Chronic mucus hypersecretion in COPD and asthma

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CHAPTER 6

Involvement of miRNAs associated with chronic mucus hypersecretion in fibroblast-epithelium crosstalk in COPD

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ABSTRACT

We recently showed that airway fibroblasts derived from chronic obstructive pulmonary disease (COPD) patients promote epithelial mucin secretion and mucociliary differentiation, and thus speculate that this crosstalk may underlie chronic mucus hypersecretion (CMH) development in COPD (chapter 5). We hypothesized that microRNAs (miRNAs) are involved in this fibroblast-epithelium crosstalk.

The present study aimed to identify which of our previously identified CMH-associated miRNAs are differentially expressed in primary bronchial epithelial cells (PBECs) and primary airway fibroblasts (PAFs) upon co-culture. PBECs from one stage IV COPD patient with CMH were cultured at air-liquid interface with and without PAFs from 6 stage IV COPD patients with CMH. Total RNA from both cell types was harvested separately and used for assessing the expression of selected candidate miRNAs with qPCR.

The miRNAs positively associated with CMH, i.e. let-7a-5p, miR-31-5p, and miR-708-5p, were detected in both PBECs and PAFs. For miRNAs negatively associated with CMH, miR-146a-5p and miR-193-5p were detected in both PBECs and PAFs, while miR-134-5p was only detected in PAFs. In PAFs, let-7a-5p and miR-146a-5p expression was significantly higher upon co-culture, and there was a trend for higher expression of miR-31-5p ($p=0.0625$). In PBECs, we observed a trend for higher miR-708-5p expression upon co-culture ($p=0.0625$).

The significantly higher expression of let-7a-5p and miR-146a-5p in airway fibroblasts upon co-culture with airway epithelial cells from COPD patients with CMH suggests their involvement in fibroblast-epithelium crosstalk. These miRNAs may thus serve as candidates for future studies that aim to elucidate the mechanisms of this crosstalk in CMH.

INTRODUCTION

One of the characteristics of chronic obstructive pulmonary disease (COPD) is chronic mucus hypersecretion (CMH), which is characterized by the presence of chronic inflammation, chronic cough and exaggerated sputum production[1]. microRNAs (miRNAs) are main regulators of many processes in cells and tissues and can be expected to also play a role in the pathogenesis of CMH. miRNAs are small non-coding RNA molecules consisting of approximately 22 nucleotides which post-transcriptionally regulate gene expression by inducing mRNA degradation or inhibiting protein translation[2]. Over 60% of mammalian genes are predicted to be targeted by miRNAs[2]. They have been reported to be involved in various respiratory diseases, including COPD[3]. Recently, we identified 10 miRNAs that are associated with CMH in COPD using miRNA gene expression profiles of bronchial biopsies[4]. Among these miRNAs, the expression of let-7a-5p, let-7d-5p, let-7f-5p, miR-31-5p, and miR-708-5p was higher with CMH and the expression of miR-134-5p, miR-146a-5p and miR-193-5p, miR-500a-3p, and miR-1207-5p was lower with CMH.

It is not yet clear in which cell types these miRNAs are active and how these miRNAs contribute to CMH. We recently demonstrated that the co-culture of primary bronchial epithelial cells (PBECs) with primary airway fibroblasts (PAFs) leads to more mucociliary differentiation and more secretion of MUC5B and MUC5AC mucins (chapter 5). These findings suggest that aberrant fibroblast-epithelial cell crosstalk may contribute to CMH development in COPD. Thus, we hypothesized that CMH-associated miRNAs previously identified in bronchial biopsies[4] are involved in aberrant fibroblast-epithelial cell crosstalk in CMH. This study aims to identify which CMH-associated miRNAs are involved in fibroblast-epithelium crosstalk in COPD using a long-term co-culture model in which PBECs are co-cultured with PAFs for 1 week during ALI differentiation.

METHODS

Air-liquid interface (ALI) culture

PBECs were isolated from explanted lungs of one COPD stage IV patient with CMH undergoing lung transplantation, as previously described[5]. PAFs were isolated from 6 COPD stage IV patients with CMH as previously published[6]. The patient characteristics are described in Table 1. The presence of CMH was defined by the patients' clinical records. PBECs were grown in Keratinocyte Serum-Free Growth Medium (KSFM, Gibco, NY, USA) supplemented with 1% Penicillin (10,000 U/mL)/ Streptomycin (10,000 µg/mL) (P/S) (Gibco, California, USA), 1 µM isoproterenol, 25 ng/ml bovine pituitary extract (BPE, Gibco) and 2.5 µg/ml epidermal growth factor (EGF, Gibco). PAFs were grown in HAMs' F12 medium supplemented with 1% pennicilin/streptomycin, 1% L-glutamine (Lonza, Switzerland) and 10% fetal bovine serum (FBS, Sigma-Aldrich, Germany). Cells were passaged at 90% confluence. In all experiments, we used PBECs in passage 3 and PAFs in passage 4-6.

ALI culture of PBECs was performed as previously described7. Transwell inserts for 24-well plates (Corning®, NY, USA) were coated with 10 µg/ml bovine serum albumin (BSA; Sigma-Aldrich, MO, USA), 10 µg/ml fibronectin (Sigma-Aldrich, MO, USA) and 30 µg/ml collagen (PureCol®, Advanced Biomatrix, San Diego, CA, USA) in Eagle's Minimum Essential Medium (EMEM, Lonza, Walkersville, MD, USA) before being used to culture PBECs. PBECs from each donor were seeded at a density of 75,000 cells/insert in ALI culture medium prepared by mixing DMEM (LONZA BE12-709F) and BEBM (Clonetics CC-3171) in 1:1 ratio supplemented with a set of BEGM Single Quots (Clonetics CC-4175) and 1.5µg/ml BSA (Sigma-Aldrich) and 15 ng/ml retinoic acid (Sigma-Aldrich). After 4-5 days, the cells were air-exposed for 14 days (day 0-14) during which the basal medium was refreshed every 2-3 days. PAFs seeded in 24-well plates in HAMs' F12 medium until 70-80% confluent were co-cultured with PBECs on the inserts from day 7 to day 14 using ALI culture medium. At the end of day 14, total RNA from PBECs and PAFs was collected separately. This experimental design is demonstrated in figure 1.

RNA isolation and qPCR

Total RNA was collected and isolated using Tri Reagent® according to the manufacturer's protocol. To assess miRNA expression, total RNA was converted to cDNA using the TaqMan microRNA reverse transcription kit (Life Technologies, Bleiswijk, Netherlands) and reverse transcription primers (Life Technologies) for let-7a-5p (assay id: 000377), miR-31-5p (002279), miR-134-5p (000459), miR-146a-5p (000468), miR-193a-5p (002281), and miR-708-5p (002341). qPCR was performed using LightCycler® 480 Probes Master according to the manufacturer's guidelines

(Roche, Switzerland). Expression of all miRNAs was normalized to the expression of small nuclear RNA, RNU48 (001006).

Table 1. Patient characteristics

donor	age	gender	pack-years	FEV1 (%predicted)	FEV1/FVC
E1	58	m	35	15	0.19
F1	53	f	40	23	0.26
F2	48	f	30	12	0.26
F3	59	m	40	15	0.29
F4	61	f	30	16	0.19
F5	58	m	35	15	0.19
F6	59	m	47	15	0.21

All patients are ex-smokers with COPD stage IV with CMH. E1 is PBEC donors; F1-F6 are PAF donors; FEV1 is forced expiratory volume in 1 second; FVC is forced vital capacity; CMH is chronic mucus hypersecretion defined by clinical records; m is male, f is female.

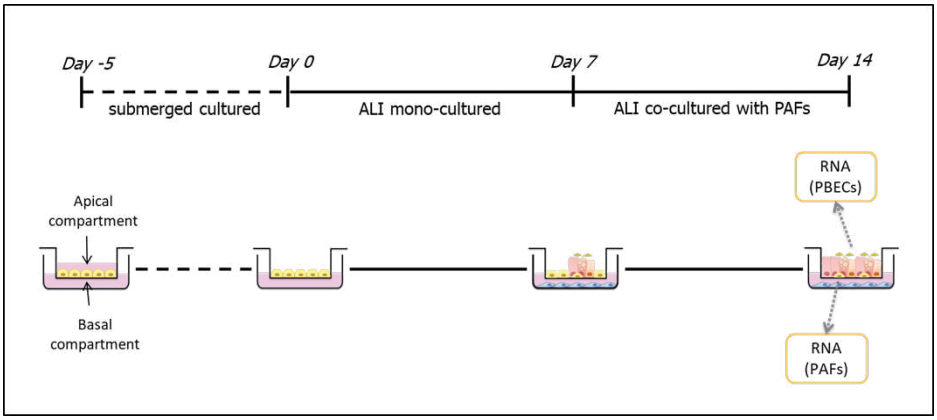

Figure 1. Co-culture of primary bronchial epithelial cells (PBECs) and primary airway fibroblasts (PAFs) at air-liquid interface (ALI). PBECs were submerged-cultured for 5 days before air-exposed for 2 weeks (day 0-14), during which the basal medium was refreshed every 2-3 days. On day 7, PAFs on basal compartments were co-cultured with PBECs for the next 7 days. On day 14, RNA from both cell types were collected separately for gene expression assessment.

Statistical analyses

One-sample Wilcoxon signed rank test was used to determine significant differences between mono-cultured and co-cultured PBECs. Paired-samples Wilcoxon signed rank test was used for comparisons between mono-cultured and co-cultured PAFs. All statistical analyses were performed on GraphPad PRISM v7.

RESULTS

To determine which of the 10 CMH-associated miRNAs previously identified in bronchial biopsies4 (Table 2) are likely involved in fibroblast-epithelial cell crosstalk, we assessed whether the expression of any of these miRNAs changes upon co-culture. Since let-7a-5p, let-7d-5p, and let-7f-5p are from the same miRNA cluster of which seed sequences are very similar, sharing several potential targets4, let-7a-5p was selected as a representative of the let-7 family. Since we previously observed that miR-500a-3p and miR-1207-5p were neither expressed in PBECs nor PAFs4, we did not include them in the current study.

Table 2. CMH-associated miRNAs identified in bronchial biopsies of COPD patients

miRNA positively associated with CMH	miRNA negatively associated with CMH
let-7a-5p	miR-134-5p
let-7d-5p	miR-146a-5p
let-7f-5p	miR-193a-5p
miR-31-5p	miR-500a-3p
miR-708-5p	miR-1207-5p

When assessing the expression of let-7a-5p, miR-31-5p, miR-134-5p, miR-146a-5p, miR-193a-5p, and miR-708-5p in mono-cultured and co-cultured PBECs and PAFs, we observed that in PAFs, all miRNAs were expressed, with a significantly higher expression of let-7a-5p and miR-146a-5p upon co-culture with PBECs and a trend for higher expression of miR-31-5p ($p=0.0625$) (figure 2A). In PBECs, all miRNAs except miR-31-5p were detected. miR-708-5p expression tended to increase upon co-culture with PAFs ($p=0.0625$), while no change was observed for expression of other miRNAs (figure 2B).

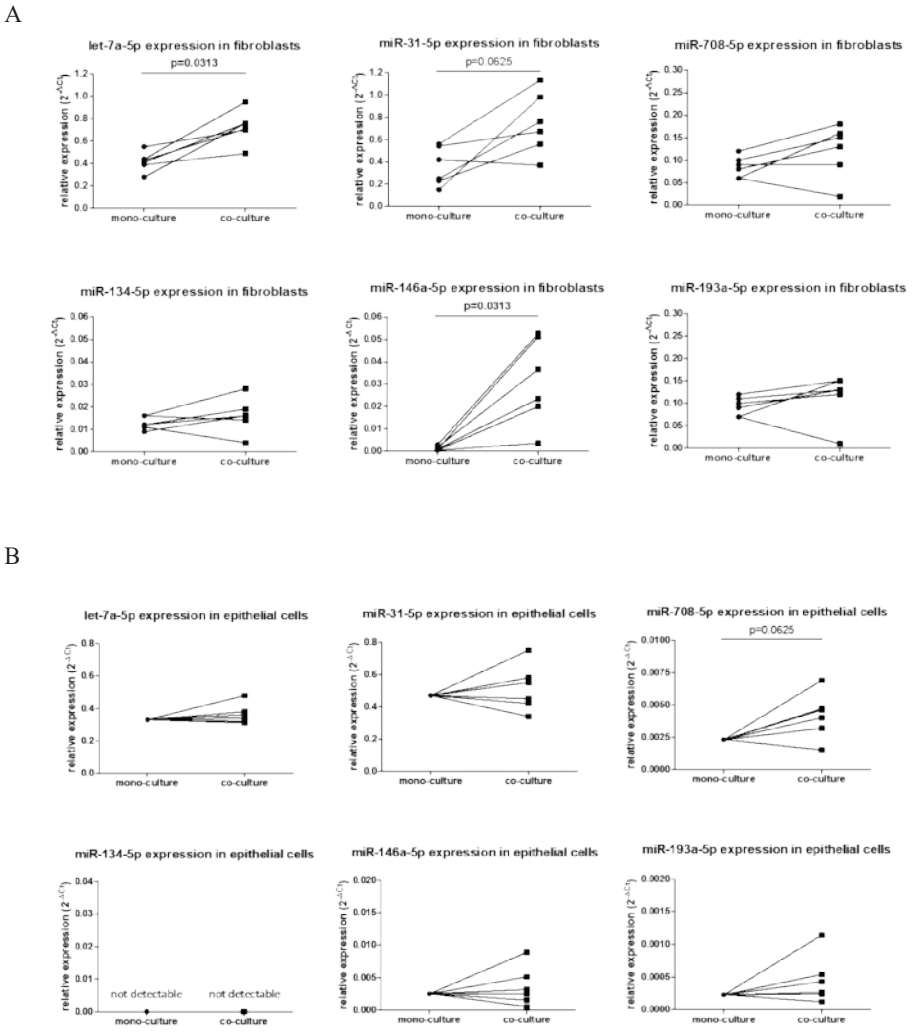

Figure 2. miRNA expression upon co-culture. Primary bronchial epithelial cells (PBECs) from a COPD patient with CMH were differentiated at air-liquid interface (ALI) for 14 days. From day 7 to day 14, the cells were continued growing in mono-culture or in co-culture with primary airway fibroblasts (PAFs) from 6 COPD patients with CMH before total RNA was collected from each cell types separately. Each condition was performed in duplicates. [A] Expression in PAFs. [B] Expression in PBECs. Expression of all miRNAs was normalized to the expression of small nuclear RNA, RNU48. Significant difference was determined by paired-samples Wilcoxon signed rank test for the expression in PAFs and by one-sample Wilcoxon signed rank test for the expression in PBECs.

DISCUSSION

In this study, we show that let-7a-5p and miR-146a-5p expression significantly increased in COPD-derived airway fibroblasts upon co-culture with COPD-derived airway epithelial cells, while miR-31-5p expression tended to increase as well. In the epithelial cells, miR-708-5p tended to increase after co-culturing with the fibroblasts, while no significant differences were observed for any of the other miRNAs. The increased expression of these CMH-associated miRNAs upon co-culture suggests that they are involved in fibroblast-epithelial cell crosstalk, which may be dysregulated in COPD patients with CMH.

We previously demonstrated that COPD patient-derived airway fibroblasts promote and mucous cell differentiation and mucin secretion in COPD patient-derived bronchial epithelial cells during ALI culture (Chapter 5). Using the same co-culture model, our findings suggest that CMH-associated miRNAs of which expression increased in the co-cultured fibroblasts may be involved in epithelial differentiation into mucus-producing cells and/or the regulation of mucin secretion. The expression of let-7a-5p increased in the co-cultured fibroblasts, and its associations with CMH in bronchial biopsies was also positive (i.e. let-7a-5p levels were higher with moderate/severe CMH compared to no-CMH controls) (Chapter 3)[4].

Little is known about the role of let-7a-5p in relation to CMH development and fibroblast-epithelial cell crosstalk. Kumar et al. showed that intranasal administration of let-7 miRNA mimic suppresses IL-13 and attenuates mucus production in bronchial biopsies (Chapter 3)[8]. It is possible that let-7a-5p upregulation in fibroblasts suppresses mucin secretion in an allergic mouse model, but a biological function of this miRNA was not shown in their report8. Since we did not observe increased expression of let-7a-5p in the co-cultured epithelial cells, let-7a-5p is more likely to be involved in a fibroblast response to the epithelial cells than directly in the increased epithelial mucus secretion observed upon co-culture with fibroblasts. Of interest, COL4A1 and COL4A2, crucial components of the basement membrane expressed by lung fibroblasts9, are predicted targets that were negatively correlated with CMH in bronchial biopsies (Chapter 3)[4]. It is possible that let-7a-5p upregulation in fibroblasts leads to lower collagen 4 production and this change in extracellular matrix (ECM) composition might consequently stimulate airway epithelium to secrete more mucus, or the other way around. This would require further investigation.

In contrast to let-7a-5p, miR-146a-5p expression was lower with CMH. We also showed that miR-146a-5p was less upregulated upon co-culture in COPD compared to control fibroblasts and proposed that this miRNA functions as a negative feedback to suppress pro-inflammatory responses, i.e. IL-6 and CXCL8 secretion (Chapter 7)[10]. A similar role was also described in another study showing that miR-146a-5p expression is upregulated by IL-1β and miR-146a-5p overexpression suppresses CXCL8 release11. Whether this miR-146 feedback mechanism is also

impaired in CMH remains to be studied. In another study, we found that both IL-6 and CXCL8 promote mucin secretion by airway epithelial cells upon differentiation at ALI culture7, and in chapter 5 we demonstrate that IL-6 is involved in increased airway epithelial MUC5B expression upon co-culture with CMH-derived fibroblasts.

Although there was just a trend of miR-31-5p upregulation in co-cultured fibroblasts and miR-708-5p upregulation in co-cultured epithelial cells, these two miRNAs are also of interest. miR-31-5p is positively associated with CMH in both asthma and COPD, suggesting that it may be a component of a shared mechanism between CMH in both diseases (Chapter 4). An interesting predicted target of miR-31-5p is ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 2 (ST3GAL2), a member of sialyltransferases which may facilitate sialylation of the core mucin structure (Chapter 4) and consequently alter the viscosity of mucus. Higher mucus viscosity means worse clearance contributing to accumulation of mucus in the airways12. Since mucins are synthesized in epithelial cells, future experiments could evaluate whether fibroblasts secrete miR-31-5p to be taken up by the epithelial cells. The expression of miR-708-5p, on the other hand, was previously shown to be negatively associated with mucociliary differentiation at ALI13, suggesting that it may negatively regulate mucociliary differentiation.

The set-up of this experiment included one epithelial cell donor and six fibroblast donors which aimed at determining fibroblast-derived factors that influence the crosstalk to promote epithelial mucus secretion. To determine epithelial cell-derived factors influencing fibroblasts or being influenced by this crosstalk, more epithelial cell donors should be included in the future. Overall, this study provides candidate miRNAs that may be involved in fibroblast-epithelial cell crosstalk and contribute to CMH development in COPD.

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