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Mucociliary Defense: Emerging Cellular, Molecular, and Animal Models

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Abstract

Respiratory tissues are bombarded by billions of particles daily. If allowed to accumulate, these particles can cause injury, inflammation, or infection, and thus may significantly disrupt airflow and gas exchange. Mucociliary defense, a primary mechanism for protecting host tissues, operates through the coordinated functions of mucus and cilia that trap and eliminate inhaled materials. Mucociliary function is also required for the elimination of endogenous cells and debris. Although defense is necessarily robust, it is also tightly regulated to minimize physiologic disruption of the host. Indeed, mucociliary dysfunction contributes to the pathogenesis of many lung diseases—including asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, and cystic fibrosis—in which airflow limitation, inflammation, persistent tissue injury, and structural remodeling occur. Here, we highlight recent advances in cilia and mucin biology, the importance of well-controlled mucociliary interactions, and the need to better understand how these regulate innate barrier and immune defense.

Keywords: airway epithelium; mucin; mucus; goblet cell

(Received in original form June 29, 2018; accepted in final form August 14, 2018)

Supported by National Institutes of Health grants R01 HL080396, R01 HL130938 (W.J.J. and C.M.E.), and P50 CA058187 (K.H.B.), Department of Defense grant PR160247 (C.M.E.), and a Boettcher Webb-Waring Biomedical Research Award (E.K.V.).

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Ann Am Thorac Soc Vol 15, Supplement 3, pp S210–S215, Nov 2018

DOI: 10.1513/AnnalsATS.201806-439AW
Internet address: www.atsjournals.org

In the lungs, the epithelial lining of the conducting airways constitutes an essential first line of innate defense. In addition to serving as a physical barrier, polarized epithelia that are oriented toward the airway lumen produce and transport a glycoprotein-rich mucus hydrogel that prevents desiccation of airway surfaces, traps potentially harmful particles, and mediates elimination of both exogenous materials and endogenous debris. Two features that are critical for these functions are motile cilia and polymeric mucins. Recent advances in cilia and mucin biology have led to a burst of new information about how mucociliary defense integrates genetic, cellular, and molecular mechanisms, the tight regulation of which is required for host protection and homeostasis. Furthermore, these insights have substantially enhanced our understanding of mucociliary dysfunction in lung injury and disease. In addition, a greater understanding of mucins as glycopolymers and potential sources of ligands for leukocyte lectin receptors has opened new fields of study that are being applied in novel animal and cell-based models.

Motile Cilia

Mucociliary clearance during homeostasis is accomplished by the directional movement of cilia on the apical surface of multiciliated cells (MCCs). The following criteria must be met for optimal ciliary clearance: *1*) the epithelium must contain specific numbers of MCCs per tissue area and numbers of cilia per cell; *2*) each cilium must possess the correct ultrastructure, and beat in the right direction, at the optimum frequency, and with the appropriate waveform; and *3*) cilia must interact with mucin macromolecules. These conditions are described subsequently here.

Because the total surface area and volume of small airways in the lungs is much greater than the total area and volume of large airways, equivalent mucus transport rates in small and large airways could overwhelm the capacity for mucus elimination. One mechanism for controlling this problem occurs by regulating the relative

numbers of MCCs along a large-to-small-airway axis. Indeed, compared with large airways, small airways have fewer MCCs relative to secretory cells. This mechanism for optimizing transport is controlled, in part, by regulating MCC differentiation. MCCs are terminally differentiated epithelial cells derived from basal stem cells or secretory cells (1, 2). MCC specification is dependent on Notch signaling during normal development and during repair after injury (Figure 1) (3). Once fated, nascent MCCs launch a gene expression program that activates expression of hundreds of structural and regulatory cilia genes (4). At the top of the hierarchy is the "EDM" (EDF4/5, DP1, MCIDAS [multiciliate differentiation and DNA synthesis associated cell cycle protein]) transcriptional complex, consisting of E2F4 or E2F5 coupled with DP1 and MCIDAS (multiciliate differentiation and DNA synthesis associated cell cycle protein) (5). Together, these drive several secondary transcriptional regulators, including FOXJ1 (6), which remains active to maintain MCC gene expression. This specification program prompts the assembly of 200–300 basal bodies (also known as centrioles) in the cytoplasm (7). Basal bodies then traffic to the apical cell membrane, dock, and elongate a microtubule-based motile ciliary axoneme.

Once assembled, coordinated ciliary motion and beat pattern derive from the sliding of microtubule filaments by dynein motor protein complexes (reviewed in Reference 8). Across species and tissue types, cilia beat at a frequency of approximately 12 Hz in the steady state, and MCCs can rapidly adjust beating in response to stimuli, including contact with mucus, pathogens, or even changes in temperature. For MCCs to drive oral-directed transport, the apical cytoskeleton is polarized via noncanonical Wnt signaling (9). This coordinates MCC directionality at the tissue level with distal-to-proximal (lung-to-mouth) anatomic regions. These features are integrated with airway liquid and mucus properties.

Cilia interact extensively with both gel-forming and membrane-tethered mucins. Airway mucus is chiefly made of hydrated gel-forming mucin macromolecules (10). Mucus is transported along airway surfaces by the insertion of cilia tips into gel matrices during the active stroke of the ciliary beat cycle. In addition to gel-forming polymeric mucins released into airspaces, membrane-tethered mucins localize to the apical membranes of MCCs, including ciliary membranes, to form a "grafted-brush" complex that sustains a semipermeable and osmotically stable periciliary layer at the airway surface (11, 12).

Divergence from these parameters, either due to environmental insults or disease, impairs optimal ciliary function and leads to compromised mucociliary clearance. Ciliary structure and function are highly vulnerable to damage from cigarette smoke, chronic inflammation, and pathogens (8, 13). Primary ciliary dyskinesia, a disorder of mucociliary clearance, involves the absence or abnormal movement of cilia due to mutations in genes responsible for cilium biogenesis or motility (14). In addition, in cystic fibrosis (CF), asthma, and chronic obstructive pulmonary disease, the excessive mucins and osmolytes on airway surfaces can impair cilium stability through osmotic stress, disruption of the grafted-brush form, and collapse or entanglement of apical cilia (15). Thus, to understand the regulation of ciliary motility, it is crucial to recognize how cilia are affected by mucus gel properties.

Polymeric Mucins

To provide an ideal matrix for ciliary transport, healthy airway mucus is kept thin through the regulation of its fluid, ionic, and macromolecular composition. The roles of osmotic flux are discussed elsewhere (15, 16). Here, we highlight the importance of two polymeric gel-forming mucin glycoproteins: MUC5AC and MUC5B. These are the chief macromolecules in airway mucus, and are hence critical regulators of mucus functions (17). Recent work by Button and colleagues (18) has shown that the concentration of polymeric mucins in airway mucus is a crucial driver of osmotic gradients that affect cilia-mediated transport.

Due to their relatedness, MUC5AC and MUC5B are typically thought of as redundant proteins. However, our laboratory showed that these mucin isoforms have distinct effects in the lungs. Using *Muc5ac* and *Muc5b* gene knockout mice ("*MUC*" in humans, "*Muc*" in mice), we found that, whereas Muc5b is required for mucociliary clearance in health, Muc5ac is dispensable (19). In humans, MUC5B content predominates over MUC5AC in healthy secretions. However, *MUC5AC* expression increases substantially in asthma, CF, and chronic obstructive pulmonary disease. Notably, deletion of *Muc5ac* is protective in murine models of asthma (20) and lung injury (21). Accordingly, because

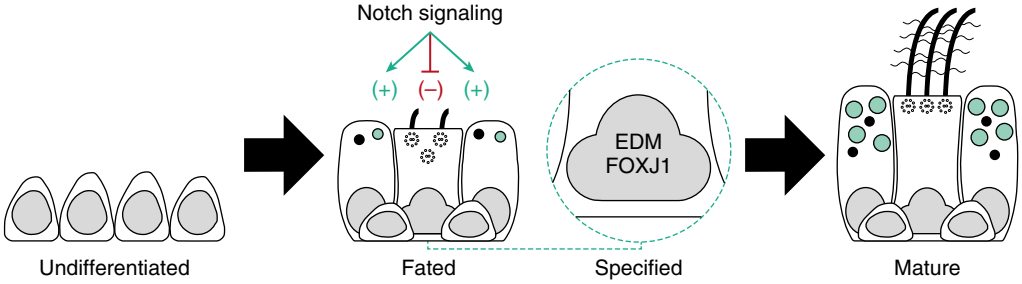

Figure 1. Mucociliary differentiation. During airway epithelial differentiation, multiciliated cell (MCC) and secretory cell fates are determined by Notch signaling, such that signal-sending cells assume the multiciliated fate and cells receptive of Notch signaling become secretory cells. Downstream of the Notch signaling event, nascent MCCs launch an MCC-specific gene expression program under the control of the EDM (EDF4/5, DP1, MCIDAS [multiciliate differentiation and DNA synthesis associated cell cycle protein]) transcriptional complex and several secondary transcription factors, including forkhead box J1 (FOXJ1) (inset). This turns on the expression of hundreds of structural and regulatory ciliary genes, and initiates the motile ciliogenesis pathway, which leads to the assembly of 200–300 cilia per MCC. Mature MCCs contain motile cilia decorated with membrane-tethered mucins. Secretory cells synthesize secretoglobins and mucins, and their transcriptional development program is reviewed by Whitsett (pp. S143–S148) (59).

of its tight regulation and significant functions, the control of *MUC5AC/Muc5ac* transcriptional induction is well studied (22). Mucin gene regulation is also discussed elsewhere (22, 23). Here, we focus on the mucin glycopolymers themselves (Figure 2).

MUC5AC and MUC5B are very large proteins (>3,000 amino acids in length) that assemble into carboxyl terminal "tail-to-tail" disulfide-linked dimers in the endoplasmic reticulum. In the Golgi, they again dimerize, or potentially multimerize, "head-to-head" at their N termini. They also become glycosylated (discussed subsequently here) (24, 25). As a result, mucins are extraordinarily large glycopolymers with viscous and elastic potentials. Reducing agents disassemble polymers, and thus decrease mucus viscoelasticity. Depolymerizing mucus is a goal for mucolytic therapy, but the field is limited by a lack of safe, tolerable, efficacious options. For example, dithiothreitol is a potent mucolytic, but it is unstable and toxic. *N*-acetylcysteine (NAC) is the only U.S. Food and Drug Administration–approved reducing agent available as an inhaled mucolytic, but efficacy is low due to NAC's weak activity at airway pH and high mucin concentrations (26, 27). Improving reducing agents is an area of focus in the field. Effective agents could "rescue" mucociliary transport (MCT) and airflow functions, and they could also enhance the deposition and penetration of inhaled bronchodilator, steroid, or antibiotics. Current areas of focus on mucin assembly mechanisms center on conserved disulfide assembly mechanisms also used by the glycoprotein polymer von Willebrand factor, an evolutionary descendent of polymeric mucins (28, 29). Although their polymeric assembly is quite similar, polymeric mucins are extensively more glycosylated than von Willebrand factor, and these carbohydrate modifications define mucus function.

Mucin glycosylation is a multistep process that occurs within domains composed of proline, serine, and threonine–rich imperfect repeats (10, 30). Glycans affect the biophysical functions of mucus gels (31–33), as well as the potential for mucin interactions with pathogens and other host cells (12, 34–37). *O*-glycosylation occurs in the Golgi, beginning with the addition of *N*-acetylgalactosamine (GalNAc) to serines and threonines in proline, serine, and threonine domains. Galactose (Gal) and *N*-acetylglucosamine (GlcNAc) glycans are then added singly or in combinations that form core structures. Cores are then further elaborated with Gal and GlcNAc extensions to form linear and branched structures (38), which can be further modified with specialized sugars, fucose and sialic acid, and with sulfate (39–42).

These heterogeneous structures impart unique physical properties on mucins. Sialic acid and sulfates provide negative charges, which introduce steric repulsion and confer rigidity between hydrated side chains (31–33, 43). On the other hand, fucose is neutral and binds less water, thereby promoting higher viscosity (31–33). High levels of mucin fucosylation are associated with increased mucus viscoelasticity in chronic rhinosinusitis (32). In addition to these effects on the biophysical properties of mucus gels, mucins also affect host cell functions. This was shown in the lungs in studies demonstrating the effects of mucin sialylation on leukocyte functions via siglec family receptors (38, 44, 45). Muc5b carries endogenous α2,3-linked sialoside ligands of Siglec-F that initiate eosinophil apoptosis in mice (38). In humans, sialoside carriers, including MUC5B supply ligands for siglecs that suppress the functions of eosinophils (Siglec-8) and neutrophils (Siglec-9) (44). The relative extents to which the functional consequences of mucin glycosylation are isoform specific, genetically determined, or environmentally controlled are beginning to be clarified. Efforts are underway to more completely characterize glycans carried by

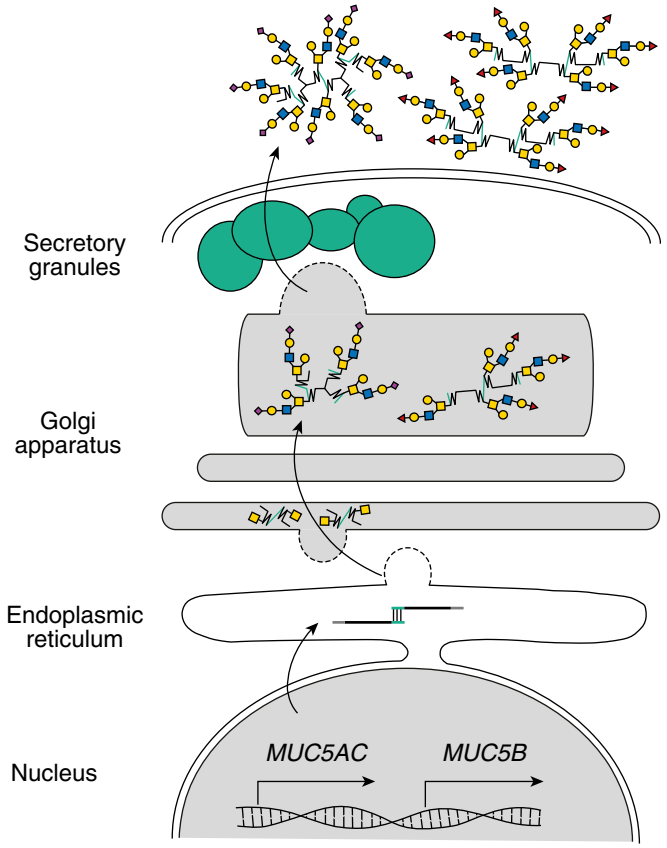

Figure 2. Assembly and secretion of polymeric mucins. Two polymeric mucin genes, *MUC5AC* and *MUC5B*, are expressed by airway surface and glandular epithelia. Translation occurs in the endoplasmic reticulum, where CTCK (C-terminal cysteine knot) regions of MUC5AC and MUC5B form interchain homodimers. Dimers are transported to the Golgi apparatus, where they are *O*-glycosylated with GalNac (yellow squares), followed by Core 1-4 glycosylation with Gal (yellow circles) and GlcNac glycans (blue squares). Core glycosylated mucins are then elaborated with Gal and GlcNac additions that form extensions or branches. Two specialized sugars, fucose (red triangles) and sialic acid (purple diamonds), can be added to Gal (and also to GlcNac in the case of fucose), creating biophysically and immunologically specialized glycoconjugates. Glycosylated complexes then multimerize via N-terminal disulfide assembly in the *trans* Golgi. Once fully synthesized, mucins are exported from the Golgi and stored in secretory vesicles for subsequent release by regulated exocytosis.

MUC5AC and MUC5B (and their mouse orthologs) across individuals and anatomic sites in respiratory tissues.

Taken together, we propose that MUC5AC and MUC5B have evolved to serve specific critical specialized functions based on host genetics, environmental stimuli, and anatomical location. Because of their sizes and complexities, there are numerous challenges to designing appropriate functional and mechanistic studies. Using animal models with gain- and loss-of-function genetic interventions, we have been able to assess significance and some important degrees of physiologic functions. With emerging technologies in animal models and in human cells, future studies will be able to apply higher-resolution mechanistic analyses, such as gene editing and microscale tissue modeling.

Model Systems for Investigating Mucociliary Functions

Several static *in vitro* culture models of human and rodent airway have been developed that allow analysis of mucin secretion, ciliary beating, and MCT (8, 13, 46, 47). Although informative, a major limitation of these systems is that *in vitro*–reconstituted mucociliary epithelia transport the secreted mucus in a continuous circular manner, and, as such, often fail to reproduce *in vivo*–observed directionality of MCT. In addition, these platforms have not been fully interrogated for their potential in performing fine analyses on ciliary behavior; rather, investigators often rely on mean cilia beat frequency (CBF) as the key (and often sole) endpoint (46–48). Recently, leveraging principles of tissue microengineering, a novel microfluidic model of human small airway has been developed (49, 50). This *in vitro*–recreated organomimetic culture (known as "human lung Small Airway-on-a-Chip") overcomes the limitations of static culture systems. More specifically, this platform reproduces unidirectional mucus transport and enables thorough analyses on high-resolution characterization of CBF per cilium, per cell, per field of view, per chip, per donor, and per condition. The platform also permits mapping the ranges of CBF values in different experimental settings, so the distributions and Gaussian normality of cilia beating can be taken into consideration, and the appropriate statistical index for comparison can be made on single-cell or population data. This allows for variability in CBF among different cells and conditions to be quantified. Thus, the Small Airway-on-a-Chip platform provides a refinement of biological modeling *in vitro* that is compatible with delineation of heterogeneous changes in CBF rates in different conditions. This *in vitro* system provides highly refined measurements that support assessments of mucociliary functions made using techniques such as microoptical coherence tomography *in vivo* (51).

In vivo testing of mucus function and dysfunction has been an area where the importance of both small and large animal models is recognized. The size and ease of genetically manipulating mice have supported their use in understanding the significance of mucociliary components in health and disease, with added advantages stemming from the ability to conditionally control gene expression in tissue-specific and temporally controlled fashions (52). However, respiratory structures and disease modeling are complex and not always optimal for studies in mice (53), so larger animal models are often required. This necessity led to the development of genetic interventions in nonmurine species, including rats, ferrets, and swine, which have become important models for studying mucociliary dysfunction and developing therapies for use in CF (54–58). In rodent and large animal models, genetic engineering has been notoriously slow. Techniques such as CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9-mediated targeting and gene editing are enhancing the potential to obtain mechanistic insights into mucociliary function at faster rates, lower cost, and greater refinement.

Conclusions

Mucociliary biology is an active and rapidly growing field. The application of advanced *in vitro* and *in vivo* tools to address our current state of understanding mucin and mucociliary interactions will lead to improved knowledge of airway epithelial cell biology and pathobiology. Future work focusing on specific sites and mechanisms of mucin assembly and mucociliary contacts could also lead to more selective intervention strategies. New therapeutics could include means for both the prevention and reversal of mucin hypersecretion and mucociliary dysfunction. These strategies could also be potentially combined with treatments targeting bronchoconstriction and inflammation with the ultimate goal of reversing or preventing the pathological effects of mucus dysfunction while protecting or enhancing lung defense. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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