Regulation of Human Airway Epithelial Tissue Stem Cell Differentiation by β-Catenin, P300, and CBP

DANIEL T. MALLESKE,a DON HAYES JR.,a,b,c SCOTT W. LALLIER,d CYNTIA L. HILL,d SUSAN D. REYNOLDSa,b,d

Key Words. Airway epithelium • Basal cell • Stem • Progenitor

ABSTRACT

The wingless/integrase-1 (WNT)/β-catenin signaling pathway is active in several chronic lung diseases including idiopathic pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease. Although this WNT/β-catenin pathway activity is associated with an increase in mucus cell frequency and a decrease in ciliated cell frequency, a cause and consequence relationship between signaling and cell frequency has not been established. We previously demonstrated that genetic stabilization of β-catenin inhibited differentiation of mouse bronchiolar tissue stem cells (TSC). This study determined the effect of β-catenin and its co-factors P300 (E1A-binding protein, 300 kDa) and cAMP response element binding (CREB)-binding protein (CBP) on human bronchial epithelial TSC differentiation to mucus and ciliated cells. We developed a modified air–liquid interface (ALI) culture system in which mucus and ciliated cell frequency is similar. These cultures were treated with the β-catenin agonist CHIR99021 (CHIR) and antagonists to β-catenin (XAV939), P300 (IQ1), and CBP (ICG001). We report that human TSC differentiation to mucus and ciliated cells can be divided into two stages, specification and commitment. CHIR treatment inhibited mucus and ciliated cell commitment while XAV939 treatment demonstrated that β-catenin was necessary for mucus and ciliated cell specification. Additional studies demonstrate that a β-catenin/P300 complex promotes mucus cell specification and that β-catenin interacts with either P300 or CBP to inhibit ciliated cell commitment. These data indicate that activation of β-catenin-dependent signaling in chronic lung disease leads to changes in mucus and ciliated cell frequency and that P300 and CBP tune the β-catenin signal to favor mucus cell differentiation. Stem Cells 2018; 36:1905–1916

SIGNIFICANCE STATEMENT

Although idiopathic pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease are characterized by increased mucus cell frequency and decreased ciliated cell frequency, a cause and consequence relationship between increased WNT/β-catenin signaling and changes in cell type frequency has not been previously demonstrated. Results of this study indicate that interactions between β-catenin and P300 or cAMP response element binding protein vary under conditions where β-catenin levels can fluctuate (normal) or are constantly high (disease). The authors suggest that chronic lung disease may increase the availability of P300 and active β-catenin resulting in mucus cell hyperplasia. These data also indicate that the increase in β-catenin signaling in chronic lung disease inhibits ciliated cell differentiation, although specific roles for P300 and CBP remain to be determined.

INTRODUCTION

Human Airway Epithelial Tissue Specific Stem Cell (TSC) Hierarchy

The human conducting airway epithelium is maintained by a TSC that was previously termed the B2 basal cell [1] and was recently redefined as a committed basal stem cell [2]. This TSC generates two intermediate (I) cell types [1] which may be related to mouse early progenitor cells [3]. The I1 cell self-renews and generates a ciliated cell. In contrast, the I2 cell produces a pair of mucus (also termed goblet) cells. In the normal bronchial airway, ciliated and secretory cell frequency are similar [4].

Airway Pathology in Chronic Lung Disease

Despite their distinct etiology and pathology, chronic lung diseases such as idiopathic pulmonary fibrosis (IPF), asthma, and chronic obstructive pulmonary disease (COPD) are characterized by a similar airway epithelial pathology, mucus cell hyperplasia, and ciliated cell hypoplasia [5, 6]. An expanding body of literature indicates that the wingless/integrase-1 (WNT)/β-catenin pathway (see below) is
activated in these chronic lung diseases and results in persistent β-catenin-dependent gene expression [reviewed in ref. [7]]. However, the relationship between WNT/β-catenin pathway activity and changes in differentiated cell frequency is unknown.

**WNT/β-Catenin Signaling**

The WNT/β-catenin signaling pathway is typically silent in the normal airway epithelium [8]. In this situation, newly synthesized β-catenin is transported to the adherens junction or targeted for degradation by the proteasome [9]. WNT signaling inhibits β-catenin degradation and results in β-catenin stabilization. This β-catenin translocates to the nucleus where it forms protein complexes and activates gene expression. WNT signaling can be modified through genetic manipulation of the mouse β-catenin gene [10,11], pharmacological regulation of β-catenin destruction complex proteins including glycogen synthase kinase 3β (GSK3β) and Axin1/2 [12,13], or inhibition of β-catenin transcriptional co-factors including P300 (E1A-binding protein, 300 kDa) and cAMP response element binding (CREB)-binding protein (CBP).

P300 and CBP are structurally similar; however, pharmacological studies suggest functional divergence [14,15]. The small molecule IQ1 prevents formation of a P300/β-catenin complex through inhibition of P300 phosphorylation [14]. In contrast, the small molecule ICG001 binds to CBP and inhibits formation of a CBP/β-catenin complex [16]. Studies in embryonic stem cells indicate that ICG001 treatment favors formation of a P300/β-catenin complex and increases stem cell differentiation [17,18].

**Cell Specification and Commitment**

Cell fate determination can be divided into two broad stages, specification and commitment [19]. A specified cell expresses cell type specific markers but its fate remains plastic. A committed cell increases expression of cell type specific markers and expresses genes that repress alternate fates. Thus, a committed cell is unlikely to alter its fate. Initiation of cell specification and the transition to commitment is regulated by WNT/β-catenin and other signaling pathways.

Our work and that of others identified a role for β-catenin in airway epithelial cell fate determination [20–28]. We showed that genetic stabilization of β-catenin in mouse bronchiolar TSC inhibited ciliated cell differentiation [21]. However, the effect of β-catenin stabilization on secretory differentiation was more complicated. Low-level expression of secretory cell markers suggested that β-catenin stabilization permitted secretory specification but inhibited commitment to the mature secretory cell fate. Collectively, these studies indicated that β-catenin regulated both cell specification and commitment but they did not determine the co-factor or co-factors that impart this complex behavior.

An analysis of alveolar and airway TSC differentiation demonstrated that chronic IQ1 treatment inhibited differentiation and suggested that a P300/β-catenin complex was necessary for this process [29]. The goal of this study was to evaluate roles for β-catenin, P300, and CBP in specification and commitment of human airway epithelial TSC.

### METHODS

#### Human Subjects

The Institutional Review Board at Nationwide Children’s Hospital (NCH) approved this study. Donors received written, informed consent.

#### Donor Demographics

This study evaluated TSC cell function in 5 normal donor samples who had no known pulmonary disease. Normal donor demographics: 4 males and 1 female; all Caucasian; mean age 35.8 ± 16.0 years (range 16–57).

#### Human Airway Cell Recovery

Excess bronchial donor tissue was recovered at the time of lung transplantation and digested with 0.15% pronase in Ham’s F12 at 4°C overnight. Bronchial airway epithelial cells were recovered as previously reported [30].

#### Cell Culture Techniques

The modified conditional reprogramming culture (mCRC) technique was used to expand the initial cell inoculum [31]. At passage 1–2, cells were plated at 6.7 × 10^4/cm^2 on polystyrene cell culture plates that contained an irradiated NIH3T3 fibroblast feeder layer, and cultured in FMed + 10 μM Y27632. These cultures contained undifferentiated airway epithelial cells [31]. At 80% confluence, epithelial cells were recovered by double-trypsinization.

Passage 2–3 cells were used to generate air–liquid interface (ALI) cultures. Cells were plated at 6.1 × 10^5 cells/cm^2 on collagen-coated 0.33 cm^2 transwell membranes and cultured in proliferation medium [32] plus 10 μM Y27632 for 24 hours. The cells were then cultured in proliferation medium for an additional 3–4 days. At confluence, the medium was changed to Half & Half [50% Wu differentiation medium [32] and 50% Pneumacult ALI without the 100x maintenance supplement]. Medium was present only in the basal compartment, which established an apical ALI.

ALI cultures were treated with various drugs at the times indicated in Results. All drugs were dissolved in dimethyl sulfoxide (DMSO) at 1000x. Final concentrations were: CHIR99021 (Tocris), 5 μM, 10 μM, or 15 μM; IQ1 (Stemcell Technologies) 20 μM; ICG001 (Cayman Chemical Company) 10 μM; XAV939 (Sellechem) 10 μM. Vehicle or drug was added to the basal compartment.

#### Immunostaining and Quantification

ALI cultures were washed, fixed, immunostained, and imaged as previously reported [31]. ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997–2016) was used to quantify the number of 4’6-diamidino-2-phenylindole (DAPI)-stained nuclei, MUC5B positive mucus cells, and acetylated tubulin-positive ciliated cells. For studies that evaluated antigen abundance, supraoptimal antibody concentrations were used [23] and images were acquired using a shutter speed optimized to the dimmest sample.

#### Cell Fractionation

Cells were recovered from transwell membranes by incubating in 0.25% trypsin/2.21 mM EDTA (GIBCO) at 37°C for 20 min.
Cells were fractionated into cytoplasmic and nuclear fractions using the Ne-Per kit (Thermo-Fisher).

**Immunoprecipitation**

These studies were performed using 400 μg of protein from each sample. Samples were incubated on a rotating platform overnight at 4°C in a 1:100 (total volume 0.3 ml) dilution of mouse Anti-phosphoserine/phospho-tyrosine (pS/pT) antibody (ECM Biosciences, #3801). The next morning, 100 μl of MACS Protein G Micro Beads (Miltenyi Biotec, #130-071-101) were added to each sample and the samples were rotated at 4°C for 1 hour. Protein/antibody complexes were recovered with Miltenyi Biotec IP Columns according to the manufacturer’s protocol. The eluate (50 μl) was divided equally between two wells of a sodium dodecyl sulfate/4%-15% polyacrylamide gel (Bio-Rad, #5671084).

**Immunoblotting**

Standard methods were used to generate Western blots [20]. Antibodies used were mouse-anti-β-actin (Abcam, #ab6276); rabbit-anti-active β-catenin (Millipore ABE208 at 1:1000); mouse-pan-C-terminal β-catenin (BD Transduction Laboratories #610154 at 1:2000); rabbit-anti-p300 (D2X6N, Cell Signaling 7389s #610154 at 1:2000); rabbit-anti-CBP (D6C5, Cell Signaling 7389s at 1:1000); rabbit-anti-αTubulin (Abcam ab4074 at 1:16,000); or rabbit-anti-topoisomerase II (Abcam ab109524 at 1:10,000).

**Animal Care and Use**

The NCH Institutional Animal Care and Use Committee reviewed and approved the studies. Care was in accordance with humane care standards published by the Public Health Service, National Institutes of Health (Bethesda, MD) in the Care and Use of Laboratory Animals (2011), and U.S. Department of Agriculture regulations outlined in the Animal Welfare Act.

**Mouse Strains**

Wild type (βcat-WT), β-catenin-stabilized (βcat-DE3, 84, 133), and β-catenin knockout (βcat-KO, 131, 137) were bred as previously reported [21, 24]. To generate WT and βcat-DE3 mice that expressed enhanced green fluorescent protein (EGFP) in SCGB1A1+ cells and their progeny, WT or DE3 mice were bred with ROSA26-LSL-EGFP mice (Jackson Laboratory).

**Gene Expression Analysis**

Mouse airway epithelial cells were recovered by elastase digestion on postnatal day 21 [33]. RNA was purified using a Qiagen micro prep kit and integrity evaluated using a Bioanalyzer. Two hundred nanograms of RNA was used as a template for preparation of cRNA using standard methods. Affymetrix Genechip Mouse Gene 1.1ST plate arrays were used to interrogate gene expression in βcat-WT, βcat-DE3, and βcat-KO mice (n = 4 mice per genotype). Standard hybridization, washing, staining, and confocal laser scanning methods were used. Raw data were log2 transformed. The data analysis platform used AffyQCReport, Bioconductor (www.bioconductor.org) [34], Robust Multichip Average method [35], and T. Statistical analysis used nonparametric statistical tests (the Mann–Whitney U test for two classes or its multi-class equivalent, the Kruskal–Wallis test). This nonparametric test was combined with the false discovery rate (FDR) correction for multiple testing [36, 37]. The FDR was set at 0.05. Statistical power was increased by pruning a priori genes that did not vary over any chips, and with other techniques (e.g., a test of replicability) as appropriate. The data have been submitted to Gene Expression Omnibus (GEO).

**Cell Sorting**

Mouse lung cells were recovered by enzymatic digestion as previously reported for mouse tracheal cells [38]. Hematopoietic cells, endothelial cells, erythrocytes, and dead cells were excluded and EGFP+ cells were sorted as previously reported [39].

**Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

RNA was prepared, reverse transcribed, and assayed as previously reported [21]. Assays on demand were purchased from Thermo-Fisher: Sgb1a1, Mm00442046_m1; CMyb, Mm00501741_m1; FoxJ1, Mm00807215_m1; and Gusb, Mm01197698_m1.

**RESULTS**

**Stabilization of β-Catenin Prevents Ciliated Cell Specification in Mice**

We previously reported that genetic stabilization of β-catenin in mouse bronchial TSC resulted in hyperplasia of immature (SCGB1A1-low) club cells and hypoplasia of ciliated cells [21]. This phenotype suggested that β-catenin expanded the bronchial TSC pool by inhibiting differentiation. To further evaluate this phenotype, we compared gene expression in lung tissue from βcat-WT, βcat-DE3, and βcat-KO mice (Supporting Information, Table 1). Unsupervised hierarchical clustering demonstrated that the βcat-WT and βcat-KO gene expression profiles were similar and distinct from the βcat-DE3 gene expression pattern (Supporting Information, Fig. 1). We found that 522 genes were differentially expressed between the βcat-WT and βcat-DE3 groups (corrected p value <.0001 and >1.5-fold change in expression level). Among these genes, 91 were upregulated in βcat-DE3 relative to βcat-WT. This gene set included 59 of the 66 β-catenin target genes that were previously validated in human or mouse tissues and cells (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). We also identified 431 genes that were downregulated in βcat-DE3 relative to βcat-WT.

Analysis of ciliation pathway genes (Fig. 1A) demonstrated that the βcat-WT and βcat-KO gene expression profiles were similar (Fig. 1B, Table 1). In contrast, each of the ciliation pathway genes was underexpressed in βcat-DE3 samples. Importantly, the underexpressed genes included proximal ciliation pathway transcription factors (cMsb, FoxJ1, Rfx) and a large number of cilia structural genes (Supporting Information, Table 1).

A concern with analysis of gene expression in whole lung tissue was that the genes of interest might be expressed in nonairway epithelial cell types. To address this issue, we generated βcat-WT and βcat-DE3 mice that expressed EGFP in SCGB1A1-expressing cells and their progeny. Flow cytometry was used to purify EGFP-positive and -negative cells and gene expression was evaluated by qRT-PCR. This analysis demonstrated significant enrichment of SCGB1A1 transcripts in EGFP+ βcat-WT cells and confirmed the SCGB1A1-low phenotype in...
EGFP+ βcat-DE3 cells (Fig. 1C). Analysis of cMyb and FoxJ1 confirmed that these genes were significantly downregulated in the SCGB1A1 lineage in βcat-DE3 mice (Fig. 1D, 1E). Collectively, these data indicate that overexpression of β-catenin inhibited ciliated cell differentiation.

Human TSC Differentiation to Mucus and Ciliated Cells in Half & Half Medium

The ALI method has been used to study differentiation of human airway TSC. However, commonly used media often skew differentiation toward the secretory or the ciliated cell fate. Consequently, these media may not allow a true assessment of the signals that regulate cell fate determination.

To address this problem, we developed a new differentiation medium, Half & Half. Like other ALI methods, our approach utilized a two-step process in which human TSC were plated at low cell density in proliferation medium [31, 32]. At confluence, the cultures were changed to Half & Half medium and an ALI was established by removing medium from the apical surface.

A time course study demonstrated that cell density was constant in Half & Half cultures over a 21-day differentiation period (Fig. 2A). Mucus cells, as assessed by MUC5B immunostaining (Fig. 2D–2G), were detected on differentiation day 2 in...
some cultures and were consistently present on day 5. Mucus cell frequency increased through differentiation day 21 (Fig. 2B, 2D–2G).

Ciliated cell differentiation was assessed by acetylated tubulin (ACT) immunostaining. As previously reported [40], a subset of cells exhibited a primary cilium on days 2 and 5 (Fig. 2D, 2E) prior to detection of multiciliated (ciliated) cells on day 7 (Fig. 2C, 2F, 2G). In addition, we observed an intermediate (bristle) cell type that was defined by the presence of multiple very short cilia (Fig. 2F). Multi-ciliated cell frequency increased through differentiation day 21.

The frequency of mucus and ciliated cells was similar on differentiation day 21 (Fig. 2B, 2C). Collectively, these data indicate that Half & Half medium avoids the biased-differentiation observed in other media and identifies differentiation days 7–14 as an optimal period for analysis of signals that regulate mucus and ciliated cell differentiation.

### Table 1. Differentially expressed ciliation pathway genes

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<th>Function</th>
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<tr>
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β-Catenin Signaling Permits TSC Commitment to the Mucus and Ciliated Cell Fates

Our next goal was to determine if β-catenin regulates the differentiation of human TSC to mucus and ciliated cells. The GSK3β inhibitor CHIR99021 (CHIR) increases β-catenin levels in human induced pluripotent stem cells [41]. Thus, we treated differentiating ALI cultures with vehicle (DMSO) or 10 μM CHIR from day 7 through day 11. This interval was chosen as both the ciliated and secretory cell differentiation processes were underway and the frequency of both cell types was increasing (Fig. 2). Western blot analysis on day 11 demonstrated that
CHIR treatment resulted in a significant increase in active β-catenin (Fig. 3A).

Histological analysis and quantification on day 11 demonstrated that CHIR inhibition of GSK3β did not alter cell density (Fig. 3B). Similarly, immunostaining and quantification of MUC5B on day 11 demonstrated that CHIR treatment did not alter mucus cell frequency (Fig. 3C). However, CHIR treatment inhibited the time-dependent increase in MUC5B expression (Fig. 3E, 3F). Thus, β-catenin stabilization on days 7–11 permitted mucus cell specification and prevented mucus cell commitment.

A similar analysis of ciliated cell differentiation demonstrated that CHIR inhibition of GSK3β prevented the time-dependent increase in multiciliated cell frequency (Fig. 3D). We found that CHIR-treated cells expressed a primary cilium on their apical surface and that rare cells expressed the bristle cell phenotype (Fig. 3G, 3H). Multiciliated cells were not observed in CHIR-treated cultures. Thus, β-catenin stabilization on days 7–11 permitted ciliated cell specification and inhibited commitment to the ciliated cell fate.

β-Catenin Destabilization Prevents Mucus and Ciliated Cell Specification

To determine if β-catenin was necessary for mucus and/or ciliated cell differentiation, we treated ALI cultures with vehicle or the tankyrase inhibitor XAV939 [13]. Disruption of tankyrase activity prevents Axin PARylation and leads to Axin stabilization. Increased levels of Axin accelerates β-catenin turnover and inhibits β-catenin-dependent gene expression. Thus, XAV939 treatment increases the level of active β-catenin.
To evaluate mucus and ciliated cell specification, ALI cultures were treated with DMSO or 10 μM XAV939 on days 0–4, 4–8, and 8–12 and cell type frequency was assayed on the last day of the treatment interval. Histological analysis and quantification demonstrated that the XAV939-mediated decrease in active β-catenin level did not alter cell density at any time point (Fig. 4A).

Mucus cell differentiation was assayed by immunostaining for Muc5B and quantification. This study showed that XAV939-dependent depletion of the active β-catenin pool on days 0–4 caused a significant decrease in the frequency of MUC5B-positive cells. However, XAV393 treatment did not change the frequency of these cells when the cultures were treated on days 4–8 or 8–12 (Fig. 4B). These data suggested...
that a period of active β-catenin signaling was necessary for mucus cell specification.

Next, we determined the effect of XAV939 treatment on ciliated cell differentiation. ALI cultures were treated with vehicle or XAV939 on days 0–4, 4–8, and 8–12. Ciliated cell frequency was assayed by ACT immunostaining on the last day of the treatment interval. Vehicle-treated cultures were used to determine the relative frequency of cells expressing a primary cilium, bristle cells, and mature ciliated cells (Fig. 4C). XAV939-dependent depletion of the active β-catenin pool decreased the frequency of cells expressing a primary cilium on day 4 (Fig. 4D), bristle cells on day 8 (Fig. 4E), and mature ciliated cells on day 12 (Fig. 4F). Collectively, these data indicated that β-catenin signaling was necessary for mucus and ciliated cell specification and that β-catenin signaling was necessary for progression through the ciliated cell differentiation program.

Active P300 and CBP are Expressed in ALI Cultures

To determine if CBP and P300 play a role in human TSC differentiation, we first evaluated expression of these proteins in ALI cultures. Immunostaining detected nuclear-localized P300 and CBP at each time point (Fig. 5A–5H). A Western blot analysis demonstrated that each protein was full length on day 11 (data not shown). In anticipation of the functional studies presented below, we next determined if P300 and CBP were present in the nucleus of cells that were treated with DMSO,
Western blot analysis of CBP and P300 in cultures that were treated in their active forms in differentiating TSC.

Immuno-fluorescence analysis of P300 and β-catenin (A–H) on day 7. Arrows indicate P300+/CBP+ nuclei. A similar pattern was observed on days 1–11. (I): Western blot analysis of CBP and P300 in cultures that were treated with vehicle (DMSO), CHIR99012 (CHIR), ICG001, IQ1, CHIR+ICG001, or CHIR+IQ1 on days 7–11. Proteins were analyzed on day 11. (J): Analysis of CBP and P300 phosphorylation on serine/threonine (pS/pT) in cultures that were treated with DMSO or CHIR on days 7–11. Proteins were recovered on day 11 and immuno-precipitated with anti-pSerine/pThreonine antibody. P300 detected both proteins in the nuclear fraction under all conditions. Nuclear and cytoplasmic proteins (Fig. 5I). A Western blot analysis of CBP and P300 were detected on Western blots. CHIR, ICG001, IQ1, CHIR+ICG001, or CHIR+IQ1 on days 7–11. Nuclear and cytoplasmic fractions were prepared and Western blot analysis of α-tubulin and topoisomerase 2 demonstrated that the cytoplasmic fraction was free of nuclear proteins and that the nuclear fraction was slightly contaminated by cytoplasmic proteins (Fig. 5I). A Western blot analysis of CBP and P300 detected both proteins in the nuclear fraction under all treatment conditions. Finally, we determined if CBP and P300 were present in their active forms. Proteins that were phosphorylated on serine or threonine were immunoprecipitated and CBP and P300 were evaluated by Western blot. This study demonstrated that P300 was phosphorylated on serine/threonine, whereas CBP was not phosphorylated (Fig. 5I). These data indicate that both CBP and P300 were nuclear and present in their active forms in differentiating TSC.

**P300 Regulates Mucus Cell Specification**

To determine if P300 is necessary for mucus cell differentiation, we treated ALI cultures with vehicle or 20 μM IQ1 on days 7–11 and quantified the frequency of MUC5B-immunopositive mucus cells on day 11. Inhibition of the P300/β-catenin complex with IQ1 significantly decreased the frequency of MUC5B-low and -high mucus cells (Fig. 6A). These data indicate that the P300/β-catenin complex is necessary for mucus cell specification.

A related P300 inhibition study evaluated mucus cell frequency in ALI cultures that were treated with IQ1 and vehicle or CHIR. Cultures were treated on days 7–11 and analyzed on day 11. This study demonstrated that inhibition of the β-catenin/p300 interaction with IQ1 led to a significant decrease in mucus cell frequency in the absence or presence of GSK3β inhibition by CHIR (Fig. 6A). These data suggest that changes in β-catenin availability do not alter the role played by P300/β-catenin in mucus cell specification.

To determine if CBP plays a role in mucus cell differentiation, we treated ALI cultures with vehicle or 10 μM ICG001 on days 7–11 and quantified the frequency of MUC5B-immunopositive mucus cells on day 11. Inhibition of the β-catenin/CBP complex with ICG001 did not alter mucus cell frequency (Fig. 6B). However, ICG001 prevented the commitment-associated increase in MUC5B expression (not shown). These data suggest that the β-catenin/CBP complex was necessary for mucus cell commitment but not specification.

A related CBP inhibition experiment evaluated mucus cell frequency in ALI cultures that were treated with ICG001 and vehicle or CHIR. Cultures were treated on days 7–11 and analyzed on day 11. This study demonstrated that inhibition of the β-catenin/CBP complex by ICG001 and inhibition of GSK3β by CHIR led to a significant decrease in the frequency of MUC5B-low and -high mucus cells (Fig. 6B). These data indicate that the β-catenin/CBP complex can regulate mucus cell specification but only under conditions in which β-catenin availability is increased by GSK3β inhibition.

**P300 and CBP Regulate Ciliated Cell Commitment**

We next determined if ciliated cell differentiation was dependent on P300. Human ALI cultures were treated on days 7–11 and ciliated cell differentiation was evaluated by ACT staining on day 11. Multiciliated cell frequency was not altered by inhibition of the β-catenin/P300 complex with IQ1 (Fig. 6C). In contrast, inhibition of the β-catenin/P300 complex with IQ1 and inhibition of GSK3β with CHIR increased multiciliated cell frequency relative to cultures that were treated with CHIR alone (Fig. 6C). These data suggest that the β-catenin-dependent inhibition of ciliated cell commitment was mediated by the β-catenin/P300 complex.

Finally, we determined if ciliated cell differentiation was dependent on CBP. Human ALI cultures were treated on days 7–11 and ciliated cell differentiation was evaluated by ACT staining on day 11. Inhibition of the β-catenin/CBP complex with ICG001 did not alter multiciliated cell frequency (Fig. 6D). A related CBP inhibition experiment evaluated multiciliated cell frequency in ALI cultures in which the β-catenin/CBP complex was inhibited by ICG001 and GSK3β was inhibited by CHIR. Multiciliated cell frequency did not vary among cultures that were co-treated with ICG001 and 5 μM CHIR or 10 μM CHIR (Fig. 6D). However, co-treatment with ICG001 and 15 μM CHIR significantly increased multiciliated cell frequency. These data indicate that the inhibitory effect of CHIR on ciliated cell commitment was dependent on CBP.
DISCUSSION

Transient β-Catenin Signaling Regulates Human TSC Differentiation

Waxing and waning of β-catenin signaling is a common developmental theme in invertebrates and in many mammalian tissues. In the mouse lung, β-catenin signaling transitions from high to low during development of the alveolar and airway epithelia [42], supports expansion of the Axin2+ progenitor cell pool [43], and sets the stage for generation of alveolar type 2 cells. We and others reported transient upregulation of β-catenin in the repairing adult mouse airway epithelium [21, 28] and that genetic stabilization of β-catenin dysregulated the replacement of differentiated cell types after injury. This study extends these observations to human airway TSC and supports the conclusion that mucus and ciliated cell differentiation requires transient activation of β-catenin signaling (Figs. 3–4).

β-Catenin Regulates Cell Specification and Commitment

In this study, we report that mucus cell differentiation involves two stages: specification which is marked by expression of a primary cilium and commitment which is indicated by expression of multiple cilia (Fig. 2). The existence of these differentiation steps is supported by the identification of a transcriptional regulator, β-catenin, that is necessary for specification and mediates the transition from specification to commitment (Figs. 3–4).

Mucus Cell Specification is Promoted by a β-Catenin/P300 Complex

A previous analysis of roles played by P300 in alveolar and airway TSC differentiation defined P300 as a necessary co-factor [29]. Our analysis of β-catenin and P300 expands the Rieger study by showing that P300 is necessary for mucus cell specification under conditions where β-catenin signaling can fluctuate (DMSO-treated cultures) and when β-catenin is persistently active (CHIR-treated cultures) (Fig. 6A). In contrast, CBP regulates mucus cell specification under a specific condition, when β-catenin is stabilized (Fig. 6B). A previous study reported that ICG001 inhibition of the β-catenin/CBP complex promoted the formation of a β-catenin/P300 complex [17, 18]. These data in combination with our finding that mucus cell specification was inhibited by IQ1 and by co-treatment with ICG001 + CHIR suggest that a β-catenin/P300 complex promotes mucus cell specification.
Ciliated Cell Commitment is Inhibited by β-Catenin and P300 or CBP

Our study indicates that the function of P300 and CBP in ciliated cell commitment is dependent on the abundance of active β-catenin. Under conditions where β-catenin levels can fluctuate, ciliated cell commitment is not altered by inhibition of P300 or CBP (Fig. 6C, 6D). This observation suggests that the active β-catenin level is low in specified ciliated cells and that there is little or no β-catenin/P300 or β-catenin/CBP complex. Thus, ciliated cell commitment can precede and changes in P300 or CBP abundance have no impact on ciliated cell frequency. In contrast, when active β-catenin is present at high levels, P300 and CBP inhibit ciliated cell commitment (Fig. 6C, 6D). Given that β-catenin typically functions as a transcriptional activator, we speculate that the β-catenin/P300 and β-catenin/CBP complexes may activate an anti-ciliation pathway such as Notch Lateral Inhibition [44–47].

Our IQ1 and ICG001 studies also suggest that the pool of active P300 is too small to compensate for a decrease in the pool of active CBP and vice versa. However, it is also possible that the β-catenin/P300 and β-catenin/CBP complexes regulate distinct β-catenin dependent functions. In this situation, the β-catenin/P300 complex may shift cell fate toward mucus cell specification and decrease the number of cells that become ciliated cells. This scenario suggests that the β-catenin/CBP complex activates an anti-ciliation pathway. Additional experiments are needed to distinguish these possibilities but are beyond the scope of this study.

CONCLUSION

Although IPF, asthma, and COPD are characterized by distinct etiology and pathology, these chronic lung diseases exhibit a shared airway epithelial phenotype, increased mucus cell frequency, decreased ciliated cell frequency, and increased WNT/β-catenin signaling. To date, a cause and consequence relationship between increased WNT/β-catenin signaling and changes in cell type frequency has not been previously demonstrated [7]. This study indicates that interactions between β-catenin and P300 or CBP vary under conditions where β-catenin levels can fluctuate (normal) or are constantly high (disease). We suggest that chronic lung disease may increase the availability of active β-catenin and the β-catenin/P300 complex resulting in mucus cell hyperplasia. Our data also indicate that the increase in β-catenin signaling in chronic lung disease inhibits ciliated cell differentiation although specific roles for P300 and CBP remain to be determined.

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AUTHOR CONTRIBUTIONS

D.T.M.: conception and design, interpretation of data, drafting of the manuscript, final approval of manuscript. D.H.: conception and design, acquisition of clinical samples, interpretation of data, drafting of the manuscript, final approval of manuscript. S.W.L. and C.L.H.: analysis of laboratory samples, final approval of manuscript. S.D.R.: conception and design, analysis of laboratory samples, interpretation of data, drafting of the manuscript, financial support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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