Supplemental Material

Cell Culture: Primary human bronchial epithelial cells were obtained per established protocols from the UNC Tissue Core Facility.(11) Non-CF human lung tissue was obtained from lungs donated for research after being deemed unsuitable for transplantation. Cells were seeded as passage 0 (P0) cultures onto 12mm Transwell polycarbonate inserts that were previously coated with a thin layer of human placental collagen. Plated cells were then incubated at 37°C for at least 3 weeks until an air-liquid interface (ALI) was achieved prior to experiments. Cells were not apically washed unless otherwise specified per protocol.

Washed cultures exhibited 1.9% mucus solids content, similar to that reported for normal subjects (29). The unwashed mucus cultures exhibited mucus concentrations that mimicked the mucus extracted from explanted CF lungs (12.2%). Mucus mass (weight) (29) for the normal cultures was 1.84 $^{+}$/- 0.03mg, whereas the mass for the CF-like mucus was 2.74 +/- 0.18mg, measured in the absence of HS.

HBE Preparation:

For confocal experiments, epithelia were first labeled with calcein-AM (Invitrogen) basolaterally for 30 minutes. For HBE preparations that were to be used in “normal” mucus experiments, visible mucus was washed from the surface of the cell culture at least 30 minutes prior to study using three serial washes with PBS followed by removal with gentle suction after 5 minutes of incubation. The concentration of the “normal” (non-CF) vs CF-like hyperconcentrated mucus culture surfaces was measured using a mesh sampling technique coupled to a computer controlled microbalance to measure % solids (2, 30). To visualize the ASL, the luminal surface was labeled with Texas Red Dextran (TRD) (Invitrogen, 70,000 MW; 10mg/ml PBS), a cell impermeable fluorescent dye. For washed culture experiments, 10µl TRD was applied 30 minutes before the study and after the third wash. For experiments with undisturbed mucus on the HBE surface, 10 µl of 70,000 MW TRD was applied apically at least 12 hours before experimentation to allow the dye to distribute in concentrated mucus. In all cases, to

eliminate edge/meniscus effects on ASL volume, the Transwell membrane was excised from its support and affixed via a hydrophobic gel to the surface of a polycarbonate ring embedded on a cell culture dish. On the basolateral surface, 10µl of PBS was placed under the culture to maintain continued hydration of the cell culture during the experiments.

Aerosol Delivery: A 7% HS solution was aerosolized utilizing an Aerogen Lab Pro Vibrating Mesh Nebulizer modified to deliver clinically relevant small volumes (nl/cm^2/min) directly onto HBE surfaces. The volumes deposited were designed to simulate the deposition of nebulized HS generated by two clinically available nebulizers (jet and vibrating mesh) onto the airway epithelial surfaces of the large and mid-sized airways *in vivo*. (**Figure S1**) This system is mounted on a confocal microscope, allowing for direct visualization of HS aerosolization to the HBE surface.

Confocal Microscopy: Following HBE preparation and labeling with TRD, cultures were housed in an environmental chamber that regulated temperature to 37˚C, humidity at 50%, and CO_2 at 5%, interfaced to a scanning confocal microscope (SP5, Leica). ASL and HBE heights were measured simultaneously by X-Z confocal microscopy. Following 2 minutes of baseline imaging (t0), 7% HS was nebulized via the nebulizer system to deposit HS onto apical surface of HBE cells at 8µg NaCl/cm^2/min. Serosal, cell, and ASL heights were measured before nebulization, every 30 seconds during the nebulization period, and until ASL volume had returned to baseline. This protocol utilized both normal and CF-like hyperconcentrated mucus HBE cultures. In another set of experiments, the total delivered mass of sodium chloride was held constant, but the rate of nebulization was varied (3µg NaCl/cm^2/min for 47.5 minutes, 8µg NaCl/cm^2/min for 15 minutes, or 18µg NaCl/cm^2/min for 6.5 minutes) to compare rate of delivery of HS on ASL volume. In a third protocol, HS was nebulized onto the apical surface of HBS at a rate of 8 µg NaCl/cm^2/min for 15 min, with administrations repeated at intervals of 15 min to examine the effectiveness of repeated dosing. These experiments were then modified to include a bolus challenge 10 µl of hypotonic (0.63%) or (isotonic, 0.9%) saline applied to HBE apical surfaces between doses. Finally, to assess the role of apical membrane water channels on ASL volume responses to HS, mercury chloride, a

non-selective aquaporin inhibitor, was incubated with HBE cells, followed by nebulized HS dosing.

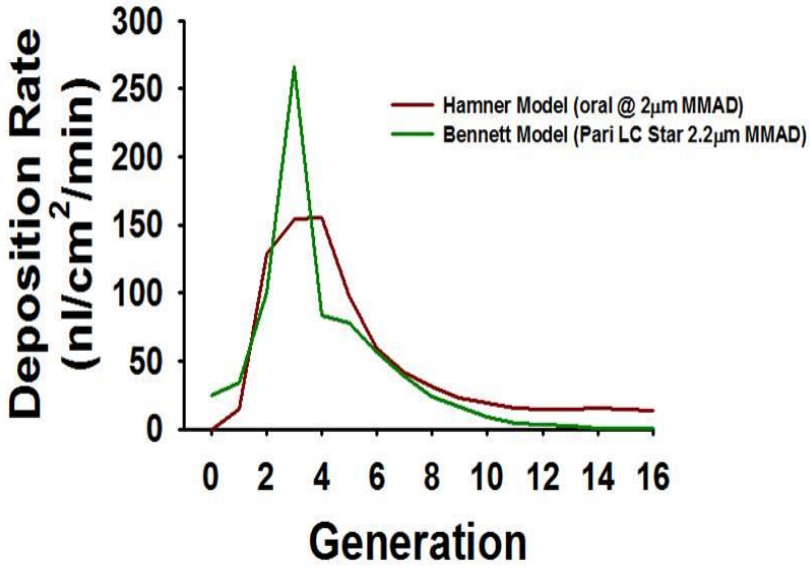

Figure S1: The deposition rate (in nl/min/cm2 of airway) is shown for the first 16 generations, based on 2 separate models of deposition calculated for the Pari LC Star (which nebulizers 0.2 ml/min). Only about 15% of the delivered dose is deposited in the airways/lungs.