



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 smoothed. These cilia also have proteins affecting cAMP-dependent signaling, including G α_i and 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 | smoothed | 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/effector system for the HH signaling pathway is complex and involves patched 1 (PTC1), smoothed (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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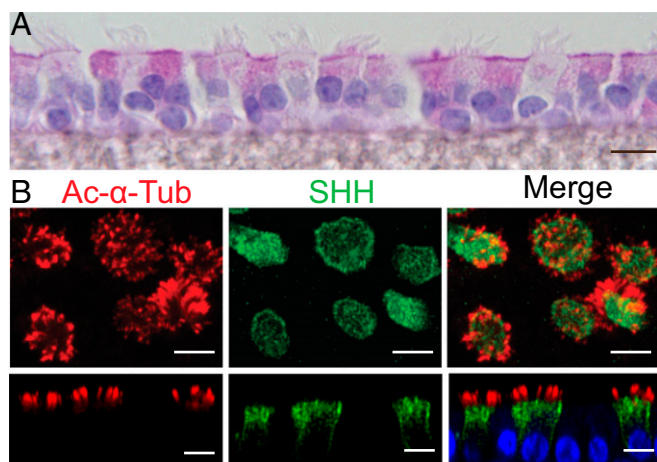


Fig. 1. SHH is expressed in ciliated airway epithelial cells. Images in this and other figures are from primary cultures of differentiated human airway epithelia, except in Fig. S4, which is lung tissue. (A) Section of periodic acid Schiff-stained epithelia. (B) Immunostaining of acetylated α -tubulin (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 μ m.)

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 *PTC1*, *SMO*, *SUFU*, and the glioma zinc-finger transcription factors *GLI2* and *GLI3* (Fig. S1A). *GLI1* transcripts were detected, but only at very low levels. Moreover, we did not detect *GLI1* 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 *PTC1*, *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 axonemes 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 $\sim 10,000$ 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 epithelia have cilia with a 9 + 0 axoneme. Therefore, it is exceedingly likely that the cilia expressing *PTC1*, *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, *PTC1*, relieving inhibition of the G protein-coupled receptor *SMO*, which signals to *GLI* transcription factors (4, 25). Immunostaining

has shown that *PTC1* is present in unstimulated primary cilia; adding SHH drives *PTC1* to exit primary cilia, *SMO* then enters (23), and that induces expression of *GLI1* and *PTC1* genes (31).

Several observations suggested that canonical HH signaling was not involved in signaling by motile cilia. First, in contrast to most primary cilia, *PTC1* and *SMO* were simultaneously present in airway motile cilia (Fig. 3A). Second, airway cilia and ciliated cells express very little if any *GLI1* (Figs. S1A and S2 A and B). Third, applying exogenous human SHH or the *SMO* agonist SAG did not trigger *PTC1* to exit cilia (Fig. S6). Fourth, neither SHH nor SAG increased *PTC1* transcripts, and *GLI1* transcripts remained very low (Fig. S7A). Fifth, neither SHH, SAG, nor cyclopamine-KAAD affected *GLI* activity assessed with a *GLI*-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 $G\alpha_i$ (32, 33), which inhibits adenylyl cyclase 5/6. Previous reports identified $G\alpha_i$ and adenylyl cyclase 5/6 in primary cilia (34, 35). We found $G\alpha_i$ 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 adding forskolin and 3-isobutyl-2-methylxanthine (IBMX) (Fig. 4A and Fig. S9). The *SMO* agonist SAG had a similar effect (Fig. 4B). Inhibiting $G\alpha_i$ 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 $G\alpha_i$ 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 ciliary 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 HCO_3^- , thereby regulating ASL pH (39, 40). Decreased HCO_3^- secretion acidifies airway liquid, which reduces antimicrobial activity and increases mucus viscosity (40, 41). SHH and SAG reduced and cyclopamine-KAAD increased HCO_3^- secretion (Fig. 5C). These changes were paralleled by acidification and alkalinization, respectively, of ASL (Fig. 5 D and E).

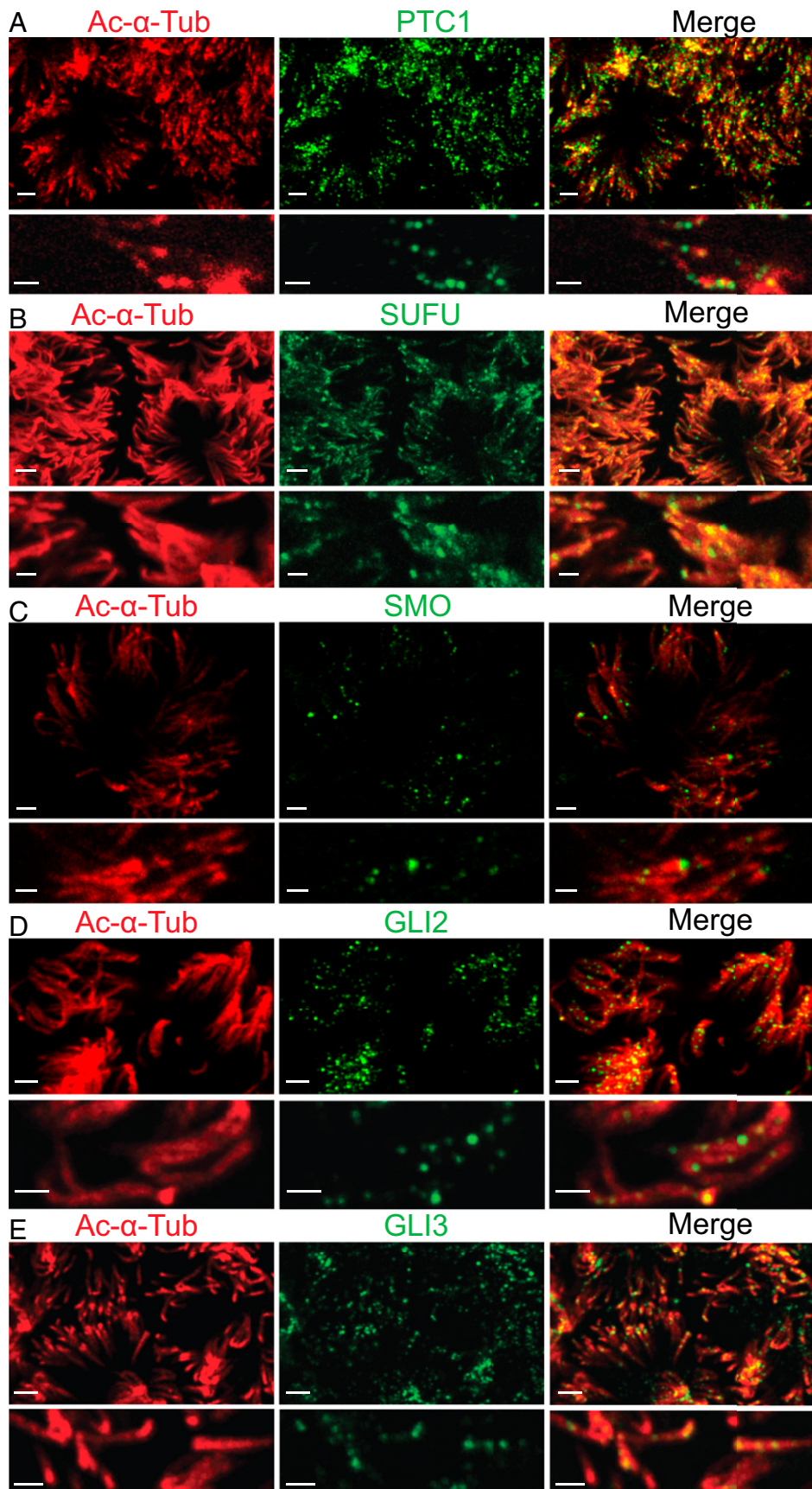


Fig. 2. HH signaling proteins are present in airway motile cilia. Staining of acetylated α -tubulin is red, and staining of other immunolabeled proteins is green. Immunostaining is for PTC1 (A), SUFU (B), SMO (C), GLI2 (D), and GLI3 (E). Lower images are expanded images from Upper. [Scale bars: 2 μ m (Upper), 1 μ m (Lower).]

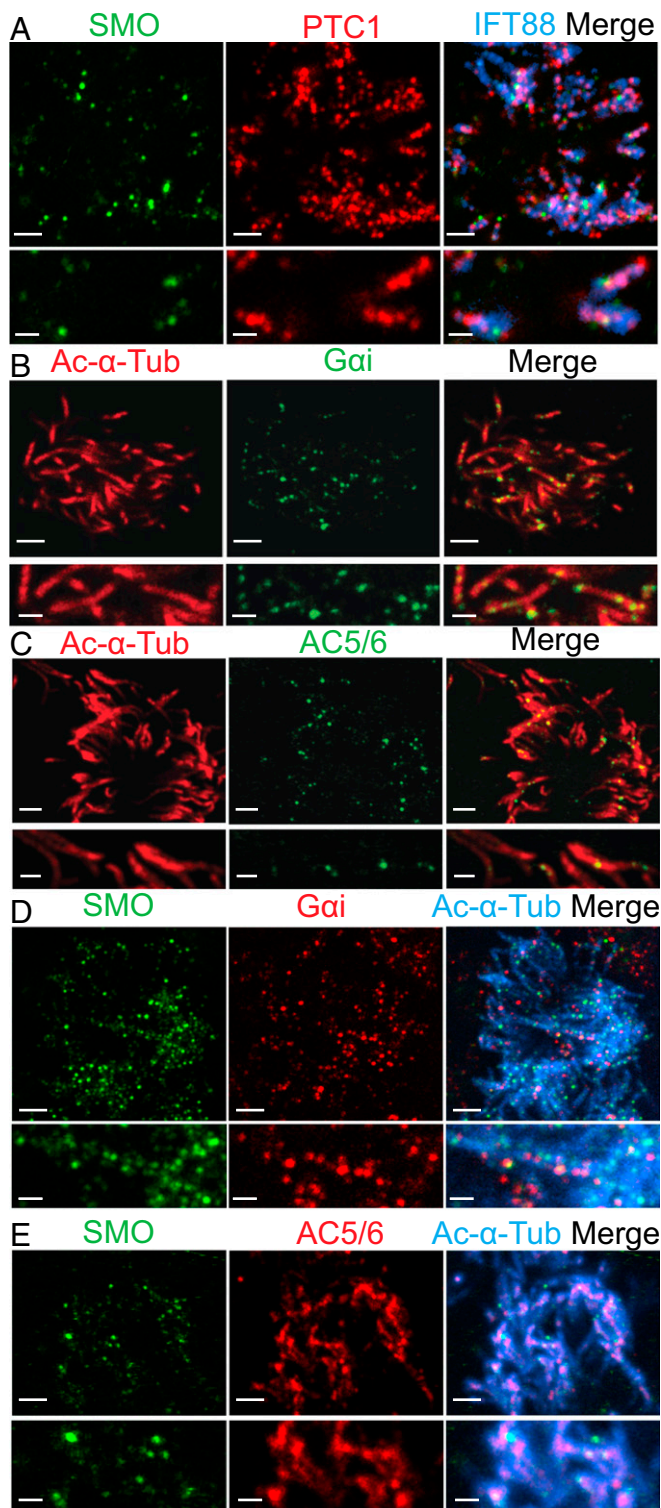


Fig. 3. $G\alpha_i$ and AC5/6 are expressed with SMO in motile cilia. (A) Staining of SMO (green), PTC1 (red), and IFT88 (cilia, blue). (B and C) Staining of acetylated α -tubulin is red, and staining of $G\alpha_i$ (B) and AC5/6 (C) is green. (D and E) Staining of SMO is green, staining of $G\alpha_i$ (D) and AC5/6 (E) is red, and staining of acetylated α -tubulin is blue. Lower images are expanded images from Upper. [Scale bars: 2 μ m (Upper), 1 μ m (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

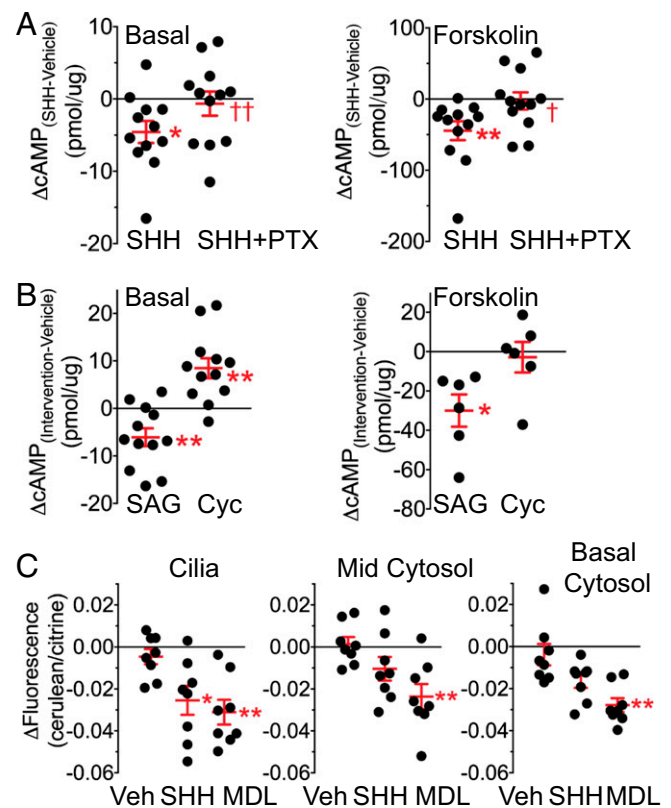


Fig. 4. SHH reduces cellular levels of cAMP. (A–C) Each dot is a measurement from epithelia from a different human donor. Bars and whiskers indicate mean \pm SEM. (A) Data are changes in cAMP concentrations measured by ELISA in differentiated human airway epithelia. Data for basal conditions (Left) and data obtained 10 min after addition of forskolin (5 μ M) and IBMX (100 μ M) (Right). Data are the change in cAMP levels compared with vehicle control. For vehicle, basal cAMP level was 11 ± 2 pmol/ μ g, and for forskolin/IBMX, cAMP level was 219 ± 9 pmol/ μ g. Epithelia received apical SHH (53 nM) or SHH plus PTX (1.7 nM). $n = 12$. In A and B, compared with vehicle, $**P < 0.01$ and $*P < 0.05$ by paired Student's t test. In A, compared with SHH, $^{++}P < 0.01$ and $^{+}P < 0.05$ by paired Student's t test. (B) Data are changes in cAMP concentrations compared with vehicle control following apical addition of the SMO agonist SAG (200 nM) or cyclopamine-KAAD (250 nM) under basal conditions (Left) ($n = 12$) or with forskolin (5 μ M) and IBMX (100 μ M) (Right) ($n = 6$). For vehicle, basal cAMP level was 15 ± 2 pmol/ μ g and forskolin/IBMX cAMP was 201 ± 11 pmol/ μ g. (C) Change in cerulean/citrine fluorescence ratio of the FRET-based cAMP sensor in ciliated cells at the level of cilia, in the midportion of the cell, and at the basal region of the cell. Measurements in nonciliated cells in the same microscopic field are in Fig. S10. Fluorescence is reported 15 min after addition of vehicle, SHH (263 nM), or MDL-12330A (100 μ M, as a positive control). Each data point is the average of two to four ciliated cells in epithelia from a different donor. $*P < 0.05$ and $**P < 0.01$ compared with vehicle by one-way repeated-measures ANOVA with Sidak multiple-comparison posttest.

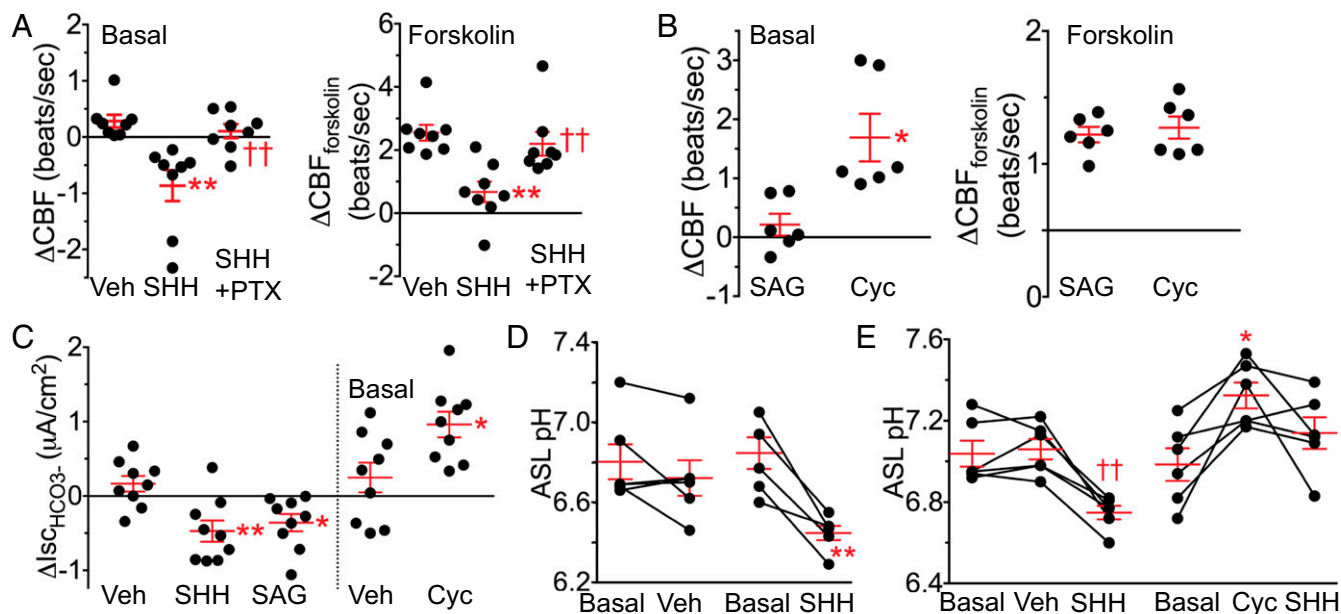


Fig. 5. Apical SHH reduces CBF and ASL pH. Each set of data points is from a different donor. Bars and whiskers indicate mean \pm SEM. (A) Change in CBF following addition of vehicle, SHH (263 nM), and SHH plus PTX (1.7 nM) under basal conditions (Left). Data (Right) are change in CBF produced by addition of forskolin (10 μ M) and IBMX (100 μ M) in presence of vehicle, SHH, or SHH plus PTX. Each data point is average of two to four cells. $n = 8$. ** $P < 0.01$ vs. vehicle and $\dagger\dagger P < 0.01$ compared with SHH by one-way repeated-measures ANOVA with Sidak multiple-comparison posttest. (B) Change in CBF after addition of vehicle or cyclopamine-KAAD (250 nM) (Left). Data (Right) are change in CBF produced by addition of forskolin (5 μ M) and IBMX (100 μ M) in the presence of vehicle or cyclopamine-KAAD. Each data point is average of two to four cells. $n = 6$. * $P < 0.05$ vs. vehicle by paired Student's t test. (C) Change in HCO_3^- secretion measured as short-circuit current ($\Delta I_{\text{scHCO}_3^-}$) with addition of vehicle, SHH (53 nM), and SAG (200 nM). Data on Left, $n = 9$, ** $P < 0.01$ and * $P < 0.05$ vs. vehicle by one-way repeated-measures ANOVA with Sidak multiple-comparison posttest. Data (Right) are from a different set of airway epithelia treated with vehicle or cyclopamine-KAAD (250 nM), $n = 9$, * $P < 0.05$ by paired Student's t test. (D) ASL pH measured before (basal) and 30 min after addition of vehicle or SHH (53 nM). Connecting lines indicate paired epithelia from an individual donor. $n = 6$, ** $P < 0.01$ compared with basal by paired Student's t test. (E) ASL pH measured before and 45 min after addition of vehicle or cyclopamine-KAAD (250 nM) followed by addition of SHH. $n = 6$, * $P < 0.05$ compared with basal and $\dagger\dagger P < 0.01$ compared with vehicle, both by one-way repeated measures ANOVA with Sidak multiple-comparison posttest.

signaling (an individual ciliated cell might release SHH and detect it) and noncell autonomous signaling (SHH released by one ciliated cell might be detected by a different ciliated cell some distance away). This contrasts with basolateral HH signaling, which is paracrine and may be more locally constrained. The apical location also makes HH signaling accessible to other substances in airway liquid and to inhaled therapeutics: for example the SMO agonist, fluticasone, prescribed for asthma (43).

Previous studies reported noncanonical HH signaling mediated through primary cilia. For example, SHH signaling regulates migration of murine embryo fibroblasts and tubulogenesis of endothelial cells in a *GLI1*-independent manner (44, 45). Previous work in cells with primary cilia has also shown that SMO, the transducer of SHH signaling, can couple with G_i subunits (32, 33), and $G\alpha_i$ can inhibit adenylyl cyclase, thereby reducing cellular 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 cilia 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 d 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 HCO₃⁻ 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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