Motile cilia of human airway epithelia contain hedgehog signaling components that mediate noncanonical hedgehog signaling

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Differentiated airway epithelia produce sonic hedgehog (SHH), which is found in the thin layer of liquid covering the airway surface. Although previous studies showed that vertebrate HH signaling requires primary cilia, as airway epithelia mature, the cells lose primary cilia and produce hundreds of motile cilia. Thus, whether airway epithelia have apical receptors for SHH has remained unknown. We discovered that motile cilia on airway epithelial cells have HH signaling proteins, including patched and smoothened. These cilia also have proteins affecting cAMP-dependent signaling, including Gα13, adenylyl cyclase 5/6. Apical SHH decreases intracellular levels of cAMP, which reduces ciliary beat frequency and pH in airway surface liquid. These results suggest that apical SHH may mediate noncanonical HH signaling through motile cilia to dampen respiratory defenses at the contact point between the environment and the lung, perhaps counterbalancing processes that stimulate airway defenses.

lung | cAMP | host defense | smoothened | sonic hedgehog

During embryogenesis and early development and shortly after seeding airway epithelial cultures, airway cells possess primary cilia (1, 2). These single cilia are essential for canonical vertebrate hedgehog (HH) signaling (3, 4). The sonic hedgehog (SHH) ligand is a morphogen that guides development of the lung, regulating pulmonary branching and mesenchyme differentiation (5–9). The receptor/effecter system for the HH signaling pathway is complex and involves patched 1 (PTC1), smoothened (SMO), suppressor of fused (SUFU), and the glioma zinc-finger transcription factors GLI1, GLI2, and GLI3 (10).

After development is completed, airway epithelia continue to produce SHH (11–13). SHH is present on the basolateral side of the epithelia, where it signals to primary cilia on mesenchymal cells, restraining their proliferation (12). This paracrine pathway signals via canonical HH signaling to regulate expression of GLI1 in the mesenchyme. SHH is also present on the apical side of the epithelia in the thin layer of liquid covering the epithelial surface (11, 13, 14). However, mature airway epithelia lack primary cilia. As the epithelia polarize and mature, primary cilia disappear, and the cells develop numerous motile cilia on the apical surface (1, 2).

These observations raise the question of whether SHH has apical receptors and what their function might be. Previous studies showed that motile cilia on respiratory epithelia not only perform mechanical work, but also perform sensory functions like primary cilia (15, 16). These findings led us to hypothesize that motile cilia might participate in HH signaling. To test this hypothesis, we studied airway epithelia using human cells because of potential differences in cell-type distribution and HH signaling across species (17, 18), and because HH signaling has been reported to contribute to human lung disease, including interstitial pulmonary fibrosis, chronic obstructive pulmonary disease, and small-cell lung carcinoma (19–22). We examined differentiated primary cultures of human tracheal/bronchial epithelia grown at the air–liquid interface.

Results

HH Signaling Components Are Located in Airway Motile Cilia. To address the hypothesis that HH signaling involves motile cilia and ciliated airway epithelial cells, we first tested for components of the HH signaling pathway. Human airway epithelia expressed mRNA transcripts for SHH (Fig. S1A). Immunostaining revealed SHH in ciliated airway epithelial cells, located beneath the apical membrane and along the upper lateral membrane (Fig. 1). We detected SHH in airway surface liquid (ASL) and basolateral medium (Fig. S1 B and C). Although the rate of basolateral secretion exceeded apical secretion, the apical SHH concentration was high because of the small volume of liquid. Whether SHH is secreted directly into the basolateral medium and whether basolateral accumulation reflects SHH movement down a concentration gradient remain unknown. These results are consistent with previous reports that ciliated airway epithelial cells produce SHH and that the ligand is present both apically and basolaterally (11–14, 20).

Significance

Previous studies localized the hedgehog (HH) signaling system to primary cilia. We discovered that motile cilia on airway epithelia also contain HH signaling proteins, indicating that like primary cilia, these motile cilia have an important sensory function. However, in contrast to the function of HH signaling in most primary cilia, sonic hedgehog (SHH) elicits noncanonical signaling, reducing cellular levels of cAMP. These findings suggest that airway SHH may quiet airway defenses. Involvement of SHH in lung disease and positioning of motile cilia where they sample SHH and other ligands in the airway lumen suggest that noncanonical HH signaling might modulate airway responses to the environment in health and disease.

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The presence of SHH in apical liquid suggested that it might signal to airway epithelia. Therefore, we tested for components of the HH signaling pathway and found transcripts of PTCl, SMO, SUFU, and the glioma zinc-finger transcription factors GLI2 and GLI3 (Fig. S1A). GLII transcripts were detected, but only at very low levels. Moreover, we did not detect GLII by immunostaining (Fig. S2 A and B), consistent with previous reports (12). Immunostaining revealed PTC1 located along the length of motile cilia (Fig. 2A and Fig. S3A), which is similar to its location in primary cilia (23). We detected lower levels of patched 2 (PTC2), also in cilia (Fig. S2 C and D). SUFU also localized to cilia, and immunostaining was most prominent distally, consistent with reports that it sits in the distal portion of primary cilia (24) (Fig. 2B and Fig. S3B). In addition, we identified SMO, GLI2, and GLI3 in motile cilia, where they displayed discrete punctate patterns (Fig. 2 C–E and Fig. S3 C–E), similar to their reported localization in primary cilia (25, 26). All of these HH signaling components were expressed in ciliated but not nonciliated cells. Overexpressed SMO confirmed its location in motile cilia (Fig. S3F). Human lung tissue also revealed PTCl, SMO, and GLI2 in motile cilia (Fig. S4 A–C).

Airway epithelial cells have ~150 motile cilia; two readers counted 147 ± 7 and 160 ± 18 cilia per cell (n = 17 cells). In contrast, cells that have primary cilia have only that one cilium (Fig. S5A) (27–30). Motile cilia have axonomes with nine microtubule doublets around the circumference and two central microtubules: a “9 + 2” axoneme (1, 29). Primary cilia generally lack the two central microtubules: a “9 + 0” axoneme. Airway epithelial cilia exhibit a 9 + 2 structure (29). By transmission electron microscopy, we examined ~10,000 airway cilia (i.e., the equivalent of ~65 cells). We found none with a 9 + 0 axoneme (Fig. S5B). These results agree with previous observations (1, 2, 29), and we are not aware of reports that mature airway epithelium have cilia with a 9 + 0 axoneme. Therefore, it is exceedingly likely that the cilia expressing PTCl, SMO, SUFU, GLI2, and GLI3 are motile cilia with a 9 + 2 axoneme. Thus, these data suggest that motile cilia have HH signaling components that might serve as receptors and effectors for SHH in the apical airway liquid.

Airway Motile Cilia Have cAMP Signaling Components. In canonical HH signaling, SHH binds its transmembrane receptor, PTCl, relieving inhibition of the G protein-coupled receptor SMO, which signals to GLII transcription factors (4, 25). Immunostaining has shown that PTC1 is present in unstimulated primary cilia; adding SHH drives PTCl to exit primary cilia, SMO then enters (23), and that induces expression of GLII and PTCl genes (31).

Several observations suggested that canonical HH signaling was not involved in signaling by motile cilia. First, in contrast to most primary cilia, PTCl and SMO were simultaneously present in airway motile cilia (Fig. 3A). Second, airway cilia and ciliated cells express very little if any GLII (Figs. S1A and S2 A and B). Third, applying exogenous human SHH or the SMO agonist SAG did not trigger PTCl to exit cilia (Fig. S6). Fourth, neither SHH nor SAG increased PTCl transcripts, and GLII transcripts remained very low (Fig. S7A). Fifth, neither SHH, SAG, nor cyclopamine-KAAD affected GLII activity assessed with a GLII-luciferase reporter expressed in airway epithelia (Fig. S7 B and C). Thus, SHH does not appear to activate the canonical HH signaling pathway in airway epithelia.

SHH can also signal through a noncanonical pathway to decrease intracellular cAMP levels (32, 33). In this context, SMO couples to Gαi (32, 33), which inhibits adenylyl cyclase 5/6. Previous reports identified Gαi and adenylyl cyclase 5/6 in primary cilia (34, 35). We found Gαi and adenylyl cyclase 5/6 localized to airway cilia (Fig. 3 B and C and Fig. S4D) in the same cilia as SMO (Fig. 3 D and E). Consistent with previous reports (15), ciliated airway epithelial cells also expressed the catalytic and regulatory subunits of cAMP-dependent protein kinase (PKA), which localized to multiple cilia and the cytoplasm (Fig. S8).

SHH Signaling Dampens cAMP-Dependent Airway Defense Functions. These data place SHH in a position to interact with cilia and regulate intracellular cAMP levels. To test this prediction, we measured cAMP levels in airway epithelia by ELISA. We applied a concentration in the range we observed in ASL (Fig. S1B) and previously reported in vivo (13). SHH reduced cAMP levels under basal conditions and when cAMP was increased by forskolin and 3-isobutyl-1-methylxanthine (IBMX) (Fig. 4A and Fig. S9). The SMO agonist SAG had a similar effect (Fig. 4B). Inhibiting Gαi with pertussis toxin (PTX) blunted the effect of SHH (Fig. 4A). In addition, the SMO inhibitor, cyclopamine-KAAD, increased cAMP levels under basal conditions, but had minimal effects when cAMP levels were elevated with forskolin (Fig. 4B).

To test for cAMP in cilia, we expressed a genetically encoded sensor of cAMP levels that localizes to cilia and also cytoplasm (Fig. S10A and Movie S1) (36). In ciliated epithelial cells, SHH reduced cAMP levels in the region of cilia, as did an adenylyl cyclase inhibitor MDL-12330A (a positive control) (Fig. 4C) (35). SHH also tended to reduce cAMP beneath cilia in the cell; a change in cytosolic levels of cAMP might result from cAMP or Gαi diffusion. In contrast, SHH did not reduce cAMP levels in nonciliated cells (Fig. S10B). The presence of HH signaling components in ciliated but not in nonciliated cells and a SHH-induced cAMP reduction in ciliated but not nonciliated cells suggest that SHH regulates cAMP levels via airway cilia. Nevertheless, we cannot exclude some HH signaling via receptors in nonciliated cells.

These findings suggest that HH signaling may influence physiological functions at the airway apical surface, the point of contact between the environment and the lung. Two host defenses at that site are mucociliary transport and bacterial killing by antimicrobial proteins. Previous studies showed that increasing cAMP accelerates cilial beating (37). We found that SHH slowed cilia beat frequency (CBF), and PTX attenuated this effect (Fig. 5A). Conversely, cyclopamine-KAAD increased CBF under basal conditions (Fig. 5B).

cAMP also regulates cAMP-dependent PKA, which phosphorylates and activates apical CFTR anion channels (38). CFTR secretes HCO3−, thereby regulating ASL pH (39, 40). Decreased HCO3− secretion acidifies airway liquid, which reduces antimicrobial activity and increases mucus viscosity (40, 41). SHH and SAG reduced and cyclopamine-KAAD increased HCO3− secretion (Fig. 5C). These changes were paralleled by acidification and alkalization, respectively, of ASL (Fig. 5 D and E).

Fig. 1. SHH is expressed in ciliated airway epithelial cells. Images in this and other figures are from primary cultures of differentiated human airway epithelia, except in Fig. S4, which is lung tissue. (A) Section of periodic acid Schiff-stained epithelium. (B) Immunostaining of acetylated α-tubulin (red), SHH (green), and DAPI (nuclei, blue). Upper images are stacks of X–Y confocal images, and lower images are X–Z images. (Scale bars, 10 μm.)
Fig. 2. HH signaling proteins are present in airway motile cilia. Staining of acetylated α-tubulin is red, and staining of other immunolabeled proteins is green. Immunostaining is for PTC1 (A), SUFU (B), SMO (C), GLI2 (D), and GLI3 (E). Lower images are expanded images from Upper. [Scale bars: 2 μm (Upper), 1 μm (Lower).]
Discussion

These findings suggest that motile airway cilia serve as a HH signaling center. Thus, in addition to propelling particulate material out of the lung, motile cilia, like primary cilia, may have a sensory function. That conclusion is consistent with previous work showing that motile cilia on airway epithelial cells express bitter taste receptors that increase the intracellular Ca\(^{2+}\) concentration (16). Our results also identify a noncanonical signaling pathway that reduces cAMP levels in ciliated airway epithelial cells.

The HH signaling components and cAMP-dependent signaling proteins were both present in motile cilia. That close proximity may be key for efficient signal transduction, as is the case in primary cilia (3, 42). A ciliary location also positions HH signaling proteins in an optimal location to detect SHH moving through the thin layer of liquid covering the airway surface. Thus, apical HH signaling could involve both cell-autonomous...
Can inhibit adenylyl cyclase, thereby reducing intracellular levels of cAMP (46). These results may raise questions about the relationship between primary and motile cilia and HH signaling. Previous studies of vertebrate HH signaling showed that primary cilium are essential for canonical HH signaling (3, 4, 47). Our results now suggest a role for motile cilia in noncanonical HH signaling. In addition, a recent report suggested the HH signaling components localize odorant receptors in mouse olfactory cilia (48); olfactory cilia have a 9 + 2 axoneme, but lack dynein arms and are immotile (30). Thus, during evolution, HH signaling components and cilia may have been adapted for a variety of functions, with variations in cilia type and the function of HH signaling components. In this regard, SUFU, GLI2, and GLI3 are essential regulators in the canonical HH signaling pathway (49, 50), but their potential function in motile airway cilia remains unknown.

Airway injury and chronic lung diseases induce a variety of inflammatory, proliferative, and defense processes (51, 52). They can also increase SHH levels (22, 51, 52). Previous studies showed that SHH in the basolateral compartment may restrain responses to injury by reducing mesenchymal cell proliferation (12). Similarly, SHH in the apical compartment might restrain, at least in part, respiratory defense responses initiated by injury and disease. By reducing intracellular levels of cAMP, SHH might dampen defenses, including cilia beating and CFTR-mediated anion secretion. However, whereas HH signaling in mesenchymal cells is through primary cilia and the canonical HH signaling pathway, in epithelial cells it is through motile cilia and a noncanonical pathway.

**Materials and Methods**

*SI Materials and Methods* contains a detailed description of the materials and methods used.

Primary cultures of differentiated human airway epithelia were prepared from trachea and bronchi of nonsmokers seeded onto collagen-coated semipermeable membranes, grown at the air–liquid interface, and studied after they had differentiated and at least 14 days after seeding (53).

We used standard immunocytochemistry methods. Primary and secondary antibodies are listed in Table S1. Samples were imaged by confocal microscopy and analyzed with NIH Fiji software. All studies were performed at least four times using epithelia prepared from different donors. Studies were approved by the University of Iowa Institutional Review Board.

Methods for transmission electron microscopy and quantitative RT-PCR (Table S2) were standard. The SHH assay used a reporter cell line. cAMP concentrations were measured by ELISA and with a FRET-based cAMP assay (36). CBF was measured with transmitted light line-scans. ASL pH
and transepithelial HCO$_3^-$ secretion were measured as previously described (40, 41). Statistical significance was tested with an unpaired or paired Student's $t$ test for comparisons between two samples. For comparisons between more than two samples, statistical significance was tested with a one-way repeated-measures ANOVA with Sidak multiple-comparison posttest. $P < 0.05$ was considered statistically significant.

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