replicate wells. The following increases were significant ($P < 0.05$ compared with unstimulated mice [time 0] by Dunnett's test): *ITLN*, *ALOX15*, and *CLCA1* at all time points; *SLC26A4* at 8–48 h; *SCIN* at 8 and 48 h; and *MUC5AC* and *AGR2* at 48 h. The decreases in *MUC5B* and *TFF1* at 24 and 48 h were also significant. Data for *CHIA* and *TFF2* are not shown because these transcripts were not detectable at any of the time points included in this experiment.

transcript expression occurred relatively late in both the human and mouse systems.

Inhibition of the EGFR Pathway Reduces Constitutive and IL-13-Induced *MUC5AC* Transcript Expression

To test the role of the EGFR pathway in mucin production in NHBE cells, we used neutralizing antibodies and pharmacologic inhibitors to block different steps in the pathway. Cells were maintained at the air-liquid interface in medium without IL-13 for 12 d, and then treated with EGFR pathway inhibitors and/or IL-13 for the final 2 d of culture. To exclude possible effects of exogenous EGF in these experiments, we used medium without supplemental EGF for the final 3 d of culture. IL-13 stimulation for 48 h induced *MUC5AC* transcript expression (Figure 4) and development of Alcian blue-stained goblet cells (data not shown). A neutralizing antibody that blocks EGFR ligand binding reduced *MUC5AC* transcript expression in both unstimulated and IL-13-stimulated cells (Figure 4a). AG1478, a pharmacologic inhibitor of EGFR tyrosine kinase activity, had similar effects on *MUC5AC* expression (Figure 4b). We analyzed the

role of specific EGFR ligands using neutralizing antibodies. An antibody specific for TGF- α reduced *MUC5AC* transcript expression (Figure 4c), whereas antibodies against amphiregulin, EGF, and HBEGF did not (data not shown).

Active TGF- α is released after cleavage of the transmembrane precursor pro-TGF- α by metalloproteinases (27). We found that the broad-spectrum metalloproteinase inhibitor, GM6001 (data not shown), and the more specific inhibitor, TAPI-1 (Figure 4d), which is active against the metalloproteinase a disintegrin and metalloproteinase domain 17 (ADAM17), each inhibited both IL-13-induced and constitutive *MUC5AC* transcript expression in differentiated NHBE cell cultures. We were able to detect TGF- α in the conditioned medium from NHBE cells, but IL-13 stimulation did not increase TGF- α levels in the medium (Figure 4e). A previous study that showed PMA-induced increases in TGF- α release used neutralizing EGFR antibody (10), which reduces ligand binding and might block ligand uptake by the receptor. We used a similar approach and found that EGFR antibody markedly increased the concentration of TGF- α in the medium, which might have been due to decreased binding and uptake, or to other effects of the antibody. However, IL-13 stimulation still had no detectable effect on the amount of TGF- α in the medium (Figure 4f). Taken together, our data indicate that there is constitutive activity of the ADAM17/TGF- α /EGFR pathway that is not affected by IL-13 stimulation. Constitutive activity of this pathway contributes both to maintaining the low level of *MUC5AC* transcript expression seen in unstimulated NHBE cells and to the higher level of *MUC5AC* expression seen in IL-13-stimulated cells. EGFR blockade did not prevent IL-13-induced increases in other transcripts, including *ALOX15*, *CLCA1*, *ITLN*, *SCIN*, and *SLC26A4*, although there were modest effects of EGFR blockade on two of these (*CLCA1* and *SLC26A4*) (Figure 4g).

Global Analysis of the Effects of IL-13 and EGFR Activity on Gene Expression in NHBE Cells

To further investigate the effects of IL-13 and the EGFR pathway on airway epithelial cells, we incubated mature NHBE cells with IL-13, anti-EGFR antibody, or both, for 2 d, and analyzed transcript expression using DNA microarrays (Figure 5). The complete microarray results are available from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>, series accession GSE4804), and a list of all differentially expressed transcripts is available in the online supplement (Table E1). IL-13 altered the expression of 50 gene transcripts in NHBE cells (48 transcripts increased and 2 transcripts decreased; adjusted $P < 0.05$). Many of the IL-13-induced genes identified in the NHBE cell microarray analysis were not detected in our previous microarray-based analysis of the direct effects of IL-13 on mouse airway epithelial cells *in vivo* (20). The most highly induced transcripts are shown in Table 1. We selected several of these for validation, and were able to confirm that these transcripts were induced by IL-13 stimulation of NHBE cells by qRT-PCR (Figure 6a). As expected, in many cases, the qRT-PCR method gave a larger estimate of the magnitude of the increase in expression than did the arrays. Apparently, seven of these genes (*CAII*, *CDH26*, *FCGBP*, *FOXA3*, *SERPINB10*, *SLC39A8*, and *STATH*) have not been previously associated with asthma or allergy. Six of these seven have known mouse orthologs, and expression of two of the mouse orthologs was also significantly increased 24 h after intranasal administration of a single dose of IL-13 (23-fold in *Fcgbp* and 10-fold in *Foxa3* compared with saline; $P < 0.05$). In addition to these two, three more of the mouse orthologs were significantly increased in the lungs of allergen-challenged mice (Figure 6b), which may reflect responses to IL-13, IL-4, or other mediators produced during allergic airway inflammation. Some

Figure 4. EGFR pathway effects on gene expression in unstimulated and IL-13-stimulated, differentiated NHBE cells. NHBE cells were cultured at the air-liquid interface for 14 d. EGF-free medium was used during the last 72 h. IL-13 (25 ng/ml) was added to the medium 48 h before the end of culture. (a–d) Effects of EGFR pathway blockade on basal and IL-13-induced *MUC5AC* mRNA expression. Anti-EGFR (a), the EGFR kinase inhibitor, AG1478 (b), anti-TGF-α (c), and the metalloproteinase inhibitor, TAPI-1 (d) were added to the medium 1 h before the addition of IL-13. (e) TGF-α release from unstimulated cells. IL-13 (25 ng/ml) was added to the medium 1–24 h before the end of culture, and TGF-α levels in the medium were measured by ELISA. None of the values were significantly different from the unstimulated (0 h) control by Dunnett's test. (f) Release of TGF-α in the presence of EGFR blockade. Anti-EGFR antibody was added to the medium, and TGF-α levels in the medium were measured after cells were cultured for 48 h in the absence or presence of IL-13 (25 ng/ml). (g) Analysis of the effects of anti-EGFR on expression of other gene transcripts. Gene transcript expression was measured by qRT-PCR. Fold difference was calculated relative to expression in NHBE cells not exposed to IL-13 or inhibitors. Data are means ($\pm$ SEM) for three replicate wells. * $P < 0.05$ for IL-13 or inhibitor (anti-EGFR, AG1478, anti-TGF-α, or TAPI-1) alone compared with unstimulated controls; † $P < 0.05$ for the combined effect of IL-13 and the inhibitor compared with the effect of IL-13 alone; ‡ $P < 0.05$ for the combined effect of IL-13 and the inhibitor compared with the effect of the inhibitor alone by Student's *t* test.

Figure 5. Global analysis of IL-13- and EGFR-dependent gene expression changes in NHBE cells. Cells were treated with IL-13 and anti-EGFR as in Figure 4a, and RNA was analyzed using microarrays. Each column represents data from one of three replicate samples for each group. Colors represent fold change compared with cells maintained in the absence of IL-13 or anti-EGFR. (a) Results for 1,667 transcripts that were differentially expressed in at least one of the three conditions (adjusted $P < 0.05$). (b) Detail from (a) highlighting the small subset of IL-13-induced transcripts.

of the IL-13-induced transcripts identified in the earlier PCR analysis, including *MUC5AC*, do not appear on the list of significantly differentially expressed transcripts from the microarray analysis. This illustrates a limitation of array studies, which may not be sufficiently sensitive to detect some changes because of low transcript abundance, suboptimal primer design, or statistical issues relating to adjustments (such as the Bonferroni correction) that are necessary when very large numbers of genes are analyzed.

Blockade of EGFR had profound effects on gene expression (Figure 5). Treatment with EGFR antibody for the final 2 d of culture changed the expression of 7% of the transcripts represented on the arrays, with increased expression of 645 transcripts and decreased expression of 636 transcripts, even using conservative statistical methods (Bonferroni adjusted $P < 0.05$). These transcript expression changes involve groups of genes involved in a very wide range of cellular processes. Gene ontology “biological processes” terms that are significantly overrepresented (false discovery rate $< 5\%$) within the group of increased transcripts include “negative regulation of cellular physiological process” (GO:0051243, 47 increased transcripts), “regulation of metabolism” (GO:0019222, 110 increased transcripts), “cell death” (GO:0008129, 38 increased transcripts), and “transcription” (GO:0006350, 99 increased transcripts). Terms that are overrepresented in transcripts with decreased expression include “mRNA metabolism” (GO:0016701, 25 decreased transcripts) and “macromolecule metabolism” (GO:0043170, 171 decreased transcripts). A recent study demonstrated that EGFR activity is important for preventing apoptosis of ciliated cells that are capable of transdifferentiating into goblet cells after IL-13 stimulation (19). We found that EGFR blockade substantially reduced

TABLE 1. IL-13-INDUCED INCREASES IN GENE EXPRESSION IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

Symbol	Description	Fold Increase*
<i>CCL26</i>	Chemokine (C-C motif) ligand 26 (eotaxin 3)	11.5
<i>ITLN</i>	Intelectin	10.6
<i>FCGBP</i>	Fc fragment of IgG binding protein	9.6
<i>FETUB</i>	Fetuin B	9.3
<i>CDH26</i>	Cadherin-like 26	6.0
<i>DPP4</i>	Dipeptidyl-peptidase 4 (CD26)	5.8
<i>ALOX15</i>	Arachidonate 15-lipoxygenase	4.9
<i>NOS2A</i>	Nitric oxide synthase 2A (inducible)	4.9
<i>IL19</i>	IL-19	4.9
<i>FOXA3</i>	Forkhead box A3	4.7
<i>SERPINB10</i>	Serpin peptidase inhibitor B10	4.5
<i>CA2</i>	carbonic anhydrase II	4.3
<i>TMEM16A</i>	Transmembrane protein 16A	3.5
<i>SLC39A8</i>	Solute carrier family 39 (zinc transporter), member 8	3.5
<i>CD36</i>	CD36 antigen (collagen type I/thrombospondin receptor)	3.4
<i>POSTN</i>	Periostin	3.2
<i>GALNT4</i>	GalNAc-T4 acetylgalactosaminyltransferase	3.1
<i>STATH</i>	Statherin	2.6

* Values represent fold increase for all mRNA transcripts with significant (adjusted $P < 0.05$) changes of at least 2.5-fold compared with untreated control cells by DNA microarray analysis.

expression of transcripts for ciliated cell markers, including tubulin- β 4 (Figure 7a). EGFR is also expressed on basal cells (28), and expression of transcripts for the basal cell-associated genes, keratin 4 and 14 (29, 30), was also reduced, suggesting that effects of EGFR signaling are also important in this population.

Figure 6. Novel gene expression changes in NHBE cells and mouse lungs. (a) Selected IL-13-induced gene transcript expression changes discovered using microarrays were validated by qRT-PCR. Cells were treated with IL-13 and anti-EGFR as in Figure 4a. (b) Expression of six orthologous gene transcripts was measured in control (saline) and allergen-sensitized and -challenged mouse lungs by qRT-PCR. Fold difference was calculated relative to expression in the control group. Data are means ($\pm$ SEM) for three replicate wells. * $P < 0.05$ compared with controls by Student's t test. (a) Open bars, control; closed bars, IL-13. (b) Open bars, saline; closed bars, ovalbumin (OVA).

We used the microarray data to look for evidence of synergistic or antagonistic interactions between the IL-13 and the EGFR pathways. Visual inspection of Figure 5 suggests that the effects of IL-13 and anti-EGFR were generally simply additive in the NHBE cell model system. To assess this rigorously, we used the two-way interaction term for the interaction of IL-13 and anti-EGFR that is included in the model used for statistical analysis of the array results (see MATERIALS AND METHODS). Only one transcript, *S100P* (which encodes a calcium-binding protein), had a significant interaction term (adjusted $P < 0.05$). *S100P* was increased 2.3-fold by IL-13 alone and 4.4-fold by anti-EGFR alone; the 4.1-fold increase seen after simultaneous IL-13 and anti-EGFR treatment is less than that predicted by combining these two individual effects. If IL-13 induced expression of transcripts by increasing the activity of the EGFR pathway, we would have expected to find that these transcripts were inhibited or unaffected by anti-EGFR alone, and that the effect of IL-13 on these transcripts was less in the presence of anti-EGFR. We found no statistically significant interactions of this type, which is consistent with the idea that IL-13 does not affect gene expression by increasing the activity of the EGFR pathway.

A recent study found that deletion of the *Foxa2* gene in mouse lung epithelial cells results in goblet cell metaplasia, indicating that this transcription factor plays a key role in bronchial epithelial cell differentiation (12). We found that treatment of NHBE cells with IL-13 at concentrations sufficient to induce *MUC5AC* expression (10–25 ng/ml) resulted in substantial decreases in expression of human *FOXA2* transcripts and protein (Figures 7b–7d). IL-13-induced decreases in *FOXA2* transcript expression were first seen 24–48 h after addition of IL-13, coincident with the rise in *MUC5AC* expression (data not shown). *FOXA2* transcript expression was markedly increased after EGFR blockade (Figure 7a), indicating that both IL-13 and the EGFR pathway are involved in maintaining the expression of this key transcription factor. Strong *FOXA2* immunoreactivity was also seen after EGFR blockade (Figure 7e).

DISCUSSION

Airway epithelial cell dysfunction is an important feature of asthma, but the underlying molecular mechanisms are not yet well understood. Direct effects of IL-13 on airway epithelial

Figure 7. IL-13 and EGFR effects on expression of markers of epithelial cell differentiation and FOXA2. (a) Effects of short-term (48 h) treatment with IL-13 or anti-EGFR on mRNAs encoding tubulin- $\beta 4$ (TUBB4), keratins (KRT) 4 and 14, and FOXA2, were determined by qRT-PCR. Fold difference was calculated relative to expression in NHBE cells not exposed to IL-13. Data are means ($\pm$ SEM) for three replicate wells. * $P < 0.05$ for IL-13 or anti-EGFR antibody-treated cells compared with unstimulated cells; † $P < 0.05$ for IL-13-stimulated and anti-EGFR antibody-treated compared with IL-13-stimulated cells; ‡ $P < 0.05$ for IL-13-stimulated and anti-EGFR antibody-treated compared with anti-EGFR antibody-treated cells by Student's t test. (b) Effects of long-term (14 d) treatment with IL-13 on FOXA2 mRNA expression. NHBE cells were maintained at the air-liquid interface for 14 d in the presence of 0, 1, 10, or 25 ng/ml of IL-13, and mRNA levels were measured by qRT-PCR. * $P < 0.05$ compared with unstimulated cells (no IL-13) by Dunnett's test. (c–e) FOXA2 protein expression in unstimulated cells (c), cells stimulated with IL-13 (25 ng/ml for 14 d) (d), and cells cultured in the presence of anti-EGFR antibody for the final 48 h of the 14 d culture (e) was visualized by immunohistochemistry. FOXA2 immunoreactivity appears as brown staining and mucin is stained with Alcian blue.

cells are necessary and sufficient for allergen-induced mucus and mucin production *in vivo* in mice (7, 8). In the studies reported here, we implemented a human cell culture system that recapitulates many of the key IL-13-induced gene expression changes seen *in vivo*. We used this system to study the kinetics of these changes, analyze the relationship between IL-13 effects and the EGFR pathway, and identify novel genes induced by effects of IL-13 on bronchial epithelial cells. These results provide new insights into how IL-13, the EGFR pathway, and the key transcription factor, FOXA2, work together to alter airway epithelial cell function in ways that are likely to be relevant to the pathogenesis of asthma.

The IL-13-stimulated NHBE cell system that we used for these experiments was a particularly valuable tool, as it emulated many of the gene expression changes produced by IL-13 stimulation *in vivo*. We and others have consistently found that *in vivo* stimulation with IL-13 or the closely related cytokine, IL-4, in-

duces mucus and mucin production in the airways (4, 5, 7, 8, 31). In contrast, the effects of these cytokines in previously reported human cell culture models have been quite variable. In many cases, these cytokines had no effect (17, 18, 32), decreased mucus production and mucin expression (16), or increased mucus and mucin production only at a low concentration (1 ng/ml), but not at a higher concentration (10 ng/ml) (14). Similar to Yoshisue and colleagues (15), we found that 14 d of stimulation with higher concentrations (10–25 ng/ml) induced substantial increases in mucus production. This was accompanied by large increases in expression of the major mucin gene, *MUC5AC*, and the human orthologs of most other IL-13-induced genes identified in mouse *in vivo* models. By administering IL-13 during the final 2–48 h of the 14-d air-liquid interface culture period, we were able to examine the kinetics of the IL-13 response in NHBE cell cultures. Genes tended to be induced more slowly in cultured NHBE cells than in the mouse *in vivo* model (compare Figures 1 and 3). The most extreme example is *TFF1*; *Tff1* transcripts increased within 16 h in mice, whereas *TFF1* transcript expression in NHBE cells was decreased at 24 and 48 h, but increased after 14 d (Figure 2). It is possible that the slower kinetics may be explained, in part, by differences in the differentiation state of the epithelium *in vivo* and *in vitro*; support for this idea comes from the observation that IL-13 failed to have any effect on *MUC5AC* expression in relatively undifferentiated NHBE cells studied immediately after establishment of an air-liquid interface (G. Zhen and D. J. Erle, unpublished observations). In any case, most transcripts that were induced very rapidly in mice were also induced rapidly in NHBE cells, and transcripts that were induced late in mice tended to be induced even later in NHBE cells.

We used this NHBE cell system to test the hypothesis that IL-13 stimulates mucin production by increasing release of EGFR ligands, as do other mucin producing stimuli that have been studied previously (9, 10). EGFR signaling was important for mucin production by IL-13-stimulated cells, as inhibitors of EGFR, the EGFR ligand, TGF- α , or metalloproteinases, which cleave pro-TGF- α , each reduced *MUC5AC* transcript expression in IL-13-stimulated cells (Figure 4). However, the effects of IL-13 cannot be explained by increased release of TGF- α , because (1) the amount of TGF- α released was unaffected by IL-13 stimulation, (2) direct stimulation with exogenous TGF- α itself at a variety of concentrations did not increase *MUC5AC* expression in these cells, and (3) IL-13 stimulation did not increase the level of EGFR phosphorylation (G.Z. and D.J.E., unpublished observations). Instead, our results indicate that constitutive TGF- α release and EGFR signaling regulate *MUC5AC* production in both unstimulated and IL-13-stimulated cells. Microarray analysis indicated that constitutive EGFR signaling plays a major role in control of a very large group of genes, including many with well established roles in cell growth, differentiation, and survival. EGFR signaling is not required for general IL-13 responsiveness, because many IL-13-induced gene expression changes were seen even in the presence of EGFR blockade. EGFR signaling might contribute to survival of cells that have the potential to differentiate into mucin-producing goblet cells after IL-13 stimulation, as suggested by elegant studies recently performed by Tyner and colleagues (19). In those studies, goblet cells were found to be derived, at least in part, from transdifferentiation of ciliated cells, which undergo apoptosis in the absence of EGFR signaling. We found that EGFR blockade reduced the expression of transcripts encoding markers for ciliated cells (e.g., β -tubulin), as well as keratins that are expressed primarily in basal cells, which have also been shown to express EGFR (28). The relative contribution of specific

precursor cell types to the development of mucus-producing cells is not yet clear.

Microarray analysis of NHBE cells allowed us to identify additional IL-13-induced epithelial cell genes that we did not identify in our previous microarray analysis of mouse models (20). Although there are many possible explanations for this, it is likely that the use of a purified epithelial cell population, instead of the complex mixture of cells present in the whole lung and tracheal samples used in the earlier study, was an important factor. Some of these genes, such as the peptidase gene, *DPP4* (CD26), and the cytokine gene, *IL19*, have already been associated with asthma, but were not known to be induced by direct effects of IL-13 on epithelial cells (33, 34). Other novel IL-13-induced genes identified here have not, to our knowledge, been previously associated with allergy or asthma. Several of these genes have known functions that would appear to be relevant. For example, the IL-13-induced genes, carbonic anhydrase II and *SLC39A8* (a metal ion transporter), are likely to affect ion transport across the epithelium. Two other IL-13-induced genes encode proteins that are likely to be important constituents of mucus: statherin, a 6-kD protein that can form complexes with the mucin, *MUC5B* (35), and the Fc γ -binding protein, *FCGBP*, which includes multiple mucin-like domains and is expected to have a role in immunity (36). Another novel IL-13-induced gene is *SERPINB10*, which encodes a protease inhibitor that is similar to plasminogen activator inhibitor-2 and can inhibit cell death in some systems (37). Although further studies will be required to determine the importance of these gene expression changes, the fact that mouse orthologs of these genes were significantly induced after *in vivo* allergen challenge suggests that the changes may be relevant to allergic airway disease.

Both IL-13 and the EGFR pathway contributed to the regulation of expression of the transcription factor, *FOXA2*. We examined *FOXA2* expression because a recent report demonstrated that conditional deletion of *Foxa2* in mouse airway epithelial cells leads to a large increase in goblet cells, and that nuclear expression of *FOXA2* protein is reduced in goblet cells from IL-13-overexpressing mice (12). We found that IL-13 stimulation resulted in marked decreases in expression of *FOXA2* mRNA and protein, indicating that the reduction in *FOXA2* nuclear staining found in the previous study is likely caused, at least in part, by decreased *FOXA2* transcription and/or mRNA stability. We also found that inhibition of EGFR increased *FOXA2* mRNA expression in IL-13-stimulated cells as well as unstimulated cells. *FOXA2* has been shown to decrease transcription from the *MUC5AC* promoter in NCI-H292 human airway mucocoeptidermoid carcinoma cells (12). Taken together with data reported here, this result suggests that IL-13- and EGFR-induced decreases in *FOXA2* expression could contribute directly to increased *MUC5AC* expression in bronchial epithelial cells. One of the novel IL-13-induced genes that we identified using microarrays is the *FOXA2* family member, *FOXA3*, which is normally expressed at high levels in the liver, stomach, and intestine, but not the lung (38). Because lung *Foxa3* expression was substantially increased after allergen challenge of mice (Figure 6b), our results raise the question of whether reciprocal effects of IL-13 on *FOXA2* and *FOXA3* contribute to the effects of this cytokine on bronchial epithelial cells in allergic airway disease and asthma.

IL-13, EGFR, and *FOXA2* are each important potential therapeutic targets in asthma and other diseases characterized by excessive mucus production. We found that IL-13 and the EGFR pathway both contribute to mucin production in airway epithelial cells. Although other stimuli reportedly increase mucin production by increasing release of EGFR ligands, IL-13 did not have this effect in our system. Instead, we found that constitutive

activity of the EGFR pathway plays a major role in regulating expression of a wide range of genes in NHBE cells. One of the very few common effects of IL-13 and EGFR signaling that we identified is reduction in expression of *FOXA2*. Because loss of *FOXA2* expression in airway epithelial cells causes mucus metaplasia (12), *FOXA2* may be an important common regulator of mucus production in response to IL-13 and other stimuli that depend upon EGFR pathway activation.

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

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References

- Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;86:245–278.
- Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 1992;101:916–921.
- Ordóñez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, et al. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* 2001;163:517–523.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261–2263.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258–2261.
- Whittaker L, Niu N, Temann UA, Stoddard A, Flavell RA, Ray A, Homer RJ, Cohn L. Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9. *Am J Respir Cell Mol Biol* 2002;27:593–602.
- Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;8:885–889.
- Kuperman DA, Huang X, Nguyen L, Holscher C, Brombacher F, Erle DJ. IL-4 receptor signaling in Clara cells is required for allergen-induced mucus production. *J Immunol* 2005;175:3746–3752.
- Lemjabbar H, Basbaum C. Platelet-activating factor receptor and ADAM10 mediate responses to *Staphylococcus aureus* in epithelial cells. *Nat Med* 2002;8:41–46.
- Shao MX, Ueki IF, Nadel JA. Tumor necrosis factor α -converting enzyme mediates *MUC5AC* mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci USA* 2003;100:11618–11623.
- Basbaum C, Li D, Gensch E, Gallup M, Lemjabbar H. Mechanisms by which gram-positive bacteria and tobacco smoke stimulate mucin induction through the epidermal growth factor receptor (EGFR). *Novartis Found Symp* 2002;248:171–176; discussion 176–180, 277–282.
- Wan H, Kaestner KH, Ang SL, Ikegami M, Finkelman FD, Stahlman MT, Fulkerson PC, Rothenberg ME, Whitsett JA. *Foxa2* regulates alveolarization and goblet cell hyperplasia. *Development* 2004;131:953–964.
- Laoukili J, Perret E, Willems T, Minty A, Parthoens E, Houcine O, Coste A, Jorissen M, Marano F, Caput D, et al. IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells. *J Clin Invest* 2001;108:1817–1824.
- Atherton HC, Jones G, Danahay H. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L730–L739.
- Yoshisue H, Hasegawa K. Effect of MMP/ADAM inhibitors on goblet cell hyperplasia in cultured human bronchial epithelial cells. *Biosci Biotechnol Biochem* 2004;68:2024–2031.
- Jayawickreme SP, Gray T, Nettekheim P, Eling T. Regulation of 15-lipoxygenase expression and mucus secretion by IL-4 in human bronchial epithelial cells. *Am J Physiol* 1999;276:L596–L603.

17. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem* 2003;278:17036–17043.
18. Thai P, Chen Y, Dolganov G, Wu R. Differential regulation of MUC5AC/Muc5ac and hCLCA-1/mGob-5 expression in airway epithelium. *Am J Respir Cell Mol Biol* 2005;33:523–530.
19. Tyner JW, Kim EY, Ide K, Pelletier MR, Roswit WT, Morton JD, Battaile JT, Patel AC, Patterson GA, Castro M, *et al.* Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 trans-differentiation signals. *J Clin Invest* 2006;116:309–321.
20. Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ. Dissecting asthma using focused transgenic modeling and functional genomics. *J Allergy Clin Immunol* 2005;116:305–311.
21. Venkayya R, Lam M, Willkom M, Grunig G, Corry DB, Erle DJ. The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyper-responsiveness through direct effects on resident airway cells. *Am J Respir Cell Mol Biol* 2002;26:202–208.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001;25:402–408.
23. Glonek GF, Solomon PJ. Factorial and time course designs for cDNA microarray experiments. *Biostatistics* 2004;5:89–111.
24. Gentleman R, Carey V, Huber W, Irizarry R, Dudoit S. Bioinformatics and computational biology solutions using R and Bioconductor. New York: Springer; 2005.
25. Beissbarth T, Speed TP. Gostat: find statistically overrepresented gene ontologies within a group of genes. *Bioinformatics* 2004;20:1464–1465.
26. Shahzeidi S, Aujla PK, Nickola TJ, Chen Y, Alimam MZ, Rose MC. Temporal analysis of goblet cells and mucin gene expression in murine models of allergic asthma. *Exp Lung Res* 2003;29:549–565.
27. Arribas J, Coodly L, Vollmer P, Kishimoto TK, Rose-John S, Massagué J. Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. *J Biol Chem* 1996;271:11376–11382.
28. Aida S, Tamai S, Sekiguchi S, Shimizu N. Distribution of epidermal growth factor and epidermal growth factor receptor in human lung: immunohistochemical and immunoelectron-microscopic studies. *Respiration (Herrlisheim)* 1994;61:161–166.
29. Fisseler-Eckhoff A, Erfkamp S, Muller KM. Cytokeratin expression in preneoplastic lesions and early squamous cell carcinoma of the bronchi. *Pathol Res Pract* 1996;192:552–559.
30. Purkis PE, Steel JB, Mackenzie IC, Nathrath WB, Leigh IM, Lane EB. Antibody markers of basal cells in complex epithelia. *J Cell Sci* 1990; 97:39–50.
31. Zuhdi Alimam M, Piazza FM, Selby DM, Letwin N, Huang L, Rose MC. Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways. *Am J Respir Cell Mol Biol* 2000;22:253–260.
32. Longphre M, Li D, Gallup M, Drori E, Ordonez CL, Redman T, Wenzel S, Bice DE, Fahy JV, Basbaum C. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J Clin Invest* 1999;104:1375–1382.
33. Kruschinski C, Skripuletz T, Bedoui S, Tschernig T, Pabst R, Nassenstein C, Braun A, von Horsten S. CD26 (dipeptidyl-peptidase IV)-dependent recruitment of T cells in a rat asthma model. *Clin Exp Immunol* 2005;139:17–24.
34. Liao SC, Cheng YC, Wang YC, Wang CW, Yang SM, Yu CK, Shieh CC, Cheng KC, Lee MF, Chiang SR, *et al.* IL-19 induced Th2 cytokines and was up-regulated in asthma patients. *J Immunol* 2004;173:6712–6718.
35. Iontcheva I, Oppenheim FG, Troxler RF. Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins. *J Dent Res* 1997;76:734–743.
36. Harada N, Iijima S, Kobayashi K, Yoshida T, Brown WR, Hibi T, Oshima A, Morikawa M. Human IgGfC binding protein (FcγBP) in colonic epithelial cells exhibits mucin-like structure. *J Biol Chem* 1997;272: 15232–15241.
37. Schleef RR, Chuang TL. Protease inhibitor 10 inhibits tumor necrosis factor α -induced cell death. *J Biol Chem* 2000;275:26385–26389.
38. Kaestner KH, Hiemisch H, Luckow B, Schutz G. The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. *Genomics* 1994;20:377–385.