Continuous mucociliary transport by primary human airway epithelial cells in vitro

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Sears PR, Yin W, Ostrowski LE. Continuous mucociliary transport by primary human airway epithelial cells in vitro. Am J Physiol Lung Cell Mol Physiol 309: L99–L108, 2015. First published May 15, 2015; doi:10.1152/ajplung.00024.2015.—Mucociliary clearance (MCC) is an important innate defense mechanism that continuously removes inhaled pathogens and particulates from the airways. Normal MCC is essential for maintaining a healthy respiratory system, and impaired MCC is a feature of many airway diseases, including both genetic (cystic fibrosis, primary ciliary dyskinesia) and acquired (chronic obstructive pulmonary disease, bronchiectasis) disorders. Research into the fundamental processes controlling MCC, therefore, has direct clinical application, but has been limited in part due to the difficulty of studying this complex multicomponent system in vitro. In this study, we have characterized a novel method that allows human airway epithelial cells to differentiate into a mucociliary epithelium that transports mucus in a continuous circular track. The mucociliary transport device allows the measurement and manipulation of all features of mucociliary transport in a controlled in vitro system. In this initial study, the effect of ciliary beat frequency and mucus concentration on the speed of mucociliary transport was investigated.

MUCOCILIARY CLEARANCE (MCC) is an important innate defense mechanism that is essential for maintaining a healthy pulmonary system (22, 45). The MCC system first traps pathogens and toxins in a mucus layer and then utilizes the coordinated beating of cilia to remove them from the airways, thus reducing the incidence of infection and exposure to harmful airborne materials. Impaired MCC is a feature of many airway diseases, including both genetic and acquired disorders. For example, in cystic fibrosis, mutations in the cystic fibrosis transmembrane conductance regulator protein result in a viscous, thick mucus that cannot be easily cleared, leading to repeated infections, bronchiectasis, and, eventually, respiratory failure (8). Primary ciliary dyskinesia is caused by genetic defects in the biogenesis, structure, or function of the motile cilia that line the respiratory tract (4, 24). The defective cilia result in impaired MCC, again leading to repeated airway infection and bronchiectasis. Mucociliary dysfunction is also a major component of other diseases, including chronic obstructive pulmonary disease (COPD) (34) and asthma (9). Furthermore, disruption of normal MCC in healthy individuals, e.g., by viral infection, can result in subsequent bacterial pneumonias (33). Thus there is a profound need for both a better understanding of the development and regulation of normal MCC and the development of therapeutic approaches to improve ciliary clearance in individuals with the disease.

Efficient MCC requires the proper regulation of three major processes, i.e., salt and water transport, mucin synthesis and secretion, and ciliary beat frequency (CBF) and coordination. While many studies have investigated the regulation of the individual components of MCC in vitro, there have been fewer investigations of the integrated system, largely because of the difficulty of performing MCC studies in vivo. The most common and least invasive technique for measuring MCC in vivo involves measuring the disappearance of radioactive tracers from the airways (2). This technique has been utilized to study MCC in mice (3, 10), dogs (23), and sheep (35), as well as in humans (2). An alternative method to study mucociliary transport (MCT) in vivo records the movement of individual radiopaque disks (20, 35). However, these techniques are both labor intensive and expensive, limiting their usefulness as a research tool or as assays for drug testing and development. In mouse models, MCT has frequently been measured by following the movement of endogenous particles or fluorescent tracers in the trachea; however, these procedures are typically more invasive (16, 26, 32, 46). Other investigators have used ex vivo preparations to study MCT (e.g., Refs. 7, 11, 12), and, while these studies have also yielded valuable information, the use of excised tissue introduces additional challenges.

Human airway epithelial (HAE) cells, when cultured under appropriate conditions at an air-liquid interface, have been observed to develop regions of locally coordinated ciliary activity that exhibit MCT over short distances. In most cultures, these areas of coordinated activity are randomly spaced around the culture. MCT rates have been measured in these cultures by tracking the movement of particles in selected fields; however, in these studies, there is no overall direction of transport (e.g., Ref. 38). In some cases, cultures have been observed to spontaneously develop regions of coordinated ciliary activity that transport mucus in a circular pattern (29). These “mucus hurricanes” have been especially useful for investigations into the pathogenesis of cystic fibrosis (5, 28, 42, 47). In these cultures, mucus is transported as a sheet, or disk, with a constant angular velocity. However, because the mucus in this system tends to accumulate in the center of the area of coordination, both the speed of MCT and the height of the mucus layer vary, depending on the distance from the center of rotation. In addition, the location and size of the area of transport is also variable, making longer term studies difficult.

In this report, we have used a modified culture system to generate HAE cell cultures that demonstrate MCT around a continuous circular track. This system provides several advantages, including the ability to study directional MCT over larger distances (~6 cm) in a standardized format that allows...
for multiple measures to be made from the same culture. Our studies show that, once established, ciliary coordination is maintained for long periods of time (i.e., weeks), and the rate of MCT is responsive to experimental manipulation. Using this system, we have documented the effects of CBF, mucus concentration, and forskolin stimulation on the rate of MCT. The system will be useful for investigations into the development of ciliary coordination and the basic mechanisms of MCT, and also for testing the effect of potential therapeutics on MCT.

**MATERIALS AND METHODS**

**Materials and Reagents**

Millicell cell culture inserts (PICM03050, 0.4 μm, 30-mm diameter) were purchased from Millipore (Billerica, MA). Fluorescently labeled carboxylate-modified polystyrene microspheres (FluoSpheres) were from Molecular Probes (Life Technologies). One- and two-micrometer yellow-green (505/515) microspheres were used for the experiments reported here. Silicone sealant was from Aqueon (Franklin, WI). Bovine submaxillary mucin (BSM) (M3895) and human placental collagen (C7521, type IV collagen) were from Sigma-Aldrich (St. Louis, MO). Other reagents and chemicals were standard laboratory grade, unless otherwise noted.

**Cell Culture and Preparation of MCT Device**

Normal HAE cells were obtained in suspension from the University of North Carolina Cystic Fibrosis Center Tissue Procurement and Cell Culture Core under protocols approved by the University of North Carolina Medical School Institutional Review Board. The isolation of cells, preparation of media, and basic culture techniques have been previously described in detail (13, 14).

Millicell cell culture inserts (Millipore, PICM03050, 0.4 μm, 30-mm diameter) are modified to make MCT devices (MCTD). To create MCTDs, a plastic central cylinder (15-mm outer diameter) is glued with silicone sealant onto the membrane in the center of the insert. The MCTD is allowed to dry for 3 days. MCTD membranes are coated with human placental collagen (Sigma C7521, type IV collagen, powder). The collagen stock is prepared by dissolving 50 mg collagen in 16.7 ml water and 33.3 μl glacial acetic acid (final collagen concentration: 3 mg/ml). The stock is then aliquoted and stored at −20°C. To coat the culture membranes, the stock is thawed and diluted 1:4 with water. The working collagen solution is layered onto the insert membranes at 115 μl/cm² (280 μl/MCTD). The cultures are allowed to dry, exposed to ultraviolet light for 20 min, and stored at 4°C until use. Inserts are seeded at a density of 500 × 10³ cells/cm² (primary cells) or 250 × 10³ cells/cm² (passage 1 cells). Each culture is placed in an individual 100 × 20 mm tissue culture dish (Corning, Falcon ref. 353003) with 9 ml of ALI media (13, 14). Primary cultures received additional antibiotic/antifungal (ampicillin 1 μg/ml, ceftazidime 100 μg/ml, tobramycin 80 μg/ml, vancomycin 100 μg/ml) treatment for the first 3 days of culture.

For the initial 2 wk of culture, cells were washed and fed three times a week. PBS (1 ml) is added to the culture apical surface and incubated for 5 min at 37°C. The apical and basolateral fluids are then removed by aspiration, and fresh ALI (0.6 – 0.8 ml). A small amount of HEPES buffer (10 – 20 ml) is added to the apical surface to prevent drying during microscopy. The experiment was then placed in an enclosed holder on the microscope stage and allowed to equilibrate at room temperature for 20 min.

**Particle Tracking and Interactions of Particles with the Culture Surface**

MCT speed was measured by reviewing videos and tracking the motion of individual particles (either endogenous debris or fluorescent beads) using in-house software, which reported positions of computer mouse clicks on paused video frames. While analyzing the videos for particle motion, some tracked particles would suddenly slow down and then resume their course, while others maintained a smooth flow. To avoid any bias in the selection of particles, the results present average data obtained by tracking all particles.

**Ciliary Coordination**

**Culture preparation.** Cultures demonstrating CCT were transferred to a culture dish with a glass bottom and top with a small amount of ALI (0.6 – 0.8 ml). A small amount of HEPES buffer (10 – 20 μl) was added to the apical surface to prevent drying during microscopy. The culture was then placed in an enclosed holder on the microscope stage and allowed to equilibrate at room temperature for 20 min.

**Microscopy.** A Nikon 40× extra-long working distance (ELWD DIC-M) objective was used with 1.5× postobjective magnification and DIC optics. Videos were taken from the center of the MCTD track at eight evenly spaced positions around the track. For each video, 256...
frames were taken at 125 frames per second and captured using the SAVA system. This yielded videos of 2-s duration.

**Video analysis.** The videos were analyzed after the experiment using software that records the position of computer mouse clicks on video images. For each field of view, a video was observed, and a single ciliated cell was chosen for analysis. This was the cell closest to the center of the image for which a discernible beat direction was observed. The computer mouse was clicked on the video at the center of the end-recovery area and then at the center of the end-effective area for that cell. From these positions, the beat direction was calculated relative to the expected flow axis for the position of the field of view on the MCTD. The expected flow axis was that which would result from perfect circular flow around the track for the global direction (clockwise or counterclockwise) observed in that culture.

**Temperature**

**General.** Three different protocols were utilized to measure the effect of temperature on CBF and MCT. Temperature was regulated with a Zeiss temperature controller (TempControl 37-2) and a microscope stage heater block. For each protocol, mock experiments were performed using an MCTD with no cells and a temperature probe placed on the membrane surface, to establish the profile of temperature change. A 10× objective was used with no postobjective magnification. MCT speed was determined by particle tracking, as described above, and CBF was determined using the whole field analysis function in the SAVA software package. Basolateral ALI media supplemented with 10 mM HEPES to provide additional pH buffering was used for all experiments.

**Temperature: Increasing Smoothly, 0 \rightarrow 37^°C**

The response of cultures to the increasing temperature was measured as follows. For each experiment, a culture was placed in a polystyrene container with ice below and above the culture, returned to the cell culture incubator, and allowed to equilibrate for 30 min. The container allowed the air around the culture to exchange with the 5% CO₂ air of the incubator. After the incubation period, the culture was quickly taken to the microscope and placed in the stage heater block, and videos were recorded immediately. Videos were taken when transport was seen and at regular intervals for CBF when transport was not seen. During this time, the cultures were exposed to a smoothly, although nonlinearly, increasing temperature as the experimental dish temperature equilibrated with the temperature of the stage heater block.

**Temperature: Decreasing Smoothly, 37 \rightarrow 25^°C**

Experiments in which the temperature was smoothly changed from 37 to 25°C were done by preheating the stage heater block to 25°C and quickly transferring the culture from the incubator to the stage heater block. After this, the experiment proceeded in the same way as in the smoothly increasing experiments. And in the same way, the rate of temperature change at the culture was dependent on the temperature gradient between the stage heater block and the culture and followed an exponential profile.

**Temperature: Changing in Steps, 24.5 \rightarrow 39.5^°C**

 Cultures were allowed to equilibrate to RT (\sim25°C) on stage for 20 min before the experiment started. Videos were recorded for an additional 20 min to establish a baseline CBF. We then recorded videos for 10 min, increased the controller temperature by 3°C, waited 10 min for equilibration, and repeated the cycle. From mock experiments using a culture transport device with no cells and a temperature probe on the membrane surface, it was determined that the temperature at the culture surface was linearly related to the setting at the temperature controller in the range from 25 to 45°C, and that equilibration after a 3° jump at the controller took less than 5 min. Initial experiments with cultures indicated that a 10-min equilibration time was sufficient to establish a level baseline in CBF. The controller temperature settings were 25, 28, 31, 34, 37, 40, and 43°C, which corresponded to the culture temperatures from 24.5 to 39.5°C.

**BSM Experiments**

BSM (Sigma M3895) stock solution (16%) was prepared by gently rocking the BSM powder in HEPES buffer for several hours at 4°C. Dilutions were made in HEPES buffer with the addition of fluorescent beads at 100 μg/ml.

To minimize the amount of endogenous mucus, cultures were washed with PBS the day before the experiment, and 60 μL HEPES buffer was added to the apical surface. On the day of the experiment, cultures were washed three times with HEPES buffer and placed back in the incubator to equilibrate (1–2 h). The BSM solution (100 μl) was pipetted onto the surface, and the insert was rolled gently to flow the mucin solution over the surface. When the surface was entirely covered, the insert was tilted to allow mucin to pool at one side, and ~50 μl were removed. The culture was then placed back in the incubator for a 5-min incubation and then moved to the heated microscope stage (37°C), where videos were quickly taken at four to eight evenly spaced positions around the track. Both high-speed transmitted light and slower fluorescence videos were taken. The high-speed videos were taken with the ES-310T camera driven by the SAVA software, as in previous experiments, and were used to analyze both CBF and transport. For these, a 20× ELWD Ph1 objective with phase optics and no postobjective magnification was used.

The fluorescence videos were taken with a DVC Intensiscan driven by Compix SimplePCI software using a 2× objective and were used to analyze transport only. Because of the high contrast in fluorescent videos and the much larger field of view presented by the 2× objective and the large DVC camera collecting area, it was sometimes possible to see the bead transport in the fluorescence videos when transport was not visible in the transmitted light high-speed videos.

For the experiment reported in Fig. 4, three cultures demonstrating CCT were studied at four BSM concentrations (2, 4, 6, and 8%). Each culture was studied with each mucin concentration, several days apart. Between experiments, each culture was washed three times with ALI containing additional antibiotics/antifungals and refed with the same media. In another series of experiments, additional cultures were studied following addition of BSM at 0, 2, 4, 6, or 8%, but each culture was not studied at all mucin concentrations. It should also be noted that 0% BSM is not equivalent to 0% mucin, as the washing procedure utilized in these experiments was not expected to completely remove endogenous mucin.

**Forskolin Studies**

Forskolin (Sigma, F-6886) was dissolved in 100% ethanol to make a 10 mM stock solution and stored at −20°C. On the day of the experiment, a working solution of 2 mL of ALI media with 4 μL of forskolin stock (20 μM forskolin) and another working (carrier) solution with 4 μL of 100% ethanol were prepared. To prevent effects of fresh media components, the ALI media used to prepare the forskolin and carrier working solutions was taken directly from the dishes of the cultures, which were going to be exposed to the corresponding solutions during the experiment. The apical surfaces of cultures demonstrating CCT were washed quickly with 1 mL PBS. Ten microliters of 1-μm polystyrene beads in HEPES buffer (10 μg/mL) were added to the apical surface, and the culture was returned to the incubator to equilibrate (20–40 min). Cultures were transferred to the microscope stage, and videos were captured immediately from four evenly spaced locations around the culture. An equal volume of ALI (2 ml) containing 20 μM forskolin or carrier (ethanol) was added to the basolateral compartment and swirled gently, and videos were again captured from four evenly spaced locations. Cultures were washed and returned to the incubator, and, after 1 wk (2 media
CONTINUOUS MUCOCILIARY TRANSPORT IN VITRO

Innovative Methodology

L102

changes), the experiment was repeated, with each culture receiving the opposite treatment (forskolin or carrier). CBF and MCT speed were determined as above and compared with baseline for both treated and untreated cultures.

Statistical Methods

To compare the number of MCTDs and millicells demonstrating CCT, a one-tailed paired t-test was performed.

Circular plots of ciliary beat tracks were constructed by calculating a beat direction unit vector for each of eight cells on a culture and then rotating the vector to line up the mean transport direction with the x-axis. For each culture, the eight unit vectors were averaged to plot the individual culture vectors, and all vectors were averaged to give an overall mean vector (see Fig. 2). The circular standard deviations (CSD) were calculated as $\text{CSD} = \sqrt{-2 \ln(m)}$, where $m$ is the magnitude of the mean vector.

Calculations of particle time and position coordinates in video images were done with custom software. All other calculations and statistical analyses were done using the Gnnumeric Spreadsheet software (version 1.10.17, www.gnumeric.org). Hypothesis testing was done at the 0.05 significance level.

The following were analyzed using least squares linear regression: transport vs. CBF for temperature experiments (see Fig. 3 and Table 1); transport rate and CBF vs. mucus concentration (see Fig. 4).

The effect of forskolin was analyzed by applying a paired one-tailed t-test to the forskolin/baseline compared with the carrier/baseline line measurements for both CBF and transport rate data (see Fig. 5). The data were paired by having cultures exposed to the carrier and the forskolin during separate experiments.

RESULTS

Initial Studies

As previously reported, early passage HAE cells, when cultured at an air/liquid interface, occasionally develop areas of circular MCT. Thus in the absence of developmental clues or external signals, ciliated cells in culture are capable of spontaneously orientating to produce directional mucus flow. We hypothesized that the circular boundary of the culture vessel and the inability of the mucus to be “cleared” from the culture dish encourage the ciliated cells to organize in a manner that provides the least resistance to their normal beating and allows for mucus flow. This suggested that providing additional boundaries and a defined path for mucus flow would further encourage ciliary coordination. To test this, we modified 30-mm millicell culture inserts by attaching a central core to the surface of the millicell membrane to create a circular track (see Fig. 1A and MATERIALS AND METHODS). Primary HAE cells cultured at the air/liquid interface, as previously described (13–15), differentiated to form a heavily ciliated epithelium containing secretory cells (Fig. 1B). The circular track of the MCTD has two bounding walls to restrict ciliary motion and mucus flow, and provides a path for mucus to flow continuously without accumulating. In addition, because the track is narrower than the original culture insert, the distance over which ciliated cells must become coordinated to generate directional flow is smaller. Indeed, preliminary experiments using several different size central cores occasionally resulted in well-coordinated cultures that transported mucus around the circular track. In these preliminary experiments, primary and first-passage HAE cells from both control and cystic fibrosis donors were observed to develop circular transport. Once ciliary coordination was established, it was maintained, usually for the lifetime of the culture. Mucus transport was observed for various lengths of time, lasting for several hours to several days between media changes and washing of the apical surface. Although the transport of endogenous mucus/cellular debris was usually visible by light microscopy (Supplemental Video S1; the online version of this article contains supplemental data), the addition of small volumes of fluorescent microspheres to the apical surface of cultures provided an easy

Fig. 1. A, left: illustration of the mucociliary transport (MCT) device (MCTD) from above. The MCTD track has an outer diameter of ~2.3 cm and an inner diameter of ~1.5 cm, with a track width of ~4 mm. Right: photograph of an MCTD showing the central cylinder and the outer track. Bottom: illustration of the MCTD in cross section. The human airway epithelial cells (blue) are cultured on a porous membrane at the air/liquid interface above the culture media (red). Drawings are not to scale. B: paraffin sections of a well-differentiated MCTD stained with hematoxylin and eosin (left) or alcian blue-periodic acid Schiff (right) showing the presence of ciliated and secretory cells. Scale bar = 10 μm. C: composite image showing the motion of 3-μm fluorescent particles in the MCTD. Images were taken over a period of 7 s.
method for visualizing and measuring the speed of MCT (Fig. 1C and Supplemental Video S2). Depending on the culture and the conditions (see below), the speed of MCT in these initial studies varied between 2 and 12 mm/min, with an average of 6.2 ± 2.3 mm/min (average ±SD; n = 20 measures from 4 donors), which is within the reported range of in vivo MCC rates (45). Between media changes (every 3–4 days), the apical surface of the cultures would often become dehydrated, and both ciliary beating and mucus transport would cease. However, after the surface was washed with buffer, MCT was usually restored. During these initial studies, we estimated that approximately one-third of the cultures examined demonstrated circular MCT. The number of successful cultures also varied, depending on the donor, with cells from some donors generating several cultures that exhibited circular MCT, whereas cells from other donors failed to produce any.

Several attempts were made to increase the percentage of cultures that became coordinated. For example, in some experiments, the amount of collagen coating on the membrane was increased. In other experiments, a small volume of fluid (PBS) was added to the apical surface, and the cultures were placed on a rotating platform to create a directional fluid flow (17) during the peak times of ciliogenesis. However, none of the modifications tested to date resulted in a clear increase in the number of coordinated cultures, suggesting that the major source of variability was the HAE cells. Based on these initial studies, we developed a standardized procedure to generate cultures for the study of MCT (see MATERIALS AND METHODS). Under these conditions, cells from 18/23 donors (78%) generated at least one culture (out of >3/donor), demonstrating complete circular transport, with 61/136 (45%) individual inserts demonstrating CCT. As noted above, cells from different donors varied in their ability to produce CCT, with most donors generating CCT in approximately one-third to one-half of cultures initiated. We examined the available information on the age, smoking status, sex, and race of the donors, but were not able to identify any correlation between these variables and the number of cultures that successfully developed transport. Importantly, during these experiments, cells from some of the same donors (n = 16) were cultured on standard millicells under identical conditions to the cells cultured on MCTDs. In these experiments, <5% of the standard millicells developed circular MCT (1 insert out of 22), compared with 25% (17 out of 60) of the cultures grown on MCTDs. Thus, although some of the standard cultures developed areas of local transport, the inclusion of the central cylinder in the MCTD increased the number of cultures demonstrating CCT by over fivefold (P = 0.035).

To examine the extent of ciliary orientation within the MCTD, high-speed videos (125 fps) were recorded from the central region of the track at equally spaced points around the culture. The orientation of ciliary beating of a centrally located ciliated cell was determined by an investigator blinded to the direction of transport (Supplemental Video S3). The direction of ciliary beating was then compared with the direction of transport, and a circular plot was used to visualize the data. Cultures from six different donors (8 fields/culture) were analyzed, and, although the extent of ciliary coordination varied between cultures, in each culture, the overall direction of beating aligned well with the direction of transport. Combining the data from all of the cultures shows that, on average, the majority of the cilia examined are well aligned with the direction of mucus transport, with a mean vector almost parallel to the direction of transport, with a magnitude of 0.69 and a CSD of 0.86 (Fig. 2A). Examination of the individual orientation data (Fig. 2, B–G) shows that, while the vast majority of ciliated cells were beating generally in the direction of flow, some were orientated in a direction greater than 90° away. These data suggest that having a majority of the ciliated cells oriented in a similar direction is sufficient to produce MCT, even when some individual cells are oriented in a different direction. We conclude that, when cultured in a MCTD, HAE cells can spontaneously coordinate their ciliary beat to generate continuous MCT transport over relatively long distances (cm) for extended periods of time, and the number of cultures demonstrating CCT is largely dependent on the initial donor cells.

Effect of Temperature on CBF and MCT

To explore the relationship between CBF and the speed of mucus transport, we took advantage of the known sensitivity of CBF to changes in temperature (40). By varying temperature, we were able to vary CBF over a wide range and measure the effect on MCT. Changing temperature also allowed us to collect data over a range of different CBFs from the same culture, without making additions to the apical fluid. For these studies, the basolateral media was replaced with a HEPES buffered solution before the experiment to better control pH during the extended periods outside of the culture incubator (see MATERIALS AND METHODS for details). In one set of experiments, cultures were allowed to cool from the incubator temperature of 37°C to that of the heated stage (25°C). CBF was initially ~12 Hz and slowed to ~6 Hz over the ~20 min of the experiment. Mucus transport speed decreased roughly in parallel with CBF, from ~140 to ~90 µm/s (Fig. 3, A and D). To investigate the dependency of mucus transport over a wider range of CBF, cultures were incubated on ice and then moved to a heated stage set at 37°C. As expected, CBF increased with increasing temperature, and MCT also increased, again, in parallel with the increase in CBF (Fig. 3, B and E). To more accurately measure the relationship between CBF and MCT, cultures were incubated at different temperatures between 24.5 and 39.5°C in a stepwise fashion. These experiments thus allowed for multiple measures of the CBF and MCT from the same culture while it was held at a constant temperature. Over this range, CBF increased 0.49 ± 0.06 Hz/°C, which is similar to the value of 0.37 ± 0.02 Hz/°C reported by Smith et al. (40). As shown in Fig. 3, C and F, the rate of MCT again increased in parallel with the increase in CBF. The approximate distance a particle travelled per ciliary beat was 8.5 µm (at 10 Hz). Interestingly, in these experiments, the distance a particle travelled per ciliary beat was relatively constant over the range of CBF observed (changing by ~0.08 µm/Hz). In each of these three protocols, the rate of MCT was dependent in a linear fashion on CBF (Fig. 3, A–C), with MCT increasing on average between 5 and 11 µm/s for every Hertz increase in CBF (Table 1). Although the absolute change in MCT per Hertz increase in CBF varied between the experiments (Table 1), this is likely due to differences in the endogenous mucus present at the start of these experiments. Overall, these data demonstrate that the rate of MCT is directly dependent on
CBF, and that small changes in CBF may have substantial effects on the rate of MCT.

Effect of Mucus Concentration on MCT

HAE cells, when cultured at an air/liquid interface, produce and secrete mucus onto the apical surface. This mucus layer, which contains both MUC5B and MUC5AC (21, 43), likely varies in composition, due to both differences in the donor cells and the time at which the culture is studied. To directly examine the relationship between mucin concentration and the speed of transport, the endogenous mucus was removed by washing and replaced with a standardized mucin solution. For these studies, we chose commercially available purified BSM, as this preparation is readily available and has been previously characterized (27). HAE cultures demonstrating circular MCT were washed to remove the endogenous mucus. An excess of purified BSM of known concentrations (sufficient to completely cover the surface) was then added to the apical surface. After removing the bulk of the added mucus, the speed of MCT was measured. Cells cultured in the MCTD maintained their coordinated transport for several weeks, providing the opportunity to measure MCT speeds at different mucin concentrations in the same cultures. Thus we measured MCT in three different cultures at each of four different mucin concentrations (Fig. 4). MCT speed clearly decreased with increasing mucin concentration. Increasing the mucin concentration from 2 to 8% reduced the speed of MCT in a linear fashion ($r^2 = 0.86$) by ~70%, showing that, under these conditions, the rate of transport depends directly on the mucin concentration. CBF decreased with increasing mucin concentration, dropping from 12.4 Hz at 2% BSM to 10.1 Hz at 8% BSM (Fig. 4, inset).

These results also demonstrate that the rate of transport when measured in the MCTD is reproducible, both between different cultures and between multiple measures in the same culture. In another set of experiments, an additional 22 measures of MCT were obtained with different concentrations of BSM; however, not all concentrations were measured in each culture. Combining these results with those above showed an almost identical effect of increasing mucin concentration, although the inclusion of multiple different cultures increased the variability.

Treatment with Forskolin Increases CBF and MCT

To validate the usefulness of the MCTD for testing the effect of therapeutic agents on MCT, we chose to measure the response of CBF and MCT to forskolin stimulation. Forskolin increases the activity of adenylate cyclase, and the resulting increase in cAMP is known to simulate CBF (1, 37). Treatment of transporting MCTD cultures with 10 μM basolateral forskolin reproducibly increased CBF by 19.3 ± 2.1% (mean ± SE; $n = 4$) over baseline (Fig. 5). This is in good agreement with previous studies that reported similar increases in CBF using well-differentiated cultures of human bronchial epithelial (HBE) cells (37). As expected, treatment with forskolin also stimulated the speed of MCT, with the average speed increasing over baseline by 24.4 ± 3.1% (Fig. 5). These data demonstrate that the transport rate in the MCTD is responsive to pharmacological treatment and further demonstrates that changes in CBF directly affect the rate of MCT.

Fig. 2. Orientation of ciliary beating in the MCTD. A: the overall direction of ciliary beating from 6 individual MCTD cultures (shaded lines) was plotted in relation to the direction of MCT. MCT is to the right (x-axis). The overall direction of ciliary activity (solid line) was closely aligned with the direction of MCT, with a vector magnitude of 0.69 and circular standard deviation of 0.86. B–G: orientation of ciliary beating in the 6 individual cultures included in A. Shaded lines indicate orientation of individual ciliated cells from 8 videos per culture, whereas solid lines indicate the overall orientation.
DISCUSSION

MCC is essential to the maintenance of pulmonary health, and impaired MCC is involved in the pathogenesis of many common respiratory diseases, including COPD and asthma (22). Efficient MCC depends on the proper regulation and integration of three main components: the quantity and type of mucins secreted, the absorption and secretion of salt and water, and the activity and coordination of ciliary beating. Thus the ideal system for studying MCC would allow study of all three components, as in a whole animal model; however, studying MCC in vivo is challenging for several reasons. For example, studies of MCC in humans typically measure the disappearance of a radioactive tracer from the lungs over time (8). While these studies are invaluable to our understanding of MCC, due to the use of radioactivity, the need for specialized equipment, and the expense of performing large scale human studies, this approach is not feasible for routine laboratory investigations or high-throughput screening. Similarly, studies in large-animal models (e.g., dog, sheep, and pig) suffer from many of the same limitations. MCC studies have also been performed in small animals using radioactive tracers or other techniques. For example, Foster et al. (10) and others (3) have measured the clearance of radioactive particles from mouse lungs under a variety of conditions, while Grubb et al. (16) have measured the transport of a fluorescent dye. Other studies have measured

Table 1. Dependence of mucus speed on CBF

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<thead>
<tr>
<th>Experimental Protocol</th>
<th>Temperature, °C</th>
<th>ΔMCT/ΔCBF, μm</th>
<th>n</th>
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<tbody>
<tr>
<td>37 → 25</td>
<td></td>
<td>5.1 ± 2.3</td>
<td>3</td>
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<tr>
<td>0 → 37</td>
<td></td>
<td>11.4 ± 4.6</td>
<td>8</td>
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<tr>
<td>24.5 → 39.5</td>
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<td>5.5 ± 3.4</td>
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Values are means ± SD; n, no. of independent cultures studied. The average change (Δ) in mucociliary transport (MCT) speed per change in ciliary beat frequency (CBF) (ΔMCT/ΔCBF) is shown. CBF was altered by varying the temperature in three different experimental protocols as in Fig. 3.
Innovative Methodology

L106 CONTINUOUS MUCOCILIARY TRANSPORT IN VITRO

Fig. 4. Relationship between mucin concentration and MCT speed. Three individual human bronchial epithelial (HBE) cultures (diamonds, squares, triangles) demonstrating continuous circular transport were washed, and different concentrations of purified bovine submandibular mucin (BSM) were added to the apical surface. After a brief equilibration period, MCT was measured. Each culture was studied with four different concentrations of BSM; each point represents transport measured from 4 separate videos. The data demonstrate a linear dependence of MCT on mucin concentration (solid black line; \( r^2 = 0.86 \)). Inset: cilary beat frequency was determined from the same cultures. Each shaded symbol represents whole field analysis of a single video, the solid circles represent the average of the 3 cultures, and the thick solid line indicates the least squares regression curve. Values are means ± SE.

Fig. 5. Effect of forskolin stimulation on CBF and MCT. Baseline measures of CBF and MCT speed were obtained, and cultures were treated basolaterally with control solution or 10 μM forskolin, and the changes in CBF and MCT speed were measured (open bars, control; solid bars, forskolin). Each culture \((n = 4; 3-4\) videos per culture) was studied under both conditions. Values are means ± SE. \( P < 0.0006 \) and \( * P < 0.002 \), compared with control.

In this work, we report that culturing HAE cells in a modified culture insert, the MCTD, results in the development of circular mucus transport, allowing the manipulation and study of MCT in vitro. Our device offers many advantages over current systems. For example, MCT is very stable once CCT is established, lasting for several weeks or months, and allowing multiple experiments to be performed in the same culture. Transport in the MCTD can be followed over longer distances (cm) and for a longer time than in other culture systems, where fluorescent beads are typically tracked over distances of only a few cells. The standardization of the width and distance of the transport path also increases the ease of performing measurements and making comparisons between studies. In addition, this system allows for the independent or combined manipulation of all aspects of MCT, including CBF, fluid absorption and secretion, and the composition/concentration of endogenous or exogenous mucus. Furthermore, the ability to generate large numbers of cultures demonstrating CCT may provide an improved method for testing the effect of therapeutic agents designed to improve MCT. Disadvantages to the current system include the time required for cultures to develop CCT (~6–8 wk) and the observation that cells from some donors did not generate CCT under our current conditions.

In vivo, it is likely that the orientation of ciliary beating is determined first by the development of planar cell polarity, and second by a hydrodynamic mechanism that refines the orientation (44). In vitro, when isolated HAE cells are plated, there are no tissue-level polarity signals, so it is perhaps not surprising that, in standard culture conditions, ciliated cells become locally coordinated, but fail to coordinate over long distances. Culturing cells in the MCTD, which allows and directs mucus flow, may encourage the development of ciliated cell coordination through hydrodynamic forces. However, we also ob-
served that, even in cultures that were demonstrating CCT, individual ciliated cells or patches of ciliated cells were not oriented in the direction of flow. Indeed, many of the cultures that did not demonstrate complete circular transport exhibited large areas of directional transport, but these were not coordinated around the entire culture. This may be due to the asynchronous development of ciliated cells in these cultures and may, in part, be responsible for the relatively low success rate with cells from some donors.

Using the described system, we first examined the relationship between CBF and MCT by utilizing changes in temperature to alter CBF. In general, measurements of CBF and MCT in this study were similar to values obtained in other studies of cultured HBE cells (6, 25, 29, 38). For example, Matsui et al. (29) reported mucus transport velocities of 20–80 μm/s, and Liu et al. (25) reported a CBF of 9–10 Hz in cultured HBE cells. Importantly, these values are consistent with estimates of MCT in vivo (45). While many agents have been shown to increase CBF of human cells, our system allowed us to measure the effect of changing CBF directly on MCT. Although changes in temperature may also affect other aspects of MCT (e.g., fluid secretion, viscosity), our data show that MCT of endogenous HBE mucus increased in a linear fashion with increases in CBF. These results demonstrate that agents that stimulate CBF will improve MCC and may be useful for treatment of a variety of respiratory diseases. For example, β-adrenergics (1) and phosphodiesterase-4 inhibitors (31), which are known to increase cAMP and CBF, have been shown to provide clinical improvement in asthma and COPD, although the precise role of increased CBF is not clear.

To examine the effect of different mucus concentrations on the rate of transport in the MCTD, the endogenous mucus was replaced with purified BSM of different concentrations. As expected, higher concentrations of BSM resulted in a reduced MCT speed, with the speed of transport decreasing ~80% over the concentration range of 0–8% exogenous mucin studied. These mucin concentrations encompass the range typically reported for normal mucus and that observed in disease states, such as cystic fibrosis and COPD (18, 19). Under these conditions, the speed of transport and the change in the speed of transport with different mucin concentrations were similar between different cultures, especially when the same cultures were studied at different concentrations (Fig. 4). These data demonstrate that the MCTD will be useful for investigating the complex relationships between the composition and concentration of mucus and speed of MCT. The system can be used for both detailed studies of the endogenous mucus produced by the HAE cells and the use of purified exogenous materials. In addition, the device will be useful for testing the effect of therapeutic agents for their ability to increase transport of well-defined mucus [e.g., BSM or purified human mucus (30)].

To determine whether the speed of MCT was sensitive to pharmacological treatment, we examined the effect of forskolin treatment on cells cultured in the MCTD. Forskolin directly increases the level of cAMP and subsequently PKA, stimulating CBF, through the phosphorylation of a dynein light chain (36), and Cl− secretion, through the phosphorylation and activation of the cystic fibrosis transmembrane conductance regulator protein (39). The addition of 10 μM forskolin to the basal media significantly increased both CBF and MCT speed, further demonstrating the usefulness of this device for the testing/development of new therapies. Because forskolin increases both CBF and chloride secretion and may increase fluid secretion (41), it is not possible from these studies to determine whether the effect on the rate of MCT is due solely to the increase in CBF. Future studies using combinations of agents that stimulate or inhibit CBF, fluid secretion, and/or mucin secretion will allow for the contribution of each component of MCT to be measured independently.

In summary, the above data demonstrate that culturing well-differentiated HAE cells in a circular track is a simple and reproducible method to study MCT in vitro. Our data show that cells from a significant fraction of donors are capable of coordinating their ciliary motility to produce mucus flow in a defined pattern, and, once established, this transport is stable for the lifetime of the culture. Using this device, it is possible to make repeated measures of the overall speed of transport, as well as many of the individual components of MCC, including CBF, fluid heights, mucus concentration and composition, etc. The device is amenable to a wide range of in vitro manipulations, as illustrated by the studies presented above, and will be useful in the development and testing of therapies targeting MCC. The device will also be useful to investigate the mechanisms responsible for the coordination of ciliary motility and determine the effects of diseases including cystic fibrosis on MCT.

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L. E. Ostrowski is listed on a patent describing the MCTD.

AUTHOR CONTRIBUTIONS
Author contributions: P.R.S., W.-N.Y., and L.E.O. performed experiments; P.R.S. and L.E.O. analyzed data; P.R.S. and L.E.O. interpreted results of experiments; P.R.S. and L.E.O. prepared figures; P.R.S. and L.E.O. edited and revised manuscript; P.R.S. and L.E.O. approved final version of manuscript; L.E.O. conception and design of research; L.E.O. drafted manuscript.

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