Temporal Relationship between Primary and Motile Ciliogenesis in Airway Epithelial Cells

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Cilia are traditionally classified as motile or primary. Motile cilia are restricted to specific populations of well-differentiated epithelial cells, including those in the airway, brain ventricles, and oviducts. Primary cilia are nonmotile, solitary structures that are present in many cell types, and often have sensory functions such as in the retina and renal tubules. Primary cilia were also implicated in the regulation of fundamental processes in development. Rare depictions of primary cilia in embryonic airways led us to hypothesize that primary cilia in airway cells are temporally related to motile ciliogenesis. We identified primary cilia in undifferentiated, cultured airway epithelial cells from mice and humans and in developing lungs. The solitary cilia in the airways express proteins considered unique to primary cilia, including polycystin-1 and polycystin-2. A temporal analysis of airway epithelial cell differentiation showed that cells with primary cilia acquire markers of motile ciliogenesis, suggesting that motile ciliated cells originate from primary ciliated cells. Whereas motile ciliogenesis requires *Foxj1*, primary ciliogenesis does not, and the expression of *Foxj1* was associated with a loss of primary cilia, just before the appearance of motile cilia. Primary cilia were not found in well-differentiated airway epithelial cells. However, after injury, they appear in the luminal layer of epithelium and in basal cells. The transient nature of primary cilia, together with the temporal and spatial patterns of expression in the development and repair of airway epithelium, suggests a critical role of primary cilia in determining outcomes during airway epithelial cell differentiation.

Keywords: cilia; differentiation, *Foxj1*; PKD1; PKD2

Cilia have increasingly been implicated in embryonic development, cell homeostasis, and human disease (1–3). Broadly, cilia are classified as motile or primary (2, 4, 5). Motile cilia typically occur as clusters of 100–300 on the apical epithelial cell surface. These cilia have motor proteins that provide machinery to propel fluid through fallopian tubes, brain ventricles, and the respiratory tract. Motile cilia also occur as solitary structures, as sperm flagella, and in the embryonic node (6). In contrast, more than a century ago, single, nonmotile cilia were first identified and were thereafter described in almost all cell types as “primary,” “rudimentary,” “solitary,” “nonmotile,” or “sensory” cilia (4, 7). The appearance of primary cilia was confirmed to be related to the cell cycle. Primary cilia are dynamic structures that nucleate from the centriole of the mother centrosome after migration to the plasma membrane after mitosis (8, 9). Primary

CLINICAL RELEVANCE

A growing number of diseases have been found to be due to mutations in genes expressed in primary cilia; however, little is known about these structures in the lung. We identified a pattern of expression of primary cilia linked to airway epithelial cell differentiation during lung development and injury repair, suggesting critical roles for primary cilia.

cilia are also found in highly differentiated cells such as in the retina, where they specialize as sensory photoreceptors, in the nasal cavity as olfactory sensors, and on luminal surfaces of renal tubules, biliary ducts, and vascular endothelial cells, as mechanosensors of fluid flow (10, 11).

An emerging interest in cilia is driven by the identification of a growing number of diseases caused by mutations in genes coding for proteins expressed in cilia (1). The only well-defined genetic motile cilia disorder is primary ciliary dyskinesia (or immotile cilia syndrome) (12). This genetically heterogeneous syndrome is attributable to mutations in genes that code for proteins within the motor complex, and it can result in situs inversus, otitis media, and respiratory tract infections (12, 13). In contrast, mutations in genes expressed in primary cilia were found to be responsible for other syndromes. Among the best described are autosomal dominant polycystic kidney disease (ADPKD), nephronophthisis (NPHP), and Bardet–Beidl syndrome (BBS) (1, 14). ADPKD is a common genetic disease attributable to mutations in *PKD1* and *PKD2*, genes that code for the proteins polycystin-1 (PC-1) and polycystin-2 (PC-2), respectively. These proteins localize to primary cilia in the renal tubules to function as flow sensors, but when mutated, result in cyst formation (5, 10). Recently, many of these diseases once thought attributable to primary cilia dysfunction were shown to have features related to the disturbance of motile cilia (e.g., bronchiectasis and situs inversus). This may be the result of the expression of primary cilia-associated proteins in motile cilia. For example, we found PC-1 expression in airway motile cilia and an increased prevalence of radiographic bronchiectasis in patients with ADPKD (15). Similarly, BBS proteins function in the biogenesis of cilia, and when mutated, result in a syndrome of obesity, retinopathy, polydactyly, cystic kidneys, and situs inversus (14). However, BBS genes 2, 4, and 8 are expressed in motile cilia in the airway and BBS2-deficient and BBS4-deficient mice have abnormal motile cilia structure and reduced ciliary beat frequency (16, 17). These observations suggest roles for primary cilia-related genes in motile cilia assembly or function.

Although the structural, temporal, and spatial expressions of motile cilia in the airways have been characterized, depictions in the literature of primary cilia in the lung are limited to a few in the past 50 years (18–21). For example, Kober in 1975 and Galliard and colleagues in 1989 published scanning electron micrographs of a solitary cilium on a rat embryonic tracheal

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epithelial cell and a human embryonic airway cell, respectively (19, 20). In a seminal ultrastructural analysis of primary and motile ciliogenesis, Sorokin noted the appearance of a “primary” or “rudimentary” cilium preceding the presence of motile cilia in epithelial cells of developing rat lungs (18). Further descriptions of the temporal and spatial characterizations of primary cilia in the airways, and their relationship to motile cilia, are lacking.

Limited reports of primary cilia in airways and the increasing recognition of cilia-related diseases led us to hypothesize that primary cilia emerge from airway epithelia, share proteins with motile cilia, and have a temporal relationship to motile ciliogenesis. Our analyses of primary cell culture models and lung development and repair revealed that primary cilia are transiently present on undifferentiated airway epithelial cells and are absent in adult airways unless injured, suggesting that motile ciliated cells originate from primary ciliated cells. These observations suggest that primary cilia play a key role in airway epithelial cell differentiation and repair.

MATERIALS AND METHODS

Cell Culture

BEAS-2B cells (CRL-9609; ATCC, Manassas, VA) and Madin Darby Canine Kidney (MDCK) cells (CCL-34; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Primary human tracheal epithelial cells (hTEC) obtained from donors for lung transplantation and mouse tracheal epithelial cells (mTEC) from C57BL/6J mice were cultured as described previously (22). Cells were seeded on supported membranes (Transwell; Costar-Corning, Corning, NY), and an air-liquid interface (ALI) was established for the induction of differentiation by removing apical compartment media. In some experiments, confluent cells were continuously cultured in a submerged condition by maintaining media in the apical compartment. The Institutional Review Committee at Washington University approved these animal and human studies.

Immunofluorescent Staining and Microscopy

Cells on membranes were fixed and processed for immunodetection, as described previously (22, 23). For anti- γ -tubulin and anti-polycystin-1 antibodies, cells were treated with PHEM buffer (45 mM PIPES, 45 mM HEPES, 10 mM EGTA, and 5 mM $MgCl_2$, pH 7.4) for 1 minute before 4% paraformaldehyde fixation (24). For anti-polycystin-2 antibodies, cells were fixed in methanol/acetone (50:50) at $-20^\circ C$ for 15 minutes before paraformaldehyde fixation. Fixed samples were blocked in 5% donkey serum and 3% BSA in PBS with 0.1% Triton-X100, and then incubated with the primary antibody. Primary antibodies and dilutions included mouse anti-acetylated α -tubulin (1:1,000; Sigma-Aldrich, St. Louis, MO), rabbit anti- γ -tubulin (1:1,000; Sigma-Aldrich), rabbit anti-Ki-67 (1:1,000; Vector Laboratories, Burlingame, CA), rabbit anti-polycystin-1 (anti-LRR, 1:50; provided by O. Ibraghimov-Beskrovnaya, Genzyme Corp, Cambridge, MA.) (25), rabbit anti-polycystin-2 (1:50, catalogue number sc-25749; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-ADP-ribosylation factor-like 13B (Arl13b, 1:1,000; provided by T. Caspary, Emory University, Atlanta, GA) (26), mouse anti-cytokeratin 14 (1:80; Vector Laboratories), mouse anti-Foxj1 (1:1,000) (23), rabbit anti-Clara cell secretory protein (CCSP, Scgb1a1, 1:10,000; provided by S. Reynolds, National Jewish Hospital, Denver, CO), and rabbit anti-secretoglobulin 3a2 (Scgb3a2, 1:1,500; provided by S. Kimura, National Cancer Institute, Bethesda, MD) (27). Antibody binding was detected using Alexa Fluor 488 or 555 secondary antibodies (Invitrogen, Carlsbad, CA). Staining was compared with isotype-matched control antibodies. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Photomicrographs were obtained using a 5000B Leica microscope (Leica, Wetzlar, Germany) equipped with a charge-coupled device camera (Retiga 200R) interfaced with QCapture Pro software (both from Q Imaging, Surrey, Canada). Images were globally adjusted for contrast and brightness, and

composed using Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

Lung Tissue Immunostaining

Mouse embryos were harvested as described elsewhere (28). Lung sections were prepared from mice infected with Sendai virus (SeV), and collected as previously described (29). Tissue sections were subjected to antigen retrieval by incubation in antigen unmasking solution at pH 6.0 (Vector Laboratories) within a pressure cooker (Biocare Medical, Concord, CA) for 30 minutes before immunostaining, as described for cells on membranes.

Electron Microscopy

Cells on membranes were prepared for electron microscopy (EM) as previously described (24). Scanning and transmission EM were performed using Hitachi S-450 (Hitachi, Tokyo, Japan) and Zeiss 902 (Zeiss, Thornwood, NY) microscopes, respectively.

Protein Blot Analysis

Cells were lysed in RIPA buffer (PBS, pH 7.4, 1.0% IPEGAL CA630, 0.5% sodium deoxycholate, and 0.1% SDS) containing proteinase inhibitors. For the detection of PC-1 and PC-2, samples containing 25 μg of protein were resolved on tris-acetate gels (NuPAGE; Invitrogen) and subjected to immunoblot analysis, as described by others for the transfer of high molecular weight proteins (25). The antibodies used were those indicated for immunostaining. Horseradish peroxidase-labeled secondary antibody binding was detected using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Statistical Analysis

Cilia types in cultured cells were quantified in three or more random fields obtained from three or four independent preparations. At least 900 cells at each time point were assessed. Differences between conditions were analyzed by Student's *t* test or ANOVA, using SPSS software (SPSS, Inc., Chicago, IL). A significant difference between conditions was established at $P < 0.05$.

RESULTS

Primary Cilia Are Present in Primary-Culture Airway Epithelial Cells

Primary cilia were described in a wide variety of cell types, including renal tubule epithelium, vascular endothelium, fibroblasts, and cell lines (4, 7). To evaluate for the presence of primary cilia in airway epithelial cells, we examined the human airway epithelial cell line BEAS-2B, using immunofluorescent staining for the expression of primary cilia marker acetylated α -tubulin and basal body marker γ -tubulin (Figure 1A). The presence of primary cilia was confirmed by scanning EM, which revealed solitary cilia on the surface of BEAS-2B cells (Figure 1B). BEAS-2B cells are transformed and incapable of differentiating into motile ciliated cells (24). To search for primary cilia in primary airway cells, we evaluated culture preparations of airway epithelial cells from tracheas of mice and humans (mTEC and hTEC) before the appearance of motile cilia. Primary cilia in cells of these preparations were demonstrated by co-staining for the expression of acetylated α -tubulin and γ -tubulin, and were confirmed by scanning EM (Figures 1A and 1B). An evaluation of the ultrastructure of solitary cilia in mTEC using transmission EM showed characteristic foot appendages composed of perpendicular fibers (Figure 1C) (7). In fixed preparations, cilia length was typically 0.5 μm , but varied from approximately 0.2–2.0 μm .

Primary Cilia Are Present in Nonproliferating Mouse Tracheal Epithelial Cells

To characterize cells with primary cilia further, we considered that their appearance is tightly linked to the cell cycle. In cell

Figure 1. Primary cilia are present on primary culture airway epithelial cells. (A) Human airway epithelial cell line BEAS-2B and tracheal epithelial cells from mice and humans (*mTEC* and *hTEC*), cultured using air-liquid interface (ALI) conditions, were immunostained with antibodies for cilia protein acetylated α -tubulin (α -*tub*, green) and basal body protein γ -tubulin (γ -*tub*, red). Nuclei were stained using 4',6 diamidino-2-phenylindole (DAPI) (blue). Overlaid photomicrographs of BEAS-2B and preparations on ALI Day 2 (*mTEC*) and Day 1 (*hTEC*) demonstrate primary cilia. (B) Scanning electron microscopy (EM) of preparations (as in A) show solitary primary cilia. Bars = 5 μ m. (C) Transmission EM of *mTEC* preparations on ALI Day 3 show solitary primary cilia on the cell surface (arrows). Bars = 1 μ m at low magnification, and 0.5 μ m at high magnification. (D) *mTEC* preparations on ALI Day 3, immunostained for α -*tub* (arrows) and cell-cycle protein Ki-67 (arrowheads), show primary cilia on cells that lack Ki-67 expression. (E) Primary cilia (arrows) are abundant in *mTEC* preparations cultured for 14 days using submerged rather than ALI conditions, immunostained as in A. Bars in A, D, and E = 10 μ m.

lines, a primary cilium is transient and most commonly detectable in the G_0 phase, preceding resorption before or during G_2 (3, 9, 30). We previously showed that *mTEC* preparations have a low level of proliferation (<3%) during the first few days of ALI culture, followed by an ongoing rate of less than 1% after ALI Day 7, as detected by Bromodeoxyuridine (BrdU)

incorporation (22). During ALI Days 1–3, primary cilia were absent in cells that expressed Ki-67, a cell cycle protein not expressed in G_0 . During this period, primary cilia were present on some but not all cells lacking Ki-67 (Figure 1D). We next evaluated *mTEC* that were confluent and cultured while continuously submerged in media for 14 days. At this time, cells are

Figure 2. Polycystin-1 and polycystin-2 are expressed in primary and motile cilia of airway epithelial cells. (A) Preparations of primary-culture tracheal epithelial cells were immunostained for cilia marker acetylated α -tubulin (α -*tub*) and polycystin-1 or polycystin-2 (PC-1 or PC-2) on ALI Day 10 (*hTEC*) or Day 1 (*mTEC*). Overlaid photomicrographs show colocalization of polycystins with α -*tub* in primary cilia. PC-1 and PC-2 were not detected in cilia incubated with normal rabbit IgG. (B) Well-differentiated *mTEC* preparations immunostained for PC-2 and α -*tub* demonstrate co-expression in motile cilia, shown *en face* (top) and in isolated cells (bottom). Bars in A and B = 10 μ m. (C) Protein blot analysis of primary culture tracheal cell preparations, harvested on indicated day of ALI culture, were analyzed for expression of PC-1 and PC-2, α -*tub*, cilia protein Arl13b, and motile ciliated cell transcription factor Foxj1. Samples from mouse kidney (*mkidney*) and MDCK cells are indicated. Membranes were re-probed to detect the indicated protein. PC-1 was detected on a separate membrane, to resolve a protein of approximately 500 kD.

quiescent, as demonstrated by the absent expression of Ki-67, and motile ciliogenesis is inhibited by the submerged condition (31). Here, primary cilia are abundant, and were evident in over 50% of cells on the surface layer (Figure 1E). Thus, consistent with previous observations in other cell types, primary cilia in airway epithelial cells were not observed in actively proliferating cells, but were present in quiescent cells and abundant in the absence of motile cilia.

Polycystins Are Expressed in Primary and Motile Cilia

We next sought to determine if primary cilia on airway epithelial cells express proteins that are considered characteristic to this class of cilia. Based on our previous studies, we were particularly interested in the expression of the polycystin proteins PC-1 and PC-2 that are expressed in the primary cilia of renal tubular epithelial cells (15, 32). We therefore used immunostaining to localize polycystin proteins and cilia markers. The expression of PC-1 was evaluated in hTEC, rather than mTEC, because of the limitations of anti-mouse PC-1 antibodies (33). Preparations of cultured airway cells showed a colocalization of acetylated α -tubulin with PC-1 (in hTEC) and PC-2 (in mTEC), seen as a punctate structure of the basal body–cilium complex, as described by others (Figure 2A) (34). We previously showed that PC-1 is expressed in motile cilia of the airway epithelium (15), and others demonstrated PC-1 expression in motile cilia lining the brain ventricles (33).

Consistent with the known interaction of PC-1 and PC-2, PC-2 is also expressed in the motile cilia of airway epithelial cells by colocalization with acetylated α -tubulin (Figure 2B). Western blot analysis showed that the antibodies used for immunostaining PC-1 and PC-2 detected proteins of the predicted size in cultured tracheal epithelial cells (Figure 2C). The expression of PC-1 and PC-2 in these cells was evaluated at an early time point, when mainly primary cilia are present, and later, when motile cilia are abundant (hTEC, ALI Day 6 versus Day 36; mTEC, Day 1 versus Day 20). Evidence of motile ciliogenesis in the late samples was confirmed by the increased expressions of motile cilia transcription factor Foxj1 (24) and Arl13b, a primary and motile cilia-specific protein (26). Acetylated α -tubulin, a protein expressed in primary and motile cilia and within the cytoplasm, increased minimally. During differentiation, PC-1 expression increased markedly whereas the increase in PC-2 was minimal, suggesting that polycystins are regulated differently in these cells. Collectively, these data indicate that primary cilia in airway cells express known primary cilia genes, and that the expression of PC-1 and PC-2 is shared by motile cilia.

A Temporal Relationship Exists between Primary and Motile Cilia

The shared expression of polycystins in primary and motile cilia led us to determine the relationship between cells with primary

Figure 3. Primary cilia appear in airway epithelial cells before motile ciliogenesis *in vitro* and in developing lungs. (A) Preparations of mTEC were analyzed on indicated day of ALI culture by immunostaining, using antibodies for acetylated α -tubulin (α -tub, green) and γ -tubulin (γ -tub, red). Cells with primary cilia on ALI Day 1 decreased in abundance as motile cilia appeared on ALI Day 4, and as cells became well-differentiated on ALI Day 14. (B) Mouse lung sections from embryonic day (E) 12.5 were immunostained with antibodies to α -tub (green) and Arl13b (red), and show primary cilia. (C) Transition from primary to motile cilia during development. Lungs on E14.5 and E15.5 were immunostained for primary and motile cilia protein Arl13b (green) and motile cilia protein β -tubulin-IV (β -tub-IV, red). Insets show detail. Primary cilia (arrows) and motile cilia (arrowheads) are indicated. All bars = 10 μ m.

cilia and those with motile cilia. We previously described the temporal stages of motile ciliogenesis during *in vitro* differentiation induced in mTEC preparations under ALI conditions (22–24). Cell preparations were assayed for the expression of acetylated α -tubulin, because this protein is present in both primary and motile cilia, and for the expression of γ -tubulin to mark basal bodies, the site of cilia nucleation. Upon the initiation of ALI (Day 0), cells are relatively undifferentiated. Primary ciliated cells are abundant on ALI Days 0–2, whereas cells with motile cilia appear on ALI Days 3 and 4 (Figure 3A). ALI preparations of mTEC ultimately result in an apical surface composed of approximately 60% motile ciliated cells by ALI Day 14, whereas primary cilia were rarely evident at this stage, indicating a shift in cilia type during differentiation *in vitro*.

Primary Cilia Precede Motile Cilia during Lung Development

To determine if a similar temporal switch occurs from primary to motile cilia *in vivo* as seen *in vitro*, tissue sections of embryonic mouse lungs were analyzed for the appearance of cilia types. Epithelial cells of the lung bud rapidly proliferate after formation on Embryonic Day (E) 9.5 (35). At the embryonic stage E12.5, primary cilia are identified in the proximal large airways by the colocalization of acetylated α -tubulin and Arl13b, both proteins expressed in primary cilia (26) (Figure 3B, *arrows*). We next used the expression of β -tubulin-IV, a protein enriched in motile cilia, to characterize cilia types further (28, 36). Only primary cilia are present on airway epithelial cells through E14.5, as indicated by the expression of acetylated α -tubulin, but not β -tubulin-IV (Figure 3C). At the late pseudoglandular stage, on E15.5, fewer primary cilia are evident, and most airway epithelial cells lack cilia of any type (Figure 3C). In the largest airways on E15.5, a transition occurs between cilia types, characterized by a decrease in primary cilia and an emergence of motile cilia, as identified by the colocalization of Arl13b and β -tubulin-IV, whereas primary cilia express only acetylated α -tubulin (Figure 3C). These data suggest that primary cilia play a specific role in airway epithelial cell differentiation during lung development.

Primary Ciliogenesis Is Foxj1-Independent

The shift in cilia types during the *in vitro* and *in vivo* differentiation of airway epithelial cells led to a further examination of markers of differentiation in cells with primary cilia. Based on observations by Sorokin (18) that primary cilia preceded motile cilia, we examined the relationship of primary cilia to the expression of Foxj1, a transcription factor required for the differentiation of motile ciliated cells (37). Our previous studies of mammalian systems showed that Foxj1 is necessary for generating multiple motile cilia, whereas others showed that in *Xenopus* and zebrafish, Foxj1 can induce solitary motile cilia (24, 37–39). To understand the role of Foxj1 further and its relationship to primary ciliogenesis, we analyzed preparations of mTEC from wild-type and Foxj1^{-/-} mice (Figure 4A). In wild-type cells, before the onset of Foxj1 expression (ALI Day 2), cells with primary cilia are associated with a centriole at the cell cortex. Centrioles multiply to become basal bodies immediately before the onset of Foxj1 (24, 37). Basal body docking follows, as indicated by an organized array of γ -tubulin-expressing structures at the apical membrane, evident on ALI Day 14. Foxj1^{-/-} cells are characterized by a halt in motile ciliogenesis, resulting in a failure of basal bodies to dock at the apical membrane (shown by clusters of γ -tubulin-expressing structures within the apical domain of the cytoplasm), and have a primary cilium, as evident on ALI Day 14 (Figure 4A) (24, 37).

Figure 4. Primary ciliogenesis is Foxj1-independent. (A) mTEC derived from wild-type (WT) and Foxj1^{-/-} mice, cultured under ALI conditions, were immunostained for acetylated α -tubulin (α -*tub*, green) and γ -tubulin (γ -*tub*, red). On ALI Day 2, primary cilia are similar in mTECs from each strain. On ALI Day 14, Foxj1^{-/-} cells show clusters of basal bodies (red) that fail to dock at the apical membrane (*arrows*, lower right) and primary cilia, unlike the motile cilia in WT mTECs. (B) Primary cilia in Foxj1-expressing cells during lung development. Lung sections from the indicated embryonic day (E) were immunostained for Arl13b (green) and Foxj1 (red). On E14.5, primary cilia are present before Foxj1 expression. At the onset of Foxj1 expression, on E15.5, primary cilia are present in some Foxj1-expressing cells, and absent in others. On E19.5, motile-type cilia are present on nearly all Foxj1-expressing cells (insets show detail). Representative airways with primary (*arrows*) and motile (*arrowheads*) cilia are shown. All bars = 10 μ m.

Primary Cilia Are Present in Cells That Express Foxj1 during Lung Development

We next sought to determine the relationship between Foxj1 expression and the occurrence of primary cilia in the developing lung. As shown in Figure 3, primary cilia are present on airway epithelial cells on E14.5, before the appearance of Foxj1 (Figure 4B, E14.5). At the onset of Foxj1 expression (E15.5), solitary cilia are evident in some cells that express Foxj1, whereas others expressing Foxj1 lack cilia of any type (on E15.5; Figure 4B). Later in development, abundant motile cilia are found on cells that express Foxj1, as previously described (e.g., on E19.5; Figure 4B) (28). Thus, the onset of Foxj1 expression occurs before the loss of primary cilia, and serves as an early marker of cells fated to become motile ciliated cells.

Motile Ciliated Cells Originate from Primary Ciliated Cells

The observed switch between the appearance of primary and motile cilia during differentiation suggested that motile ciliated cells originate from cells with primary cilia. The mTEC cultures allowed us to quantify cells more precisely with primary and motile cilia on a day-by-day basis during differentiation. The assessment of cilia types from ALI Day 0 through the formation of mature, motile ciliated cells (ALI Day 14) revealed a distinct and significant shift in the number of cells with the two different cilia types (Figure 5A). Approximately 65% of cells in mTEC preparations express primary cilia on ALI Day 0. The primary cilia are transiently present, as indicated by a drop to 10% by Day 2, and to less than 5% of cells by ALI Day 5. In contrast, motile cilia are nearly absent until ALI Day 3, when they rapidly achieve levels above 50% by ALI Day 7. These observations suggest an abrupt loss of primary cilia approximately 1 day before motile cilia formation, similar to what was observed between E14.5 and E15.5 in developing lungs. To analyze this shift in cilia type further, we followed the relationship between cells with primary cilia and markers of cells fated to generate motile cilia. As in development, cells with primary cilia initially lack Foxj1 expression (Figure 5B, *top left*). However, as differentiation proceeds, a consistent relationship is evident between cells with primary cilia and Foxj1. Careful analysis of the transition of primary to motile cilia reveals that Foxj1-expressing cells initially have a solitary cilium, as seen *in vivo*, likely representing an early low Foxj1 state (Figure 5B, *top right*). After this stage, many Foxj1-expressing cells lack primary cilia, suggesting cilia resorption (Figure 5B, *bottom*). Thereafter, cells expressing Foxj1 have multiple motile cilia. The Foxj1-expressing cells (on ALI Days 2–4) of each phenotype (primary cilia, no cilia, or motile cilia) were quantified and analyzed using ANOVA, revealing that the numbers of cells with each phenotype were significantly different ($P < 0.05$) within each day and between each type of cell. We also analyzed relationships between cells with primary cilia and markers of early motile ciliogenesis that are present before the onset of Foxj1 (23). At an early stage, clusters of γ -tubulin (i.e., nascent basal bodies) are observed in cells with primary cilia (Figure 5D, *left*). When basal bodies dock at the apical membrane, just before the assembly of motile cilia, primary cilia are absent (Figure 5D, *right*).

We next examined the phenotypes of cells that did not have primary cilia, using developing lung tissue, because mTECs do not provide a robust model for Clara cell differentiation (22). Cells that express secretoglobin Scgb3a2, a marker of early Clara cell differentiation (40), lack primary cilia (Figure 5E, *left*). Likewise, mature Clara cells, identified by their expression of Scgb1a1 (CCSP), also lack primary cilia (Figure 5E, *right*). Examinations of adult mouse lung tissue using scanning EM confirm this lack of primary cilia in nonciliated cells and in those with Clara cell morphology (Figure 5F). Together, these data suggest that cells with primary cilia are present during a specific temporal stage of differentiation, and are associated with motile ciliogenesis.

Primary Cilia Are Present in Adult Lungs after Injury

The disappearance of solitary cilia during development led us to determine whether primary cilia may reappear in adult lungs. Cells with a solitary cilium are distinctly absent in scanning and transmission EM surveys of adult lungs from many species (41). However, we hypothesized that primary cilia may be transiently present in the postnatal lung after injury, because the repair process recapitulates features of differentiation during development (29, 42). Respiratory virus injury provides an ideal

model for assessing epithelial cell repair, because these pathogens specifically target airway epithelium, including motile ciliated cells (29). We previously showed that SeV infection in a mouse model results in marked airway epithelial cell injury by 8 days after infection (29). At this time, epithelial cells elongate to cover areas of denuded epithelium (29). Immunostaining for primary and motile cilia showed that few cilia of any type are present 8 days after infection in injured airways (Figure 6A). Similar to the patterns of differentiation observed in the developing lung, we found that at 12 days after infection, epithelial cells expressing Foxj1 have primary cilia, no cilia, or short, multiple cilia (Figure 6B, *SeV d 12*). At recovery, 21 days after infection, abundant motile cilia are found on cells expressing Foxj1 (Figure 6B, *SeV d 21*).

Interestingly, during the repair process at 12 days after infection, primary cilia are also present on epithelial cells in the basal position. We previously reported that cell proliferation and an increase in Foxj1 expression marked this time. However, Foxj1-expressing cells did not label with BrdU (29). Cells that expressed cytokeratin 14 (K14), a protein previously reported to be upregulated after injury in basal cells, had primary cilia (Figure 6B) (43). K14-expressing cells are absent in injured airways on Day 8, abundant in repairing large airways on Day 12, diminished on Day 15, and infrequent by Day 21 after infection (Figure 6B). These cells in the basal layer with primary cilia do not express Foxj1 (Figure 6C). These findings further support a role for primary cilia in replication and differentiation within a separate epithelial niche, identified by unique locations and cell markers. The additional role of primary cilia in cells with progenitor potential is consistent with descriptions of primary cilia in stem cells (44).

DISCUSSION

To the best of our knowledge, this is the first comprehensive characterization of primary cilia in airway epithelium. We demonstrate that the appearance of primary cilia is common and is related to cell cycle, differentiation, injury, and repair. We identified the presence of primary cilia in nonproliferating tracheal epithelial cells during early differentiation, but found that primary cilia are not present in well-differentiated airway epithelial cells. Instead, primary cilia are transiently present during the differentiation of airway epithelial cells, and give way to motile cilia. This phenomenon is demonstrated during differentiation in lung development (Figures 3 and 4), in a cell-culture model (Figure 5), and in a mouse injury-repair model (Figure 6). In addition, those cells fated to become secretory types ultimately lack a primary cilium (Figure 5), but this process was less well-characterized. We also found that primary cilia were present in basal cells expressing K14 that were previously proven to exhibit progenitor capacity by generating secretory and motile ciliated cells (43). Taken together, our observations suggest that the varied temporal and spatial appearances of primary cilia in cells of different phenotypes lead to different fates. We incorporated these observations into a model of airway epithelial cell differentiation that positions the primary cilium in a lineage commitment scheme (Figure 7).

Several of our observations suggest that epithelial cells along the airway lumen and possessing primary cilia are fated to become motile ciliated cells within a specific stage of airway epithelial cell differentiation. Although the temporal relationship of primary and motile ciliogenesis is consistent with observations by Sorokin (18), we identified a sequence of primary cilia appearances and disappearances by linking events in multiple models and using multiple markers of motile cilio-

Figure 5. Motile ciliated cells originate from primary ciliated cells. (A) mTEC preparations were immunostained for acetylated α -tubulin on the indicated ALI day, and ciliated cell types were quantified. Shown are the means $\pm$ SDs of cilia types in three random fields of supported membranes from at least three independent preparations (* $P < 0.05$, primary versus motile ciliated cells). (B) Primary cilia are lost during early motile ciliogenesis in mTECs (ALI Days 1–4). Primary cilia (Arl13b, red) are evident in cells before the expression of Foxj1 (green) (Day 1). Primary cilia are evident in Foxj1-expressing cells at time of onset of Foxj1 expression (Day 2). Loss of primary cilia occurs in cells that express Foxj1 as motile ciliogenesis proceeds (Day 3), and motile cilia develop (Day 4). (C) Quantification of cilia type in cells that express Foxj1 in *B*. Shown are the means $\pm$ SDs of Foxj1-expressing cells in one inset each day, from each of four independent preparations. Foxj1-expressing cells were evaluated for the totals: Day 2, $n = 795$; Day 3, $n = 1,184$; and Day 4, $n = 1,413$. (D) Primary cilia (α -tub, green) in mTECs undergoing early motile ciliogenesis marked by clusters of centrioles (basal bodies, γ -tub, red) within the cytoplasm (ALI Day 2). Primary cilia are

absent after the docking of basal bodies and before motile cilia formation (ALI Day 3). (E) In the developing lung, primary cilia (α -tub, green) are absent in E16.5 cells that express early Clara cell marker Scgb3a2 (red; adjacent cell with cilium is outlined) and in adult cells that express Scgb1a1 (red, Clara cell secretory protein). (F) Scanning EM of adult mouse airways shows an absence of primary cilia in nonciliated cells, with the typical domed appearance of Clara cells. All bars = 10 μ m.

genesis. First, using an *in vitro* differentiation model, we found that the number of cells with primary cilia early in differentiation was nearly equal to the number of cells with motile cilia after differentiation (Figure 6A). Second, *in vitro* and *in vivo*, the number of cells with primary cilia decreased after the commencement of motile ciliogenesis (as marked by Foxj1 expression) (Figures 2, 4, and 5). Third, cells with primary cilia bear two early markers of nascent motile ciliated cells, i.e., multiple, newly replicated centrioles (basal bodies) and Foxj1

expression (Figures 4–6). Fourth, the failure to undergo motile ciliogenesis was marked by the persistence of primary cilia in cultured, submerged, wild-type mTECs (Figure 1D) and in Foxj1 null mice (Figure 4A). Finally, cells that expressed the early Clara cell protein Scgb3a2 lacked primary cilia.

Our findings lead to the question of what role primary cilia may play in motile ciliogenesis. The observation that primary cilia are present mainly during development and repair suggests that at least one role is to control programs of differentiation.

Figure 6. Primary cilia are transiently present in adult mouse airway during repair after respiratory virus injury. Lung sections from mice were administered Sendai virus (SeV), harvested on the indicated day after infection. (A) Cilia type relative to Foxj1 expression was determined by immunostaining for Arl13b (green) and Foxj1 (red). On Day 8, SeV-infected mice lack cilia and Foxj1 expression. On Day 12, Foxj1-expressing cells have primary cilia (arrows) or no cilia. On Day 21, Foxj1-expressing cells have motile cilia (arrowheads). (B) Primary cilia in basal cells were determined by immunostaining for Arl13b (green) and basal cell marker cytokeratin 14 (K14, red). On Day 8, K14-expressing cells are not present. On Days 12 and 15, primary cilia are found in K14-expressing cells. By Day 21, motile cilia and fewer K14-expressing cells are present in airways. (C) Basal cells with primary cilia (arrows) are independent of Foxj1-expressing cells (immunostained as in A). Representative airways are shown. Bars = 10 μ m.

Figure 7. Proposed model for relationship of primary cilia to motile ciliogenesis during airway epithelial cell differentiation. A progenitor airway epithelial cell with primary cilia generates daughter cells that enter motile cilia and secretory cell lineage pathways. Within the appropriate environment, the presence of a primary cilium marks cells that generate multiple centrioles (nascent basal bodies) and then express *Foxj1*, indicating commitment to a motile ciliated cell. As motile ciliogenesis proceeds to a high *Foxj1* state, primary cilia are lost. In the Clara cell lineage, an undefined cell (*in brackets*) differentiates cells identified by markers of early commitment (*Scgb3a2*) and maturation (*Scgb1a1*, CCSP) that lack a primary cilium.

The loss of primary cilia soon after the onset of *Foxj1* expression also suggests that *Foxj1* regulates a program for primary cilia resorption, possibly by promoting the phosphorylation of the protein Aurora, which normally functions during mitosis (30). At present, we cannot be certain that primary cilia reappear in motile ciliated cells. Tracking primary cilia in cells that generate hundreds of cilia is difficult. We did not identify a solitary, taller, central cilium among small, emerging, motile cilia using scanning EM or immunostaining. Importantly, unique markers for primary cilia have not been identified. Proteins previously thought to be primary cilia markers, i.e., BBS and renal tubule cell cilia proteins (e.g., PC-1 and NPHP1), were also identified in motile cilia (15, 16, 45). We propose that the loss of the primary cilium in a cell committed to a specific phenotype (e.g., motile ciliated) is required to enforce the maintenance of differentiation, so that continued signaling through a primary cilium does not occur. Such signaling may occur through pathways known to use primary cilia as receptors, including Sonic Hedgehog, platelet-derived growth factor receptor- α , and others (3, 4, 9, 14), or else signaling may involve a mechanosensory signal through a calcium-dependent PC-1/PC-2 complex (10, 46).

We also found that primary cilia appear on K14-expressing basal cells in SeV-injured airways, suggesting a role in repair, likely through conducting a proliferative response. These cells were located in the basal position, and their cilia were exposed to a distinct environment compared with *Foxj1*-expressing cells on the luminal surface. K14 cells were shown to be pleuripotent when dividing, to provide both motile ciliated and Clara cells that are localized within the apical layer of the lumen (43). Accumulating evidence suggests a role for primary cilia in repair, including decreased rates of wound repair in studies of *Tg737^{orp}* mutant mice that lack fully developed primary cilia (47). A report that primary cilia in human embryonic stem cells can signal through the Sonic Hedgehog pathway may imply a conserved cilia-dependent pathway for renewing cell populations in many organs (44).

In addition to the need to identify primary cilia signaling pathways in the airway, our conclusions cannot be fully supported without specific future studies. First, genetic proof is required to determine ultimately whether primary cilia uniquely fate cells to become motile ciliated cells. Likewise, genetic proof is required to prove that during the stage of motile ciliated cell commitment, cells without primary cilia become Clara cells. We cannot test this proposal in mTEC preparations because of their relatively low number of cells that express CCSP (22). We attempted to remove primary cilia from mTECs, using chloral hydrate treatment, as demonstrated by others in MDCK cells (11). However, the drug significantly

altered tubulin organization and cell shape (as in MDCK cells) (11), leading to a leakage of medium into the apical compartment, a condition that inhibits motile ciliogenesis. Studies may instead be performed in tissues from genetically targeted mice with abnormal primary cilia architecture or function (e.g., deficient in *Tg737^{orp}*, BBS2, BBS4, or RFX3) that are reported to have defects in motile cilia structure, function, or numbers (16, 47, 48). Finally, we showed that polycystin proteins are expressed in airway primary cilia, implying a function for flow sensing in the developing lung, although this finding calls for further investigation.

In conclusion, this study provides the first detailed characterization of the temporal and spatial presence of primary cilia in the airways, and serves as the basis for future studies. Based on our observations, we propose a new model for airway epithelial cell differentiation, where primary cilia serve as antennae to coordinate signals from the environment to drive both cell division, in the case of K14-expressing cells, and in the fate of motile ciliated cells directly lining the airway lumen (Figure 7). Although this model is in line with the proposal that primary cilia direct motile ciliogenesis, a recent study suggests that Notch pathway signaling controls decisions about the fates of motile ciliated or Clara cells (49). Given the existence of redundant and overlapping pathways for critical processes in higher organisms, additional non-cilia-dependent factors are also likely to be important in airway epithelial cell differentiation.

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