Chronic mucus hypersecretion in COPD and asthma
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CHAPTER 1

General introduction
Chronic mucus hypersecretion (CMH) is a feature observed in various chronic respiratory diseases, particularly chronic obstructive pulmonary disease (COPD)\(^1,2\) and asthma\(^3,4\). While COPD is one of the top leading causes of death worldwide especially in elderly\(^5\), asthma is the most common chronic disease among children\(^6\) and responsible for more than 400,000 deaths overall each year worldwide\(^7\). Both genetic and environmental factors contribute to COPD and asthma\(^8-10\), and cigarette smoke is widely recognized as a major cause of COPD\(^10\). COPD and asthma patients may experience similar symptoms, e.g. breathing difficulty, chest tightness, wheezing, and cough; however, these symptoms are driven by different—though partly overlapping—mechanisms\(^11\). In COPD, exposure to air pollutants or irritant particles (e.g. cigarette or wood smoke) triggers inflammatory responses characterized by infiltration of neutrophils, macrophages, T lymphocytes and innate lymphoid cells, which in the long-term causes damage to the lung\(^12\). Abnormal tissue repair is a key pathophysiological feature of COPD which leads to small airway wall thickening and/or destruction of alveolar tissue (emphysema)\(^13\), resulting in airflow limitation that is not fully reversible and accelerated lung function decline\(^13,14\). In asthma, the most common type is allergen-induced asthma characterized by chronic eosinophilic airway inflammation, airway remodeling with increased smooth muscle mass, subepithelial fibrosis and goblet cell hyperplasia\(^14,15\). In patients with allergic asthma, allergen-specific type-2 T-helper cells drive the airway inflammatory response that gives rise to clinical symptoms such as wheezing, variable airflow limitation and airway hyperresponsiveness\(^16\). As both are highly heterogeneous diseases, COPD and asthma patients can be categorized into different subgroups based on their clinical features. One of the important features shared by both COPD and asthma patients is CMH.

CMH in COPD is often referred to as chronic bronchitis\(^1,2\). Here, it is associated with lower quality of life, an accelerated decline of lung function, more severe airflow obstruction and an increased risk of exacerbations and mortality\(^17\). In asthma, CMH is most prevalent in patients with severe asthma\(^4\) and associated with acute exacerbations\(^18\). Mucus plugging in the airways is found in the vast majority of fatal asthma cases and may be an important, but underappreciated, cause of respiratory failure\(^19\). Although some anti-inflammatory drugs, bronchodilators and antibiotics have been reported to reduce mucus production or improve mucus clearance, the results of the studies were either inconsistent or not yet proven to be beneficial in humans\(^20\). The lack of effective CMH-targeted therapy, while it represents a high burden, reflects an urgent need to better understand mechanisms underlying CMH pathophysiology in order to find better treatment options.
Factors contributing to chronic mucus hypersecretion

Patients with CMH suffer from chronic cough and sputum expectoration as a result of increased mucus accumulation in their airways, a process attributed to exaggerated mucin secretion and/or impaired mucus clearance. Increased mucin secretion in COPD and asthmatic airways likely results from increased numbers of goblet cells and enlargement of mucous glands, increased mucin synthesis, and/or increased degranulation of goblet cells. Ineffective mucus clearance can be driven by defective cilia function, changes in mucus composition, mucus dehydration and/or higher viscoelasticity resulting from more mucin cross-linking or impaired mucin degradation. All these factors are commonly used as markers for studying CMH, as summarized in figure 1.

Mucins are heavily glycosylated proteins responsible for mucus viscoelasticity. MUC5AC and MUC5B are the most common mucins present in the respiratory tract and they are associated with both COPD and asthma. When external pathogens or noxious particles are inhaled, the airway epithelium is the first line of defense protecting the lungs from these harmful stimuli. It is composed of various epithelial cell types including basal cells and more differentiated cells, e.g. club cells, goblet cells and ciliated cells. Mucins are synthesized by goblet cells as a constituent of the airway epithelium and by mucous cells in submucosal glands. After being synthesized, mucins are stored in intracellular granules before being released into the airway lumen. Here, secreted mucins are mixed with lipids, antimicrobial proteins, electrolytes, and water to form a mucus layer, which can then be transported from distal to proximal airways by the rhythmic beating of cilia before entering the larynx and being expectorated by a sudden opening of vocal cords.

Mucus which is accumulated in the airways requires effective clearance, a process that is often impaired in patients with COPD or asthma. As described above, one of the factors contributing to ineffective mucus clearance is ciliary dysfunction. Cilia on airway epithelium of COPD smokers are shorter, more vulnerable and show disorderly beating, while lower cilia beating frequency has been observed in asthmatic airways. In addition, smoking causes squamous metaplasia in the airways, that even further worsens mucus clearance. The type of secreted mucins may also influence mucociliary clearance. A recent study suggested that coating of secreted MUC5B with MUC5AC may provide anchoring activity that slows down mucus transport. In asthma, impaired mucus transport has been proposed to be caused by tethering of MUC5AC to the epithelium rather than by cilia dysfunction. Other studies suggest a negative impact of airway surface dehydration on mucus clearance. Even without an increase of mucin synthesis and goblet cell number, dehydration of constitutively secreted mucus alone is sufficient to cause mucus plugging. Apart from dehydration, impaired cleavage of mucins or extracellular DNA can increase mucus viscosity and therefore impair mucus clearance.
acute asthma, more cross-linking of mucin polymers is associated with reduced cough clearance, while mucin degradation is suppressed possibly due to inhibition of protease by excess plasma proteins. These mechanisms can contribute to ineffective mucus clearance leading to CMH in asthma and COPD.

Molecular mechanisms involved in mucus production
Various well-known transcription factors play a crucial role in mucin synthesis/secretion and cilia function. The first one is SAM pointed domain-containing ETS transcription factor (SPDEF). Expression of SPDEF is dependent on Signal transducer and activator of transcription 6 (STAT6). Goblet cells are absent in SPDEF-deficient mice, while SPDEF overexpression in club cells leads to goblet cell differentiation without proliferation, suggesting that club cells can function as a progenitor for goblet cells in these mice. The second transcription factor is Forkhead box protein A2 (FOXA2), a winged helix protein known to play an important role in embryonic development. FOXA2-deleted mice developed goblet cell hyperplasia and increased MUC5AC synthesis in the airways. Upon mucociliary differentiation of airway epithelial cells, SPDEF is upregulated while FOXA2 is downregulated, suggesting their opposite roles in mucociliary development. Another transcription factor involved in epithelial differentiation is Forkhead box protein J1 (FOXJ1) which plays an essential role in epithelial polarization and differentiation towards ciliated cells and is often used as a marker to assess ciliated cell differentiation in vitro. These molecular processes may be regulated by pro-inflammatory cytokines, which are also known to play a role in CMH pathophysiology. IL-13, for instance, is an important T cell-derived mediator of type 2 inflammatory responses in allergic asthma, which is also upregulated in COPD and is well known for its central role in regulating mucus production through induction of SPDEF. In addition,
IL-1 family members, e.g. IL-1α and IL-1β, can promote mucus hypersecretion. Inhibition of IL-1 receptor 1 (IL-1R1) in βENaC-overexpressing (βENaC-Tg) mice, which develop spontaneous mucus plugging associated with cystic fibrosis/COPD-like phenotypes, alleviates airway mucus obstruction. IL-1β acts on IL-1R1 and has been shown to stimulate MUC5AC and MUC5B expression in human airway epithelial cells via NF-κB activation.

**Role of stromal cells in chronic mucus hypersecretion**
Recent reports suggest that stromal cells, such as airway smooth muscle cells (ASMCs) and fibroblasts, play a crucial role in epithelial homeostasis and differentiation and therefore may also be involved in CMH development. ASMCs respond to various pro-inflammatory cytokines, which are secreted by other cell types including epithelial cells, e.g. IL-1β and TGF-β. Co-culturing tracheal epithelium with fibroblasts stimulates epithelial proliferation, mucin production and basement membrane formation in vitro. It is still unknown which mechanisms mediate this crosstalk and whether these findings are relevant in the context of airway diseases. Our group previously observed that during epithelial-fibroblast co-culture, epithelial-derived IL-1α increases CXCL8 and IL-6 production by fibroblasts. Furthermore, we observed a significant association between a polymorphism in the Frizzled (FZD) 8 region and CMH. FZD8 is a receptor for WNT growth factors, which are involved in lung development and regeneration as well as pro-inflammatory responses. Lung fibroblasts responded to epithelial-derived stimuli (IL-1 and EGF) by upregulating FZD8 expression and this upregulation was more pronounced in fibroblasts from patients with CMH than those without CMH. Fibroblasts derived from COPD patients with CMH, compared to ones without CMH, secreted higher levels of CXCL8 and IL-6 cytokines, which have been shown to promote MUC5AC production in differentiated epithelial cells. Together, these findings suggest a potential involvement of fibroblasts in CMH development. How abnormalities in the complex interplay between structural cells in the airways lead to CMH and which gene networks are involved is currently unknown.

**Role of microRNAs in chronic mucus hypersecretion and stromal cell-epithelium crosstalk**
As microRNAs (miRNA) play a key role in many cellular processes and have been implicated in a variety of diseases, it is likely that they also are involved in CMH pathogenesis. miRNAs are small non-coding RNA molecules that regulate messenger RNA (mRNA) expression at post-transcriptional level by targeting the 3’ untranslated region (3’ UTR) of their mRNA target, leading to mRNA degradation or translational inhibition. It is predicted that miRNAs regulate the expression of over 60% of all
genes in mammalian genome. Expression patterns of miRNAs in differentiated airway epithelial cells differ from those in basal cells. Several miRNAs have been shown to be associated with asthma or COPD. The expression of 7 miRNAs were higher and of 15 miRNAs were lower in bronchial brushings of asthma compared to healthy controls. IL-13 stimulation alters expression of several miRNAs in this list, including suppression of miR-34/449 family, consistent with lower expression in asthma. In addition, the expression of 5 miRNAs was higher and of 23 miRNAs, including miR-146a-5p, was lower in bronchial epithelium derived from current-smokers than from never-smokers. miRNAs may also be involved in the crosstalk between epithelial cells and fibroblasts. The induction of miR-146a-5p by pro-inflammatory cytokines was lower in primary lung fibroblasts from COPD patients compared to those from healthy controls. Interestingly, miR-146a-5p silencing promotes MUC5AC secretion by 16HBE cells.

Altogether, these findings led us to the hypothesis that abnormal stromal-epithelial crosstalk contributes to CMH pathophysiology and that miRNAs act as mediators of disturbed molecular mechanisms underlying abnormalities in mucociliary differentiation, pro-inflammatory responses, and stromal cell-epithelium crosstalk in CMH (figure 2).

Figure 2. Hypothesis overview.
The scope of this thesis

We started this thesis with a comprehensive review discussing the role of miRNAs and exosomes in asthma pathogenesis (chapter 2). As to date no studies on differential miRNA expression profiles in relation to CMH or chronic bronchitis have been reported, we applied an unbiased approach to identify novel candidate miRNAs potentially involved in CMH pathophysiology. Moreover, since most in vitro studies of CMH focused solely on airway epithelial cells, we developed co-culture models using patient-derived cells that take cell-cell interactions into consideration to shed new light on our current understanding of CMH regulatory mechanisms.

The aims of this thesis were to; 1) identify CMH-associated miRNAs and their associated biological pathways in COPD (Chapter 3) and asthma (Chapter 4) using miRNA and mRNA expression profiles of patient-derived bronchial biopsies; 2) to investigate whether and how COPD patient-derived fibroblasts promote mucin secretion and mucociliary differentiation by airway epithelial cells from COPD patients with CMH using a long-term air-liquid interface (ALI) co-culture model (Chapter 5); 3) to assess the expression of selected CMH-associated miRNAs identified in bronchial biopsies in COPD patient-derived airway fibroblasts and epithelial cells co-cultured at ALI (Chapter 6); 4) to elucidate the function of miR-146a-5p in disturbed fibroblast-epithelial cell crosstalk in COPD using a submerged co-culture model of lung fibroblasts and airway epithelial cells from controls and COPD patients (Chapter 7), and 5) to compare gene expression profiles of asthma- and control-derived ASMCs and to determine the subsequent role of a selected soluble factor on mucin production using ALI-cultured Calu-3 bronchial adenocarcinoma cells and primary airway epithelial cells (Chapter 8).
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General introduction


