β -Catenin Dosage Is a Critical Determinant of Tracheal Basal Cell Fate Determination

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The purpose of this study was to determine whether β -catenin regulates basal cell fate determination in the mouse trachea. Analysis of *TOPGal* transgene reporter activity and Wnt/ β -catenin pathway gene expression suggested a role for β -catenin in basal cell proliferation and differentiation after naphthalene-mediated Clara-like and ciliated cell depletion. However, these basal cell activities occurred simultaneously, limiting precise determination of the role(s) played by β -catenin. This issue was overcome by analysis of β -catenin signaling in tracheal air-liquid interface cultures. The cultures could be divided into two phases: basal cell proliferation and basal cell differentiation. A role for β -catenin in basal cell proliferation was indicated by activation of the *TOPGal* transgene on proliferation days 3 to 5 and by transient expression of Myc (alias c-myc). Another peak of *TOPGal* transgene activity was detected on differentiation days 2 to 10 and was associated with the expression of Axin 2. These results suggest a role for β -catenin in basal to ciliated and basal to Clara-like cell differentiation. Genetic stabilization of β -catenin in basal cells shortened the period of basal cell proliferation but had a minor effect on this process. Persistent β -catenin signaling regulated basal cell fate by driving the generation of ciliated cells and preventing the production of Clara-like cells. (Am J Pathol 2011, 179:367–379; DOI: 10.1016/j.ajpath.2011.03.016)

The human tracheobronchial region is characterized by a pseudostratified epithelium and the presence of smooth muscle and cartilage.¹ This anatomy extends from the trachea through the first six intrapulmonary generations. Thus, the mouse trachea serves as a model for identifi-

cation of pathways that regulate repair of the human tracheobronchial epithelium (TBE).

Pulse-chase and lineage tracing analyses have demonstrated that the mouse basal cell,^{1–4} similar to its human counterpart,^{5,6} serves as a progenitor for all differentiated cell types in the mouse tracheal epithelium. In the mouse trachea, parenteral naphthalene (NA) exposure depleted the secretory progenitor cell pool (termed Clara-like cells) and the ciliated cell population within 3 days.¹ Basal cells, defined by the expression of keratin (K) 5, proliferated on recovery days 3 to 9. Nascent Clara-like cells, which were defined by the expression of Clara cell secretory protein (CCSP) and nascent ciliated cells, that expressed forkhead box protein J1 (FoxJ1) or acetylated tubulin (ACT) were detected between recovery days 6 and 13. The basal cell-mediated reparative process was uniform along the proximal to distal axis of the trachea, suggesting that the basal cell progenitors were uniformly distributed. The signals that may regulate the reparative process include developmentally important pathways such as Notch, Sonic hedgehog, and Wnt/ β -catenin.⁷

Wnt/ β -catenin signaling waxes and wanes during lung development, suggesting that this signaling pathway regulates similar processes over time or that it mediates multiple but distinct components of organ formation.^{8–11} Loss- and gain-of-function studies have demonstrated that β -catenin is necessary and sufficient to alter lung branching morphogenesis. Okubo and Hogan¹⁰ demonstrated distalization of the foregut endoderm using a Lef1- β -catenin fusion protein and suggested that excess β -catenin signaling altered specification of proximal endodermal lineages. Li et al¹² stabilized β -catenin early in lung epithelial development (approximate embryonic day 9.5) using the Nkx2.1-cre transgene and the floxed exon 3 β -catenin allele.¹³ Cre recombinase-mediated

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excision of exon 3 resulted in generation of a transcriptionally active β -catenin protein that lacked the GSK3 β phosphorylation sites. This β -catenin mutant is "stabilized." The study by Li et al¹² demonstrated polyp formation in the trachea and upper airways. These polyps were devoid of ciliated and Clara-like cells, suggesting that excess β -catenin blocked generation of the tracheal secretory/ciliated lineage.

In contrast to the tracheal phenotype, stabilization of β -catenin during the pseudoglandular phase of lung development (approximate embryonic day 15.5) using the CCSP-cre transgene and the floxed exon 3 β -catenin allele⁸ attenuated postnatal maturation of bronchiolar Clara cells. β -Catenin stabilization did not alter Clara cell proliferation in response to NA injury but did block Clara to ciliated cell differentiation. These studies indicated that β -catenin did not drive Clara cell proliferation. However, β -catenin did play an important role in Clara cell fate determination.

Clara cell-specific knockout of β -catenin demonstrated that β -catenin was not necessary for embryonic development after the pseudoglandular stage, for postnatal maturation of bronchiolar Clara cells, or for repair of the NA-injured bronchiolar airways.¹⁴ Collectively, the gain- and loss-of-function studies indicated that a threshold level of β -catenin signaling was important for Clara and ciliated cell differentiation through the pseudoglandular stage and that an overabundance of β -catenin signaling altered Clara cell fate in the adult.

Analysis of β -catenin signaling in basal cells and its effect on basal cell fate has not been reported. We demonstrate that NA-mediated tracheobronchial injury results in transient β -catenin stabilization during the period of basal cell proliferation/differentiation. This observation led us to hypothesize that β -catenin serves as a signal that toggles the basal cell life cycle between cell division and generation of differentiated ciliated and Clara-like cells. Analysis of the β -catenin reporter transgene TOPGal¹⁵ demonstrated that β -catenin stabilization was associated with basal cell proliferation and differentiation in tracheal epithelial cell cultures. Genetic stabilization of β -catenin demonstrated that β -catenin did not promote basal cell proliferation. However, β -catenin down-regulation was required for generation of appropriate numbers of ciliated cells and for basal to secretory cell differentiation. We conclude that β -catenin is a critical determinant of basal cell fate determination in the tracheal epithelium.

Materials and Methods

Animal Strains

All the animals were cared for and treated according to procedures approved by the National Jewish Health Institutional Animal Care and Use Committee. All the experiments used adult mice 6 to 8 weeks old. TOPGal-C57Bl/6 congenic (TOPGal-B6) mice were generated by backcrossing CD1-TopGal¹⁵ mice with C57Bl/6 mice for 10 generations. C57Bl/6 mice were used for *in vivo* gene expression. Air-liquid interface (ALI) cultures were estab-

lished from TOPGal-B6 mice for X-Gal reporting experiments. Fvb/n mice were used for *in vitro* gene expression analysis. K14/rtTA/TRE-cre/DE3 mice were generated by breeding transgenic mice harboring the K14–reverse tetracycline (T) transactivator (K14-rtTA),¹⁶ T response element-cre recombinase transgenic mice (TRE-cre),¹⁷ to mice homozygous for the floxed β -catenin exon 3 allele (DE3).¹³ Bitransgenic mice that were homozygous for the floxed allele were used to establish the ALI cultures used for β -catenin stabilization experiments.

NA Exposures

Dose-response experiments were used to determine the dose of NA needed to cause 95% depletion of Clara cells on posttreatment day 3 in TOPGal-B6 mice. This dose was 275 mg/kg. NA was delivered i.p. as previously reported.¹ Mice were weighed daily. Survival was >95%. Tissue was recovered for histologic and gene expression analyses on recovery days 3, 6, 9, and 13.

Primary Tracheal ALI Cultures

ALI cultures were established based on the method of You et al¹⁸ using tracheae from 6- to 8-week-old mice. Tracheae were carefully cleaned of excess tissue and glands by blunt dissection. Cells were recovered by digestion with 0.15% pronase (from *Streptomyces griseus*) in Ham's F-12 supplemented with L-glutamine and 5% penicillin/streptomycin. Digestion was overnight at 4°C. The protease was inactivated by the addition of 10% fetal bovine serum. Cells were removed from the trachea by gentle agitation, were pelleted at 300 $\times g$, and were plated in Dulbecco's modified Eagle's medium/5% penicillin/streptomycin/10% fetal bovine serum for 3 hours at 37°C with 5% CO₂ to remove macrophages and fibroblasts. The cells were pelleted at 300 $\times g$ and were plated in collagen I-coated Transwells (Corning Inc., Corning, NY) at a density of 1.0 $\times 10^5$ cells/cm².

The proliferation phase of ALI cultures is defined as the period when cells are proliferating and creating a polarized epithelium. During the proliferation phase, cultures were grown in MTEC+, Dulbecco's modified Eagle's medium/F-12 (1:1) (Gibco, Grand Island, NY) supplemented with 2 mmol/L L-glutamine (Mediatech Inc., Manassas, VA), 0.25 μ g/mL of amphotericin B (Sigma-Aldrich Corp., St. Louis, MO), 5% penicillin/streptomycin, 7.5% NaHCO₃, 5% fetal bovine serum (HyClone, Logan, UT), insulin-transferrin-selenium (Gibco), 10 μ g/mL of insulin, 5 μ g/mL of transferrin, 5 μ g/mL of selenite, 0.1 mg/mL of cholera toxin (Sigma-Aldrich Corp.), 0.1 μ g/mL epithelial growth factor (BD Biosciences, Franklin Lakes, NJ), 25 ng/mL of bovine pituitary extract (Gibco), 0.03 mg/mL of hydrocortisone (MP Biomedicals, Solon, OH), and 50 nmol/L retinoic acid (Sigma-Aldrich Corp.). Proliferation day 0 is defined as the day the cells are seeded. Transepithelial resistance is measured beginning on proliferation day 4. Cultures that reach >330 Ω -cm² are fed proliferation media and are cultured for 1 additional day. At this time, transepithelial resistance averaged 2000 Ω -cm².

Gene Expression Analysis

Tracheal RNA was isolated for gene expression analysis according to previously described methods.¹ Tracheae were isolated as indicated previously herein. Tissue was stored in RNAlater (Ambion, Foster City, CA) at 4°C overnight and at –80°C until RNA isolation. RNA from ALI cultures was isolated for gene expression using the Absolutely RNA microprep kit (Agilent Technologies, La Jolla, CA) according to manufacturer protocols. Pools of RNA from normal tracheae were used as a calibrator for tracheal and ALI gene expression. Real-time analysis of gene expression was completed as previously reported.¹ Assays on demand used in this study were purchased from Applied Biosystems (Carlsbad, CA) and included CCSP (Mm00442046_m1), β -glucuronidase (Mm00446953_m1), FoxJ1 (Mm00807215_m1), Lef1 (Mm00550265_m1), Myc (alias c-myc) (Mm00487803_m1), Tcf7 (Mm00493445_m1), K5 (Mm00503549_m1), Axin 2 (Mm00443610_m1), and Plunc (Mm00465064_m1). Standard reagents and protocols from Applied Biosystems were used. Relative gene expression was calculated using the delta-delta cycle threshold method.¹⁹

Immunofluorescence and Histologic Staining

Tissue staining was as previously reported.¹ ALI cultures were fixed for 20 minutes at 4°C with 3.0% sucrose/3.2% paraformaldehyde/1X PBS solution. Membranes were rinsed with 1X PBS, removed from the plastic insert, placed in a 24-well plate, and stained using standard methods and previously validated antibodies.^{1,8} Images were captured using a Zeiss AxioVision microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) using 11 steps on the Z-axis and the extended focus function.

Morphometry

Regions of interest were identified in the DAPI channel followed by imaging in the red or green channels. At least three images were acquired at $\times 200$ or $\times 400$. Cells were defined by the presence of a DAPI-stained nucleus. The number of nuclei per field was determined and was set as the denominator. The number of cells expressing the marker of interest in each field was determined and was set as the numerator. Experiments were repeated up to three times and included a minimum of three replicates. Data are presented as mean $\pm$ SEM.

Western Blot

Tracheae were collected into ice-cold radioimmunoprecipitation assay buffer [1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), 0.35 mol/L SDS, 0.15 mol/L NaCl, and 0.05 mol/L Tris (pH 8)] with 1X Sigma-Fast protease inhibitor tablets (#S8820; Sigma-Aldrich, St Louis, MO), 1X Halt phosphatase inhibitor cocktail (#78420; Thermo Scientific, Waltham, MA), and 1 mmol/L phenylmethanesulfonyl fluoride (#78830; Sigma-Aldrich). Tracheae were homogenized by alternating bead-beating for 60 seconds at 4000 rpm (Tomy Micro Smash

MS-100, CS Bio Co, Menlo Park, CA) and incubating on ice for 5 minutes. Homogenates were centrifuged at 2000 rpm at 4°C for 5 minutes, and supernatants were recovered for protein analysis. Twenty micrograms of sample was added to each lane of BioRad Criterion XT precast 4% to 12% Bis-Tris gels (BioRad Laboratories, Hercules, CA), and electrophoresis and transfer (polyvinylidene difluoride membranes) were completed on BioRad's Criterion gel box system according to manufacturer protocols. Membranes were blocked overnight at 4°C in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE). Primary antibodies were added overnight at 4°C at 1:1000 in Odyssey blocking buffer with 0.2% Tween 20 (Roche Diagnostics GmbH). Membranes were washed three times for 10 minutes at room temperature in PBS/0.1% Tween 20. Secondary antibodies were applied 1:20,000 in Odyssey blocking buffer with 0.2% Tween 20 and 0.01% SDS for 1 hour in the dark. Membranes were washed as described previously and then were scanned using the Li-Cor Odyssey imager. The primary antibodies used in this study were mouse monoclonal anti-actin (C-2) (#sc-8432; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti- β -catenin (amino-terminal) (#9581; Cell Signaling Technology, Danvers, MA), and mouse anti- β -catenin (C-terminal) (#610154; BD Biosciences Pharmingen, San Diego, CA). The secondary antibodies used were goat anti-mouse IR 800 (#926-32210; Li-Cor) and goat anti-rabbit IR 680 (#926-3222; Li-Cor).

Statistical Analysis

Analysis of variance with Tukey comparisons and 2-way analysis of variance with Bonferroni comparisons were calculated using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA). A $P < 0.05$ was considered statistically significant.

Results

NA Injury in C57Bl/6 Strain Mice

The goal of this study was to evaluate β -catenin-dependent gene expression in steady state and in response to NA-mediated injury. To benchmark this analysis to previous studies, the NA dose response and injury and repair patterns were documented. A previous analysis using female Fvb/n mice demonstrated that NA exposure resulted in depletion of tracheal Clara-like and ciliated cells.¹ The abundance of CCSP mRNA, a Clara-like cell marker, was 5% of control levels on recovery day 3 and returned to 80% of control levels on recovery day 13. Preliminary analysis demonstrated that the original TOPGal-CD1 strain¹⁵ was insensitive to NA relative to the Fvb/n strain mice. Minimal injury was observed at an NA dose of 325 to 350 mg/kg body weight, and the lung epithelial injury pattern was inconsistent (data not shown). In contrast, an NA dose-response analysis in TOPGal (C567Bl/6 congenic, TOPGal-B6) mice demonstrated that 275 mg/kg of NA resulted in a 97% decrease in CCSP mRNA abundance on recovery day 3 and a return to 85% of control on recovery day 9 (see Supple-

Figure 1. β -Catenin stabilization in NA-treated TOPGal mice. Male TOPGal (B6 congenic) mice were treated with 275 mg/kg of NA and recovered at 2 to 13 days. Transgene activity (blue) and basal (K5+) or secretory (CCSP+) cell recovery (brown) were assessed over time. **A, B, F, and G:** Untreated controls. Transgene (Tg) negative (**A** and **F**) and positive (**B** and **G**) tracheal sections. Endogenous eukaryotic β -gal staining is indicated by asterisks. **C-E** and **H-J:** Recovery time points. **C** and **H:** Three days after NA. **D** and **I:** Six days after NA. **E** and **J:** Thirteen days after NA. **K-N:** NA-induced injury results in variable repair on day 6. **K** and **M:** Slow recovery. **L** and **N:** Rapid recovery.

mental Figure S1A at <http://ajp.amjpathol.org>). This analysis detected more variation in CCSP mRNA abundance on recovery days 6 and 9 than previously reported for Fvb/n mice. Body weight change paralleled changes in CCSP mRNA abundance (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). A sexually dimorphic response to NA treatment was not observed for the C57Bl/6 background (see Supplemental Figure S1C at <http://ajp.amjpathol.org>).

Trachea Morphology in NA-Treated Mice

The number and distribution of basal and Clara-like cells in the tracheae of steady state and NA-injured TOPGal-B6 mice was assessed by histologic analysis. A single layer of K5+ basal cells was positioned adjacent to the basement membrane (Figure 1, A and B). Rare K5+ cells co-expressed K14 (data not shown). Basal cell shape varied from tall/pyramidal to short/squamous. This variation did not differ between wild-type and transgenic positive (data not shown). Clara-like cells expressed CCSP and exhibited an apical surface that projected into the airway lumen (Figure 1, F and G). Columnar and cuboidal Clara-like cells were noted, and the frequency of these morphologic variants did not vary between wild type and transgenic positive. Three days after NA administration, the epithelium was severely disrupted. Most regions had an intact basal cell layer containing squamated K5+ cells (Figure 1C). Clara-like cells were absent (Figure 1H), as were ciliated cells (data not shown).

In most animals, epithelial repair was evident by recovery day 6, but three subpatterns were noted. An intermediate repair pattern was characterized by increased density of basal cells (Figure 1D), with some areas of basal cell hyperplasia. Moderate restoration of Clara-like cells was detected (Figure 1I). A slower repair process was

noted in ~25% of animals. This “slow repair” was typified by a broken line of basal cells and some regions that were devoid of basal cells (Figure 1K). Clara-like cells were not detected on recovery day 6 in the slow repair group (Figure 1M). The remaining 25% of animals underwent a “rapid repair” process in which numerous Clara-like cells were detected on recovery day 6 (Figure 1N). These differences in repair kinetics could not be attributed to the level of injury because depletion of CCSP mRNA and body weight loss on recovery day 3 was similar for all groups (see Supplemental Figure S1 at <http://ajp.amjpathol.org>; data not shown). Regardless of the repair pattern, the epithelium regained much of its pseudostratified appearance by recovery day 13 and was populated by basal (Figure 1E), Clara-like (Figure 1J), and ciliated (data not shown) cells.

β -Galactosidase Expression in Control C57Bl/6 Mice

Analysis of transgene-negative C57Bl/6 mice demonstrated a high level of endogenous (eukaryotic) β -galactosidase (β -gal) activity in the normal tracheal epithelium (Figure 1, A and F). This β -gal activity persisted despite careful adjustment of the buffer pH.²⁰ The eukaryotic β -gal activity was limited to a “belt-like” staining pattern in CCSP+ Clara-like cells (compare Figure 1, A and F). An independent flow cytometry analysis used a fluorescent β -gal reporter and demonstrated that cells from control C57Bl/6 mice contained a high level of β -gal activity. This activity could not be inhibited by chloroquine diphosphate and suggested that the enzyme was lysosomal. Other strains, such as Fvb/n, also demonstrated this high level of endogenous β -gal activity (data not shown). Therefore, it is important to evaluate individual strains for endogenous β -gal activity when using a β -gal reporter.

β -Catenin-Dependent Gene Expression in Control and NA-Treated TOPGal-B6 Mice

Control TOPGal-B6 transgene-positive mice exhibited a β -gal pattern that was indistinguishable from that observed in control C57Bl/6 wild-type mice (compare Figure 1, A and F, with Figure 1, B and G). Consequently, we were unable to use the TOPGal reporter to evaluate β -catenin-dependent gene expression in the tracheal epithelium under steady state conditions.

NA treatment resulted in ablation of Clara-like cells on recovery day 3 and complete loss of the steady state β -gal staining pattern (Figure 1, C and H). The absence of β -gal activity on recovery day 3 indicated that any subsequent changes in β -gal activity could be attributed to reexpression of the β -gal reporter in previously negative cells. On recovery day 6, intense β -gal activity was detected in mice exhibiting intermediate repair kinetics (Figure 1, D and I). This activity was pH dependent (data not shown), indicating that it was due to expression of the prokaryotic β -gal reporter. On recovery day 6, β -gal activity was detected throughout the epithelial layer and was present in the previously negative K5+ basal cells. β -Gal activity was also detected in nascent CCSP+ cells. Transgene activity was not detected in glandular structures (data not shown) or in nonepithelial tissues. Mice exhibiting slow repair were negative for β -gal activity on recovery day 6 (Figure 1, K and M), and mice undergoing rapid repair exhibited a β -gal pattern that was similar to that of control on recovery day 6 (Figure 1, L and N). On recovery day 13, all the mice exhibited the control β -gal activity pattern (Figure 1, E and J). This analysis indicated that β -catenin-dependent gene expression was transiently activated in basal cells after NA-mediated Clara-like and ciliated cell depletion.

Expression of WNT/ β -Catenin Pathway Target Genes after NA Injury

TOPGal transgene activation implied expression of β -catenin target genes. Genes in this analysis were selected on the basis of a preliminary survey of 86 WNT/ β -catenin pathway genes (SuperArray; data not shown), which demonstrated differential expression of Lef1, TCF7, Myc, and Axin 2 after NA treatment. Transcript abundance in total tracheal RNA from control C57Bl/6 mice and those exposed to 275 mg/kg of NA and recovered for 5 to 9 days was quantified by real-time RT-PCR. Lef1 mRNA decreased fourfold between control and recovery days 5 and 6 and returned to control levels on recovery days 7 to 9 (Figure 2A). TCF7 mRNA was also detected in control RNA and was depleted approximately twofold on recovery days 5 to 9 (Figure 2B). TCF7 mRNA levels were significantly different on recovery days 5, 7, and 8. Differences in the Lef1 and TCF7 gene expression patterns suggested a complex and variable role for β -catenin in the repair process.

In contrast to the β -catenin interaction partner results, Myc mRNA abundance increased 1.7-fold on recovery day 5 (Figure 2C). This increase was transient, and Myc

Figure 2. WNT pathway gene expression after NA administration. Expression of WNT/ β -catenin target genes was assayed during the period of TopGal transgene activity. Control C57Bl/6 mice were treated with 275 mg/kg of NA and recovered at 5 to 9 days. WNT pathway gene expression was evaluated by real-time RT-PCR. Data are presented as mean $\pm$ SEM. Columns with nonidentical superscript letters are significantly different ($P < 0.05$) as determined by analysis of variance using the Tukey postcomparison test. **A:** Lef1. **B:** TCF7. **C:** Myc. **D:** Axin2.

mRNA abundance decreased to control levels on recovery days 6 to 9. Because Myc expression is most commonly associated with changes in mitotic state, these data suggest that β -catenin-dependent gene expression plays a role in proliferation.

Significant differences in Axin 2 mRNA, a negative regulator of the Wnt/ β -catenin pathway, were not detected (Figure 2D). Given the complexity of the *in vivo* injury/repair system¹ and the variability in the rates of recovery between animals (Figure 1), small changes in mRNA levels, particularly mRNAs that oscillate,^{21,22} may be difficult to detect using whole tissue preparations. Overall, changes in the abundance of Lef1, TCF7, and Myc suggested that β -catenin target genes were expressed in the steady state and that these genes were reactivated in response to NA injury. However, the high level of β -gal activity in steady state Clara-like cells and simultaneous proliferation and differentiation after NA injury demanded cautious interpretation of these results.

Separation of Proliferation and Differentiation in ALI Cultures

To evaluate roles for β -catenin in epithelial repair, this process was reduced to its component parts, basal cell proliferation and basal cell differentiation. These processes were evaluated in primary cultures of tracheal epithelial cells, termed ALI cultures. ALI cultures were first evaluated for their ability to recapitulate key aspects of the NA injury and repair process. Histologic analysis was used to determine the cell types responsible for establishment of the ALI culture. Cytospin preparations from 15 strains of mice were evaluated for representation of basal cells (K5+ and/or K14+), Clara-like cells (CCSP+), and ciliated cells (ACT+). This analysis dem-

Figure 3. Representation of cell types recovered from tracheae by pronase digestion. Immunofluorescence staining of cytospin preparations was used to determine the representation of cells recovered after pronase digestion of the trachea. Fifteen different strains of mice were analyzed. The cell type mean values centered around the following: K5+ basal cells, 37%; K14+ basal cells, 4.2%; CCSP+ Clara-like cells, 28.8%; ACT+ ciliated cells, 12.8%; and other cells (not expressing any of the assayed markers), 21.5%. Data are presented as mean $\pm$ SEM. Columns with nonidentical superscript letters are significantly different ($P < 0.05$) as determined by analysis of variance using the Tukey postcomparison test.

onstrated that 37% of cells recovered by pronase digestion were basal cells (Figure 3). Of this population, 37% were K5+/K14– and 4.2% were K14+. Significant differences were not detected among mouse strains. Clara-like cells were 28.8% and ciliated cells were 12.8% of the pronase cell preparation. These three cell types accounted for 78.5% of nucleated cells.

Proliferation and differentiation occurred simultaneously *in vivo*, and the repair rate was variable among individuals (Figure 1). Thus, we determined whether proliferation and differentiation could be separated in ALI cultures. Cell proliferation was evaluated by pulse labeling with bromodeoxyuridine (BrdU) (Figure 4). The mitotic index was 78% on ALI day 0, decreased to ~30% on ALI day 2, and was 2% to 5% on ALI days 4 to 10. These data indicate that mitotic activity decreases precipitously after the shift to growth factor–reduced medium and establishment of the ALI. These data are consistent with previous reports¹⁸ and indicate that the submerged culture period through ALI day 2 could be termed the proliferation stage.

During the proliferation phase, basal cells, defined by K5, accounted for >98% of cells in culture (see Supplemental Figures S2 and S3 at <http://ajp.amjpathol.org>). These data, and co-localization of BrdU and K5 (data not shown), indicate that basal cell proliferation drives establishment of the ALI culture. When the culture media was switched to promote differentiation, there was a 24- to 48-hour period during which basal cells were the dominant cell type (see Supplemental Figures S2, A-D, and S3, A-D, at <http://ajp.amjpathol.org>). On ALI day 3, ciliated cells begin to appear (see Supplemental Figure S3D at <http://ajp.amjpathol.org>). This cell type increased in number through ALI day 10 (see Supplemental Figure S3, D-H, at <http://ajp.amjpathol.org>). Ciliated cell fre-

quency did not vary after ALI day 10 (see Supplemental Figure S3, G and H, at <http://ajp.amjpathol.org>). Clara-like cells were first detected on ALI day 6 (see Supplemental Figure S3E at <http://ajp.amjpathol.org>) but were not highly prevalent until ALI days 10 to 12 (see Supplemental Figure S3, F and G, at <http://ajp.amjpathol.org>). Additional analysis demonstrated that the abundance of Clara-like cells peaked on ALI day 10 and that representation of this cell type decreased through ALI day 20 (data not shown). Thus, ciliated cells differentiated first and were followed by Clara-like cells.

Analysis of nine ALI experiments indicated that the kinetics of proliferation and differentiation were consistent among different cultures and that these variables were independent of mouse strain. These data are in agreement with previous reports¹⁸ and indicated that ALI days 4 to 12 could be termed the differentiation stage. These data, combined with previous lineage tracing analysis,²³ demonstrated that the ALI culture system faithfully represented the differentiated cell types generated as part of the *in vivo* repair process (basal, ciliated, and Clara-like), the order in which they were regenerated (basal then ciliated then Clara-like), and the source of differentiated cells (direct basal to ciliated, basal to Clara-like, and Clara-like to ciliated).

Gene Expression Changes in the ALI System Parallel Those Associated with NA-Mediated Injury and Repair

To determine whether the ALI model system recapitulated gene expression changes associated with NA-mediated injury and subsequent repair, expression of cell type–specific mRNAs was compared in control and NA-treated tracheal tissue and in ALI cultures. *In vivo*, expression of K5 increased after NA exposure, but this increase was not significantly different from control at any recovery time point (Figure 5A). A similar pattern was noted in ALI cultures with the exception that K5 mRNA abundance was significantly increased relative to control on ALI day 1 (Figure 5B). This increased gene expression was as-

Figure 4. Proliferation in ALI cultures. Proliferation in ALI cultures was evaluated by pulse labeling with BrdU (10 μ mol/L). Cultures were fixed on ALI days 0 to 10 and were evaluated for BrdU labeling using immunofluorescence. Data are presented as mean $\pm$ SEM. Columns with nonidentical superscript letters are significantly different ($P < 0.05$) as determined by analysis of variance using the Tukey postcomparison test.

Figure 5. Comparison of cell type-specific marker gene expression *in vivo* and *in vitro*. **A, C, and E:** Fvb/n mice were treated with 300 mg/kg of NA and recovered at 3 to 13 days. **A:** Basal cell marker, K5. **C:** Ciliated cell marker, FoxJ1. **E:** Clara-like cell marker, PLUNC. **B, D, and F:** ALI cultures were established from Fvb/n mice and were collected for gene expression on proliferation days (P2 to P4) and on differentiation days (ALI1 to ALI15). **B:** Basal cell marker, K5. **D:** Ciliated cell marker, FoxJ1. **F:** Clara-like cell marker, PLUNC. Data are presented as mean $\pm$ SEM. Columns with nonidentical superscript letters are significantly different ($P < 0.05$) as determined by analysis of variance using the Tukey postcomparison test.

sociated with a change in cell shape and the organization of cytoplasmic K5 (see Supplemental Figure S2, D and E, at <http://ajp.amjpathol.org>).

As previously reported,¹ FoxJ1 mRNA, which encodes a ciliated cell-specific transcription factor, was severely reduced after NA treatment, and its abundance increased rapidly after day 3 (Figure 5C). In ALI cultures, the pattern of FoxJ1 mRNA abundance (Figure 5D) paralleled histologic detection of cilia (see Supplemental Figure S3D at <http://ajp.amjpathol.org>). The Clara-like cell marker PLUNC was severely depleted after NA injury and rebounded on recovery days 9 to 13 (Figure 5E). In ALI cultures, PLUNC exhibited a biphasic expression pattern, with peaks on ALI days 3 and 15 (Figure 5F). Previous analysis indicated that the early peak correlated with re-expression of the Clara-like marker CCSP by rare Clara-like cells that seeded the culture.²³ In contrast, the late peak coincided with basal to Clara-like cell differentiation. These data indicated that ALI cultures separated basal to ciliated cell differentiation from basal to Clara-like cell differentiation.

β -Catenin Is Stabilized during the Late Proliferation Stage and throughout the Differentiation Phase in Vitro

Because the ALI model allowed temporal separation of proliferation and differentiation, expression of the TOPGal transgene was evaluated in ALI cultures generated from TOPGal-B6 tracheal cells. X-Gal staining was used to detect β -gal activity (see Supplemental Figure S4, A–J, at <http://ajp.amjpathol.org>). β -Gal enzyme activity was not detected in cultures derived from transgene-negative mice (see Supplemental Figure S4K at <http://ajp.amjpathol.org>).

Analysis of TOPGal-B6 ALI cultures identified a dynamic pattern of β -catenin signaling during the proliferation phase (Figure 6). Less than 10% of cells were X-Gal+ on proliferation day 2 (see Supplemental Figure S4A at <http://ajp.amjpathol.org>). Transgene activity increased to include ~40% of cells on day 4 and ~80% of cells on day 5 (see Supplemental Figure S4, C and D, at <http://ajp.amjpathol.org>). Staining intensity increased between days 3 and 4, suggesting accumulation of the reporter enzyme. This activity correlated with establishment of a confluent monolayer and polarization of the epithelium. These data suggest a role for β -catenin in the regulation of basal cell proliferation.

TOPGal transgene activity was diminished on ALI day 0 (see Supplemental Figure S4E at <http://ajp.amjpathol.org>), when the cultures were switched to growth factor-deficient medium. The diffuse X-Gal staining on ALI day 0 may indicate turnover of the reporter enzyme. Transgene activity resumed as the cultures progressed toward ciliated or Clara-like cell differentiation. On ALI day 2, 95% of cells were X-Gal+ (see Supplemental Figure S4F at <http://ajp.amjpathol.org>). On ALI days 4 to 10, 99% of cells were positive for X-Gal (see Supplemental Figure S4, G–J, at <http://ajp.amjpathol.org>). The X-Gal reaction product did not co-localize with a specific cell type (data not shown). This analysis supports a role for β -catenin in basal cell differentiation.

Figure 6. β -Catenin stabilization in TopGal-B6 ALI. ALI cultures were established from TopGal-B6 mice. β -Catenin stabilization was determined throughout the proliferation (P2 to P4) and differentiation (ALI0 to ALI10) days by X-Gal staining. The frequency of cells that expressed the β -gal reporter was determined in five randomly selected regions from each time point. The percentage of cells positive (black) or negative (white) for the reporter is reported. Data are presented as mean $\pm$ SEM.

Figure 7. WNT pathway gene expression in ALI cultures. Expression of WNT/ β -catenin target genes was assayed by real-time RT-PCR during the period of TopGal-B6 transgene activity in ALI cultures. Data are presented as mean $\pm$ SEM. Columns with nonidentical superscript letters are significantly different ($P < 0.05$) as determined by analysis of variance using the Tukey postcomparison test. P2 to P4, proliferation days 2 to 4; ALI0 to ALI10, differentiation days 0 to 15. **A:** Lef1. **B:** TCF7. **C:** Myc. **D:** Axin2.

Dynamic Pattern of WNT/ β -Catenin Pathway Gene Expression in Vitro

The WNT/ β -catenin pathway target genes that were evaluated *in vivo* (Figure 2) were analyzed at various time points in ALI cultures. Lef1 was expressed on ALI days 0 to 7 but was not detected on proliferation days 2 or 4 or ALI days 9 and 15 (Figure 7A). Transcript levels were low relative to the tracheal tissue calibrator and were variable. This pattern was similar to that detected *in vivo* and placed *Lef1* gene expression in the early to middle differentiation phase (ALI days 0 to 7). TCF7 was detected at all time points tested in the ALI system, but expression levels peaked on day 4 (Figure 7B). This pattern suggested that TCF7 was expressed in basal cells, which were the prevalent cell type during this stage (see Supplemental Figure S2, A and B, at <http://ajp.amjpathol.org>). Low levels of TCF7 were detected during the differentiation stage. This pattern was distinct from that detected *in vivo*. Differences in the expression patterns of Lef1 and TCF7 suggest that they differentially regulated the pattern of gene expression in the proliferation and differentiation phases of epithelial repair.

Myc mRNA was most abundant during the proliferation phase. Myc mRNA abundance peaked on culture days 2 and 3 but decreased when the growth factor-rich medium was replaced (Figure 7C). As the cultures differentiated, Myc mRNA was detected, but at reduced levels. These data further support a role for Myc in the proliferation phase. This pattern was distinct from that of other WNT/ β -catenin pathway targets (see later herein) and will be pursued in a later study.

The WNT/ β -catenin pathway regulator Axin 2 was detected at all time points tested *in vitro* (Figure 7D). Transcript abundance increased approximately fourfold between proliferation day 2 and ALI day 3 and persisted

through ALI day 9. Detection of Axin 2 throughout the differentiation phase suggested that WNT/ β -catenin signaling occurred during this stage. The ALI pattern was distinct from that in the *in vivo* analysis, where significant changes in Axin 2 mRNA abundance were not detected (Figure 2D). These data suggest that analysis of the WNT/ β -catenin pathway *in vivo* was compromised by overlapping signaling processes.

Genetic Stabilization of β -Catenin in Basal Cells in Vitro

The ALI analysis suggested that WNT/ β -catenin regulated basal cell proliferation and differentiation. To evaluate the role of β -catenin in basal cell behavior, ALI cultures were generated from K14-rtTA/TRE-cre/DE3 (BiTg) mice. Western blot analysis of β -catenin in ALI day 5 cultures generated from BiTg or monotransgenic TRE-cre/DE3 cells demonstrated recombination of the β -catenin locus and generation of the mutant β -catenin (see Supplemental Figure S5 at <http://ajp.amjpathol.org>). This recombination was doxycycline independent. A sexual dimorphism related to the X-linked status of the K14-rtTA transgene¹⁶ was noted. K14-rtTA/DE3 monotransgenic cells did not undergo recombination. To determine whether recombination ceased after the proliferation phase, ALI cultures from ALI days 0 to 10 were analyzed for β -catenin by Western blot analysis (see Supplemental Figure S6 at <http://ajp.amjpathol.org>). Quantification demonstrated that recombination occurred early and that the level of modified β -catenin did not change after day 5. These results were confirmed by dual-immunofluorescence analysis of N- and C-terminal β -catenin as a func-

Figure 8. Stabilization of β -catenin shortens the proliferation stage *in vitro*. ALI cultures derived from K14/rtTA/TRE-cre/DE3 mice generated a mosaic epithelium composed of β -catenin wild-type and β -catenin-stabilized (recombined) regions. Mitotic activity was evaluated using BrdU pulse labeling. The number of BrdU+ cells is presented as a percentage of all cells in the region. Wild-type regions, solid circles; recombined regions, open squares. Data are presented as mean $\pm$ SEM. Significance ($P < 0.05$) was determined by 2-way analysis of variance using the Bonferroni postcomparison test.

Figure 9. Cellular distribution of β -catenin. Cells and cultures were derived from K14/rTA/tre-cre/DE3 mice. β -Catenin cellular distribution was evaluated by dual immunofluorescence on cells grown on glass coverslips, cytopins from ALI day 3 cultures, and confocal microscopy of ALI membranes. **A:** Cells grown on glass coverslips β -catenin (β -cat; green) and BrdU (red). **Yellow arrows** demonstrate co-localization of β -catenin and BrdU. **White arrows** indicate cells with nuclear localized β -catenin in nonmitotic cells. **B:** Schematic of the K14/rTA/tre-cre/DE3-stabilized allele. Wild-type cells have alleles with both the N-terminal (N-term) and C-terminal (C-term) epitopes; therefore, they are labeled red and green when both antibodies are used for dual immunofluorescence. Stabilized cells do not have the N-terminal epitope and are, therefore, identified by single staining. **C:** Cytopins from ALI day 3 membranes. **White arrowheads** indicate localization of stabilized β -catenin (green only). **Yellow arrowheads** show wild-type cells with nuclear β -catenin (red and green). **White arrows** indicate stabilized cells with cytoplasmic β -catenin. **Yellow arrows** indicate wild-type cells with cytoplasmic β -catenin. **D:** Schematic diagram of fluorescence intensity as a function of β -catenin distribution. **E:** β -Catenin (red) and nuclear (blue) signal in stabilized and wild-type regions. **White arrow** indicates junctional β -catenin. **Yellow arrow** indicates nuclear and cytoplasmic β -catenin. **F:** β -Catenin distribution in wild-type regions. **G:** β -Catenin distribution in stabilized regions.

tion of time (data not shown). Clara-like and ciliated cells were not detected during the proliferation phase of culture. These data indicate that the BiTg model could be used *in vitro* to evaluate the impact of β -catenin stabilization in basal cells and secondary effects on basal to ciliated and basal to Clara-like cell differentiation.

β -Catenin Signaling in Basal Cells Decreases the Mitotic Index

ALI cultures generated from BiTg tracheal cells produced a mosaic epithelium. Regions that were wild type for at least one β -catenin allele were identified by reactivity with the anti-N-terminal β -catenin antibody (see Supplemental Figure S7 at <http://ajp.amjpathol.org>): these regions are termed wild type. Regions that had undergone recombination on both β -catenin alleles were identified by an absence of N-terminal β -catenin immunofluorescent staining: these regions are termed stabilized. In general, the area of stabilized regions was less than that of wild-type regions. To determine whether this size difference was a consequence of proliferation, the mitotic index in wild-type and stabilized regions was compared

(Figure 8). The mitotic index did not vary between wild-type and stabilized regions on ALI days 0, 4, 6, 8, and 10. However, a significant difference was detected on ALI day 2. At this time point, the mitotic index in stabilized regions was approximately half that in wild-type regions. Analysis by 2-way analysis of variance indicated that this difference was a consequence of stabilizing β -catenin rather than a consequence of time. These data indicate that stabilization of β -catenin decreased the proliferation period by 1 day.

Nuclear Localization of β -Catenin in β -Catenin-Stabilized Cells

To determine whether β -catenin stabilization resulted in increased nuclear translocation, we used several histologic methods. Cells were grown in the presence of doxycycline on glass coverslips and were labeled for 6 hours with BrdU. Dual immunofluorescence demonstrated co-localization of β -catenin (green) and BrdU (red), providing direct evidence of nuclear β -catenin in stabilized cells (Figure 9A). However, cultures grown on glass coverslips are submerged and are limited by the fact that they

cannot be differentiated. To confirm the presence of nuclear β -catenin in stabilized cells grown in ALI, cells from ALI day 3 were recovered by trypsinization, and cytopins were generated. Dual immunofluorescence and confocal imaging of cytopins demonstrated a diverse culture composed of stabilized (green) and wild-type (red) cells with nuclear and/or cytoplasmic localized β -catenin (Figure 9, B and C). This method was excellent for demonstrating the dynamic pattern of β -catenin in stabilized and wild-type cells grown in ALI conditions, but it was limited by the fact that cytopins resulted in loss of culture morphology.

To determine the extent of nuclear, junctional, and cytoplasmic β -catenin in ALI cultures, we used confocal microscopy to quantify the cellular distribution of β -catenin. The histogram function was used to compare fluorescence signal intensity for β -catenin (red) and DNA (blue) along the diameter of wild-type and stabilized cells (Figure 9D). In wild-type cells, β -catenin was highly enriched in junctional complexes (Figure 9, E and F). In contrast, in stabilized cells, β -catenin was increased in the cytoplasmic and nuclear compartments (Figure 9, D and E). These data indicate increased free β -catenin in cells with stabilized β -catenin and suggest an increase in availability of β -catenin for the regulation of gene expression.

β -Catenin Signaling in Basal Cells Enhances Basal to Ciliated Cell Differentiation

To determine whether β -catenin stabilization in basal cells effected their differentiation to ciliated cells, the number of ACT+ cells in wild-type and stabilized regions was compared on differentiation day 3. At this time, lineage tracing studies demonstrated that all ciliated cells were derived from basal cells.²³ On differentiation day 3, ciliated cells were present in wild-type and stabilized regions (Figure 10A). The percentage of ciliated cells in wild-type regions was half that in stabilized regions (Figure 10E). Ciliated cell differentiation was also evaluated on day 13, when ciliated cells are derived from basal and Clara-like cells. The percentage of ciliated cells increased twofold in wild-type regions between ALI days 3 and 13 but did not change significantly in stabilized regions. On ALI day 13, ciliated cells were twofold more abundant compared with in stabilized regions (Figure 10, B and E). These data indicate that β -catenin stabilization in basal cells enhanced basal to ciliated cell differentiation.

β -Catenin Signaling in Basal Cells Blocks Basal to Clara-Like Cell Differentiation

To determine whether β -catenin stabilization in basal cells effected their differentiation to Clara-like cells, the number of CCSP+ cells in wild-type and stabilized regions was compared on ALI day 8, the first time point that Clara-like cells were reliably detected in these cultures. At this time point, Clara-like cells were located exclusively in wild-type regions (Figure 10C). Of cells in wild-type

Figure 10. β -Catenin signaling in basal cells alters cell fate determination. ALI cultures were derived from K14/rtTA/ter-cre/DE3 mice and were evaluated for basal to ciliated and basal to Clara-like cell differentiation using immunofluorescence. **A–D:** Dual immunofluorescence was used to analyze differentiation in ALI cultures. **White arrows** indicate cells of interest in wild-type regions. **Yellow arrows** indicate cells of interest in β -catenin-stabilized regions. **E and F:** Number of cells expressing the differentiation markers ACT and CCSP is presented as a percentage of all cells in the region. Wild-type (WT) regions, closed circles; recombined (Rec.) regions, open squares. Data are presented as mean $\pm$ SEM. Significance ($P < 0.05$) was determined by 2-way analysis of variance using the Bonferroni postcomparison test. d3 and d13, days 3 and 13. **A:** ALI day 3 ACT. **B:** ALI day 13 ACT. **C:** ALI day 8 CCSP. **D:** ALI day 13 CCSP. **E:** Quantification of ciliated cell differentiation. **F:** Quantification of Clara-like cell differentiation.

regions, ~4% expressed CCSP (Figure 10F). No CCSP+ cells were detected in stabilized regions (Figure 10F). By differentiation day 13, ~2% of cells in wild-type regions expressed CCSP (Figure 10F). Stabilized regions remained devoid of the Clara-like cell type (Figure 10D). These results demonstrate that β -catenin stabilization blocked basal to Clara-like cell differentiation.

Discussion

The purpose of this study was to determine whether β -catenin regulated basal cell fate determination in the mouse trachea. *In vivo* analysis indicated a complex role for β -catenin in epithelial repair after NA injury. Use of tracheal epithelial ALI cultures permitted temporal separation of basal cell proliferation and basal cell differentiation into ciliated and Clara-like cells. Stabilization of β -catenin did not alter basal cell proliferation other than to shorten the proliferation phase by 1 day. Stabilization of β -catenin enhanced basal to ciliated cell differentiation twofold and was associated with a complete blockade of basal to Clara-like cell differentiation. These results sug-

gest that β -catenin was a critical determinant of tracheal basal cell fate determination.

Advantages and Disadvantages of the in Vitro Model

The complexity of β -catenin signaling during injury and repair limited mechanistic analysis of β -catenin effects on basal cell proliferation and differentiation. We demonstrated that tracheal epithelial cell cultures recapitulate critical features of the NA injury and repair model but that they allow temporal segregation of proliferation and differentiation. In addition, this model partitions the basal to ciliated cell and basal to Clara-like cell replacement processes.

Injury and repair *in vivo* is a dynamic process involving several cell and tissue types that signal and respond to signals that alter the behavior of their environment and of the cells in that environment. The *in vitro* model is limited in its ability to recapitulate this complexity. The finding that basal cells generate numerous ciliated cells and fewer Clara-like cells may reflect this deficiency. However, consistent generation of Clara-like cells indicates that the tracheal epithelial culture model is superior to the tracheosphere model²⁴ for analysis of Clara-like cell differentiation. Identification of a distinct Clara-like cell differentiation period permitted delineation of the role played by β -catenin in the generation of ciliated and Clara-like cells by basal cell progenitors.

Roles for β -Catenin in Basal Cell Proliferation

Accumulation of β -catenin in highly mitotic, rapidly renewing epithelium, such as the blood, intestine, and skin, results in stem cell proliferation and, when left unchecked, often results in the development of cancer and/or progression to metaplasia.^{25–28} Analysis of β -catenin-regulated cell function in these tissues has been facilitated by several naturally occurring mutations that impact progenitor cell function.²⁹ However, the role of β -catenin stabilization in tissues with a low mitotic index and slow regeneration rates (eg, lung, liver, and pancreas) is less clear.⁷ The germ line mutations that severely alter progenitor cell proliferation and differentiation in the gut have little effect on the respiratory epithelium. These results suggest that β -catenin is a proximal regulator of cellular proliferation in some tissues, whereas it plays a more subtle role in the lung.

The β -catenin–TCF/LEF transcription complexes typically function as positive regulators of genes involved in progenitor cell proliferation, including Myc and cyclin D.^{30,31} Several cancers have mutations in the adenomatosis polyposis coli protein, resulting in overexpression of downstream β -catenin targets, such as Myc. Myc is widely recognized for its ability to promote cell proliferation by regulating cell cycle progression through G(1) into S phase.^{32,33} The present data demonstrate concurrent expression of Myc and stabilization of β -catenin *in vivo*

and *in vitro*. Although compelling, cautious interpretation of the β -catenin–Myc pathway is required because proliferation was not strongly altered by β -catenin stabilization *in vitro* (Figures 6 and 7; see also Supplemental Figure S4 at <http://ajp.amjpathol.org>) or in previous *in vivo* analyses.^{8,14} Thus, other signals, including growth factors, may function through or in parallel with β -catenin to regulate proliferation.

Roles for β -Catenin in Basal to Ciliated Cell Differentiation

Tracheal basal cells are the facultative progenitor pool.¹ In the steady state, K5+ cells and the K14-expressing subset²³ maintain the basal cell pool. After NA injury, K14+ basal cells serve as a progenitor for ciliated and Clara-like cells.^{3,4} The direct differentiation of basal cells to ciliated cells²³ is similar to the progenitor-progeny relationships reported for the adult human bronchial epithelial cells *ex vivo*^{5,6} and for the developing human and mouse lung (reviewed by Daniely et al³⁴). A second cohort of ciliated cells is generated from the Clara-like cell progenitor in adult mice³⁵ and rats.²

Several studies have reported activation of β -catenin reporter genes during the period of lung development in which basal cells are the sole source of ciliated cells (reviewed by De Langhe and Reynolds¹¹). These data associated basal to ciliated cell differentiation with β -catenin signaling but did not establish cause and effect. The present analysis of ciliated cell differentiation demonstrated that β -catenin stabilization in basal cells increased ciliated cell differentiation. Minimally, this study demonstrated that basal to ciliated cell differentiation was not inhibited by excess β -catenin. The increase in ciliated cell differentiation may be a consequence of β -catenin-dependent promotion of basal to ciliated cell differentiation. However, an alternative explanation could be that β -catenin inhibited Clara-like cell differentiation. In this scenario, the pool of basal cell progenitors available to become ciliated cells would be increased. Differentiating between these two possible mechanisms requires further evaluation.

Roles for β -Catenin in Basal to Clara-Like Cell Differentiation

Analysis of β -catenin reporter transgene expression during lung development suggests a reciprocal relationship between β -catenin-dependent gene expression and differentiation of Clara-like and Clara cells.⁸ This observation was refined to a “just right” paradigm by the finding that low β -catenin signaling was important for Clara cell maturation but that deletion of the β -catenin signal after establishment of the prenatal Clara cell population did not alter the maturation process.¹⁴ These data suggested that β -catenin was critical for maintenance of an embryonic Clara cell progenitor but that β -catenin was not a positive mediator of the differentiation program. The present study supports this view by demonstrating a similar just right paradigm in the *in vitro* model.

ALI cultures expressing wild-type levels of β -catenin underwent basal to Clara-like cell differentiation in the presence of β -catenin signaling as identified by expression of the TOPGal transgene. These data indicate that β -catenin signaling is permissive for Clara-like cell differentiation. Regions in the mosaic cultures that expressed at least one wild-type β -catenin allele produced Clara-like cells, a result comparable with cultures expressing only wild-type β -catenin. However, the regions that expressed only stabilized β -catenin did not generate Clara-like cells. The loss of basal to Clara-like cell differentiation may be the result of too much or prolonged β -catenin signaling. In either case, the just right hypothesis is supported by the results.

Differential β -Catenin Signaling in Ciliated and Clara-Like Cells

The differential effect of β -catenin on the generation of basal cell–derived differentiated cells begs the question of whether ciliated and Clara-like cells have distinct mechanisms to regulate β -catenin availability. Recent evidence indicates that Chibby is a potent inhibitor of β -catenin activity. Chibby regulates nuclear partitioning and β -catenin–dependent signaling via direct binding and nuclear export of β -catenin.^{36–38} The nuclear to cytoplasmic shuttling of β -catenin via Chibby uses interactions with 14-3-3 zeta.³⁸ Chibby is abundant in ciliated cells but is absent in Clara-like cells (data not shown). It is possible that the blockade of Clara-like cell differentiation in the presence of stabilized β -catenin is due to the inability of this cell type to export β -catenin via the Chibby–14-3-3 pathway. It is unlikely that increased β -catenin dose was toxic to the Clara-like cell because survival and proliferation of bronchial Clara cells that expressed stabilized β -catenin were not different from those of wild-type cells. In contrast, expression of Chibby in ciliated cells may give this cell type a competitive advantage and may result in ciliated cell hyperplasia.

Clinical Relevance

Many upper respiratory tract diseases are the result of defective wound repair.³⁹ Repair of the TBE, as modeled by NA injury in mice, is a two-part wound-healing process. Basal cells proliferate for the purpose of reepithelialization and then differentiate to reestablish a functional secretory/ciliated epithelium. Disruption of the TBE is a common pathologic alteration that is observed in numerous chronic lung diseases, including cystic fibrosis, asthma, chronic obstructive pulmonary disease, bronchopulmonary dysplasia, and idiopathic pulmonary fibrosis.⁴⁰ Cellular alterations in the TBE lesion include basal cell hyperplasia and secretory and ciliated cell hypoplasia. In idiopathic pulmonary fibrosis, this abnormality is associated with reactivation of the embryonic signaling pathway Wnt/ β -catenin pathway.⁴¹ The present study supports the hypothesis that the activation of β -catenin in the human TBE alters basal cell fate determination. An

understanding of the basal cell lineage and the role of β -catenin in the selection of ciliated versus Clara-like cell fate is an important clue to understanding the cellular and molecular underpinnings of airway disease and may be the basis for redirection of basal cell differentiation toward normal cellular phenotypes.

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