Goblet Cells Are Derived from a *FOXJ1*-Expressing Progenitor in a Human Airway Epithelium

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The overproduction of mucus is a key pathology associated with respiratory diseases, such as asthma and chronic obstructive pulmonary disease. These conditions are characterized by an increase in the number of mucus-producing goblet cells in the airways. We have studied the cellular origins of goblet cells using primary human bronchial epithelial cells (HBECs), which can be differentiated to form a stratified epithelium containing ciliated, basal and goblet cells. Treatment of differentiated HBEC cultures with the cytokine IL-13, an important mediator in asthma, increased the numbers of goblet cells and decreased the numbers of ciliated cells. To determine whether ciliated cells act as goblet cell progenitors, ciliated cells in HBEC cultures were hereditably labeled with enhanced green fluorescent protein (EGFP) using two lentiviral vectors, one which contained Cre recombinase under the control of a *FOXJ1* promoter and a second Cytomegalovirus (CMV)–floxed-EGFP construct. The fate of the EGFP-labeled ciliated cells was tracked in HBEC cultures. Treatment with IL-13 reduced the numbers of EGFP-labeled ciliated cells compared with untreated cultures. In contrast, IL-13 treatment significantly increased the numbers of EGFP-labeled goblet cells. This study demonstrates that goblet cells formed in response to IL-13 treatment are in part or wholly derived from progenitors that express the ciliated cell marker, *FOXJ1*.

Keywords: ciliated cell; FOXJ1; goblet cell; IL-13; MUC5AC

Excessive mucus production in the airways has been linked to several of the hallmark features of respiratory diseases, such as asthma (1) and chronic obstructive pulmonary disease (COPD) (2). Increased frequency and duration of infections, decline in lung function, and increases in morbidity and mortality in COPD have all been associated with mucus hypersecretion (2–4). Additionally, the progression of COPD has been reported to be strongly associated with accumulation of mucus in the lumen of the small airways (5, 6). Both asthma and COPD are characterized by an increase in the number of goblet cells that are mucus-producing cells of the respiratory epithelium (7, 8). Whereas, in the large airways, mucus is produced by goblet cells and submucosal glands, in the small airways, the only source of mucus is the goblet cell (7).

Goblet cells can rapidly secrete mucins to form a mucus layer that protects against inhaled pathogens, toxins, and other foreign particles. Although this mucus layer normally plays a protective role, abnormal mucus production and clearance can contribute to respiratory disease pathologies (9, 10). Despite the importance of goblet cells in respiratory diseases, the origins of goblet cells and processes regulating their formation are poorly understood. The mucin, MUC5AC, is one of the major mucins found in airway secretions in asthma and COPD (7, 11, 12). A candidate implicated in the control of MUC5AC expression and goblet cell formation is the T helper (Th) type 2 cytokine, IL-13. Indeed, IL-13 gives rise to increased goblet cell numbers in both murine models of allergic asthma (13, 14) and *in vitro* models of the human respiratory epithelium (15). Moreover, IL-13 has been demonstrated to induce MUC5AC mucin expression *in vivo* and *in vitro* (15, 16).

CLINICAL RELEVANCE

The understanding of the origins of goblet cell progenitors contributes to our understanding of processes regulating goblet cell formation. This may ultimately aid the identification of new therapeutic targets to treat excessive mucus in respiratory diseases.

Studies in the mouse have implicated Clara or ciliated cells as progenitors of goblet cells via a process of transdifferentiation (17, 18). Data supporting transdifferentiation of Clara cells as a mechanism of goblet cell formation include a study using an ovalbumin (OVA)-induced mouse model of goblet cell formation, where mucin expression was found in a subset of Clara cells in the proximal, but not distal, airways (17). In a separate study, an ultrastructural analysis of mouse lung after OVA challenge showed mucus-producing cells in the airways, with many characteristics of Clara cells (19). Additional indirect evidence of Clara cell transdifferentiation comes from an analysis of Clara and goblet cell numbers in the lung of a mouse OVA model, where a decrease in the numbers of Clara cells was accompanied by an increase in the number of goblet cells (20). In contrast, in a Sendai virus model, transdifferentiation of ciliated cells was implicated as a mechanism for the observed goblet cell metaplasia, involving IL-13 and EGFR pathways (18). One limitation of the studies to date examining the origins of goblet cells in the airways is that the mouse has been used as the model organism. Differences in human and mouse airway epithelial cell biology (21, 22) indicate the importance of performing studies to elucidate goblet cell progenitors in human as well as mouse model systems. In addition, a direct, lineage-tracing approach would help to clarify the confusion over mechanisms underlying mucus metaplasia.

Here, we have investigated the role of the ciliated cell as a progenitor of the goblet cell using a cell lineage–tagging approach and primary human bronchial epithelial cells (HBECs). A lentiviral cotransduction approach employing the *FOXJ1* promoter was used to deliver constructs for permanent Cre/loxP-mediated enhanced green fluorescent protein (EGFP) tagging of ciliated cells. Subsequently, the fate of the EGFP-tagged ciliated cells after treatment with IL-13 to induce goblet cell formation was monitored by histology. By increasing our understanding of cell types that contribute to goblet cell formation in human model systems, new therapeutic approaches

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for targeting excessive mucus production in respiratory diseases may be identified.

MATERIALS AND METHODS

Generation of Plasmids

A *FOXJ1*-EGFP lentiviral construct (Figure 1A) containing a 1,008-bp fragment of the human *FOXJ1* promoter upstream of EGFP was generated as described in the supplemental MATERIALS AND METHODS section online. Cytomegalovirus (CMV)–floxed-EGFP (CMV loxP-lacZ-STOPx3-loxP) and *FOXJ1*-Cre constructs (Figure 1B) for use in Cre recombinase–mediated recombination for ciliated cell lineage tagging were generated as described in the supplemental MATERIALS AND METHODS.

Lentivirus Production and Transduction of HBECs

HBECs were cultured essentially as described previously (23), except that cells were cultured submerged for 7 days, after which they were exposed to air–liquid interface (ALI) and cultured for a further 14–28 days. IL-13 (Peprotech, London, UK) was added to the basolateral medium at a final concentration of 1 ng/ml. Lentivirus production, concentration, and titer was performed as described in the supplemental MATERIALS AND METHODS. HBECs were transduced with concen-

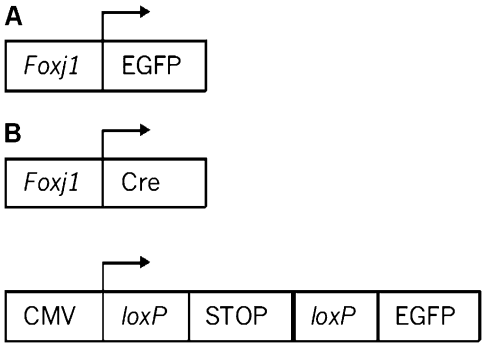

Figure 1. Schematic representation of transgene constructs. A *FOXJ1*–enhanced green fluorescent protein (EGFP) expression construct designed to determine cell-type specificity of the *FOXJ1* promoter in differentiated human bronchial epithelial cells (HBECs) contained EGFP under the control of the *FOXJ1* promoter (*A*). The lineage-tagging constructs designed for hereditable labeling of ciliated cells were Cre recombinase under the control of the *FOXJ1* promoter (*FOXJ1*-Cre) and a Cytomegalovirus (CMV)–floxed-EGFP–labeling construct (*B*).

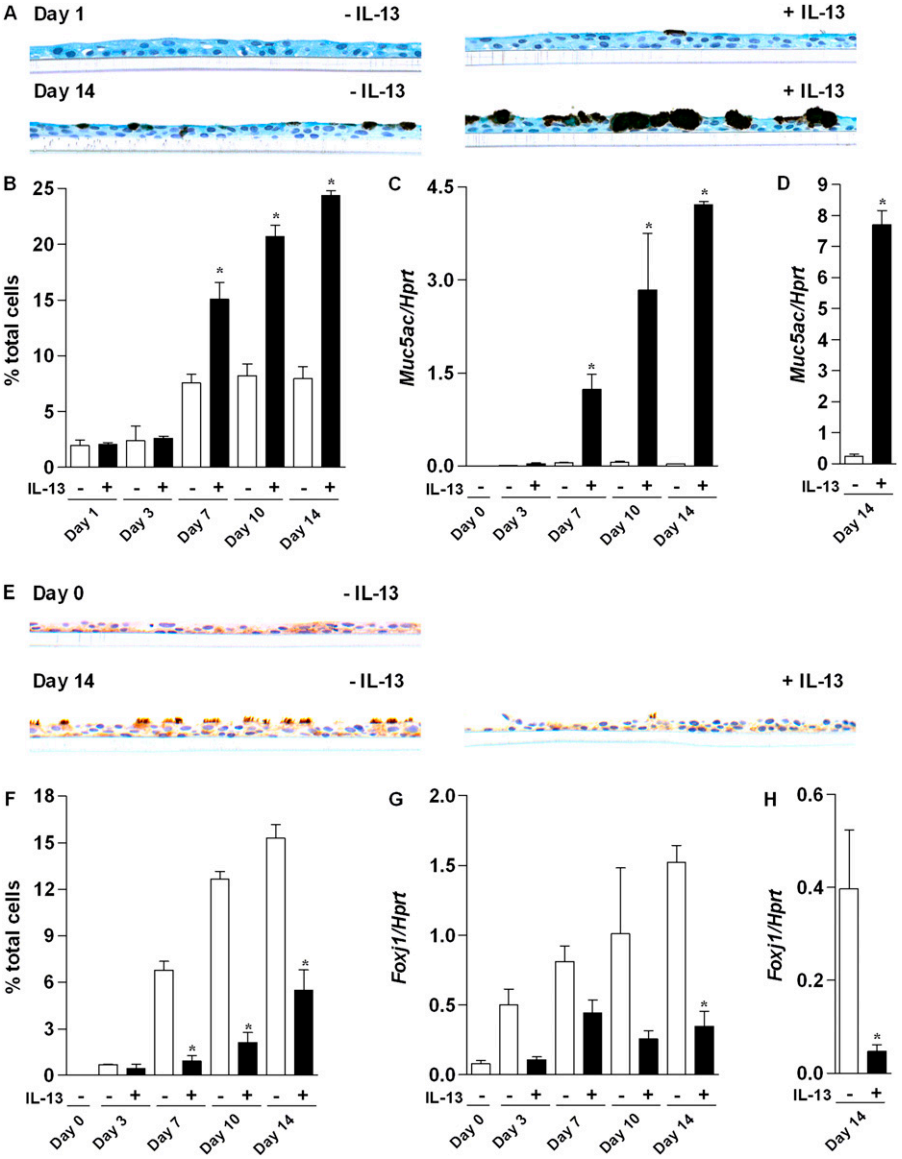

Figure 2. IL-13 increases goblet cell density and suppresses ciliated cell formation. HBECs were cultured in the presence or absence of 1 ng/ml IL-13 for 14 days at air–liquid interface (ALI). Cells were immunostained with an anti-MUC5AC antibody for the identification of goblet cells ([*A*], *brown*) or an anti-tubulin antibody for the identification of ciliated cells ([*E*], *brown*). The numbers of MUC5AC-positive goblet cells (*B*) and tubulin-positive ciliated cells (*F*) were counted in sections from five time points throughout the culture period, treated with or without IL-13. Expression of *Muc5ac* and *FOXJ1* were analyzed by TaqMan quantitative PCR and normalized to that of the housekeeping gene, *Hprt*, throughout the ALI culture period (*C* and *G*) and at Day 14 ALI in four separate HBEC donors (*D* and *H*). Data are shown as mean cell number as a percentage of total cells or mean mRNA expression (±SEM) from three Transwell inserts. *Open bars* are untreated cultures; *closed bars* are those treated with IL-13. Differences between treated and untreated groups at the same time point were tested using two-tailed *t* tests (*$P < 0.05$). Data are representative of two independent experiments. Original magnification, 400×.

trated virus added to the apical chamber of cells grown on transwell inserts as described in the supplemental MATERIALS AND METHODS.

Immunohistochemistry

After differentiation at ALI, HBECs were fixed, processed, and embedded in paraffin wax before 3-μm sections were cut as described previously (23). All immunohistochemistry was performed on an automated Ventana Discovery XT immunostainer (Ventana Inc., Tucson, AZ). The primary antibodies and dilutions used were as follows: mouse IgG_1 anti-MUC5AC (Lab Vision Products/Thermo Fisher Scientific, Runcorn, UK) at 1:1,000; mouse IgG_{2b} anti-acetylated tubulin (Sigma, Gillingham, UK) at 1:2,000; mouse IgG_{2a} anti-p63 (Abcam plc, Cambridge, UK) at 1:40; and rabbit anti-EGFP (Abcam plc) at 1:400 (fluorescence) or 1:500 (RedMap).

Details of the chromogenic DABMap staining of tubulin and MUC5AC are described in the supplemental MATERIALS AND METHODS. Fluorescence costaining of EGFP with either tubulin or MUC5AC in HBECs is also described in the supplemental MATERIALS AND METHODS. To costain for p63 and EGFP, samples were stained as detailed in the supplemental MATERIALS AND METHODS, with RedMap reagents (Ventana Inc.) applied to stain sites of EGFP antibody binding red.

Confocal microscopy was performed using a Leica TCS SP2 UV confocal laser scanning microscope (Leica Microsystems Ltd., Milton Keynes, UK). Brightfield and epifluorescence microscopy was performed using a Zeiss Axioplan 2 microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). To quantify cell populations, cell counts were performed manually along the length of each insert (~10-mm sections). The total cell number was scored by counting 4′,6-diamidino-2-phenylindole–labeled nuclei. Stained cell numbers are expressed as a percentage of the total nuclei (n = 3–6 Transwell inserts per treatment group). Differences between treatment group means were tested with two-tailed t tests using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Quantitative RT-PCR

The preparation of total RNA from HBEC cultures, first-strand cDNA synthesis and *Muc5ac* and hypoxanthine phosphoribosyl transferase (*Hprt*) 1 quantitative PCR was performed as described previously (23). Preprepared Assay-On-Demand primer and probe sets (Applied Biosystems, Foster City, CA) were used to analyze *FOXJ1* expression. Expression of *FOXJ1* and *Muc5ac* were normalized to the housekeeping gene *Hprt*. Three independent HBEC samples were prepared for each condition, and each sample was analyzed in duplicate.

RESULTS

Effect of IL-13 on Epithelial Cell Populations in a Differentiated Human Airway Epithelium

To characterize differentiated HBECs, the number of ciliated and goblet cells present in cell cultures and expression of their respective cell type–specific markers were analyzed over a 14-day differentiation time course at ALI in cultures treated with or without IL-13. Goblet cells expressing the mucin MUC5AC were observed in HBEC cultures located at the apical surface of the epithelium (Figure 2A). In untreated cultures, few goblet cells were present, but the numbers were raised by IL-13 treatment at 14-day ALI compared with untreated controls (Figure 2A). Quantification of goblet cells in HBEC ALI cultures showed that IL-13 significantly increased the numbers of goblet cells from Day 7 to Day 14 ALI compared with untreated controls (Figure 2B). At Day 14 ALI in IL-13–treated cultures, 24% of the total cell population were goblet cells, consistent with previous reports (15). The increases in goblet cells induced by IL-13 are mirrored by increases in *Muc5ac* mRNA assayed by quantitative PCR (Figure 2C). The significant increase in *Muc5ac* expression induced by IL-13 treatment at Day 14 ALI was confirmed in four independent HBEC donors (Figure 2D).

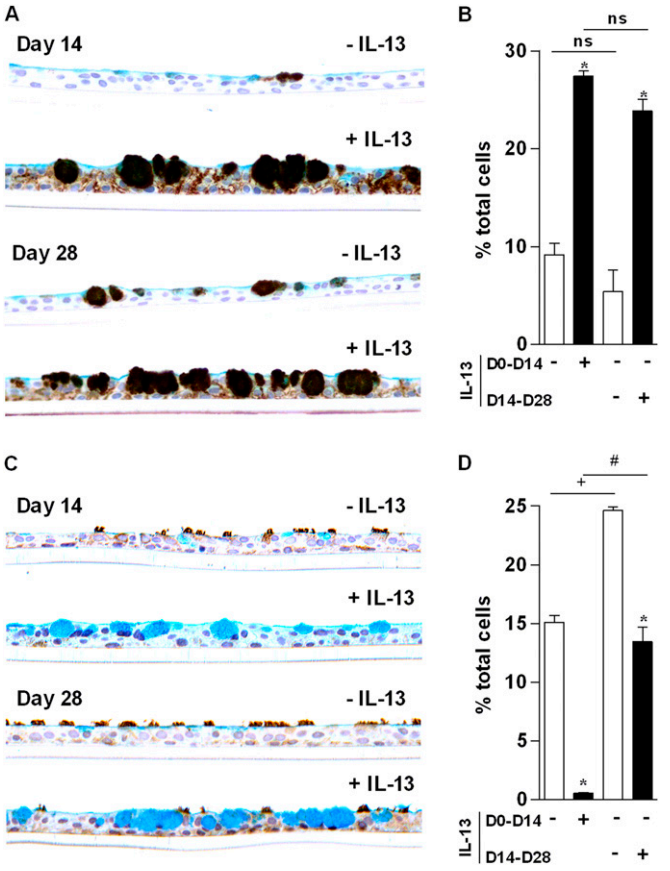

Figure 3. IL-13 increases goblet cell density and suppresses ciliated cell formation in a predifferentiated epithelium. HBECs were either cultured in the presence or absence of 1 ng/ml IL-13 for 14 days at ALI (14-d model) or in the absence of IL-13 for 14 days at ALI, followed by a further 14 days at ALI in the presence or absence of 1 ng/ml IL-13 (28-d model). Goblet cells (*A*) and ciliated cells (*C*) were stained as described in Figure 2. Samples here were also counterstained for expression of mucins with alcian blue. Images are from Day 14 ALI of the 14-day model or Day 28 ALI from the 28-day model. The numbers of goblet cells (*B*) and ciliated cells (*D*) were quantified as described in Figure 2 on the final day of the 14- and 28-day models. Data are shown as mean cell number as a percentage of total cells from three Transwell inserts. *Open bars* are untreated cultures; *closed bars* are those treated with IL-13. Differences between untreated and IL-13–treated cultures ($^*P < 0.05$) or untreated cultures ($^+P < 0.05$; ns, not significant) or IL-13–treated cultures ($^\#P < 0.05$) between the 14- and 28-day models were tested using two-tailed t tests. Data are representative of two independent experiments. Original magnification, 400×.

Histological analysis of ciliated cells by staining for tubulin expression first revealed the presence of ciliated cells at the apical surface of untreated cultures at Day 3 ALI, with numbers increasing over the time course up to Day 14 ALI (Figures 2E and 2F). However, in cultures treated with IL-13, fewer ciliated cells were present, indicating that, in contrast to goblet cell formation, IL-13 suppresses ciliated cell formation (Figure 2F). At Day 14 ALI, in untreated cultures, ciliated cells comprised 15% of the total cell population, consistent with previous reports (24), whereas IL-13 caused a significant reduction in ciliated cell numbers (Figure 2F). Quantitative PCR demonstrated that expression of the ciliated cell–associated transcription factor *FOXJ1* mRNA increased from Day 1 to Day 14 ALI. This increase was also suppressed by IL-13 treatment, and was significantly reduced by Day 14 ALI compared with untreated

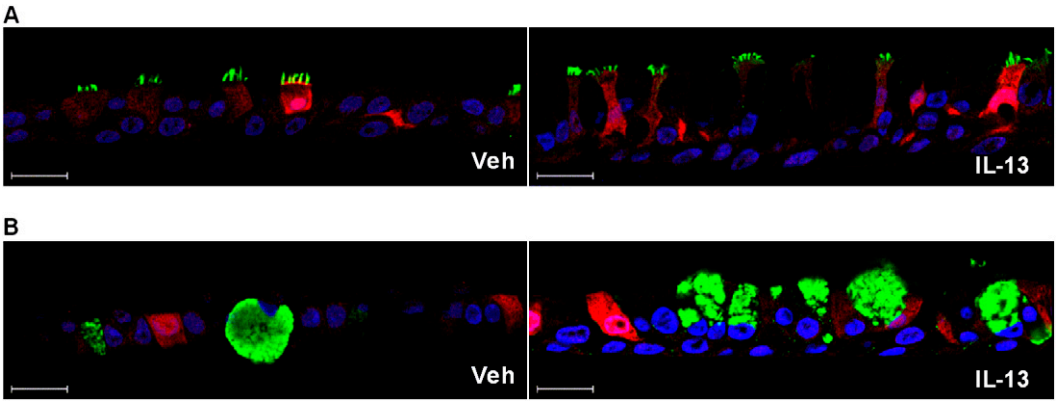

Figure 4. The *FOXJ1* promoter directs transgene expression to ciliated cells in differentiated HBECs. HBECs were transduced with a lentiviral vector containing EGFP under the control of a *FOXJ1* promoter, and cultured in the absence of IL-13 for 14 days at ALI, followed by a further 14 days at ALI in the presence or absence of 1 ng/ml IL-13 (28-d model). Samples taken at Day 28 ALI were analyzed by confocal immunofluorescence microscopy with anti-EGFP (*red*) and either anti-tubulin ([*A*] *green*) or anti-MUC5AC ([*B*] *green*) antibodies. Data are representative of two independent experiments. *Scale bar*, 20 μm. Veh, vehicle.

cultures (Figure 2G), an observation confirmed in four independent donors (Figure 2H). Thus, changes in *FOXJ1* expression correlate with changes in ciliated cell number in response to IL-13. Differentiated HBEC cultures were also stained for the Clara cell marker, Clara Cell Secretory Protein (CCSP), but no staining was obtained in either untreated or IL-13–treated cells (as shown in Figure E1 in the online supplement), indicating that HBEC cultures contain no Clara cells, as anticipated. Basal cells were also detected at the basaloteral side of HBEC cultures using an antibody against p63 or K14, although there was no apparent change in basal cell numbers with IL-13 treatment (data not shown).

IL-13–Induced Goblet Cell Formation in a Differentiated Airway Epithelium

As airway remodeling *in vivo* takes place with a fully differentiated epithelium, we developed a modified model of goblet cell formation, which allows for the differentiation of HBECs before exposure to IL-13. Cultures were differentiated at ALI for 14 days, after which IL-13 was added for a further 14 days. Formation of goblet and ciliated cells in this extended 28-day model was compared with the shorter 14-day model.

Histologically, there was no significant difference in the amount of MUC5AC-positive goblet cells between the two models (Figures 3A and 3B). IL-13–induced increases in goblet cells were similar in both models, and were significantly higher than untreated controls (Figure 3B). In the longer, 28-day model, goblet cells represented 5% of the total cell population in untreated cultures compared with 24% in the IL-13–treated cultures (Figure 3B). Analysis of tubulin staining demonstrated that IL-13 reduced the numbers of ciliated cells in cultures of both models compared with untreated controls (Figure 3C). However, although ciliated cells were largely absent from IL-13–treated cultures of the 14-day model, ciliated cells were observed in IL-13–treated cultures of the 28-day model. In untreated cultures, the number of ciliated cells was significantly increased from 15 to 25% on going from the 14-day to the 28-day model (Figure 3D), consistent with previous reports (25). In IL-13–treated cultures, there was also a significant increase in ciliated cells, from 0.5 to 13% of the total cell population when comparing the 14- and 28-day models. This probably reflects differences in the two models: in the 14-day model, IL-13 is present at the beginning of and during the process of epithelial differentiation, and, as such, may suppress the formation of ciliated cells. In contrast, in the 28-day model, ciliated cells are allowed to form before the addition of IL-13, which is only added for the last 14 days of the differentiation period.

Ciliated Cell Lineage Tagging

To investigate the origins of the goblet cells in IL-13–treated HBEC cultures, we investigated whether ciliated cells can act as progenitors of goblet cells formed in response to IL-13 by a direct cell-tagging approach. The extended 28-day model was used for these studies, as this model allows for the full differentiation of ciliated cells before IL-13–induced goblet cell formation.

The *FOXJ1* promoter was used to direct exogenous gene expression in ciliated cells. In the first instance, HBECs were transduced with lentiviral vectors containing EGFP directly under the control of the *FOXJ1* promoter to confirm that expression directed by this promoter is restricted to ciliated cells. Undifferentiated HBECs were transduced with a *FOXJ1* promoter–EGFP lentivirus (Figure 1A) and cultured over 28 days at ALI, in the presence or absence of IL-13 for the final 14 days at ALI. For histological analysis, sections were costained either with anti-EGFP and anti-tubulin or anti-EGFP and anti-MUC5AC antibodies. Confocal microscopy revealed that EGFP was exclusively expressed in apical cells positive for tubulin expression and in no other cell types (Figure 4), indicating that the *FOXJ1* promoter specifically directs exogenous gene expression to ciliated cells. Staining with the appropriate isotype control antibody for MUC5AC, tubulin, or EGFP performed on transduced HBECs gave the anticipated negative staining, confirming the specificity of the antibodies used in this study (data not shown).

For ciliated cell lineage tagging, undifferentiated HBECs were cotransduced with two lentiviral vectors before differentiation at ALI. A *FOXJ1*-Cre lentivirus contained Cre recombinase under the control of the *FOXJ1* promoter, and a second vector, CMV–floxed-EGFP, contained a floxed-EGFP expression cassette under the control of the constitutive CMV promoter (Figure 1B). Expression of Cre in ciliated cells results in recombination of the floxed-EGFP expression cassette and, thereby, expression of EGFP under control of the CMV promoter. In HBEC cultures, the CMV promoter was shown to be active in all cell types (data not shown). Hence, ciliated cells, and any cells derived from ciliated cells, are irreversibly tagged with EGFP.

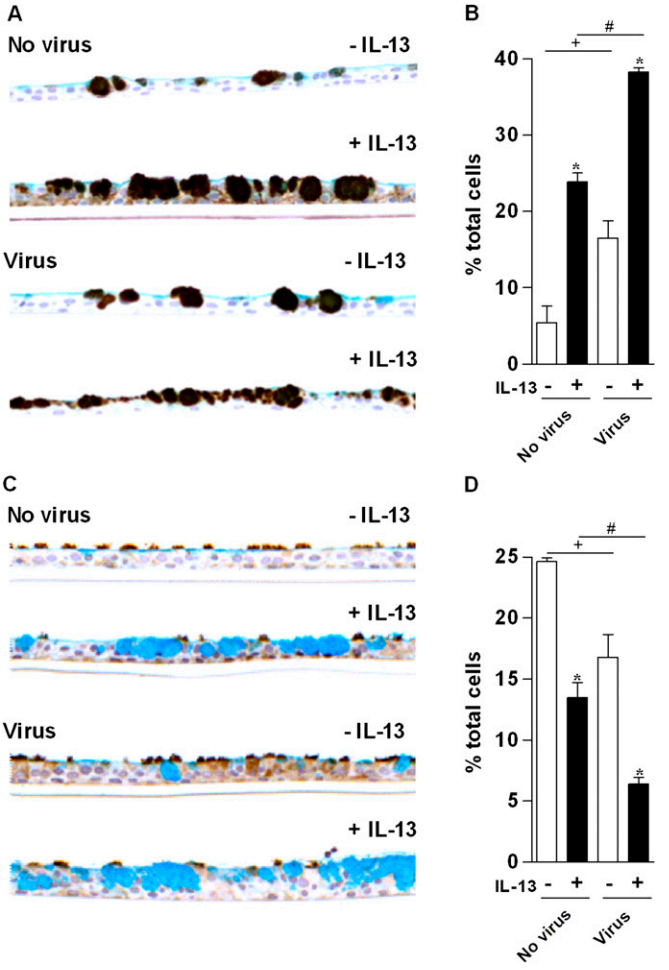

Figure 5. The effect of transduction of HBECs with lineage-tagging lentiviruses on epithelial differentiation and response to IL-13. HBECs were transduced with a lentiviral vector containing Cre recombinase under the control of a *FOXJ1* promoter and a CMV–floxed-EGFP construct. After transduction cells were cultured in the absence of IL-13 for 14 days at ALI, followed by a further 14 days at ALI in the presence or absence of 1 ng/ml IL-13 (28-d model). Goblet cells (*A*) and ciliated cells (*C*) were stained as described in Figure 2. Samples here were also counterstained for expression of mucins with alcian blue. Images are from Day 28 ALI. The numbers of goblet cells (*B*) and ciliated cells (*D*) were quantified as described in Figure 2 at Day 28 ALI. Data are shown as mean cell number as a percentage of total cells from three Transwell inserts. *Open bars* are untreated cultures; *closed bars* are those treated with IL-13. Differences between untreated and IL-13–treated cultures ($^{*}P < 0.05$) or untreated cultures ($^{+}P < 0.05$) or IL-13–treated cultures ($^{\#}P < 0.05$) between untransduced or virus-transduced cultures were tested using two-tailed *t* tests. Data are representative of two independent experiments. Original magnification, 400×.

Transduction of HBECs with ciliated cell–tagging lentiviruses resulted in no apparent changes in epithelial morphology compared with untransduced controls (Figures 5A and 5C). Treatment with IL-13 significantly elevated goblet cell number (Figure 5B), and significantly suppressed ciliated cell number (Figure 5D), regardless of whether cultures were transduced with lentivirus or not. However, in both IL-13–treated and untreated cultures, transduction with lentivirus resulted in increased numbers of goblet cells and decreased numbers of ciliated cells compared with nontransduced controls (Figures 5B and 5D). Thus, although virus transduction of HBECs caused an increase in goblet cell numbers and reduction of ciliated cell numbers, epithelial cell morphology was apparently unchanged.

In cultures of HBECs cotransduced with *FOXJ1*-Cre and CMV–floxed-EGFP ciliated cell–tagging lentiviruses, confocal microscopy of cells costained for EGFP and tubulin at Day 28 ALI revealed numerous EGFP-labeled ciliated cells (Figure 6A). Quantitative analysis of these sections revealed that 4.2 and 3% of the total cell population was labeled with EGFP in the absence and presence of IL-13, respectively, although regulation of the total EGFP-labeled cell population by cytokine treatment was not found to be significant (Figure 6C). As the *FOXJ1* promoter is used to drive the expression of Cre recombinase only in ciliated cells, only the ciliated cell population and any cells arising from these tagged cells should be labeled with EGFP after double viral transduction with *FOXJ1*-Cre and CMV–floxed-EGFP lentiviruses. In untreated cultures, 1.8% of the total cell population coexpressed EGFP and tubulin (Figure 6C), which amounts to 13.6% of the ciliated cells labeled with EGFP. IL-13 treatment resulted in a reduction in EGFP-labeled ciliated cells from 1.8 to 0.5% of the total cell population (Figure 6C), as well as reducing the number of non–EGFP-labeled ciliated cells (Figure 6D).

In contrast, cultures cotransduced with *FOXJ1*-Cre and CMV–floxed-EGFP lentiviruses and costained for EGFP and MUC5AC identified multiple EGFP-labeled goblet cells in cultures treated with IL-13, with few or none in the absence of IL-13 (Figure 6B). Indeed, IL-13 stimulation caused a significant ninefold increase in the number of EGFP-labeled MUC5AC-positive goblet cells, from 0.06 to 0.52% of the total cell population (Figure 6C). It can be concluded that EGFP-labeled goblet cells are expressing, or have expressed, *FOXJ1* and, therefore, these data suggest that goblet cells formed *de novo* within the respiratory epithelium in response to IL-13 exposure can be derived from progenitor cells that express *FOXJ1*.

In addition to EGFP-labeled ciliated and goblet cells, cultures transduced with lineage-tagging lentiviruses also contained EGFP-labeled cells located at the basolateral surface of differentiated HBEC cultures (Figures 6A and 6B). Such cells were only observed in cultures transduced with FOXJ1-Cre and CMV–floxed-EGFP lentiviruses used for ciliated cell tagging, and not in cultures transduced with lentiviruses containing EGFP directly under the control of the *FOXJ1* promoter. To further characterize these cells, sections from cultures transduced with FOXJ1-Cre/CMV–floxed-EGFP lineage–tagging lentiviruses were costained with antibodies against both EGFP and the basal cell marker, p63. Histological analysis of p63 expression showed p63-positive cells exclusively along the basolateral surface of differentiated HBEC cultures (Figure 7A). When quantified, EGFP-negative basal cells represented 15 and 14% of the total cell population in untreated and treated cultures, respectively, with no significant regulation by IL-13 (Figure 7C). Expression of EGFP in basolaterally located cells in cultures transduced with ciliated cell lineage–tagging lentiviruses colocalized with p63 (Figure 7A), identifying these cells as basal cells. In untreated and IL-13–treated cultures, EGFP-labeled basal cells represented 0.9 and 1.0% of the total cell population, respectively, and were not regulated by IL-13 (Figure 7B). The average number of cells counted per insert costained with EGFP and either tubulin, MUC5AC or p63 is shown in Table 1.

DISCUSSION

Elevated goblet cell numbers and excessive mucus production contribute to the pathology of respiratory diseases, such as

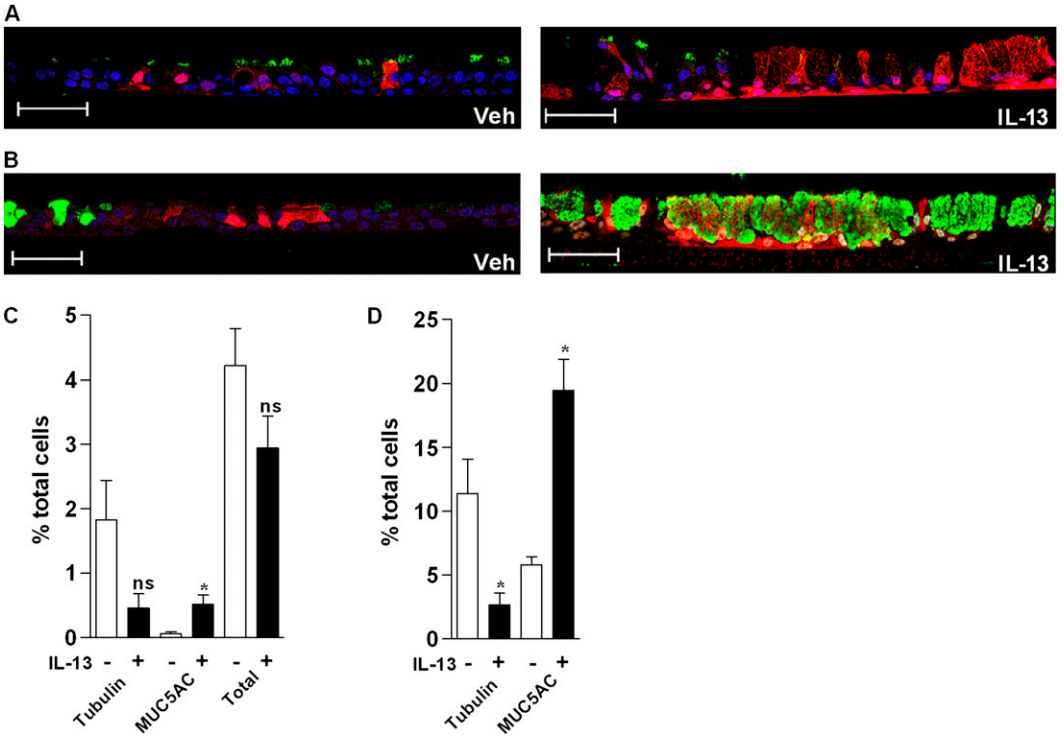

Figure 6. Goblet cells formed in response to IL-13 are derived from cells that express *FOXJ1*. HBECs were transduced with lentiviral vectors containing Cre recombinase under the control of the *FOXJ1* promoter and a CMV–floxed-EGFP construct. After transduction, cells were cultured in the absence of IL-13 for 14 days at ALI, followed by a further 14 days at ALI in the presence or absence of 1 ng/ml IL-13 (28-d model). Samples taken at Day 28 ALI were analyzed by confocal immunofluorescence microscopy with anti-EGFP (*red*) and either anti-tubulin ([*A*] *green*) or anti-MUC5AC ([*B*] *green*) antibodies. *Scale bar*, 50 μm. The total cell number and numbers of EGFP-positive cells (*C*) and EGFP-negative cells (*D*) stained with MUC5AC or tubulin counted in sections from cultures treated with or without IL-13 at Day 28 ALI are indicated. Data are shown as mean (±SEM) cell number as a percentage of total cells from six Transwell inserts derived from two independent experiments. *Open bars* are untreated cultures; *closed bars* are those treated with IL-13. Differences between treated and untreated groups were tested using two-tailed *t* tests (*$P < 0.05$).

asthma and COPD, but the cellular origins of goblet cells in the disease state are poorly understood. In the present study, we demonstrate using a ciliated cell lineage–tagging approach that one source of goblet cells formed in response to exposure to IL-13 in the human respiratory epithelium are *FOXJ1*-expressing cells resident within the epithelium.

We examined the cellular composition of a human airway epithelium differentiated *in vitro* at ALI. The numbers of ciliated and goblet cells at the apical surface of differentiating HBECs were found to increase throughout 2 weeks of culture at ALI. Consistent with previous studies using human or murine tracheobronchial ALI cultures, we find that culturing in the presence of IL-13 substantially increases the numbers of goblet cells present in the cultured epithelium (15, 25). Conversely, we observed that IL-13 treatment suppressed the numbers of ciliated cells, which is consistent with previous findings using differentiated human respiratory epithelia (25, 26). To establish a model that more closely resembles a differentiated human epithelium *in vivo*, we examined the effects of IL-13 on a differentiated epithelium, by culturing HBECs for 2 weeks at ALI before a further 2 weeks of treatment with the cytokine. In this extended, 28-day model, ciliated cells were first allowed to form for 14 days before treatment with IL-13, which stimulated goblet cell formation to a similar extent to that previously reported (15). Thus, in the 28-day model, IL-13 treatment suppressed the formation of ciliated cells and stimulated the formation of goblet cells, suggesting an inverse relationship between these two epithelial cell populations.

To determine whether ciliated cells can act as progenitors of goblet cells, we used a lentiviral-mediated lineage–tagging approach using the human promoter *FOXJ1*, a transcription factor the expression of which is restricted to ciliated epithelial cells (27, 28). In the mouse, the expression of Foxj1 has been shown by immunohistochemistry to be restricted to ciliated epithelial cells in the late embryo and in adult tissues (27, 28), and this transcription factor has been proposed to play a role in the regulation and maintenance of the ciliated cell phenotype. Indeed, targeted deletion of Foxj1 (also known as hepatocyte nuclear factor–3/forkhead homolog 4) in mice resulted in absence of cilia in the airway (29). We found that the *FOXJ1* promoter directed transgene expression to ciliated cells in the human *in vitro* model, which is consistent with data from a transgenic mouse model using the same promoter (30). For lineage-tagging experiments, we used the modified differentiation model described previously here that allows for the formation of ciliated cells before exposure to IL-13. By cotransducing such cultures with lentiviruses containing Cre recombinase under the control of the *FOXJ1* promoter, together with a floxed-EGFP construct under the control of the CMV promoter, ciliated cells were irreversibly labeled with EGFP. The fate of the tagged ciliated cells was monitored after IL-13 treatment, and we demonstrate the presence of goblet cells derived from progenitors that express the ciliated cell marker *FOXJ1*. The number of EGFP-expressing goblet cells was significantly increased in IL-13–treated cultures, whereas the number of EGFP-expressing ciliated cells was decreased. This provides the first direct evidence that suggests that one origin of goblet cells arising in response to IL-13 in human respiratory epithelium may be through transdifferentiation of ciliated cells. Several previous studies have identified a population of airway epithelial cells that were suggested to be in transition from ciliated to goblet cell phenotype. In one such study, using a mouse model of IL-13–dependent goblet cell metaplasia inducible by viral infection, Tyner and colleagues (18) identified airway epithelial cells in which the ciliated cell marker, β-tubulin, is colocalized with the goblet cell mucin, MUC5AC. Cells positive for goblet cell marker MUC5AC and the ciliated cell markers, FOXJ1 or β-tubulin, have also been re-

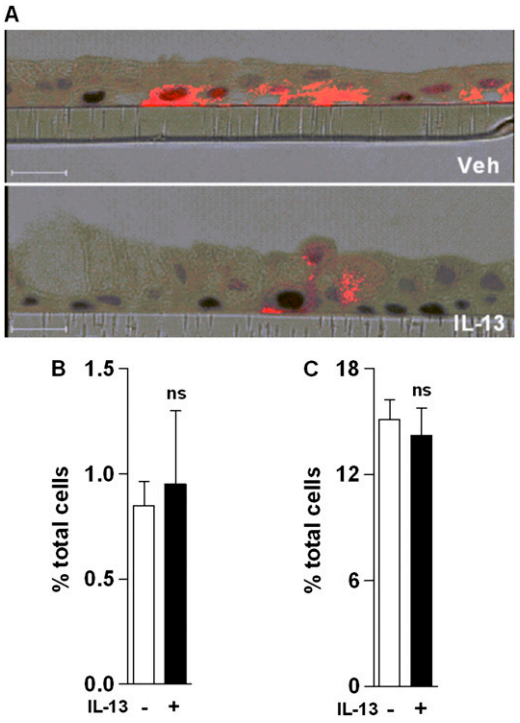

Figure 7. IL-13 does not regulate numbers of EGFP-labeled basal cells. HBECs were transduced with lentiviral vectors containing Cre recombinase under the control of the *FOXJ1* promoter and a CMV–floxed-EGFP construct. After transduction, cells were cultured in the absence of IL-13 for 14 days at ALI, followed by a further 14 days at ALI in the presence or absence of 1 ng/ml IL-13 (28-d model). Samples taken at Day 28 ALI were analyzed by a combination of confocal fluorescence and transmitted light microscopy, with images overlaid (*A*). The *red color* represents sites of anti-EGFP antibody binding, and p63-positive nuclei at the base of the epithelium are stained *dark brown* with an anti-p63 antibody. *Scale bar*, 20 μm. The total cell number and numbers of EGFP-positive cells (*B*) and EGFP-negative cells (*C*) stained with p63 were counted in sections from cultures treated with or without IL-13 at Day 28 ALI. Chart data are mean cell number (±SEM) as a percentage of total cells from six Transwell inserts derived from two independent experiments. *Open bars* are untreated cultures; *closed bars* are those treated with IL-13. Differences between treated and untreated groups were tested using two-tailed *t* tests.

ported in IL-13–treated human bronchial airway epithelial cells cultured at ALI (18, 26), suggesting the plasticity of these cells. Moreover, lung explants from patients with COPD also exhibited evidence of β-tubulin/MUC5AC coexpression (18). Such histological analyses are suggestive of the transdifferention of ciliated cells into goblet cells, and support the observations that we have made through use of a direct cell lineage–tagging approach. Further evidence supporting a relationship between ciliated and goblet cells has been reported by Kondo and colleagues (31) who analyzed the effects of IL-13 on guinea pig tracheal epithelial cell cultures. Guinea pig tracheal epithelial cell cultures were grown initially in the presence of IL-13 and when this then omitted from the cultures a reduction in goblet cell number and an increase in ciliated cells was observed (31), with transitional cells also detected in the cultures.

In the current study a Cre/loxP mediated lentiviral approach has been used to specifically label ciliated cells with EGFP using the *FOXJ1* promoter to drive the expression of Cre recombinase in ciliated cells. Cre-mediated recombination results in expression of EGFP in the ciliated cells under the control of the CMV promoter rather than the *FOXJ1* promoter, which may

TABLE 1. AVERAGE CELL COUNTS IN COSTAINED INSERTS

	Cytokine Treatment	
Staining	Vehicle	+IL-13
Tubulin and EGFP	1,315 ± 44	1,274 ± 113
MUC5AC and EGFP	1,442 ± 53	1,239 ± 116
p63 and EGFP	1,124 ± 82	1,019 ± 70

Definition of abbreviation: EGFP, enhanced green fluorescent protein.

Data are mean (±SEM) number of 4′,6-diamidino-2-phenylindole –stained nuclei counted from six Transwell inserts derived from two independent experiments.

give rise to the reduced expression of EGFP observed compared with that reported in by Schmid and colleagues (32) who expressed EGFP directly under the control of the *FOXJ1* promoter. The CMV promoter has previously been described to give reduced expression in ciliated cells compared with the *FOXJ1* promoter (32). Additionally, the efficiency of the Cre-mediated recombination may also partly explain the reduced efficiency of ciliated cell labeling in HBEC cultures compared with that reported by Schmid and colleagues (32).

In IL-13–dependent murine models of allergic asthma, remodeling of the epithelium induced by the allergen OVA occurs rapidly with the first appearance of goblet cells 6 hours after allergen challenge, with numbers peaking 7 days after challenge (17). There is no significant change in epithelial proliferation at the time when goblet cells first appear in response to allergen, indicating cell division does not play a role in this process (17). Such observations are consistent with the hypothesis that goblet cells formed during airway remodeling *in vivo* may arise through transdifferentiation of cell populations resident within the airways, although they do not exclude goblet cell progenitors from other sources.

In mice, in addition to the ciliated cell, the Clara cell may also act as a goblet cell progenitor. In the respiratory epithelium of allergen-challenged mice, detailed ultrastructural analysis revealed the presence of cells sharing the characteristics of both goblet and Clara cells not present in the epithelium of control mice (19). Similarly, in a second report, mucin expression was found in a subset of Clara cells located specifically to the proximal, but not distal, airways of allergen-challenged mice (17). More recently, lineage tracing experiments have implicated Clara cells as progenitors of goblet cells in an allergen exposure model in mice (33). However, such studies in mice cannot be readily extrapolated to humans, because whereas, in mice, Clara cells are found throughout the bronchi and bronchioles, in humans Clara cells are located only in the bronchioles (22). Therefore, Clara cells cannot have a major contribution to the increased goblet cell numbers found in the proximal airways in human respiratory diseases, such as asthma and COPD. The differentiated HBEC cultures used in the present study do not contain Clara cells, based on immunohistological analysis of expression of the Clara Cell Secretory Protein (CCSP), as expected based on the known distribution of Clara cells in human airways. Which cell types act as progenitors of goblet cells may vary between species, and may depend on their location within the lung and the specific stimulus received. Thus, differences remain between human and mouse airway epithelial biology indicating the importance of studying these processes in human model systems.

In addition to EGFP-tagged ciliated and goblet cells, we also identified EGFP-tagged, p63-positive basal cells in cultures cotransduced with lineage-tagging lentiviruses. Expression of p63 has previously been shown to be restricted to basal cells of human conducting airway epithelium (34) and in *in vitro* differ-

entiated human tracheobronchial epithelial cells (35). The contribution of these cells to IL-13–driven changes in epithelial phenotype is unclear, as their numbers were not regulated by IL-13. The HBECs used in the present study are collected post-mortem from human lung tissue and, therefore, at source, the cells collected are derived from a fully differentiated epithelium. In view of this, the differentiation of HBECs in ALI cultures may be more accurately viewed as a redifferentiation of the epithelium analogous to processes of lung repair. In mice, Park and colleagues (36) suggest that, after lung injury induced by naphthalene, which ablates Clara cells, Foxj1-expressing cells derived from ciliated cells assume a squamous phenotype, and redifferentiate into both ciliated and nonciliated cells types. However, this contrasts with data from a similar study using lineage-tagged bitransgenic mice expressing a tamoxifen-inducible Cre recombinase under the control of the human *FOXJ1* promoter and a LacZ floxed reporter gene (37). Rawlins and coworkers (37) reported that, after naphthalene injury, cells positive for the heritable LacZ reporter showed no ability to self-renew or transdifferentiate. Neither of these studies specifically addressed ciliated-to-basal cell transdifferentiation, and it is possible that the tagged basal cells that we observed in lineage-tagged HBEC cultures are derived from ciliated cells present within the donor lung. Alternatively, they may represent basal cells committed to differentiation into ciliated cells. A precedent for this process has been demonstrated in mice, where cells positive for the basal cell marker, K14, are capable of giving rise to ciliated cells after naphthalene injury (38, 39). Also, more recently ciliated cells derived from K5-positive basal cells have been identified in steady-state mouse airway epithelium (40).

Direct *in vivo* evidence for ciliated-to-goblet transdifferentiation using a cell lineage–tagging approach is thus far absent. The generation of transgenic mice, which express Cre recombinase under the control of the *FOXJ1* promoter, have recently been described (37, 41) and used to label ciliated cells in mouse airways via Cre/loxP-mediated recombination. When *FOXJ1*-Cre estrogen receptor $(ER)^{T2}$ transgenic mice expressing a tamoxifen-inducible Cre recombinase ER fusion protein were crossed with a ROSA26 floxed-lacZ reporter strain, subsequent exposure of the adult bitransgenic mice to tamoxifen resulted in lineage-labeling of ciliated cells in the lung which could be tracked in models of lung repair (37). The use of such *FOXJ1*-$CreER^{T2}$/ROSA26 floxed-lacZ bitransgenic mice in mouse models of goblet cell formation would allow the contribution of ciliated cells to goblet cell formation to be studied *in vivo*.

In summary, we have described a lentiviral-based approach for irreversibly tagging specific cell types in HBEC cultures. Using this approach, we have demonstrated that goblet cells formed in response to IL-13 treatment are in part or wholly derived from progenitors, which express the ciliated cell marker, *FOXJ1*. Identification of the adult progenitors of goblet cells contributes to our understanding and aids the examination of the processes that direct goblet cell formation. By studying such processes in models of respiratory disease, novel therapeutic targets may ultimately be identified.

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