Assessing mucociliary transport of single particles in vivo shows variable speed and preference for the ventral trachea in newborn pigs


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Mucociliary transport (MCT) is an innate defense mechanism that removes particulates, noxious material, and microorganisms from the lung. Several airway diseases exhibit abnormal MCT, including asthma, chronic bronchitis, and cystic fibrosis. However, it remains uncertain whether MCT abnormalities contribute to the genesis of disease or whether they are secondary manifestations that may fuel disease progression. Limitations of current MCT assays and of current animal models of human disease have hindered progress in addressing these questions. Therefore, we developed an in vivo assay of MCT, and here we describe its use in newborn wild-type pigs. We studied pigs because they share many physiological, biochemical, and anatomical features with humans and can model several human diseases. We used X-ray multidetector-row–computed tomography to track movement of individual particles in the large airways of newborn pigs. Multidetector-row–computed tomography imaging provided high spatial and temporal resolution and registration of particle position to airway anatomy. We discovered that cilia orientation directs particles to the ventral tracheal surface. We also observed substantial heterogeneity in the rate of individual particle movement, and we speculate that variations in mucus properties may be responsible. The increased granularity of MCT data provided by this assay may provide an opportunity to better understand host defense mechanisms and the pathogenesis of airway disease.

Mucociliary transport (MCT) depends on the coordinated beating of cilia to propel mucus out of the lung (1–4). In large mammalian lungs, multiple cell types participate in MCT: goblet cells and submucosal glands secrete mucus, airway epithelia and submucosal glands control the quantity and composition of the airway surface liquid, and ciliated epithelial cells propel mucus through ciliary beating. The importance of MCT as a defense mechanism is demonstrated in people with primary ciliary dyskinesia; their cilia are uncoordinated or lack an effective stroke (5–7). As a result, MCT is disrupted, and progressive airway infections and bronchiectasis ensue. Defective MCT is also thought to contribute to other airway diseases, including cystic fibrosis (8–11), asthma (1, 12), and chronic bronchitis (1, 13). Although MCT can be defective in these diseases, it remains uncertain whether MCT is abnormal from the outset and contributes to disease initiation or whether MCT becomes abnormal after the onset of disease and then accelerates injury. Understanding the contribution of abnormal MCT to the origins of airway disease has been hindered by both the limitations of current MCT assays and current animal models of disease.

In vivo assays of MCT have the advantage that transport is examined with physiological populations of airway cell types including submucosal glands and with natural airway humidification. Currently, the most commonly used in vivo MCT assay involves inhalation of aerosols containing radiolabeled particles, and then retention of radioactivity in the lung is recorded over time (14). This procedure has aided understanding of airway disease and is being used to assess therapeutic interventions in humans (15, 16). Although there are attempts to improve the assay, current methods have limitations. For example, spatial resolution is limited to descriptions of central vs. peripheral lung regions with little detailed anatomical information, temporal resolution is limited, only a fraction of the radioaerosol is cleared from the lung during the study, and cough-induced clearance can confound assays. As a result, detection of abnormalities in disease can be difficult. MCT has also been assessed by placing Teflon disks on the tracheal surface and visually assaying their movement through a bronchoscope (17–20) or through chest X-ray (21). The limitations of these in vivo assays have hindered the ability to investigate lung disease at its onset.

In vitro and ex vivo assays of MCT offer advantages in the ability to control the basolateral solution and perform pharmacological manipulations (22–24). In cultured airway epithelia, the lack of submucosal glands, the constraint that liquid and mucus cannot enter or leave the culture, meniscus effects caused by the structures holding cultured epithelia, artificial control of humidity, and the requirement that investigators must add liquid to cultures to assess ciliary movement of material are limitations (22). For ex vivo preparations, washing of the apical surface and artificial humidification can pose challenges.

In this study we investigated MCT in pigs, which offer advantages over mice for assessing MCT in health and disease. For example, the cell types and anatomy of porcine airways are much closer to those of humans than are murine airways (25–30).

Significance

Mucociliary transport (MCT) defends lungs by removing particulates, and defective MCT is hypothesized to contribute to the onset of lung diseases such as asthma, chronic bronchitis, and cystic fibrosis. However, testing those hypotheses has been limited by current MCT assays and mouse models of human disease. We developed an in vivo MCT assay in newborn pigs, which share physiological and anatomical features with humans. The X-ray–computed tomographic-based method provided high spatial and temporal resolution. We discovered that particles preferentially travel up the ventral airway surface. We also discovered substantial heterogeneity in rates of individual particle movement, indicating that MCT does not likely involve homogeneous mucus blankets. The granularity of the data may aid understanding of MCT and disease pathogenesis.


The authors declare no conflict of interest.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1323633111/-/DCSupplemental.
Ciliated cells predominate in human airways, whereas Clara cells comprise ~60% of mouse airway epithelial cells (25, 31). Newborn mouse airways have few ciliated cells and no discernable MCT (32). Mouse intralobular airways lack mucus and serous cells found in humans (31). The pattern of maturation and development of submucosal glands in pigs is comparable with humans (25, 27, 28, 33). Pigs have submucosal glands in the trachea and bronchi, whereas mice have only a few submucosal glands near the larynx and upper trachea (34, 35). In addition, the size of pigs is closer to that of humans than mice, the general anatomic distribution of porcine airways recapitulates that of humans (25), and porcine and human submucosal glands appear to use the same pathways to control and regulate secretion (36, 37). Pigs can also provide models of airway diseases such as cystic fibrosis (38–43).

Our goal was to develop an in vivo assay of MCT that met several criteria: adapts to study of newborn pigs, has the spatial resolution to measure individual particles, obtains data in three dimensions, achieves good temporal resolution, registers MCT to airway anatomy, and uses natural airway humidification.

Results
High-Resolution Chest MDCT Tracks Individual Tantalum Particles in Vivo. To measure MCT we developed a multidetector-row–computed tomographic (MDCT)-based assay. In sedated newborn pigs, we placed a catheter just through the vocal cords, insufflated radiopaque disks into the airways with a puff of air, and immediately removed the catheter (Fig. S1A). We used 350 × 25-μm tantalum disks because they are radiodense and chemically inert. Piglets were supine and breathed spontaneously through the nose. Three to sixteen particles were present in the airways at a position beneath the opening to the right cranial lobe, which we used as an anatomical landmark. We acquired volumetric MDCT scans of the apical–basal extent of the lung every 15 s for 10 min for a total of 40 scans (Fig. S1B). We then identified individual particles in three dimensions (Fig. 1A–C).

We reconstructed the airway anatomy and particle positions; Fig. 2A and Movie S1 show ventral–dorsal images from one animal. Symbols and lines of a single color represent the path of individual particles. Symbols indicate a particle’s position at 15-s intervals, and lines connect the dots. Particles either moved toward the larynx or were stationary; they never moved distally. A lateral view of the same airway reconstruction suggested that particles preferentially traveled to the ventral surface of the trachea (Fig. 2B and Movie S2).

Speed of Individual Particle Movement Shows Substantial Heterogeneity. Individual particles traveled at an average speed of 6.9 ± 0.7 mm/min (mean ± SEM, 57 particles in seven pigs). This rate is similar to that previously reported in excised porcine trachea [2.3 mm/min by particle transport (24) and 5.4 mm/min by optical coherence tomography (44)]. However, the average speed masks substantial heterogeneity. Fig. 3A shows the mean speed for individual particles. In one animal (red circles) the mean speed of the fastest moving particle was ~4 times faster than the mean speed of the slowest-moving particle. The maximum speed of individual particles showed the same variation (Fig. 3B), and data for all of the animals showed variation in maximal and mean particle speed.

All particle movement had a component directed toward the larynx, but at times, some particles were immobile. In six of seven animals all of the particles exhibited some movement. However, in one pig, half of the particles advanced toward the larynx, and half exhibited no movement. Most particles remained in motion during the entire tracking period, but a few particles were stationary during some portion of the tracking period (Fig. 4A). For those particles that were stationary for at least 1 min, that period of immobility occurred at the start of the tracking period in 52% of the cases. Fig. 4B shows an example of six particles in one animal. Two of the particles (#4 and #6) were stationary for more than 5 min and then began to move. Moreover, the speed of individual disks changed during the scanning time. Often the rate of movement increased during the course of the run (Fig. 4B). For example, the speed during the first minute of tracking was slower than during the third minute for 33% of particles (n = 43 particles). However, we also observed that particles occasionally slowed and/or temporarily stopped during the course of a run (Fig. 4B).

Particles Preferentially Travel to the Ventral Trachea. To assess particle position around the circumference of the airways, we established a polar coordinate system in which the airway lumen viewed through a transverse section is treated as a circle, the most ventral point is 0 degrees, and the most dorsal point of the circle is 180 degrees (Fig. 5A and Movie S3). We plotted position on the airway circumference of particles in the first scan and in the last scan before they exited the field toward the larynx or at the end of the 10-min scanning period (Fig. 5B). In the first scan, particles were distributed evenly throughout the airway circumference. However, by the last scan, they were grouped toward the ventral part of the airway. Angular position had little influence on the speed of particles (Fig. 6).
For these studies, animals were supine, and thus the ventrally directed movement was in the opposite direction of gravity. To further test the effect of gravity, we studied two animals in the prone position. The ventral preference persisted in that position (Fig. 5C). Thus, particles demonstrated a strong gravity-independent ventral preference in their movement.

**Orientation of Cilia Beating Directs Particles to the Ventral Airway Surface.** We hypothesized that the direction of cilia beating was responsible for the ventral particle transport. To test this, we removed segments of trachea, opened them with longitudinal cuts either ventrally or dorsally, mounted them flat under Krebs HCO3−/CO2 buffered saline, and used reflected light video microscopy to determine the direction of cilia beating. We plotted the angle between the rostral–caudal axis and the direction vector of ciliary beat (Fig. 7A). At the ventral tracheal surface, cilia were oriented parallel to the rostral–caudal axis (Fig. 7B). However, as we measured ciliary orientation at positions away from the ventral surface, a component of beat directionality was directed toward the ventral trachea. We observed similar directionality irrespective of whether the trachea was opened ventrally or dorsally.

We also found that cilia beat frequency was relatively similar around the tracheal circumference (Fig. 7C), consistent with the observation that the speed of particle movement was little affected by position on the tracheal circumference. Thus, the orientation of cilia beating directs particles toward the ventral surface and the larynx.

**Discussion**

In this study, we developed a MDCT-based assay to measure MCT in vivo in newborn pigs. The ability to track individual particles in 3D with high spatial and temporal resolution allowed us to discover substantial heterogeneity in MCT, with variation in the speed of particle movement between animals, between individual particles in an animal, and in the movement of single particles at different times. The methodology also revealed that particles travel preferentially to the ventral surface of the trachea.

**Movement of Individual Particles Was Highly Variable.** The ability to track discrete particles in the lung revealed heterogeneity in MCT speed between individual particles and during the transit of single particles. There are several factors that might contribute to heterogeneity. First, variation in cilia beat frequency could affect MCT speed. However, that seems unlikely because we found that beat frequency was relatively similar across multiple regions of the trachea. Second, variable depth of periciliary liquid might be responsible. In earlier work, we showed substantial variability in the depth of periciliary liquid in newborn pigs (45). Because we observed that individual particles moved over the same area of trachea at different speeds, either periciliary liquid depth is not an important factor responsible for heterogeneity in MCT, or periciliary liquid depth varies in a highly dynamic and unrecognized manner. Third, variations in the properties or quantity of mucus or association of discs with mucus might cause heterogeneity in MCT speed. For example, there are reports of substantial variability in rates of secretion from individual submucosal glands (46), viscosity of submucosal gland secretions (47), and tracheal mucus thickness (45, 48). We also speculate that local variations in mucus properties or abundance might explain the observation that particles sometimes slow as they journey toward the larynx. Perhaps disks reach an area where mucus being secreted from a submucosal gland has not yet detached from its origin and thus slows progress of the particle. This variability suggests that the trachea, at least in the newborn, is not likely covered by a continuous, homogeneous blanket of mucus (49). If that were the case, we would have expected a more uniform rate of movement. Instead, mucus might function as islands or discrete units, and the rates of movement may vary even in similar airway regions.

**Ciliary Orientation Preferentially Moves Particles to the Ventral Tracheal Surface for Removal from the Lung in Newborn Pigs.** We discovered that particles traveled to the ventral tracheal surface as they progressed toward the larynx. This ventral-directed flow pattern is reminiscent of the flow of cars from an on-ramp onto an interstate highway. That is, the ventral tracheal wall is analogous to the highway, and the dorsal and lateral walls are analogous to on-ramps. However, in contrast to a highway system, particle speed was independent of position.

The direction of ciliary beating must have been established in the fetus because it was present immediately after birth. Thus, it cannot be attributed to gravity during development, and it was not gravity-dependent during testing. Although a core set of planar cell polarity proteins are known to establish the orientation of cilia...
in the airways, the global directional cues remain uncertain (50). Perhaps the pattern of cilia orientation in the pigs could facilitate discovery of the responsible signals.

The ventral preference for MCT in pigs contrasts with earlier studies in dogs; bronchoscopic observations suggested that when viewed from above, particles moved in a clockwise (or counterclockwise in a few dogs) orientation around the trachea, and some accumulated on the dorsal surface where they traveled cephalad (18, 19, 51). In the pig, submucosal glands are most abundant in the dorsal wall, followed by the ventral and then lateral walls (52). This distribution and frequency are relatively unique among experimental laboratory animals in that the preferential dorsal vs. ventral distribution is similar to that in humans (53). We speculate that dorsally secreted mucus sweeps around the trachea to maximally capture particulates and then concentrates in a smaller area ventrally. Gathering mucus in this way rather than having it spread evenly around the tracheal circumference might make it more susceptible to the shear force of air generated by coughing and hence facilitate its expulsion from the lung. In the dog, ventrally secreted mucus might have the reverse pattern.

This Method for Measuring MCT Has Advantages and Limitations. Our approach has several advantages. In this in vivo approach, piglets breathed spontaneously through their noses so normal airway humidification was maintained. By using volumetric MDCT scans and recording the position of discrete particles at 15-s intervals, we were able to obtain high spatial and temporal resolution. The use of MDCT scans also allowed us to register the position of particles to the airway anatomy. As with other imaging methods, increasing the granularity of the data revealed interesting patterns of MCT.

This method also has a number of limitations. (i) The amount of radiation exposure used in this study is greater than with radionuclide aerosols and thus not acceptable for human use. (ii) The animals were sedated. We used ketamine and acepromazine for initial sedation and then propofol to maintain sedation. Previous studies indicate that these agents are not likely to alter MCT, ciliary beat frequency, or mucus secretion (54–56). (iii) The size of the tantalum particles (350 × 25 μm) is larger than bacteria and some particulates that enter the lung. However, studies using other methods and larger and smaller particles suggest that MCT is not markedly altered by particle size, although small particles might be ingested by macrophages (51, 57, 58). Moreover, earlier studies reported transport rates that encompass the range of MCT we measured (59, 60). (iv) The method does not assess small airways; we currently have only deposited particles from the trachea to third generation airways. However, studying more distal airways could be of value. Although most airway diseases involve both small and large airways after the disease is established, much less is known about the distribution of abnormalities at the onset of disease. (v) The number of particles deposited in an airway is small and could therefore miss additional complexity. However, we used a small number to adequately discriminate individual particles as they advanced up the airway.

Concluding Comments. We anticipate that this method may be adapted for several applications. Because of its sensitivity, it may be of value in revealing differences between wild-type animals and those with disease, including cystic fibrosis, asthma, and chronic bronchitis. These methods may be especially helpful early in the course of disease, and the ability to assess heterogeneity may provide insight into pathophysiological mechanisms. It may also be of use for assessing the response to interventions that may alter the function of individual components of the MCT process, including cilia, mucus-producing goblet cells and submucosal glands, and transepithelial electrolyte transport by airway epithelia. Finally, probing MCT at greater resolution in pigs may help guide efforts to improve MCT assays for humans.

Materials and Methods

Animals. Newborn wild-type domestic pigs were used for these studies. Pigs were euthanized with IV euthasol (Virbac) injection followed by bilateral
we determined how many particles moved less than 3 mm over the course of more than 3 mm were included in speed vs. angular position assessments. Is seen in the speed vs. angular position assessment. Only particles that traveled the highest value for speed generated by an individual particle. In other cases, we used the following equation to calculate particle speed. These approaches generated multiple measurements of speed for each particle. 

\[
\text{Particle Speed} = \sqrt{\left(\frac{x_{n+1} - x_n}{t_{n+1} - t_n}\right)^2 + \left(\frac{y_{n+1} - y_n}{t_{n+1} - t_n}\right)^2 + \left(\frac{z_{n+1} - z_n}{t_{n+1} - t_n}\right)^2}
\]

where \(n\) refers to the number of the scan, \(x, y, z\) represent Cartesian coordinates (\(x\), left-right; \(y\), dorsal-ventral; \(z\), rostral-caudal), and \(t\) represents time in minutes.

In some examples, we show speed as the maximum particle speed, which is the highest value for speed generated by an individual particle. In other cases, we show the mean speed, which is the average of all speed values generated by an individual particle. Multiple measurements of particle speed for an individual animal allows us to display more speed values than there are total particles; this is seen in the speed vs. angular position assessment. Only particles that traveled more than 3 mm were included in speed vs. angular position assessments.

To assess how many of the particles delivered to an airway were immobile, we determined the average position of three anatomical landmarks with a time value depending on the scan (\(x, y, z\), time) using ImageJ for DICOM image analysis. To avoid alterations in the airway surface due to the presence of the delivery catheter, particles were not tracked once they traveled above the position of the lung apex. Only particles that were delivered beyond the right cranial lobe were tracked to provide enough distance to appropriately analyze transport. To determine particle speed, we measured the distance particles moved over a known period. Movement from spontaneous breathing could confound measures of transport rate. To avoid this, we determined the average position of three anatomical landmarks in each pig: the right cranial lobe bronchus (RC), the carina (CA), and the lung apex (AP). We discarded any scan in which the position of the three landmarks deviated more than 1 mm from the average position of the landmarks. We then used the subsequent scan to calculate speed. This correction removed 2.5% of the scans.

Calculation of speed using 15-s intervals exaggerates the contribution of any animal movement to speed. Therefore, to reduce noise in calculating speed, we measured particle position every 15 s and used 60-s intervals to calculate speed. These approaches generated multiple measurements of speed for each particle. We used the following equation to calculate particle speed.

Radial Coordinate Position = \(\arccos\left(\frac{a^2 + b^2 - c^2}{2ab}\right)\)

\(a = \sqrt{\left(x_n - x_0\right)^2 + \left(y_n - y_0\right)^2 + \left(z_n - z_0\right)^2}\)

\(b = \sqrt{\left(x_n - x_{n+1}\right)^2 + \left(y_n - y_{n+1}\right)^2}\)

\(c = \sqrt{\left(x_n - x_{n-1}\right)^2 + \left(y_n - y_{n-1}\right)^2}\)

The absolute value of the radial coordinate is represented with no designation for the right or left side of the airway. For one set of experiments, we examined the radial coordinates of disks at time 0 and 10 min with the pig in the prone and supine positions. During the experiments, some particles likely left the tracking area and were not included in the 10-min time points.

**In Vivo Mucociliary Transport Assay.** Tantalum disks (350 × 25-μm) were punched from tantalum foil (Sigma). The average particle mass was 55 μg. Newborn pigs were anesthetized with 20 mg/kg ketamine and 2 mg/kg acepromazine delivered IV, and then intubation was maintained with IV propofol. Pigs breathed spontaneously through the nose. To deliver tantalum particles, animals were briefly intubated, and 15–30 tantalum disks were insufflated into the lungs with a puff of air (Fig. 5IA). Immediately after particle delivery, the catheter was removed. To assess particle transport, serial three-dimensional images of the chest were acquired with a high-resolution multirow detector computerized tomography scanner (Siemens Somatom, Definition Flash Dual Source 128-slice computed tomography (CT) Scanner; Fig. 5IB). MDCT-generated Digital Imaging and Communications in Medicine (DICOM) images were taken with a slice depth of 0.6 mm and a slice interval of 0.3 to ensure visualization of tantalum particles. Unless specified, scans were performed with the pig in the supine position. Over a 10-min acquisition period, a total of 40 scans were taken with a 15-s interval between scans. The tantalum particles, with typical Hounsfield units 550, were easily discerned from surrounding airway tissue, which had Hounsfield units of approximately −200 (Fig. 1A–C). Individual particles were manually tracked by assigning three-dimensional Cartesian coordinates with a time value depending on the scan (\(x, y, z\), time) using ImageJ for DICOM image analysis. To avoid alterations in the airway surface due to the presence of the delivery catheter, particles were not tracked once they traveled above the position of the lung apex. Only particles that were delivered beyond the right cranial lobe were tracked to provide enough distance to appropriately analyze transport. To determine particle speed, we measured the distance particles moved over a known period. Movement from spontaneous breathing could confound measures of transport rate. To avoid this, we determined the average position of three anatomical landmarks in each pig: the right cranial lobe bronchus (RC), the carina (CA), and the lung apex (AP). We discarded any scan in which the position of the three landmarks deviated more than 1 mm from the average position of the landmarks. We then used the subsequent scan to calculate speed. This correction removed 2.5% of the scans.

Calculation of speed using 15-s intervals exaggerates the contribution of any animal movement to speed. Therefore, to reduce noise in calculating speed, we measured particle position every 15 s and used 60-s intervals to calculate speed. These approaches generated multiple measurements of speed for each particle. We used the following equation to calculate particle speed.

**Ex Vivo Cilia Orientation and Beat Frequency Analysis.** To assess cilia orientation ex vivo, animals were euthanized, and 1-cm-long rings of trachea immediately proximal to the right cranial lobe bronchus were excised and placed in Krebs Ringers solution containing (in mM) 115 NaCl, 25 NaHCO3, 1.2 CaCl2, 1.2 MgCl2, 6.6 K+ PO4, pH = 7.3 in 5% (vol/vol) CO2 on ice for 1–4 h. For studies, a longitudinal incision spanning the length of the tracheal ring was made on either the most ventral or dorsal aspect of the ring to make a rectangular sheet. Tracheal sheets were mounted flat by pinning to dental wax, rinsed, and then submerged in 37 °C Krebs Ringers solution pH = 7.35% CO2. We used reflected light to visualize cilia motion (Nikon A1R Resonant Scanning Confocal microscope, 25× objective [charge-coupled device (CCD) camera and Nikon Imaging System (NIS) elements software, 200 frames]). We analyzed 1-s video clips (NIS Elements) to determine cilia orientation and beat frequency.

**In Vivo Thoracotomy.** All animal protocols were approved by the University of Iowa Institutional Animal Care and Use Committee.

**Fig. 6.** Angular position had little influence on particle speed. Data are polar coordinate position on the x axis and particle speed on the y axis. n = 55 particles in 7 animals.

**Fig. 7.** Cilia orientation determines ventral directed particle movement. Cilia orientation and beat frequency were determined ex vivo using reflected light video microscopy on flat mounts of trachea. (A) Schematic shows orientation of cilia. (B) Data are cilia orientation (ω) at indicated positions on tracheal circumference. Data are from one animal. n = 7–16 cells per data point; mean ± SD. Similar results were obtained in three other animals. (C) Data are cilia beat frequency at indicated positions on tracheal circumference. Data are from one animal. n = 15–30 cells per data point; mean ± SD. Similar results were obtained in three other animals.
frequency. To quantify the orientation of cilia we determined the angle between the axis of ciliary beat and the rostral-caudal plane (Fig. 7A). To quantify cilia beat frequency we counted the number of reflected light-intensity changes in regions of ciliated cells over a known time period.

**Statistical Analysis.** Assessment of ventral preference was performed by paired t test with the assumption that dorsal–ventral movement was independent of individual piglets.

**Acknowledgments.** We thank Elizabeth Allard, Lucas Askland, Sufiang Mao, Theresa Mayhew, James McNemnien, Andrew Michalski, Sean Molchew, Thomas O Moninger, Lynda Ostedgaard, Leah Reznikov, Viral Shah, Jared Sieren, and Peter Taft. This work was supported by the National Institutes of Health (NHL) (HL051670, HL091842, DK054759), the Cystic Fibrosis Foundation, and the Cystic Fibrosis Foundation Musculoskeletal Clearance Consortium. D.A.S. is supported by the Gilead Sciences Research Scholars Program in Cystic Fibrosis and the NHL (DP2 HL117744). M.J.W. is an investigator of the Howard Hughes Medical Institute.