IL-13 Regulates Cilia Loss and *foxj1* Expression in Human Airway Epithelium

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Mucociliary clearance is essential to the defense mechanisms of the respiratory system. Loss of normal mucociliary clearance contributes to the pathogenesis of genetic and acquired lung diseases. Treatment of cultured differentiated human airway epithelial tissue with IL-13 resulted in a loss of ciliated epithelial cells and an increase in mucus-secreting cells. The loss of ciliated cells was characterized by mislocation of basal bodies and loss of ezrin from the apical cell compartment. In addition to the loss of ciliated cells and increase in mucous cells after IL-13 treatment, cells with characteristics of both ciliated and mucous cells were observed in the airway epithelium. In association with the decrease in ciliated cells after IL-13 treatment, there was noted a decrease in *foxj1* expression in the airway epithelium, characterized by a decrease in the number of *foxj1*-expressing cells. Within the *foxj1* promoter, a STAT-binding element was identified and inhibition of *foxj1* expression by STAT-6 and IL-13 was demonstrated. These findings suggest molecular and cellular mechanisms for cilia loss in pulmonary disease. Inhibition of *foxj1* expression results in loss of apical localization of ezrin and basal bodies with subsequent loss of axonemal structures. These findings have important implications for the pathogenesis and treatment of airway diseases.

Keywords: cilia; *foxj1*; IL-13; basal bodies; airway epithelium

Mucociliary clearance is an essential aspect of the innate defense mechanisms of the lung. Ciliated cells and mucus-secreting cells within the pulmonary epithelium act in concert to remove potentially harmful substances, such as bacteria, toxins, and particulates, from the lungs (1). Disruption of normal mucociliary clearance in hereditary and acquired respiratory diseases results in mucus plugging of the airways, decreased pulmonary function, and an increased risk of secondary infection. In asthma, which affects an estimated 15 million people in the United States alone, a decreased ratio of ciliated cells to mucous cells is observed (2, 3). Respiratory infections with the paramyxovirus respiratory syncytial virus (RSV) result in significant seasonal morbidity and mortality, particularly in individuals with underlying respiratory or cardiac disease. One hallmark of RSV infection is hyperplasia of mucus-secreting cells and loss of ciliated cells from the respiratory epithelium (4). Loss of ciliated cells and mucous cell hyperplasia also occurs in response to environmental toxins such as smoke (5). After injury to the airway, regeneration of the normal airway epithelial barrier may occur through proliferation and differ-

CLINICAL RELEVANCE

Our findings demonstrate molecular and cellular mechanisms for the loss of cilia in respiratory diseases. These findings have important implications for the pathogenesis and treatment of airway diseases.

entiation of populations of airway epithelial cells. The specific population of cells required has not been clearly identified, although potential precursor populations have been proposed (6–8).

Foxj1 is a member of the forkhead box family of transcription factors and is expressed in the ciliated cells of the lung, choroid plexus, and reproductive tracts, as well as in the embryonic kidney and the node of the pre-somite stage embryo (9–14). In the developing mouse and human lung, *foxj1* expression is restricted to the proximal pulmonary epithelium and is first expressed during the late pseudoglandular stage of lung development before the appearance of ciliated epithelial cells (10, 12). Targeted mutation of the mouse *foxj1* gene results in an absence of 9+2 cilia and defects in left-right asymmetry (15, 16). The pulmonary epithelium of *foxj1*^{−/−} mice demonstrates a complete absence of 9+2 ciliary axonemes. Basal bodies are not localized to the apical cell compartment as in wild-type epithelium, but are instead distributed throughout the cytoplasm (15, 17). The absence of *foxj1* also results in a decrease in the cytoskeletal anchoring proteins ezrin and EBP-50 in the apical cell compartment. Ezrin and EBP-50 thus act by anchoring basal bodies to the apical cytoskeleton (17, 18). Although *foxj1* is essential for cilia formation, mutations in the *foxj1* gene have, as of yet, not been identified in human diseases with abnormalities of cilia formation (19). Decreased *foxj1* expression, however, has been reported in paromyxovirus infection in mice as well as in human respiratory epithelium after RSV infection (20).

The Th2 cytokines IL-4 and IL-13 play central roles as molecular mediators of the pulmonary epithelial changes seen in asthma, as well as in acquired pulmonary diseases (21). The secretion of Th2 cytokines has pleiotropic effects on the pulmonary epithelium. These effects include inflammation with the recruitment of lymphocytes and eosinophils, mucous cell metaplasia and hyperplasia, loss of ciliated cells and altered ciliary beat frequency, and fibrosis (22, 23). Increased airway levels of IL-13 have been demonstrated in asthma, as well as in RSV infection (24–26). Phosphorylation of STAT-6 in response to IL-13 results in STAT-6 translocation to the nucleus, where it can activate or inhibit gene expression (27, 28). Distinct changes in the transcriptional programs of airway cells have been demonstrated in response to IL-13 (29). Understanding the molecular and cellular regulation of these airway epithelial changes in response to cytokines is essential to elucidating the pathogenic mechanisms of asthma and respiratory infections, as well as the development of novel therapeutic approaches to lung disease.

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Normal mucociliary clearance requires both the appropriate secretion of mucus and functional cilia in the airways. Given the central role of disturbed mucociliary clearance in lung disease, elucidating the cellular and molecular mechanisms of mucous cell hyperplasia and cilia loss is essential to understanding lung pathology. Significant insight has been gained regarding cytokine-mediated mucous cell hyperplasia and increased mucus secretion with asthma, pulmonary infection, or lung injury (2, 23, 25, 26, 30). Although loss of cilia, in addition to mucous cell hyperplasia, has been described in asthma, respiratory infections, and toxin exposure, little is known regarding the mechanisms of cilia loss or the repair mechanisms of the ciliated epithelium (3–5, 20, 31–33). Cytokine regulation of gene expression is well established and provides a possible molecular mechanism for the loss of ciliated epithelial cells (27–29). Considering its role in ciliogenesis, *foxj1* provides a possible target gene for cytokine regulation in cilia loss (15, 16). We hypothesize that cytokine-mediated changes in the pulmonary epithelium include the loss of ciliated epithelial cells. Furthermore, we propose that the loss of ciliated epithelial cells is mediated through cytokine inhibition of *foxj1* expression. In this report, the cellular and molecular mechanisms of cilia loss in the airway epithelium in response to IL-13 are examined.

MATERIALS AND METHODS

Differentiated Human Airway Epithelial Tissue Culture

EpiAirway differentiated human airway epithelial tissue was obtained from MatTek (Ashland, MA) and cultured on 0.8-cm Millipore Millicell CM cell culture inserts (Millipore, Billerica, MA) at air-liquid interface according to the manufacturer's specifications. The basal culture medium consisted of serum-free Dulbecco's modified Eagle's medium plus proprietary growth factors and hormones. Tissue was cultured at 37°C in 5.0% CO₂. The EpiAirway tissue provides a model of the differentiated human airway epithelium. Histologic, immunohistochemical, and electron microscopic examination of the tissue demonstrated a differentiated mucociliary phenotype (Figures 1A, 2A, and 3B). To examine the effect of IL-13 on the differentiated phenotype, IL-13 at 50 or 100 ng/ml was added to the basal culture medium after culture of the tissue at air-liquid interface for 24 hours after arrival from MatTek. The tissue was then cultured at 37°C in 5.0% CO₂ for 3 to 8 days. The IL-13 concentrations and lengths of treatment were based on previous studies by other investigators demonstrating

IL-13-mediated changes in the pulmonary epithelium with IL-13 concentrations and lengths of treatment in this same range (22, 29, 34).

Electron Microscopy

For scanning electron microscopy, cultured human airway epithelial tissue was fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate and postfixed using the osmium-thiocarbohydrazide-osmium (OTO) method (35). Samples were dehydrated in ascending concentrations of ethanol and critical point dried in liquid CO₂. Mounted samples were sputter-coated with 50 nm of gold and examined with a Hitachi H-450 scanning electron microscope (Hitachi, Pleasanton, CA).

To determine the percentage of ciliated cells present in untreated and treated tissues, three scanning electron micrographs each from control tissue and treated tissue were examined. Ciliated cells were identified by the presence of axonemal structures on the cell surface. Cells with any number of axonemal structures were considered ciliated. The percentage of ciliated cells was calculated with respect to the total number of cells present in the micrograph. For calculating the percentage of the cell surface covered with cilia, morphometric analysis was performed on scanning electron micrographs of untreated and treated tissues. A grid was superimposed over the surface of ciliated cells, and the percentage of cell surface covered with cilia was determined.

For transmission electron microscopy, cultured human airway epithelial tissue was fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate, postfixed in 1.0% (vol/vol) osmium tetroxide, and then stained with 1.0% (wt/vol) uranyl acetate. Tissue was dehydrated in ascending concentrations of ethanol before embedding in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with an Hitachi H-600 transmission electron microscope.

Western Blot Analysis

Protein was isolated from cultured human airway epithelial tissue in the presence of protease inhibitors as described (36). Ten milligrams of the cell extract were electrophoresed in 7.0% (wt/vol) polyacrylamide-SDS and transferred to a PVDF membrane (Amersham, Pittsburgh, PA). The membrane was blocked for 1 hour at room temperature with 5.0% (wt/vol) nonfat dry milk in Tris-buffered saline with 0.1% (vol/vol) Tween-20. Primary antibodies used were 1:1,000 rabbit polyclonal anti-phospho STAT-6 (Santa Cruz, Santa Cruz, CA), 1:1,000 mouse monoclonal anti-HFH-4 (Foxj1) clone MA5-3-19 (Active Motif, Carlsbad, CA), and 1:5,000 mouse monoclonal anti-GAPDH (Research Diagnostics, Concord, MA). Secondary antibodies were either anti-mouse or anti-rabbit IgG antibody linked to horseradish peroxidase (Amersham). A chemiluminescent signal was generated with ECL Plus (Amersham) and quantified with a Molecular Dynamics Phosphorimager and Storm

Figure 1. IL-13 results in loss of cilia from cultured human airway epithelial cells. Scanning electron micrographs of untreated human airway epithelial cells cultured for 3 days (A), or cultured human airway epithelial cells treated with IL-13 at 50 ng/ml for 3 days (B), 100 ng/ml for 3 days (C), or 100 ng/ml for 8 days (D). Scale bar = 10 μ m. * P = 0.005.

Figure 2. IL-13 results in basal body mislocalization in human airway epithelial cells. Immunofluorescence for pericentrin (Texas Red) and Muc5AC (fluorescein) of untreated human airway epithelial cells cultured for 3 days (A) or cultured human airway epithelial cells treated with 50 ng/ml IL-13 for 3 days (B) or 100 ng/ml for 8 days (C). Solid arrows indicate cells with apical localization of basal bodies; open arrows indicate cells with nonapical basal bodies. Transmission electron micrographs of untreated human airway epithelial cells cultured for 3 days (D) or cultured human airway epithelial cells treated with 50 ng/ml IL-13 for 3 days (E) or 100 ng/ml IL-13 for 8 days (F). Arrowheads indicate basal bodies. Red, pericentrin; green, Muc5AC; blue, DAPI. Scale bar = 5.0 μm in A, B, and C; 1.0 μm in D, E, and F.

Scanner (Molecular Dynamics, Sunnyvale, CA). GAPDH was used as a loading control for standardization of blots. Western blot analysis was performed in triplicate.

Immunofluorescence

Cultured human airway epithelial tissue was fixed in 10% (vol/vol) buffered formalin for 12 hours at 4°C, dehydrated in ascending concentrations of ethanol, paraffin embedded, and sectioned. Antigen unmasking was performed by heating sections in 10 mM sodium citrate (pH 6.0) with 0.05% (vol/vol) Tween 20 to boiling for 20 minutes in a microwave oven. Sections were then blocked with appropriate animal sera for 30 minutes at room temperature. Primary antibodies were polyclonal rabbit anti-foxj1 (1:200; CeMines, Golden, CO), monoclonal mouse anti-Muc5AC (1:200; Neomarkers, Fremont, CA), polyclonal rabbit anti-ezrin (1:100; Upstate Biotechnology, Chicago, IL) and polyclonal rabbit anti-pericentrin (1:1,000; Abcam, Cambridge, MA). For sequential, double immunofluorescence, sections were incubated with the first primary antibody for 30 minutes at room temperature, followed by incubation with the appropriate biotinylated secondary antibody for 45 minutes at room temperature. Sections were then incubated with fluorescein avidin D or Texas red avidin D (Vector, Burlingame, CA) for 30 minutes. After blocking with an avidin/biotin blocking reagent (Vector), sections were incubated with the second primary antibody overnight at 4°C. Sections were then incubated with

the appropriate biotinylated secondary antibody for 45 minutes and then with fluorescein avidin D or Texas red avidin D.

For immunofluorescence, a minimum of three independent sections from untreated or treated tissue was examined. Duplicate samples were included for each antibody and tissue as well as control samples with no primary antibody.

Transient Transfection

2.2 kilobases of the mouse foxj1 promoter region were cloned into the pCAT3e reporter vector (Invitrogen, Carlsbad, CA). This region includes a consensus STAT binding element from -1260 to -1268 bp (Table 1). Site-directed mutagenesis of the STAT-binding element was performed with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. DNA sequence analysis was performed to confirm the sequence of the mutated site (Table 1). A full-length STAT-6 expression vector in pcDNA3.1 was a kind gift from Dr. Y. Abu-Ahmer.

BEAS 2B bronchioepithelial cells were used for transient transfections, as no ciliated transformed cell lines are currently available and BEAS 2B, although not ciliated, are derived from airway epithelium. In addition, in preliminary studies, BEAS 2B cells were able to support the expression of foxj1 promoter-reporter vectors. For transient transfections, BEAS 2B cells were grown to 75% confluence at 37°C

Figure 3. IL-13 results in decreased foxj1 expression in cultured human airway epithelial cells. (A) Western blot of protein isolated from human airway epithelial cells cultured in the absence or presence of 50 ng/ml IL-13 for 3 days. Primary antibody to foxj1 gives a band of ~ 65 kD. Primary antibody to GAPDH gives a band of ~ 35 kD. Control lane is protein isolated from adult mouse lung. (B and C) Immunofluorescence of cultured

human airway epithelial cells either untreated (B) or treated with 50 ng/ml IL-13 for 3 days (C). Cells were incubated with antibody to foxj1 (fluorescein) or Muc5AC (Texas red). Green, foxj1; red, Muc5AC. Scale bar = 50 μm.

TABLE 1. SEQUENCES OF MOUSE AND HUMAN FOXJ1 STAT-BINDING ELEMENTS

Mouse <i>foxj1</i>	AGCTTCCGGGAACCTT
Human <i>FOXJ1</i>	AGCTTCTCTGGAACTT
Mutated mouse <i>foxj1</i>	AGCTGCCGGGCACTT

in 5.0% CO₂ in bronchial epithelial growth medium (BEGM; Clonetics, Lonza, Basel, Switzerland). Transfections were performed with a total of 5.0 µg of DNA. A β-galactosidase expression vector was included in all transfections to correct for transfection efficiency. Cells were incubated with Lipofectin Reagent (Invitrogen) containing plasmid DNA for 4 hours. Cells were then washed and incubated in BEGM in the absence or presence of 50 ng/ml of IL-13. After culture for 48 hours, cells were lysed and the cell extract incubated with [¹⁴C] chloramphenicol and n-butyl CoA to assay for chloramphenicol acetyltransferase (CAT) activity. β-galactosidase activity was also assayed in aliquots of the cell lysate. The β-galactosidase activity was used to correct for transfection efficiency.

Statistical Analysis

Experimental data were analyzed by one-way ANOVA. Data are expressed as the mean ± standard error.

RESULTS

IL-13 Results in a Loss of Cilia from Cultured Differentiated Airway Epithelial Tissue

To test if the cytokine IL-13 mediated the loss of ciliated epithelial cells from cultured, differentiated human airway epithelium, cells were treated with IL-13 at 50 ng/ml or 100 ng/ml (Figure 1). By scanning electron microscopy, 48 ± 7% (mean ± SE) of untreated airway epithelial cells cultured for 3 days were ciliated (Figure 1A). In tissue treated with 50 ng/ml IL-13 for 3 days, the number of ciliated cells was reduced to 35 ± 9% (Figure 1B). When tissue was treated with 100 ng/ml IL-13 for 3 days, the number of ciliated cells was reduced to 10 ± 3% (Figure 1C). Treatment of differentiated airway epithelial cells with 100 ng/ml IL-13 for 8 days resulted in a complete loss of ciliated epithelial cells (Figure 1D). There is thus a dose-dependent decrease in ciliated cells in cultured airway epithelium in response to IL-13 (Figure 1E). This decrease was statistically significant by ANOVA ($P = 0.005$).

In addition to the decrease in the number of ciliated epithelial cells after IL-13 treatment, there was also a decrease in the number of cilia per ciliated cell. Untreated, cultured ciliated cells had nearly 100% of the cell surface covered with cilia. This decreased to 78 ± 9% after treatment with 50 ng/ml of IL-13 for 3 days and decreased to 31 ± 8% after treatment with 100 ng/ml of IL-13 for 3 days. The decrease in the percentage of cell surface covered with cilia was statistically significant by ANOVA ($P < 0.001$).

IL-13 Results in Mislocalization of Basal Bodies in Cultured Differentiated Airway Epithelial Tissue

Stability of cilia requires the anchoring of basal bodies to the apical cytoskeleton (17, 18). The loss of basal body anchoring with subsequent loss of axonemal structures provides a potential mechanism for the loss of ciliated epithelial cells. To test the hypothesis that the loss of cilia in response to IL-13 was associated with disrupted basal body anchoring to the apical cytoskeleton, basal body position was identified by immunofluorescence with anti-pericentrin antibody, a marker for basal bodies and centrosomes, and transmission electron microscopy of IL-13-treated cells.

Immunofluorescence with antibody to pericentrin of untreated airway epithelial tissue cultured for 3 days reveals basal bodies in a linear arrangement at the apical surface of cells (Figure 2A). Staining of centrosomes can also be seen throughout the section. After treatment of cultured airway epithelium with IL-13 at 50 ng/ml for 3 days or 100 ng/ml for 3 or 8 days, cells can be observed that have lost the apical localization of the pericentrin-positive basal bodies (Figures 2B and 2C, and data not shown).

Transmission electron microscopy confirms the loss of apical basal body localization. In untreated airway epithelium cultured for 3 days, basal bodies were localized to the apical cell compartment, where they anchor the axonemal structures (Figure 2D). After treatment with IL-13 at 50 ng/ml for 3 days or 100 ng/ml for 3 or 8 days, basal bodies were distributed throughout the cytoplasm and not localized to the apical cell compartment (Figures 2E and 2F, and data not shown). The number of basal bodies observed was markedly reduced in the tissue treated with 100 ng/ml of IL-13 for 8 days. This phenotype of axonemal loss and basal body mislocalization is similar to the respiratory epithelial phenotype of mice with a targeted mutation of the *foxj1* gene (15, 17).

IL-13 Decreases *foxj1* Expression by Airway Epithelial Cells

Because of the similarity in phenotype between the IL-13-treated airway tissue and the airway of *foxj1*^{-/-} mice, we hypothesized that the loss of cilia in response to IL-13 was mediated through changes in *foxj1* expression. Thus, expression of *foxj1* in cultured human airway epithelial tissue treated with IL-13 was examined. By Western blot analysis, there was a 50% decrease in the 65-kD *foxj1* protein after treatment of airway epithelial tissue with 50 ng/ml of IL-13 for 3 days (Figure 3A). To determine if the decrease in *foxj1* protein was the result of a decrease in *foxj1* expressing cells, tissues were examined by immunofluorescence. In untreated airway epithelial tissue, 39 ± 9% of the cells had nuclei positive for *foxj1* (Figure 3B). After treatment of the airway epithelial tissue for 3 days with 50 ng/ml IL-13, *foxj1* localization remains nuclear, but the percentage of *foxj1* positive nuclei was decreased to 8 ± 5% of the cells ($P = 0.01$) (Figure 3C; see also Figures 4B, 4C, and 6B). As has been previously reported, mucous cell hyperplasia was also noted after treatment of the airway epithelial cells with IL-13 (22, 23, 37, 38). In untreated airway epithelial tissue, 11 ± 5% of the cells were positive for the mucous cell marker Muc-5AC (Figure 3B). The percentage of Muc-5AC-positive cells increased to 28 ± 10% of cells after 3 days of treatment with 50 ng/ml IL-13 ($P = 0.04$) (Figure 3C). A decrease in *foxj1*-positive nuclei and increase in Muc-5AC-positive cells was also observed with 100 ng/ml IL-13 for 3 days (data not shown).

In addition to the decrease in *foxj1*-positive cells and mucous cell hyperplasia, cells with a mucociliary phenotype were observed after treatment with IL-13 (Figure 4). In untreated airway epithelial tissues cultured for 3 days, no cells were observed that were positive for both *foxj1* and Muc-5AC (Figure 4A). After treatment with either 50 ng/ml or 100 ng/ml IL-13, cells were observed in the airway epithelium that were positive for both markers (Figures 4B and 4C). Cells with a mucociliary phenotype have been previously reported after treatment with IL-13 (34).

To confirm the mucociliary phenotype, double immunofluorescence was performed with antibody to Muc-5AC and pericentrin (Figure 5). In untreated airway epithelial cells cultured for 3 days, cells with apically localized pericentrin can be seen as separate from Muc-5AC-positive cells (Figure 5A). After treatment with either 50 ng/ml or 100 ng/ml IL-13 for 3 days, cells with a mucociliary phenotype are observed (Figure 5B and data not shown). These cells contain both mislocalized basal bodies,

Figure 4. Cells with a mucociliary phenotype after treatment of cultured human airway epithelial cells with IL-13. Immunofluorescence of cells treated with 50 ng/ml IL-13 for 3 days (A) or 100 ng/ml IL-13 for 3 days (B). Cells were incubated with primary antibody to foxj1 (fluorescein) or Muc5AC (Texas red). Solid arrows indicate cells positive for both foxj1 and Muc5AC; open arrows indicate cells positive for Muc5AC only; asterisks indicate cells positive for foxj1 only. Green, foxj1; red, Muc5AC. Scale bar = 5 μ m.

as indicated by the pericentrin staining, and are positive for the mucous cell marker Muc5AC.

IL-13 Decreases Ezrin Expression by Airway Epithelial Cells

In *foxj1*^{-/-} mice, loss of axonemal structures and mislocalization of basal bodies from the apical cell compartment was associated with the loss of ezrin from the apical cell compartment (17, 18). We hypothesized that a similar mechanism might be involved in the IL-13-mediated loss of cilia. Treatment of cultured human airway epithelial tissue with IL-13 also resulted in a loss of ezrin from the apical cell compartment (Figure 6). By immunofluorescence of untreated tissue, ezrin is localized to the apical cell compartment of foxj1-positive cells (Figure 6A).

Figure 5. Cells with a mucociliary phenotype following treatment of cultured human airway epithelial cells with IL-13. Double immunofluorescence of either untreated airway epithelial cells cultured for 3 days (A) or airway epithelial cells treated with 50 ng/ml of IL-13 for 3 days (B). Cells were incubated with primary antibody to pericentrin (Texas red) and Muc5AC (fluorescein). Arrowhead indicates cell positive for pericentrin only; asterisk indicates cell positive for Muc5AC only; arrow indicates cell positive for both pericentrin and Muc5AC. Red, pericentrin; green, Muc5AC; blue, DAPI. Scale bar = 2 μ m.

After treatment with IL-13 at 50 ng/ml or 100 ng/ml, there is a loss of ezrin from the apical cell compartment in cultured human airway epithelial tissue (Figure 6B and data not shown). This loss of apical ezrin coincides with the loss of foxj1-positive nuclei after IL-13 treatment.

IL-13 Regulates Expression of the foxj1 Promoter

Because of the similarity in phenotype between IL-13-treated airway epithelial tissue and the airway epithelium of *foxj1*^{-/-} mice, as well as decreased foxj1 expression in response to IL-13, we hypothesized that IL-13 regulated foxj1 expression through binding of STAT protein to the foxj1 promoter. Sequence analysis of the mouse *foxj1* promoter revealed a consensus STAT-binding element (SBE) at -1260 to -1268 bp (Table 1). Examination of the sequence of the human *FOXJ1* promoter revealed a conserved SBE at -1431 to -1439 bp (Table 1). Co-transfection of BEAS 2B cells with a STAT-6 expression vector and CAT reporter plasmid with 2.2 kilobases of the mouse *foxj1* promoter region resulted in a 50% decrease in CAT activity compared with the foxj1 promoter-reporter plasmid alone (Figure 7A). Site-directed mutagenesis of the conserved SBE abrogated completely the STAT-6 inhibition of foxj1 promoter activity (Table 1 and Figure 7A).

To analyze the effect of IL-13 on foxj1 promoter activity in BEAS 2B cells, it was first necessary to demonstrate that BEAS 2B cells could support the phosphorylation of STAT-6 in response to IL-13. To test for IL-13-induced phosphorylation of STAT-6 in BEAS 2B cells, Western blot analysis of cell extract from IL-13-treated and untreated BEAS 2B cells was performed with antibody specific to phosphorylated STAT-6 (Figure 7B). In the untreated cells, no product was detected with primary antibody to phosphorylated STAT-6. In the

Figure 6. IL-13 results in loss of apical ezrin in cultured human airway epithelial cells. Immunofluorescence of untreated cultured human airway epithelial cells (A) or cells treated with 50 ng/ml IL-13 for 3 days (B). Cells were incubated with primary antibody to foxj1 (fluorescein) or ezrin (Texas red). Green, foxj1; red, ezrin. Scale bar = 50 μ m.

treated cells, a 105-kD protein was detected with antibody to phosphorylated STAT-6 (Figure 7B). The control lane contains cell extract from IFN- γ -treated Daudi cells. Transfection of BEAS 2B cells with a 2.2-kb foxj1 promoter-reporter plasmid in the presence of 50 ng/ml IL-13 resulted in a 50% decrease in promoter activity (Figure 7C).

DISCUSSION

Changes in the pulmonary epithelial cell population are integral to the pathogenesis of a variety of pulmonary diseases, including asthma and infection. Frequently, these changes include an increase in mucous cells and decrease in ciliated cells. The increased levels of airway cytokines, such as IL-13, associated

Figure 7. STAT-6 and IL-13 inhibit expression of the foxj1 promoter. (A) Relative CAT activity from BEAS 2B cells transfected with wild-type or mutant foxj1-CAT reporter constructs in the absence or presence of a STAT-6 expression vector. The control vector is the empty pCAT3e vector. (B) Western blot of protein from BEAS 2B cells cultured in the absence or presence of 50 ng/ml IL-13 for 3 days. Primary antibody to phosphorylated STAT-6 gives a band of ~105 kD. Control cell extract is from IFN- γ -treated Daudi cells. (C) Relative CAT activity from BEAS 2B cells transfected with a foxj1-CAT reporter construct in the absence or presence of 50 ng/ml IL-13. The control vector is the empty pCAT3e vector.

with asthma and pulmonary infection suggest a role for these mediators in the changes observed in the airway epithelial phenotype (39, 40). The central role played by IL-13 in mucous cell metaplasia and hyperplasia has been well described (30, 37, 38, 41). Treatment of cultured differentiating nasal epithelial cells with IL-13 resulted in an increased number of mucous cells and inhibition of ciliogenesis (22). We have demonstrated here that in differentiated airway epithelial cells, treatment with IL-13 resulted in a loss of ciliated epithelial cells (and an increase in mucous cells). Moreover, after treatment of differentiated human airway epithelial cells with IL-13, cells were noted that still retained basal bodies although the basal bodies were not apically localized. Mislocalization of basal bodies was also observed in cultured differentiating nasal epithelium treated with IL-13 (22). Thus, the inability of basal bodies to anchor or to remain anchored to the apical cytoskeleton in response to IL-13 provides a cellular mechanism for cilia loss or inhibition of ciliogenesis.

Polarization of epithelial cells into apical and basal compartments is essential for their normal function. Localization of proteins to the apical or basal compartment requires cellular mechanisms for segregating these proteins and then maintaining their appropriate localization (42). Members of the ERM family of proteins, which includes ezrin, act to maintain anchoring of proteins to the apical cytoskeleton (43). In the pulmonary epithelium, ezrin and the associated scaffold protein EBP-50 anchor basal bodies to the apical cytoskeleton (17, 18). In cultured human airway epithelium, the loss of cilia with IL-13 treatment was associated with a loss of apical ezrin. A similar observation was made after IL-13 treatment of differentiating nasal epithelial cultures (22). These observations are consistent with the loss of basal body anchoring to the apical cytoskeleton as the mechanism of cilia loss in response to IL-13. Changes in cytoskeletal proteins in response to IL-13 have also been observed during macrophage fusion (44). In addition to its role in basal body anchoring, ezrin acts to anchor a number of other airway epithelial proteins, such as the CFTR, to the apical cytoskeleton (45). The loss of apical ezrin in response to IL-13 may thus result in a more generalized airway epithelial dysfunction.

Targeted mutation of the mouse *foxj1* gene resulted in a phenotype similar to that observed in airway epithelial cells in response to IL-13. Axonemal structures are absent with mislocalized basal bodies and loss of apical ezrin, as well as EBP-50 (17, 18). This similarity in phenotype led us to examine the possible regulation of *foxj1* expression by IL-13. After IL-13 treatment of cultured airway epithelial cells, decreased *foxj1* expression was detected. This appeared to be due primarily to a decrease in the number of *foxj1*-positive cells in the epithelium. Regulation of *foxj1* expression by IL-13 is mediated by a conserved SBE located in the mouse and human *foxj1* promoter. This suggests a molecular mechanism for cilia loss, with decreased *foxj1* expression resulting in a failure of basal body anchoring to the apical cytoskeleton and subsequent loss of axonemal structures. A similar decrease in *foxj1* expression associated with loss of cilia was noted in mouse pulmonary epithelial cells during paramyxoviral infection (20). The decrease in *foxj1* after paramyxoviral infection may be mediated by the increased airway levels of IL-13 observed after such infection (24, 39, 46). The proposed mechanisms for cilia loss in response to IL-13 are summarized in Figure 8.

The presence of cells with characteristics of both ciliated and mucous cells in the airway epithelium suggests transdifferentiation as the mechanism for the decrease in ciliated cells and increase in mucous cells in response to IL-13. Cells with characteristics of both ciliated and mucous cells have been previously observed in cultured mouse airway epithelial cells

Figure 8. Proposed model for the loss of cilia in response to IL-13. Recruitment of Th2 lymphocytes in response to infection, asthma, or injury results in increased IL-13 in the airway. Binding of IL-13 to its receptor results in phosphorylation of STAT-6 (STAT-6*), which then translocates to the nucleus. Phosphorylated STAT-6 binds to the STAT-binding element in the *foxj1* promoter and inhibits *foxj1* expression. Decreased *foxj1* results in a loss of ezrin binding to the apical cytoskeleton through an as yet unidentified mechanism. This results in basal body mislocalization and the loss of axonemal structures.

treated with IL-13 (34). Evidence for transdifferentiation of ciliated cells to mucous cells does not eliminate the possibility that other airway epithelial cell types also contribute to the increase in mucous cells observed after IL-13. It does, however, support the concept of plasticity of airway epithelial cells and suggests a mechanism for rapid changes in the airway epithelial phenotype that may be important in airway injury and repair. After naphthalene-induced injury of the mouse airway epithelium, for example, transdifferentiation of the ciliated cells provides the mechanism for the recovery of other airway epithelial cell types (8). Changes in *foxj1* expression provide at least a part of the mechanism for the transdifferentiation of ciliated epithelial cells. Elucidating in more detail the changes in other transcriptional programs required for the transdifferentiation of airway epithelial cells will provide greater insight into the pathogenesis of airway diseases as well as potential therapeutic approaches.

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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