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Chronic mucus hypersecretion in COPD and asthma

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CHAPTER 7

MiR-146a-5p plays an essential role in the aberrant epithelial-fibroblast crosstalk in COPD

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ABSTRACT

We previously reported that epithelial-derived IL-1 α drives fibroblast-derived inflammation in the lung epithelial-mesenchymal trophic unit. Since miR-146a-5p has been shown to negatively regulate IL-1 signalling, we investigated the role of miR-146a-5p in the regulation of IL-1 α -driven inflammation in chronic obstructive pulmonary disease (COPD).

Human bronchial epithelial (16HBE14o-) cells were co-cultured with control and COPD -derived lung fibroblasts (PHLFs) and MiR-146a-5p expression was assessed with and without IL-1 α neutralizing antibody. Genomic DNA was assessed for the presence of the Single Nucleotide Polymorphism (SNP) rs2910164. MiR-146a-5p mimics were used for over-expression studies to assess IL-1 α -induced signalling and IL-8 production by PHLFs.

Co-culture of PHLFs with AECs significantly increased the expression of miR-146a-5p, and this induction was dependent on epithelial-derived IL-1 α . MiR-146a-5p over-expression decreased IL-1 α -induced IL-8 secretion in PHLFs via down-regulation of IRAK-1. In COPD PHLFs, the induction of miR-146a-5p is significantly less compared to controls, and was associated with the SNP, rs2910164 (GG allele), in the miR-146a-5p gene.

Our results suggest that induction of miR-146a-5p is involved in epithelial-fibroblast communication in the lungs, and negatively regulates epithelial-derived-IL-1 α induction of IL-8 by fibroblasts. The decreased levels of miR-146a-5p in COPD fibroblasts may induce a more pro-inflammatory phenotype, contributing to chronic inflammation in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive disease in which chronic neutrophilic inflammation is associated with destruction of the lung parenchyma (emphysema) and small airway disease, which both contribute to airflow limitation and lung function decline¹. The inhalation of noxious particles, specifically from cigarette smoke, is the most common risk factor for COPD and smoking is known to induce the recruitment of neutrophils^{1,2}. However, not all smokers develop COPD, indicating a genetic susceptibility to the disease process³. The inconsistency in replicating target genes related to COPD in different study populations points to an important role of epigenetic regulation⁴. Epigenetic mediators such as miRNAs can regulate the transcriptional activity of various genes involved in lung function and inflammation that are thought to be involved in the pathogenesis of COPD⁵.

MicroRNAs (miRNAs) are small noncoding RNAs of approximately 19 to 25 nucleotides that cause post-transcriptional gene repression by increasing mRNA degradation or by inhibiting protein translation of specific mRNA targets⁶. They are involved in various biological processes and alterations in their expression can result in pathological conditions, including pulmonary diseases⁷. We recently provided an up-to-date review of studies showing the role of miRNA dysregulation in COPD and how it is associated with the various features of COPD⁵. In particular, it has been shown that exposure to cigarette smoke alone can change miRNA expression in the lungs⁸. Several studies have shown that various miRNAs are differentially expressed in whole lung tissue, serum and/or sputum of smoking COPD patients compared to smokers without COPD⁹⁻¹². Of these, miR-146a-5p has been shown to regulate the release of interleukin (IL)-1-induced inflammatory mediators from pulmonary epithelial cells, including the neutrophil chemo-attractant IL-8¹³. We recently showed in a co-culture model that cigarette smoke extract (CSE)-induced IL-1 α expression, is higher in airway epithelial cells from COPD patients compared to control-derived epithelial cells¹⁴. Further, the higher levels of epithelial IL-1 α induce a stronger release of the pro-inflammatory cytokines including IL-8 from lung fibroblasts¹⁴. Interestingly, others have shown that when lung fibroblasts are stimulated with IL-1 β and Tumour necrosis Factor- α , miR-146a-5p expression is induced to a lesser extent in lung fibroblasts from COPD patients when compared to control fibroblasts¹².

In this study, we hypothesized that a failure to upregulate miR-146a-5p in lung fibroblasts contributes to the disturbed communication within the epithelial-mesenchymal trophic unit in COPD, leading to increased inflammation in the disease. Therefore we studied the expression of miR-146a-5p in COPD and non-COPD control -derived primary human lung fibroblasts (PHLFs) in our co-culture model, and conducted functional assays to investigate the mechanism of regulation of pro-inflammatory activity by miR-146a-5p in pulmonary fibroblasts.

METHODS

Subjects and cell culture conditions

The human bronchial epithelial cell line 16HBE14o- was kindly donated by Dr D.C. Gruenert (University of California, San Francisco, CA) and cultured as previously described (1) in Eagle's minimal essential medium (EMEM)/10% Fetal Calf Serum (Lonza, BioWhittaker®, Verviers, Belgium) on collagen/BSA-coated flasks.

Fetal lung fibroblast cells (MRC-5, BioWhittaker) were cultured in EMEM/10% FCS on 24 well plates before experiments. Primary human lung fibroblasts (PHLFs) were isolated from peripheral parenchymal lung tissue of 8 non-COPD control donors undergoing tumour resection surgery and 12 COPD patients with severe disease undergoing lung transplantation using the explant technique as previously described [15, 16]. PHLFs from controls were isolated from histologically normal tissue taken as far away as possible from the tumour. The tissue was checked for cancerous abnormalities by an experienced pathologist and found to be cancer-free. Clinical information on the COPD patients and non-COPD control subjects is presented in table 1. The study protocol for this project was consistent with the Research Code of the University Medical Center Groningen (<http://www.rug.nl/umcg/onderzoek/researchcode/index>) and national ethical and professional guidelines (<http://www.federa.org>). PHLFs were cultured in HAMS-F12 medium/10% FCS (Lonza) on 24 well culture plates before experiments.

Co-culture model

We used 16HBE14o- and MRC-5 co-cultures for the mechanistic studies. 16HBE14o- cells were co-cultured with COPD and control-derived PHLFs to determine the disease-specific effects.

Briefly, 16HBE14o- cells were plated on coated 0.4µM pore 6.5mm transwell membranes (Costar, Corning Inc., New York, NY), while fibroblasts were cultured separately on a 24 well plate. After a confluent layer was obtained for both cell types, the transwell with 16HBE14o- cells (upper compartment) was placed in co-culture with lung fibroblasts in the 24 well-plate (lower compartment) and left for 72 hours in EMEM/10% FCS or HAMS-F12 medium/10% FCS (Lonza) for co-culture with MRC-5 cells or PHLFs respectively¹⁴. Before experimentation, cells were serum-deprived overnight.

Cigarette smoke extract (CSE) preparation

CSE experiments were performed in serum-free, hormone-supplemented media (Lonza). CSE was prepared before each experiment by bubbling 2 filter-less cigarettes through 25ml of media to make 100% CSE, which was then diluted to 20% CSE as

previously described¹⁷.

Conditioned medium and neutralizing antibody experiments

16HBE14o- cells were serum-deprived overnight and stimulated for 6 hours with or without 20% CSE, which we have previously reported did not affect cell viability [14]. After stimulation, the CSE was thoroughly washed off and CSE-free conditioned medium (CM) was collected after a further 24 hour incubation. CSE-free CM was pre-incubated for 1 hour with or without 4µg/ml IL-1α neutralizing antibody (AB-200-NA, MAB601, R&D Systems, Abingdon, UK) and was used to stimulate serum-deprived fibroblasts for 24 hours. For experiments with recombinant human (Rh) IL-1α (R&D Systems), a 1ng/ml concentration of rhIL-1α (R&D Systems) was chosen which is comparable to levels previously reported in sputum of COPD patients [18]. Cell-free supernatants were collected and analyzed by ELISA and cell lysates were harvested with TRIreagent for RNA isolation.

MiR-146a-5p mimic transfection

MRC-5 fibroblasts were transfected with the miR-146a-5p mimic at 25nM (MirVana miRNA mimic, assay ID= MC10722; Life Technologies) and scrambled small RNA at 25nM (AllStars Negative Control siRNA; QIAGEN) as a non-targeting control to assess the effects of miR-146a-5p induction on IL-1 signaling in lung fibroblasts. MiR-146a-5p expression was analyzed by qPCR, protein lysates were assessed by Western blot and IL-8 concentrations were determined by ELISA (R& D) in the cell-free supernatant as described in the online attachment.

SNP Genotyping

Genomic DNA for genotyping was extracted from lung tissue of PHLF donors assessed for the presence of the SNP rs2910164. A detailed description is available on the online supplement.

Statistical analysis

SPSS software was used for data analyses. Differences between COPD patients and non-COPD control subjects were analyzed with a Mann-Whitney U test. Differences between treatments within a group were analyzed using paired Students t-tests for the cell lines and Wilcoxon signed-rank tests for the primary cells. P<0.05 was considered statistically significant.

Table 1. Characteristics of COPD patients and non-COPD controls from whom primary human lung fibroblasts (PHLFs) were obtained

| No. | Age (years) | Gender | Smoking status | Pack-years | FEV ₁ % predicted | FEV1/FVC % | Experiment |
|-----------------------|-------------|--------|----------------|------------|------------------------------|------------|----------------|
| Control-derived PHLFs | | | | | | | |
| 1 | 74 | M | ES | 50 | 100 | 71 | Co-culture |
| 2 | 50 | M | ES | 31 | 97 | 78 | Co-culture |
| 3 | 65 | M | ES | 40 | 97 | 76 | Co-culture&CM |
| 4# | 68 | M | ES | 51 | NA | 74 | Co-culture&CM |
| 5 | 67 | F | NS | 0 | 101 | 81 | Co-culture& CM |
| 6 | 65 | F | CS | 38 | 98 | 76 | Co-culture&CM |
| 7 | 46 | M | ES | 32 | 97 | 82 | CM |
| 8\$ | 60 | M | ES | NA | 85 | NA | CM |
| COPD-derived PHLFs | | | | | | | |
| 7 | 58 | F | ES | 30 | 18 | 28 | Co-culture |
| 8 | 60 | M | ES | 30 | 37 | 49 | Co-culture |
| 9 | 62 | M | ES | 44 | 22 | 19 | Co-culture |
| 10 | 48 | M | ES | 27 | 12 | 23 | Co-culture |
| 11 | 57 | F | ES | 33 | 25 | 33 | Co-culture |
| 12 | 44 | M | ES | 25 | 60 | 50 | Co-culture |
| 13 | 44 | M | ES | 27 | 14 | 28 | CM |
| 14 | 56 | M | ES | 38 | 23 | 35 | CM |
| 15 | 53 | F | ES | 40 | 24 | 26 | CM |
| 16 | 52 | M | ES | 20 | 18 | 62 | CM |
| 17 | 51 | M | ES | 42 | 28 | 29 | CM |
| 18 | 59 | F | ES | 37 | 21 | 21 | CM |

FEV₁= Forced expiratory volume in 1 second, FVC= Forced Vital Capacity, F=female M= male CS= current smoker, ES= ex-smoker, NS= never smoker. #FEV₁% Predicted not available, \$Pack years and FEV₁/FVC not available. CM= conditioned medium. All COPD donors were on inhaled or oral steroids before transplantation. There was a significant difference in FEV₁%predicted (p=0.04) and FEV₁/FVC% (p=0.04), as well as in age between the COPD patients and controls, with the COPD patients being slightly younger (p=0.03). There was no significant difference in pack-years between the groups.

RESULTS

Epithelium-derived IL-1 α is responsible for the increased miR-146a-5p expression in lung fibroblasts

As our previous study showed that IL-1 α derived from the airway epithelium (primary and 16HBE14o- cells) is an important regulator of pulmonary fibroblast-derived inflammation [14], we first determined whether epithelial-derived IL-1 α causes an induction of miR-146a-5p in lung fibroblasts. We stimulated PHLFs from non-COPD controls and COPD patients with 16HBE14o- conditioned medium whose IL-1 α levels had been previously determined [14] in the presence or absence of the IL-1 α neutralizing antibody. We found that the expression of miR-146a-5p was increased in PHLFs treated with conditioned media from 16HBE14o- cells, and this was further enhanced by pre-stimulation of the 16HBE14o- cells with cigarette smoke extract (CSE) (figure 1a). Furthermore, the induction of miR-146a-5p was completely abrogated by the addition of IL-1 α neutralizing antibody (figure 1a). We also assessed the direct effects of rh-IL-1 α stimulation on miR-146a-5p expression in PHLFs, and found as with the 16HBE14o- cell conditioned media, a significant increase in miR-146a-5p expression in PHLFs (figure 1b). Lastly, stimulation with 1ng/ml (figure 1c) or 0.01ng/ml (supplemental Figure 1) rh-IL-1 α led to a significant release of IL-8 concentration from PHLFs (figure 1c), which significantly correlated with the increased expression of miR-146a-5p in PHLFs (figure 1d). Neither the expression level of miR-146a-5p nor the release of IL-8 was significantly different between COPD and control-derived PHLFs after 16HBE14o- CM or rh-IL-1 α stimulations.

MiR-146a-5p expression is decreased in primary lung fibroblasts from COPD patients in co-culture with 16HBE14o- cells

We have previously shown in our co-culture model that COPD-derived airway epithelial cells, through higher induction of IL-1 α , induce lung fibroblasts to be more pro-inflammatory [14]. Hence, we were interested in the regulation of miR-146a-5p expression in control and COPD-derived PHLFs. In our co-culture model, 16HBE14o- cells significantly upregulated miR-146a-5p expression in PHLFs from both control and COPD donors. Interestingly, there was a significantly lower induction of miR-146a-5p in COPD-derived PHLFs compared to those from control donors upon co-culture with 16HBE14o- cells (figure 2).

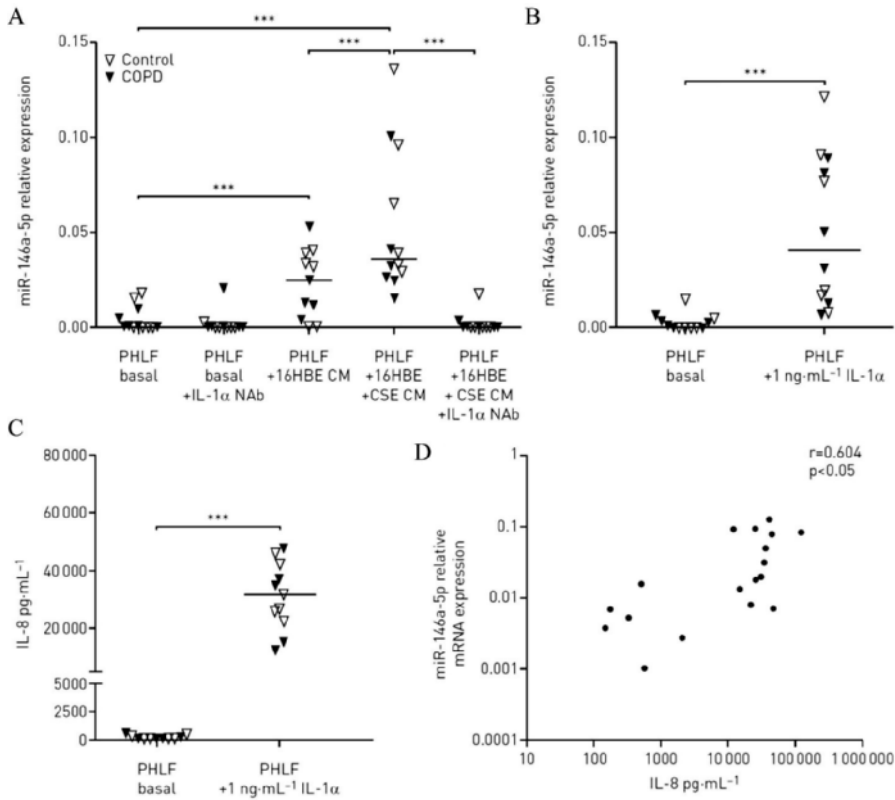


Figure 1. Epithelial-derived IL-1 α is responsible for increased miR-146a-5p expression in lung fibroblasts. PHLFs from control donors (open triangles, n=6) and COPD patients (closed triangles, n=6) were grown to confluence and serum deprived overnight. [A] miR-146a-5p (with median) expression in PHLFs after stimulating with conditioned medium (CM) from 16HBE14o- cells pre-treated with or without cigarette smoke extract (CSE) in the presence or absence of 4 μ g/ml IL-1 α neutralizing antibody (NAb). [B] MiR-146a-5p expression levels in serum-deprived PHLFs after stimulation with/without 1ng/ml recombinant human IL-1 α for 24 hours. MiR-146a-5p expression levels were related to the housekeeping non-coding RNA, RNU48 and expressed as 2-dCt. [C] IL-8 concentration (with median) released from PHLFs and [D] Correlation of IL-8 concentration released from PHLFs to miR-146a-5p expression in PHLFs after IL-1 α stimulation. Data are shown in a Log scale. *** and ### =p<0.001 between the indicated values.

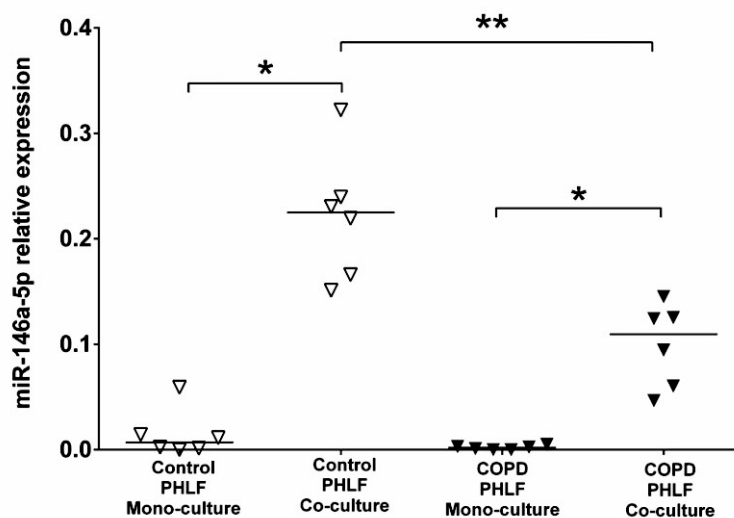


Figure 2. Expression of miR-146a-5p in primary human lung fibroblasts upon co-culture with 16HBE140- cells. Primary human lung fibroblasts from control donors or COPD patients were cultured alone or co-cultured with 16HBE140- cells for 72 hours after which they were serum deprived. miR-146a-5p expression in primary human lung fibroblasts from control donors (open triangles) and COPD patients (closed triangles) in mono-culture and co-culture with 16HBE140- cells was related to the housekeeping non-coding RNA, RNU48 and expressed as 2-DCt. **= $p < 0.01$ and *= $p < 0.05$ between the indicated values.

Lower induction of miR-146a-5p in COPD fibroblasts is associated with single nucleotide polymorphism (SNP) rs2910164

To explain the difference in miR-146a-5p induction between COPD and control fibroblasts we considered two possibilities. The first was a difference in RelB expression, a member of the nuclear factor (NF)- κ B family of transcription factors, since it has been shown to be responsible for the up-regulation of miR-146a-5p in PHLFs [19]. The second possibility was a difference in the presence of the single nucleotide polymorphism (SNP) rs2910164 in the primary mir-146a-5p sequence as this SNP has been shown to cause a reduction in the expression of mature miR-146a-5p [20].

First, we examined the expression of RelB in our PHLFs from the co-culture model and we found no significant difference in the expression of RelB between control and COPD-derived PHLFs (figure 3a). Secondly, we compared the genotypes for the rs2910164 SNP of the PHLFs used in our co-culture experiments and found that donors with the GG genotype had a lower miR-146a-5p induction after co-culture than those with the CG genotype (figure 3b).

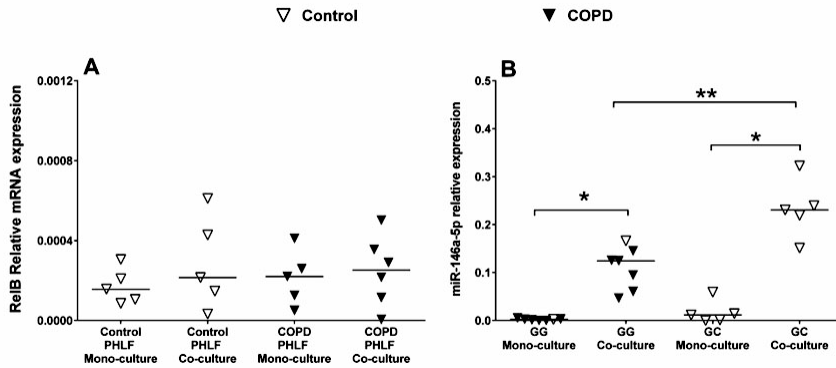


Figure 3. Effect of RelB expression and rs2910164 polymorphism on miR-146a-5p expression in co-culture. Primary human lung fibroblasts (PHLFs) from control donors or COPD patients were cultured alone or co-cultured with 16HBE14o- cells for 72 hours after which they were serum deprived. [A] RelB expression in PHLFs from control donors (open triangles) and COPD patients (closed triangles) in mono-culture and co-culture with 16HBE14o- cells. [B] Effect of the single nucleotide polymorphism rs2910164 on miR-146a-5p expression in PHLFs before and after co-culture with 16HBE14o- cells. mRNA levels were normalized to the housekeeping genes β 2-microglobulin and protein phosphatase 1 α , and expressed as $2^{-\Delta Ct}$. *= $p < 0.05$ and **= $p < 0.01$ between the indicated values.

MiR-146a-5p over-expression has anti-inflammatory effects on lung fibroblasts

MiR-146a-5p has been reported to exert anti-inflammatory properties by targeting the proteins of interleukin 1 receptor-associated kinase (IRAK)-1 and TNF receptor-associated factor (TRAF)-6, which are key downstream mediators in the cellular response to IL-1²¹. Hence, we were interested in examining the anti-inflammatory mechanism of miR-146a-5p in human lung fibroblasts. For over-expression of miR-146a-5p experiments we used a human lung fetal fibroblast cell line (MRC-5). First, we examined the expression of miR-146a-5p in MRC-5 fibroblasts co-cultured with 16HBE14o- cells and found a similar induction of miR-146a-5p in MRC-5 fibroblasts upon co-culture (figure 4a) as in the PHLFs. Next, we successfully over-expressed miR-146a-5p levels in MRC-5 fibroblasts by treatment with the miR-146a-5p mimic compared to the scrambled non-targeting control mimic (figure 4b). We then assessed the protein expression levels IRAK-1 and TRAF-6 in MRC-5 fibroblasts after over-expressing miR-146a-5p. We found the protein expression of IRAK-1 was significantly reduced after miR-146a-5p over-expression compared to scrambled control, but TRAF-6 was unaffected (figures 4c & 4d). This indicates that miR-146a-5p indeed regulates IL-1 receptor downstream signaling in human lung fibroblasts.

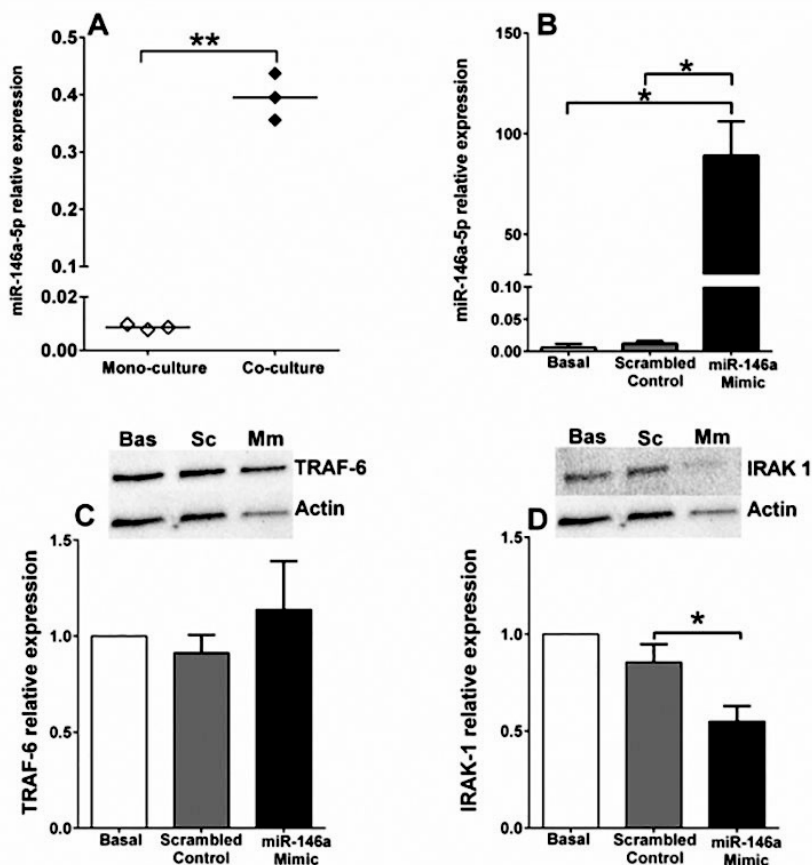


Figure 4. miR-146a-5p has anti-inflammatory effects in lung fibroblasts. [A] MRC-5 fibroblasts were cultured alone or co-cultured with 16HBE14o- cells. miR-146a-5p expression in the fibroblasts was related to the housekeeping non-coding RNU48, expressed as 2-DCt (median indicated). [B] MRC-5 fibroblasts were seeded and immediately transfected with 25nM of miR-146a-5p mimic or the scrambled control for 48 hours. MiR-146a-5p expression in the fibroblasts was related to the housekeeping non-coding RNU48, expressed as 2-DCt, and presented as mean ± SEM (n=3/4). [C] TRAF-6 protein expression with representative blot and densitometry. [D] IRAK-1 protein expression with respective representative blot and densitometry in MRC-5 fibroblasts after transfection with miR-146a-5p mimic or scrambled control. β-actin was used as the loading control for protein expression and data are represented mean ± SEM (n=3/4). N indicates the number of independent experiments. *= $p < 0.05$ and **= $p < 0.01$ between the indicated values.

To determine the role of miR-146a-5p in the suppression of IL-1 α -induced-IL-8 release, we treated MRC-5 fibroblasts with rh-IL-1 α after miR-146a-5p over-expression (figure 5a). Here, we found a significant decrease of IL-8 release from MRC-5 fibroblasts when miR-146a-5p was over-expressed compared to cells treated with the scrambled control (figure 5b). Together our data show that miR-146a-5p regulates IL-1 α -induced pro-inflammatory responses in lung fibroblasts (figure 6).

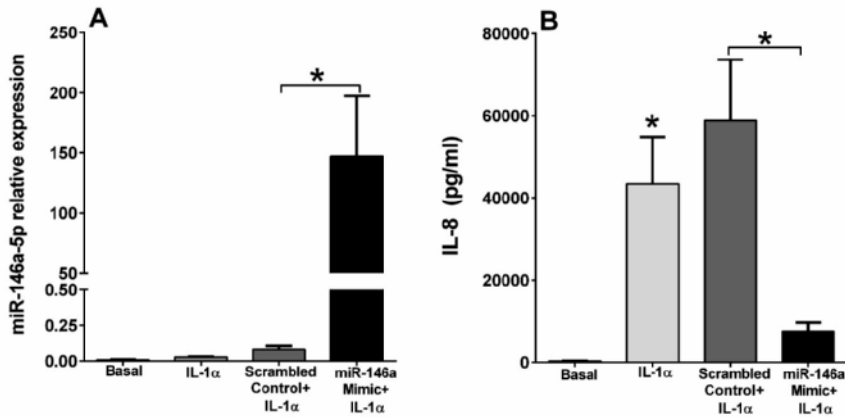


Figure 5. MiR-146a-5p reduces IL-1 α -induced IL-8 release in lung fibroblasts. MRC-5 fibroblasts were seeded and immediately transfected with 25nM of miR-146a-5p mimic or the scrambled control for 48 hours. MRC-5 fibroblasts were then serum deprived overnight and stimulated with/without 1ng/ml recombinant human IL-1 α for 24 hours. [A] miR-146a-5p mRNA expression in MRC-5 fibroblasts was related to the housekeeping non-coding RNU48, expressed as 2- Δ Ct, and presented as mean \pm SEM. [B] IL-8 release from MRC-5 fibroblasts. Data is presented as mean \pm SEM (n=3/4). N indicates the number of independent experiments. *= p <0.05 between the indicated values.

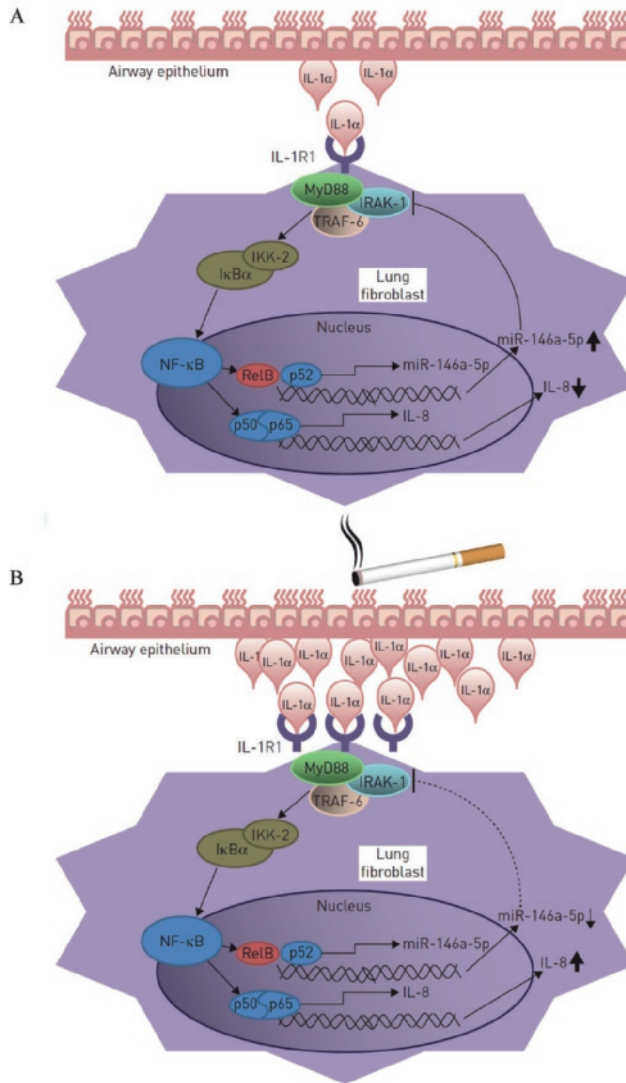


Figure 6. Proposed role of miR-146a-5p in the crosstalk between airway epithelial cells and lung fibroblasts. [A] Epithelial-derived IL-1 α causes an induction of miR-146a-5p expression as well as release of IL-8 from lung fibroblasts. MiR-146a-5p then binds to and down-regulates the expression of IRAK-1 down-stream of the IL-1 pathway in a feedback loop to dampen the NF- κ B activation and the inflammatory effects of the epithelial-derived IL-1 α on pulmonary fibroblasts.[B] In COPD, cigarette smoke exposure causes an increased IL-1 α release from airway epithelial cells which further increases IL-8 release from fibroblasts. However in COPD -derived fibroblasts, the IL-1 α induced increase in miR-146a-5p expression is lower compared to control-derived fibroblasts, which then contributes to lower feedback inhibition of the NF- κ B activation and an exaggerated pro-inflammatory response.

DISCUSSION

We investigated the role of miR-146a-5p in aberrant IL-1 α signaling between the airway epithelium and lung fibroblasts in COPD. We found that co-culture of primary human lung fibroblasts with airway epithelial cells significantly increases the expression of miR-146a-5p, which is completely dependent on epithelial-derived IL-1 α . We demonstrate that miR-146a-5p expression has an anti-inflammatory role, by down-regulating the expression of IRAK-1, which is downstream of the IL-1 pathway, and subsequently reduces IL-8 release from lung fibroblasts. Further, we shown that the induction of miR-146a-5p is significantly less in COPD fibroblasts and this was associated with the SNP, rs2910164r (GG allele), in the miR-146a-5p gene.

MiR-146a-5p has been well studied as a regulator of cellular function in both innate and adaptive immunity²². Specifically, miR-146a-5p has been suggested to target various inflammatory pathways including Toll-like receptor and IL-1 receptor signaling^{22, 23}. Over-expressing miR-146a-5p in the liver prevents the release of pro-inflammatory cytokines and protects mice from ischemia-reperfusion injury by targeting and reducing the protein expression of IRAK-1 and TRAF-6, which are downstream of the IL-1 pathway²¹. Perry and colleagues showed that miR-146a-5p over-expression reduces IL-1 β -induced IL-8 production in mono-cultures of alveolar epithelial cells¹³. Additionally, Bhaumik and colleagues found a high expression of miR-146a-5p in senescent human neonatal foreskin fibroblasts compared to quiescent cells²⁴. This high expression was shown to reflect a negative feedback mechanism that modulates the secretion of IL-1 α -induced IL-6 and IL-8 release due to a robust senescent-associated secretory phenotype activity in fibroblasts²⁴. In line with our present study, this effect was linked to the inhibition of IRAK1, but not TRAF-6²⁴. This finding is particularly important since senescence of various cell types such as epithelial cells and fibroblasts in the lung has been shown to contribute to COPD pathogenesis²⁵.

IL-1 α is an important driver of innate immune responses²⁶. This cytokine is constitutively present in the lung epithelium as part of the immune defense against inhaled particles and is responsible for the release of chemokines, such as IL-8, responsible for neutrophilic recruitment^{26, 27}. In COPD, there is an increased release of IL-1 α as indicated by the increased levels in sputum and broncho-aveolar lavage fluid compared to control subjects²⁸. We have previously shown that exposure to CSE induces a stronger expression of IL-1 α in airway epithelium from COPD patients compared to controls, leading to enhanced release of pro-inflammatory mediators such as IL-8 and IL-6 from lung fibroblasts upon their co-culture¹⁴. In addition, we showed that IL-1 α was secreted at baseline and was responsible for a pro-inflammatory switch in lung fibroblasts¹⁴.

In the present study, over-expression of miR-146a-5p reduced the IL-1 α -induced IL-8 secretion from lung fibroblasts. Several regulatory mechanisms to modulate the effects of IL-1 α are present *in vivo*, such as the secretion of the naturally occurring IL-1 receptor antagonist (IL-1Ra) and the decoy IL-1R2 receptor^{26,29}. Apart from these mechanisms, miR-146a-5p has emerged as a crucial regulator of the IL-1 pathway²⁴. In line with previous studies^{21,24}, we show that the induction of miR-146a-5p is dependent on IL-1 α stimulation and also causes a down-regulation in the protein expression of IRAK-1. IRAK-1 is an important serine/threonine kinase that associates with the IL-1R1 receptor complex upon stimulation³⁰. This eventually leads to the activation of transcription factors such as activator protein (AP)-1 and nuclear factor (NF)- κ B which subsequently leads to the induction and release of several inflammatory mediators, including IL-8³⁰. Thus, we hypothesize that the IL-1-induced increase in miR-146a-5p acts in a negative feedback loop to regulate the observed lung fibroblast pro-inflammatory activity upon co-culture with airway epithelial cells. The induction of miR-146a-5p was further enhanced when epithelial cells were pre-stimulated with CSE. This indicates an increased demand for miR-146a-5p induction as a negative feedback mechanism to counteract the enhanced release of IL-1 α from the airway epithelium in smokers.

Of interest, the observed increase in miR-146a-5p expression upon co-culture with epithelial cells was smaller in COPD-derived lung fibroblasts compared to control-derived lung fibroblasts. In line with our previous study [14], this reduced induction of miR-146a-5p may lead to an impaired feedback inhibition of the fibroblast-derived inflammation resulting from a higher production of IL-1 α from COPD-derived epithelium exposed to CSE (figure 6). Sato and colleagues additionally found less induction of miR-146a-5p in fibroblasts from COPD patients compared to healthy subjects after IL-1 β /TNF- α stimulation¹². This down-regulation was associated with increased expression of the COX-2 enzyme and an increased production of the inflammatory mediator PGE2 in the sputum of COPD patients¹². The difference in the induction of miR-146a-5p in PHLFs from our model was only seen after 72 hours of co-culture, but not after the 24 hour stimulation of PHLFs with epithelial-CM. This suggests that prolonged periods of exposure to IL-1 α are required to induce differential miR-146a-5p induction between COPD and control-derived fibroblasts which may be representative of the lung EMTU in COPD where there is a chronic exposure to cigarette smoke and the resultant epithelial-derived IL-1 α ^{14,18,31}. This is also in line with Perry et al, who suggested a time and concentration dependent effect of IL-1 on miR-146a-5p expression¹³.

To elucidate the underlying mechanism responsible for the lower induction of miR-146a-5p by COPD-derived PHLFs upon co-culture with epithelial cells, we investigated the possible involvement of RelB, a family member of the NF- κ B family, which has been shown to regulate miR-146a-5p expression¹⁹. Although Zago et al¹⁹

reported a lower expression of RelB in PHLFs from smokers with and without COPD compared those from non-smokers, we did not find a difference in expression in RelB mRNA expression between non-COPD and COPD –derived PHLFs in our co-culture model. A common G>C SNP rs2910164 in the primary miR-146a-5p sequence has been associated with a reduction in the expression of mature miR-146a-5p [20]. Of interest we found that PHLFs from donors homozygous for the GG allele of SNP rs2910164 had a lower miR-146a-5p expression in co-culture than fibroblasts from donors heterozygous for this allele. This indicates that the expression of this SNP is associated with a lower miR-146a-5p induction in COPD-derived lung fibroblasts in our co-culture model. Of interest, Wang et al showed that this particular SNP is also associated with a lower miR-146a-5p expression in relation to COPD³².

In conclusion, this study demonstrates that the pro-inflammatory phenotype of COPD lung fibroblasts resulting from a dysregulated epithelium-fibroblast interaction in our co-culture model may, at least in part, be due to the reduced ability of COPD-derived fibroblasts to up-regulate miR-146a-5p to counter-regulate pro-inflammatory activity. MiRNAs are likely to have therapeutic potential [5] with miRNA therapies recently making it through to clinical trials³³. Hence our finding could provide a basis for further investigations to target chronic inflammation in COPD.

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SUPPLEMENTARY DATA

miR-146a-5p mimic transfection

MRC-5 fibroblasts were seeded at a density of 1×10^5 cells per well in 24-well plates in EMEM/10% FCS and transfected shortly after seeding, using the HiPerFect reagent (QIAGEN, Venlo, Netherlands) and the miR-146a-5p mimic at 25nM (MirVana miRNA mimic, assay ID= MC10722; Applied Biosystems, Carlsbad, CA, USA). A scrambled small RNA at 25nM (AllStars Negative Control siRNA; QIAGEN) was used as a control. After 48 hours, cells were washed and placed in serum-free medium, followed by 24 hour stimulation with or without 1ng/ml IL-1 α (R&D Systems) and harvested with TriReagent for RNA isolation or Laemmli buffer for protein lysates preparation and cell-free supernatants were collected for IL-8 measurement.

rs2910164 Genotyping

To investigate if the lower induction of miR-146a-5p by COPD-derived primary human lung fibroblasts (PHLFs) in co-culture was caused by a common G>C SNP rs2910164, genotyping for this SNP was performed. A subset of samples were previously genotyped on Illumina Human1M-Duo BeadChip array and imputed using MACH program for genotype imputation using HapMap release 22 template, as previously described [1]. Five samples which were not genotypes on arrays had their DNA extracted from lung tissues of the same subjects from which PHLFs were obtained, according to the standard salt-chloroform extraction method. The rs2910164 polymorphism was identified using TaqMan® SNP Genotyping assay (C_15946974_10) according to the manufacturer's protocol. Each PCR was done in duplicates with 10 ng DNA template and 40 cycles of amplification was used. The PCR was carried out on a ABI7800HT machine (Applied Biosystems).

RNA isolation and qRT-PCR

RNA was harvested from cells with the standard TRIreagent method. For miRNA expression analysis, miRNA-specific cDNA synthesis was done using the TaqMan microRNA reverse transcription kit (Life Technologies, Bleiswijk, Netherlands) together with reverse transcription primers (Life Technologies) for miR-146a (000468). qRT-PCR was performed on the LightCycler 480 II (Roche, Almere, Netherlands) and expression of the miRNAs of interest was normalized to the expression the small nuclear RNA, RNU48 (001006) as an endogenous control with approximately equal amplification efficiency. For expression analysis of the NF- κ B family member RelB, the iScript cDNA kit (Biorad, Herts, UK) was used for cDNA synthesis, after which qRT-PCR was performed using the Taqman® Gene expression assay RelB (Hs00232399_m1) with the protein phosphatase 1, catalytic subunit,

alpha isoenzyme (Hs00267568_m1) and β 2- microglobulin (Hs00984230_m1) as the housekeeping genes. The reaction was followed for 40 cycles and the Ct value calculated using the LightCycler software. DeltaCt (Δ Ct) values were calculated (Average triplicate Gene of Interest – average triplicate Housekeeping gene) and the data were represented as $2^{-\Delta$ Ct.

IL-8 ELISA and Western blotting

Protein levels of IL-8 were measured with a sandwich ELISA (R&D Systems) according to the manufacturer's instructions.

Total cell lysates of fibroblasts were obtained by harvesting cells in 1X Laemmli buffer (containing 2% SDS, 10% glycerol, 2% β -mercapto, 60mM Tris-HCl (pH 6.8) and bromophenol blue) and boiling for 5 minutes after transfection. Samples were then blotted on a nitrocellulose membrane (Schleider and Schuell GmbH, Einbeck, Germany) after being subjected to SDS-PAGE. Expression of the interleukin 1 receptor associated kinase (IRAK)-1 and TNF receptor-associated factor (TRAF)-6 was analysed using mouse anti-human IRAK-1 (Sc-5288) and mouse anti-human TRAF-6 (Sc-8409) with goat anti-human β -Actin (all antibodies from, Santa Cruz Biotechnology, Santa Cruz, CA) as loading control as previously described². Detection of bands were done by enhanced chemiluminescence according to the manufacturer's instruction (ECL, Amersham) and imaged with the ChemiDocTM MP system (Biorad, Veenendaal, Netherlands)

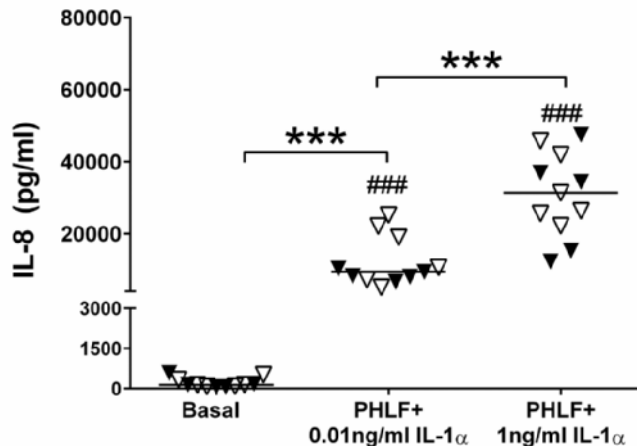


Figure S1. Recombinant human IL-1 α caused IL-8 release from primary human lung fibroblasts (PHLFs). PHLFs from control donors (open triangles, n=6) and COPD patients (closed triangles, n=6) were grown to confluence and serum deprived overnight. IL-8 concentration (with median) as released after PHLFs IL-1 α stimulation. *** = $p < 0.001$

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