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Human bronchial and parenchymal fibroblasts display differences in basal inflammatory phenotype and response to IL-17A

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Running Head: Fibroblasts differ in inflammatory phenotype

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Abstract

Background: Chronic inflammation, typified by increased expression of IL-17A, together with airway and parenchymal remodeling are features of chronic lung diseases. Emerging evidence suggests that phenotypic heterogeneity of repair and inflammatory capacities of fibroblasts may contribute to the differential structural changes observed in different regions of the lung.

Objective: To investigate phenotypic differences in parenchymal and bronchial fibroblasts, either in terms of inflammation and remodeling or the ability of these fibroblasts to respond to IL-17A.

Methods: Four groups of primary fibroblasts were used: normal human bronchial fibroblast (NHBF), normal human parenchymal fibroblast (NHPF), COPD human bronchial fibroblast (CHBF) and COPD human parenchymal fibroblast (CHPF). Cytokine and extracellular matrix (ECM) expression were measured at baseline and after stimulation with IL-17A. Actinomycin D was used to measure cytokine mRNA stability.

Results: At baseline, we observed higher protein production of IL-6 in NHPF than NHBF, but higher levels of IL-8 and GRO- α in NHBF. IL-17A induced a higher expression of GRO- α (CXCL1) and IL-6 in NHPF than in NHBF, and a higher level of IL-8 expression in NHBF. IL-17A treatment decreased the mRNA stability of IL-6 in NHBF when compared with NHPF. CHPF expressed higher protein levels of fibronectin, collagen-I and collagen-III than CHBF,

NHBF and NHPF. IL-17A increased fibronectin and collagen-III protein only in NHPF and collagen-III protein production in CHBF and CHPF.

Conclusions and Clinical Relevance: These findings provide insight into the inflammatory and remodeling processes that may be related to the phenotypic heterogeneity of fibroblasts from airway and parenchymal regions and in their response to IL-17A.

Introduction

Interleukin-17A (IL-17A) is a pro-inflammatory and pro-fibrotic cytokine mainly expressed by Th17 cells [1]. IL-17A acts on a wide range of cells, including hematopoietic, endothelial and mesenchymal cells to produce cytokines and chemokines which, in turn, influence granulopoiesis and neutrophil recruitment to inflammatory sites [2, 3]. In lung structural cells such as epithelial cells, airway smooth muscle (ASM) cells and fibroblasts, IL-17A triggers the expression of numerous chemokines and cytokines such as GRO- α , IL-8 and IL-6 [4, 5]. There is increasing evidence that IL-17A is involved in the pathogenesis of severe asthma and chronic obstructive pulmonary disease (COPD), both of which are characterized by air flow obstruction, airway inflammation and remodeling. It has been reported that IL-17A is increased in the sputum and serum of COPD patients, and that IL-17A expression positively correlates with the severity of COPD (based on GOLD classification), sputum neutrophil count, and is inversely correlated with FEV1 [6]. IL-17A is increased in the bronchial submucosa of COPD patients [7] and increased numbers of IL-17A-positive cells are found in bronchial submucosa and parenchymal regions of COPD lungs [8, 9]. Studies have also shown an increase in IL-17A in BAL fluid of idiopathic pulmonary fibrosis (IPF) patients [10]. Induction of fibrosis in murine models by either bleomycin or IL-1 β is IL-17A-dependent [10], suggesting that IL-17A participates in the

pathogenesis of pulmonary fibrosis [11]. Our laboratory recently demonstrated that IL-17A deficiency significantly reduced cigarette smoke-induced lung inflammation, including a decrease in neutrophilia, and reduced MMP-12 and IL-6 expression in the lung [12]. Moreover, neutralization of IL-17A promoted the resolution of bleomycin-induced inflammation and attenuated fibrosis [11]. Thus, IL-17A has a key role in lung disease pathogenesis by orchestrating neutrophilic recruitment, inflammation and remodeling [11-13].

Among the pulmonary cell types capable of driving the pathogenesis of obstructive lung diseases such as asthma and COPD, fibroblasts are key contributors to tissue remodeling. Fibroblasts are the main producers of extracellular matrix (ECM) proteins [14] and their activation can impact directly the repair/remodeling process via ECM turnover. There is now ample evidence that fibroblasts contribute to chronic inflammation by producing an array of cytokines, chemokines, lipid mediators and proteases [15]. The remodeling and inflammatory characteristics of fibroblasts are also dramatically altered during disease states. Fibroblasts from COPD patients (GOLD stage II-III) for example have a pro-inflammatory phenotype (IL-6 and IL-8) and a reduced capacity to repair, a feature not observed in fibroblasts isolated from COPD stage I subjects [16]. Fibrotic lung fibroblasts also produce more ECM proteins than fibroblasts isolated from normal lungs [17]. Finally, fibroblasts isolated from asthmatic airways retain a fibrotic phenotype and secrete excess ECM proteins compared to non-asthmatics [18], supporting the contention that fibroblasts contribute to inflammatory and remodeling processes during disease pathogenesis.

Like many types of immune cells, fibroblasts consist of sub-populations with distinct phenotypes and/or functions, a feature that may partially explain the heterogeneity of presentation for diseases such as IPF and COPD [19]. Fibroblast heterogeneity in the lung- based

on the site of origin (*i.e.*, bronchial vs. parenchymal)- manifests into at least two distinct phenotypes. As first reported by Kotaru and colleagues [20], in asthmatics, parenchymal fibroblasts are more proliferative than bronchial fibroblasts, whereas bronchial fibroblasts from normal or asthmatic patients expressed more ECM proteins (pro-collagen1, eotaxin-1) than did parenchymal fibroblasts [20]. Other studies have revealed that parenchymal fibroblasts isolated from normal subjects express higher α -SMA (a marker of myofibroblasts) than bronchial fibroblasts, in part due to constitutive activation of the TGF- β 1/Smad pathway [21]. Hallgren et al [22] have recently described heterogeneity in proteoglycan expression between distal lung vs. central airway-derived fibroblasts from COPD patients. While there was more biglycan production from distal lung fibroblasts than central fibroblasts derived from normal subjects, this heterogeneity was not seen in fibroblasts from COPD subjects [22]. Finally, Preobrazhenska et al. reported regional heterogeneity in murine lung fibroblasts with regard to matrix production and wound repair under basal conditions or after *in vivo* cigarette smoke exposure [23].

Thus, lung fibroblast heterogeneity may contribute to distinct remodeling processes commonly observed in chronic lung diseases. However, relatively little is understood about phenotypic differences in sub-populations of fibroblasts, either in terms of inflammation and remodeling or the ability of these fibroblasts to respond to IL-17A. We hypothesized that bronchial and parenchymal fibroblasts differ in the pattern of cytokine induction and ECM expression at baseline and in response to IL-17A stimulation. Defining the phenotypic differences between bronchial and parenchymal fibroblasts may help to identify the contribution of fibroblast subpopulations in driving inflammation and remodeling during the pathogenesis of chronic lung diseases.

Methods

Isolation of normal primary human fibroblasts from bronchial or parenchymal lung region

Fibroblasts were isolated from lung parenchyma and small airway tissue from control subjects (never-smoker or smokers who quit smoking 16.4 ± 10.8 years ago) or COPD patients at McMaster University or University of Manitoba. Subjects were recruited by a research nurse who obtained informed consent and compiled clinical characteristics and demographic data relevant to cigarette smoking status (online supplement Table 1). Samples were obtained in accordance with a protocol approved by the Research Ethics Boards of the University of Manitoba and St Joseph's Healthcare Hamilton (McMaster University). Fibroblasts were isolated and cultured as described in the online supplement method [24, 25]. Given the report that senescence can lead to an increase in basal expression of cytokines [26] experiments were conducted using fibroblasts at the same passages (P4 to P6).

Treatment of fibroblasts with rhIL-17A

NHBF and NHPF were treated with various concentrations (0.25, 2.5, 25 or 250 ng/ml) of recombinant human IL-17A for 24 h (for mRNA) or 48 h (for protein). Based on the preliminary results, the optimal concentration of 25 ng/ml of IL-17A was selected for the remaining studies. In our experiments bronchial and parenchymal fibroblasts were always plated at the same cell number and experiments on these cells were performed at the same time point.

qRT-PCR analysis of mRNA expression

Fibroblasts were treated at 70-80% confluence with 25 ng/ml rhIL-17A (R&D systems, Minneapolis, MN, USA) for 3, 6, 12, 24 h, and total RNA was extracted using the RNeasy Mini kit (QIAGEN; Mississauga, ON, Canada). Quality control and RNA concentrations were

determined using a spectrophotometer (NanoDrop Products, Wilmington, DE, USA). Reverse transcription was performed using SuperScriptIII (Bio-Rad Laboratories, Mississauga, Canada) and cDNA amplified using the 7500 Fast Real-Time PCR system thermal cycler using SYBR Green (Applied Biosystems, Foster City, CA). Primers are in Table 1. Gene expression was analyzed using the $\Delta\Delta C_t$ method and results are presented as fold-differences normalized to housekeeping gene (GAPDH). All results were expressed as fold change compared with non-stimulated controls, or as relative value for basal expression comparison between subjects.

SDS-PAGE and immunoblotting

Proteins were separated (10 μ g/lane) by 7.5% SDS-PAGE, and transferred onto a nitrocellulose blotting membrane, blocked for 1 hour at RT and incubated overnight at 4°C with antibodies specific to Collagen-1 (polyclonal rabbit anti-human; CL50111AP-1; Cederlane), Collagen-3 (polyclonal goat anti-human; SC-8781; Santa Cruz), Fibronectin (polyclonal goat anti-human; AF1918; R&D systems) and GAPDH for normalization (monoclonal rabbit anti-human; MAB374; Millipore). The blots were developed by using streptavidin-biotinylated horseradish peroxidase-conjugated antibody and detected with an enhanced chemiluminescence kit (BIO-RAD).

Enzyme-linked immunosorbent assay (ELISA) for cytokine/chemokines

The concentration of IL-6, IL-8 and GRO- α were measured by means of a human immunoassay kits (Biolegend San Diego, CA, USA) in accordance with the manufacturer's instructions. In preliminary experiments, we measured cell proliferation using ^3H -thymidine incorporation assay. There was no difference in cell number after 24, 48, 72, 96 hours in culture, between NHBF and

NHPF with or without exposure to IL-17A. Therefore, ELISA data is presented as pg/ml without being adjusted for cell number.

Determination of IL-6 mRNA stability

Fibroblasts were cultured in 12-well culture plates until 70 to 80% confluence and switched to serum-free media overnight. Cells were then treated with IL-17A (25 ng/ml) for 6 hours followed by two consecutive washes with sterile PBS and treatment with Actinomycin D (1 μ g/ml), an inhibitor of RNA synthesis, for 0.5, 1 or 3 hours. Total RNA was harvested and qPCR performed as described above to determine the remaining levels of IL-6 mRNA.

Statistical analysis

All data are presented as means $\pm$ SEM of 3 to 8 independent experiments. Data were analyzed with one-way ANOVA followed by Dunnett's multiple comparison or two-way ANOVA followed by Bonferroni's multiple comparison test for statistical analysis of the data using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA).

Results

Fibroblast heterogeneity in basal cytokine expression

To determine the regional heterogeneity of basal expression of cytokines from lung fibroblasts, we examined the mRNA expression and protein production of select cytokines in quiescent NHBF and NHPF (Fig. 1). Under basal conditions, there was no significant difference between NHBF and NHPF in relative expression of GRO- α (CXCL1) and IL-8 at the mRNA level (Fig. 1A and C). In contrast, there was higher expression of IL-6 mRNA in NHPF than in NHBF (Fig. 1B and E, respectively). Despite these observations on mRNA expression of cytokines, NHBF showed significantly higher secretion of GRO- α (CXCL1) and IL-8 than NHPF (Fig. 1D and F). Importantly, NHPF produced higher amounts of IL-6 than NHBF that was consistent with its mRNA expression level. Overall, these data indicate that there are marked differences between NHBF and NHPF in terms of the basal level of cytokine expression.

Fibroblast heterogeneity in basal expression of ECM

Under basal conditions, NHBF and NHPF did not exhibit differences in ECM (collagen-I, collagen-III and fibronectin) levels, either in terms of mRNA expression or protein production (Fig. 2A - F). Conversely, COPD fibroblasts displayed differences in ECM production at the protein level but not at the mRNA expression level (Fig. 2AF). We observed a higher basal protein production of fibronectin, collagen-I and collagen-III in CHPF than in CHBF (1.6-, 2- and 3.8-fold higher respectively) (Fig. 2D-F). Comparison between fibroblasts from normal subjects and COPD patients showed statistically significant ($p < 0.01$) differences in protein

production only between NHPF and CHPF, for collagen-I (a 2-fold higher production in CHPF than NHPF).

IL-17A differentially increases the expression of cytokines between NHBF and NHPF

Next, we assessed the effect of IL-17A on pro-inflammatory cytokine expression between bronchial and parenchymal fibroblasts. Although the kinetics of induction varied, IL-17A significantly increased the mRNA levels of GRO- α mRNA (Fig. 3A), IL-6 (Fig. 3B) and IL-8 (Fig. 3C) in NHBF. In NHPF, there was a trend towards an increase in mRNA expression of GRO- α (Fig. 3E) but there was a significant increase in IL-6 (Fig. 3E) and IL-8 mRNA (Fig. 3F) after IL-17A treatment. It is noteworthy that the increase in IL-6 mRNA in NHPF remained significantly elevated through 12 hours of exposure in the NHPF but was near baseline levels in the NHBF.

Next, we evaluated whether the differences in cytokine mRNA levels mirrored those of the protein. Figure 4 shows that there is differential cytokine secretion by fibroblasts in response to IL-17A. Here, IL-17A significantly increased GRO- α for both NHBF and NHPF fibroblasts, with the increase being significantly higher in NHPF compared to NHBF (Fig. 4A). Despite the significant increase in IL-6 mRNA in NHBF, there was no change in IL-6 protein after exposure to IL-17A (Fig. 4B). Basal levels of IL-6 were significantly higher in NHPF compared to NHBF (Fig. 4B; compare to mRNA levels in Fig. 1B), and we observed higher secretion of IL-6 in response to IL-17A in NHPF (Fig. 4C). IL-8 expression was also increased in response to IL-17A in both NHBF and NHPF (Fig. 4C); there was no significant difference in IL-8 production between NHBF and NHPF.

Interleukin-17A increases IL-6 mRNA stability in normal parenchymal lung fibroblasts

As NHBF and NHPF showed differential expression in IL-6 mRNA expression versus protein production in response to IL-17A, we next investigated whether this difference between IL-6 mRNA expression and protein production was due to changes in mRNA stability, a post-transcriptional regulatory mechanism that not only allows for flexible control of gene expression but also control over the initiation and resolution of the inflammatory response. To evaluate this, NHPF and NHBF were treated with 25 ng/ml of IL-17A for 6 h, followed by actinomycin D (ActD) (1 µg/ml) for various times (30, 60, and 180 minutes). As seen in Figure 5, the amount of IL-6 mRNA remaining after 30 min of treatment with ActD was significantly higher in NHPF than in NHBF (Fig. 5), indicating that IL-6 mRNA stability is differentially regulated between fibroblasts from different anatomical locations and may account for the lack of IL-6 protein production by IL-17A in NHBF despite an induction in mRNA levels.

Effect of interleukin-17A on ECM expression

We next evaluated whether there is heterogeneity between NHBF and NHPF with respect to ECM expression. Of the three ECM mRNA evaluated (Fig. 6), only collagen-III mRNA was significantly increased by IL-17A in the NHBF (Fig. 6C). For NHPF, fibronectin (Fig. 6D) and collagen-I (Fig. 6E) were increased after 12 h of treatment with IL-17A; there was no change in collagen-III (Fig. 6F). In CHBF and CHPF, IL-17A had no effect on mRNA expression of collagen-I, collagen-III or fibronectin (data not shown).

There was no increase in protein production of fibronectin, collagen-I and collagen-III in the NHBF (Fig. 7A-C). Only fibronectin (Fig. 7A) and collagen-III (Fig. 7C) were increased

after IL-17A in the NHPF. IL-17A treatment increased collagen-III protein production in both CHPF and CHBF (1.6- and 1.4-fold, respectively) (Fig. 7F). In COPD fibroblasts, IL-17A had no effect on fibronectin (Fig. 7D) and collagen-I (Fig. 7E) protein production.

Discussion

Fibroblasts represent a heterogeneous cell population which display differences in morphology, proliferation rate, wound healing, collagen-I and α -SMA expression [20, 21, 27, 28]. Because of their inherent ability to produce ECM proteins fibroblasts are key cells in repair and remodeling of lung matrix in several lung diseases. Fibroblasts also produce inflammatory cytokines that implicate them in immune and inflammatory responses. However, the phenotypic heterogeneity between bronchial and parenchymal fibroblasts in terms of inflammatory cytokine expression is not well delineated. In this study we show for the first time that NHBF and NHPF exhibit considerable heterogeneity in terms of inflammatory cytokine expression under basal conditions. We observed higher expression of the pro-inflammatory and pro-fibrotic cytokine IL-6 in NHPF than in NHBF, which could potentially modulate the wound repair capacity of fibroblasts within the parenchyma. Conversely, we noted a higher expression of the chemokines IL-8 and GRO- α in bronchial fibroblasts. The difference in the ability of bronchial and parenchymal fibroblasts to produce inflammatory cytokines may reflect differences in the sensitivities of these cells to inflammatory stimuli during disease development in chronic lung diseases.

Tissue injury leads to accumulation and activation of several inflammatory cells in the lung. A variety of cytokines and chemokines are implicated in recruiting inflammatory cells into pulmonary tissue after injury. IL-8 and GRO- α are C-X-C chemokines that have many biological

effects on cells, including the ability to attract and activate neutrophils [29, 30]. These chemokines also induce transendothelial migration of leukocytes; stimulate degranulation and subsequent release of a battery of neutrophil mediators. Furthermore, T-lymphocytes and eosinophils migrate in response to IL-8 [31]. It could be that the increased production of IL-8 and GRO α by bronchial fibroblasts directly mediates neutrophil migration into the air space; exposure of the lung to neutrophil-generated mediators, such as elastase and reactive oxygen species may lead to tissue destruction, obstructive pulmonary disease, and eventually respiratory failure. We also noted that constitutive mRNA expression and protein production of IL-6 was higher in the parenchymal fibroblasts compared with bronchial fibroblasts at baseline values. This is intriguing, and may indicate that parenchymal fibroblasts, along with macrophages, are a major cellular source for IL-6. IL-6 has been implicated in the progression of COPD [32]; enhanced levels of IL-6 have been reported in the induced sputum of patients with COPD, particularly during exacerbation [32]. Higher IL-6 levels are found in bleomycin-induced fibrosis and interstitial lung diseases [33], and increased levels of IL-6 may play a role in subepithelial fibrosis and airway remodeling in asthma [34]. Thus, parenchymal fibroblasts can actively take part in the airway/lung inflammation by secreting chemokines such as IL-8 and IL-6, indicating a role for this structural cell type in the pathogenesis of airway/lung diseases.

The inflammatory capacity of IL-17A is largely mediated by its ability to induce expression of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and chemokines (IL-8, GRO- α), which leads to the recruitment and activation of neutrophils, monocytes and macrophages. The higher basal expression of inflammatory cytokines in NHPF in IL-17A-treated NHPF, suggests that these parenchymal cells may play a role in the immune and inflammatory responses leading

to parenchymal destruction typical of the emphysema component of COPD. Interestingly, in response to IL-17A, the kinetics of IL-6 expression was prolonged in NHPF; IL-6 mRNA expression remained significantly higher at 12 h after IL-17A stimulation. There was also more IL-6 protein production from IL-17A-stimulated NHPF than NHBF. IL-17A can increase gene expression by extending the half-life of cytokine and chemokine mRNA [35, 36]. Our data show for the first time there is increased stability, albeit transiently, of IL-6 mRNA caused by IL-17A in NHPF. It is difficult to be certain of the relevance of this transient effect on protein production, although it is certainly plausible. We were unable, however, to find evidence in the literature to this effect. We believe that post-transcriptional regulation and protein translation efficiency may be important in determining the strength and duration of gene and protein induction by IL-17A yet is poorly understood. Future studies using pulse-label and chase techniques with radioactively labeled protein precursors will help resolve this question. The distinct phenotype exhibited by parenchymal fibroblasts may underlie the susceptibility of the parenchymal regions of the lung to interstitial lung diseases and/or alveolar destruction.

Although we observed a trend towards a difference in fibronectin and collagen III mRNA transcripts between NHPF and NHBF, there was no difference in ECM protein production between these cells under basal conditions. This is in accordance with Pechkovsky et al.(21), we did not see differences in collagen-I, collagen-III or fibronectin protein production between NHBF and NHPF at baseline. In COPD fibroblasts we observed heterogeneity in ECM production. Specifically, we observed higher levels of collagen-I, collagen-III and fibronectin in CHPF than in CHBF at baseline. In addition, collagen I protein level was higher in CHPF than in NHPF, suggesting a cellular basis for the ECM differences in normal vs COPD lungs. The

abundance of type I collagen in COPD fibroblasts highlights the importance of this protein in remodeling and repair processes in the diseased lung. The mechanism for the elevated collagen production may be related to autocrine stimulation by inflammatory/fibrotic mediators secreted by the COPD fibroblasts themselves. In the literature it has been shown that CHPF expressed higher pro-collagen I in COPD stage IV, and that CHPF from stage II-III patients expressed higher level of soluble elastin, MMP-1, collagen-I and versican, than NHPF (16, 37). This increased expression of ECM was observed in COPD fibroblasts isolated from late stage COPD and was postulated to be a response to loss of parenchymal tissue. In the same study, the authors also showed that, despite the increase of ECM production, the COPD fibroblasts were less able to repair the damaged tissue due to alterations in cell-matrix interactions (16, 37). Our findings suggest that modulation of ECM production by parenchymal fibroblasts is also present in the earlier or moderate stage of COPD, which could result in an imbalance between ECM degradation and synthesis as has been reported for late stage disease. The degree to which differential expression of ECM proteins can be translated into regional differences in the regulation and outcome of lung injury and repair is presently unknown.

In this study, we also demonstrate, for the first time, that IL-17A induced ECM production by primary human lung fibroblasts. IL-17A increased the expression of fibronectin and collagen-III in NHPF, but not in NHBF. Despite the fact that we did not find differences in ECM gene expression under basal conditions between NHBF and NHPF, we observed a different capacity of these fibroblasts to express ECM proteins in response to IL-17A stimulation. It has been reported recently that IL-17A did not affect collagen production or even decreased the expression of collagen type I in fibroblasts or fibroblast progenitors from normal subjects and patients with inflammatory or fibrotic diseases. For example, the study by Nakashima et al [38]

showed that IL-17A reduced collagen-I protein levels in normal dermal fibroblasts. Belini et al [39] reported that in vitro stimulation of fibrocytes with IL-17A did not increase collagen expression by these cells. Hayashi et al [40] reported that IL-17A alone did not increase collagen I in terms of the mRNA and protein levels in fibrocytes. The differences in collagen production in response to IL-17A between our study and those of Nakashima et al. [38] or Belini et al [39] and Hayashi et al [40] may be due to differences in cell/tissue type, concentration of IL-17A used and/or duration of stimulation with IL-17A. While lung fibroblasts were used in our study, Nakashima used dermal fibroblasts obtained from scleroderma subjects and stimulated these cells with 250 ng/ml of IL-17A for 24 h. In the case of Bellini et al [38], these authors studied the effect of IL-17A at 15 ng/ml on collagen expression by circulating fibrocytes. Hayashi et al [39] used a concentration of 10 ng/ml of IL-17A to stimulate fibrocytes. These authors treated fibrocytes for 1 h with IL-17A at 10 ng/ml and then stimulated with CD40L. Thus our findings suggest that IL-17A can either stimulate or inhibit collagen production depending upon the experimental conditions, and in a cell type-dependent manner.

We also report that parenchymal and bronchial fibroblasts from COPD patients displayed heterogeneity in terms of ECM production. Furthermore, IL-17A induced collagen-III protein production in both CHBF and CHPF; the capacity of CHBF to increase collagen-III in response to IL-17A stimulation is consistent with the observation of increased collagen deposition in the airways of COPD patients. Interestingly, at baseline, fibroblasts isolated from parenchyma or airways of severe COPD patients display differences in pro-collagen-I expression (37), similar to our present observations in CHPF (COPD stage II). The ability of IL-17A to modulate ECM production by parenchymal and bronchial fibroblasts may result in a population or subpopulation

of fibroblasts with an altered phenotype and may have important implications for our understanding of the pathogenic mechanisms involved in matrix remodeling in chronic inflammatory lung diseases.

Our study has some limitations. In our study normal parenchymal fibroblasts were derived from subjects who were either non-smokers or ex-smokers without COPD, whereas bronchial fibroblasts were all derived from ex-smokers without COPD. This raises the possibility that previous exposure to cigarette smoking may have influenced our findings. Smokers on average had discontinued smoking 16.44 ± 10.86 years previously. Further the expression levels of cytokines and ECM proteins, under basal conditions and after IL-17A stimulation, were similar in normal parenchymal fibroblasts whether they were derived from non-smokers or ex-smokers. As such we believe that the differences observed in the pattern of cytokine and ECM production between bronchial vs parenchymal fibroblasts represent a true reflection of their phenotypic heterogeneity, rather than an effect of remote exposure to cigarette smoking.

Our data on mRNA expression for cytokines/ECM proteins did not necessarily correlate with the expression of protein products. The use of mRNA expression data *per se* is not adequate to understand the expression patterns of proteins because post-transcriptional factors, such as protein translation, post-translational events, and protein degradation may all affect the protein abundance in cells or tissues. Additionally, measurement of secreted cytokines rather than their intracellular levels, or measurement of intracellular levels of ECM proteins instead of secreted levels may also have added to the discrepancy between mRNA expression and protein levels. However, less is understood regarding the mechanisms that regulate the expression patterns of

protein products in lung fibroblasts. Additionally, how bronchial and parenchymal fibroblasts control the synthesis and degradation rate of a particular protein and whether heterogeneity exists even within these cells for a given protein needs to be examined.

In summary, we have characterized the inherent differences between bronchial and parenchymal fibroblasts obtained from normal and COPD subjects in response to IL-17A. Our results show that bronchial and parenchymal fibroblasts from normal subjects demonstrated heterogeneity in terms of baseline inflammatory cytokine (IL-6, IL-8 and GRO- α) expression. COPD parenchymal fibroblasts showed a greater capacity to express ECM than bronchial fibroblasts from COPD or normal subjects. IL-17A increased cytokine expression to a greater extent in parenchymal than in bronchial fibroblasts. These phenotypic differences may help elucidate the role of fibroblasts in mediating pathological changes between the bronchial (peribronchiolar fibrosis) and parenchymal (alveolar wall destruction) regions in lung diseases characterized by remodeling such as asthma and COPD. Future studies are needed to investigate the cellular and molecular events that drive site-specific fibroblast function, how phenotypically different fibroblast populations direct local physiology and pathology, and how cells from different anatomical sites of the diseased lung respond to pharmacological treatment may impact on the phenotype of fibroblasts from a given lung site.

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Disclosures

No conflicts of interest, financial or otherwise are declared by the authors.

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Table 1: List of primer sequences used in RT-PCR

Genes	Forward primer 5' to 3'	Reverse primer 3' to 5'
Collagen- I	TCGGAGCAGACGGGAGTTTC	GTCATCGCACAAACACCTTGC
Collagen-III	ATGTTGTGCAGTTTGCCAC	TCGTCCGGGTCTACCTGATT
Fibronectin	CCCCAACTGGTAACCCTTCC	GTGTAGGGGTCAAAGCACGA
GAPDH	AGTCAACGGATTTGGTCGTATT	ATGGGTGGAATCATATTGGAAC
GRO- α	AGTGACAAATCCAACCTGACCAGA	ATGCTCAAACACATTAGGCACA
IL-6	ACCTTCCAAAGATGGCTGAAA	GCTCTGGCTTGTTCCCTCACTAC
IL-8	CAGTTTTGCCAAGGAGTGCTAA	CCCGTGCAATATCTAGGAAAATC
TGF- β 1	ATGAACCGGCCTTTCCTG	GGCAGAAGTTGGCATGGTAG

Figure legends

Figure 1. Basal mRNA expression and protein production of cytokines are different in NHBF and NHPF. Basal mRNA expression of GRO- α (A), IL-6 (B) and IL-8 (C) in NHBF and NHPF (n=6-7) were analyzed by real-time RT-PCR. Results were normalized by GAPDH and expressed as relative value. Basal protein production of GRO- α (D), IL-6 (E) and IL-8 (F) in NHBF and NHPF (n=4-6) were measured by ELISA and were expressed as pg/ml. Data were analyzed and expressed as mean $\pm$ SEM by Student t test, *p<0.05, **p<0.01, ***p<0.001,

Figure 2: Basal mRNA expression and protein production of ECM components between NHBF, NHPF, CHBF and CHPF. Basal mRNA expression of fibronectin (A), collagen-I (B) and collagen-III (C) in NHBF and NHPF (n=7-8), and CHBF and CHPF (n=5) were analyzed by real-time RT-PCR. Results were normalized by GAPDH and expressed as relative value. Basal protein production of fibronectin (D), collagen-I (E) and collagen-III (F) in NHBF (n=5-6), NHPF (n=5-6), CHBF (n=5) and CHPF (n=5) were analyzed by Western-blot and were expressed as relative value normalized by GAPDH. Data were analyzed and expressed as mean (SEM) by two-way ANOVA with Bonferroni's multiple comparison test, *p<0.05, **p<0.01, compared between the indicated group.

Figure 3: IL-17A increases cytokine mRNA expression in NHBF and NHPF. NHBF and NHPF mRNA expression (right and left panel, respectively, n=5-8) of GRO- α (A and D), IL-6 (B and E) and IL-8 (C and F) in response to IL-17A treatment at 25 ng/ml for 3, 6, 12 and 24 h were

analyzed by real-time RT-PCR. Results were normalized by GAPDH and expressed as fold-change in comparison with control value (media only). Data were analyzed and expressed as mean $\pm$ SEM by one-way ANOVA with Dunnett's multiple comparison test, * p <0.05, ** p <0.01, compared with the control.

Figure 4: Differences in IL-17-induced cytokine expression from lung fibroblasts based on lung origin. Fibroblasts cytokine production of GRO- α (A), IL-6 (B) and IL-8 (C) in NHBF and NHPF (n=5-8) in response to IL-17A treatment at 25 ng/ml for 48 h were analyzed by ELISA method and expressed as pg/ml. Data were analyzed and expressed as mean $\pm$ SEM by two-way ANOVA multiple comparison test, * p <0.05, *** p <0.001, significant effect of IL-17A treatment, ### p <0.001, significant differences between parenchymal and bronchial fibroblasts.

Figure 5. IL-17A differentially modulates IL-6 mRNA stability in NHBF and NHPF. Fibroblasts were treated with IL-17A (25 ng/ml) for 6 hours followed by exposure to ActD (1 μ g/ml) or DMSO (control condition) for the indicated time-points. IL-6 mRNA expression was first normalized by GAPDH and then folding to the control condition (DMSO condition). Data were analyzed and expressed as mean $\pm$ SEM of 3–4 independent experiments by two-way ANOVA with Bonferroni's multiple comparison test, *** p <0.001, compared for each time point between group.

Figure 6. Interleukin-17A modulates mRNA expression of ECM components in NHBF and NHPF. NHBF and NHPF mRNA expression (right and left panel, respectively, n=5-8) of fibronectin (A and D), collagen-I (B and E) and collagen-III (C and F) in response to IL-17A treatment at 25 ng/ml for 3, 6, 12 and 24 h were analyzed by real-time RT-PCR. Results were normalized by GAPDH and expressed as fold-change in comparison with control value (fibroblast no-treated). Data were analyzed and expressed as mean $\pm$ SEM by one-way ANOVA with Dunnett's multiple comparison test, *p<0.05, **p<0.01, compared with the control.

Figure 7. Interleukin-17A modulates ECM protein production in NHBF, NHPF, CHBF and CHPF. Protein levels of fibronectin (A, D), collagen-I (B, E) and collagen-III (C, F) in NHBF and NHPF (n=5-6) and in CHBF and CHPF (n=5) treated or not with IL-17A at 25 ng/ml for 48 h, were analyzed by Western-blot. Results were normalized by GAPDH and were represented as fold-change by folding to the basal condition. Data were analyzed and expressed as mean $\pm$ SEM by two-way ANOVA with Bonferroni's multiple comparison test, *p<0.05, **p<0.01, compared between the indicated group.

FIGURE-1

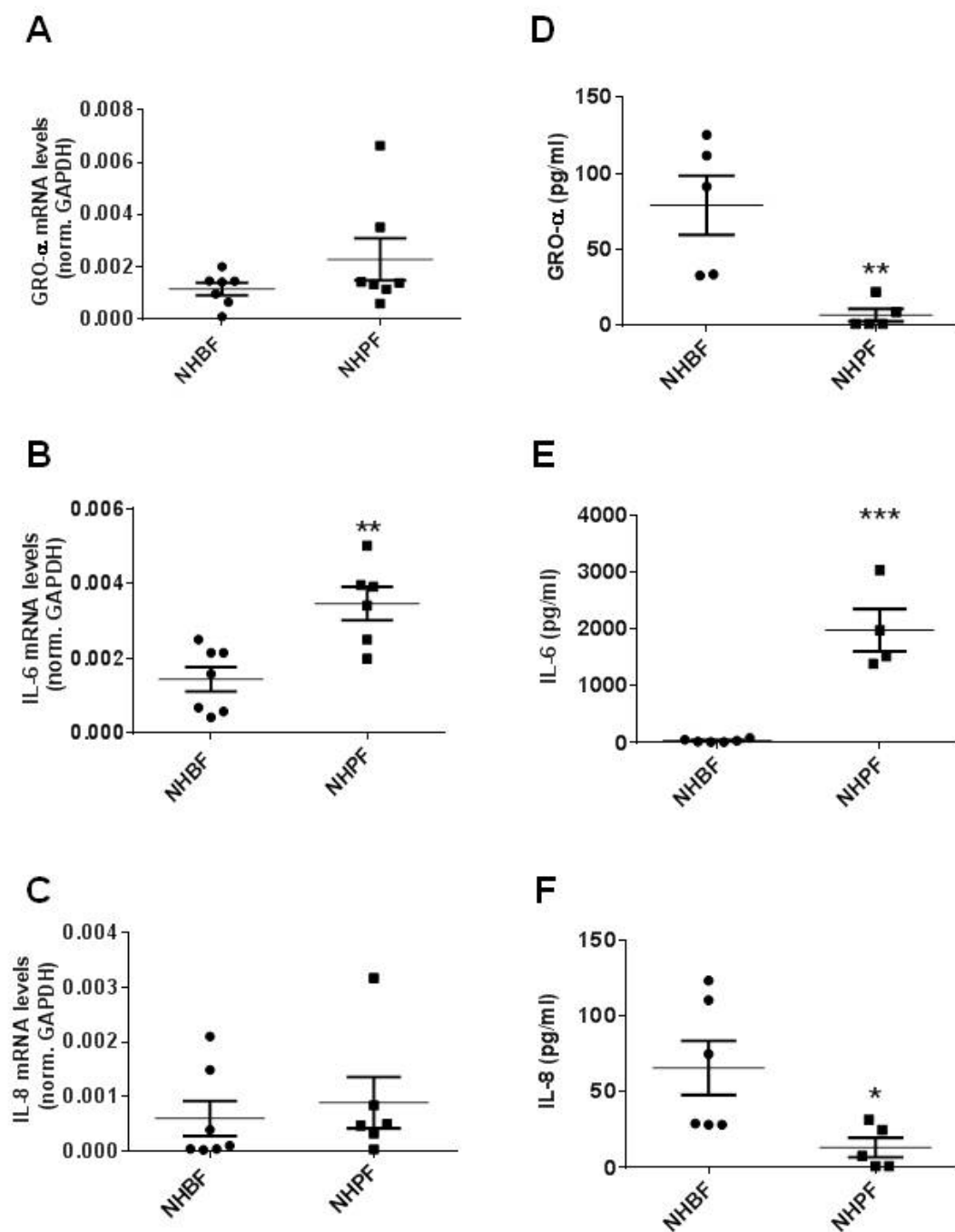


Figure 1. Basal mRNA and protein expression of cytokines are different in NHBF and NHPF.

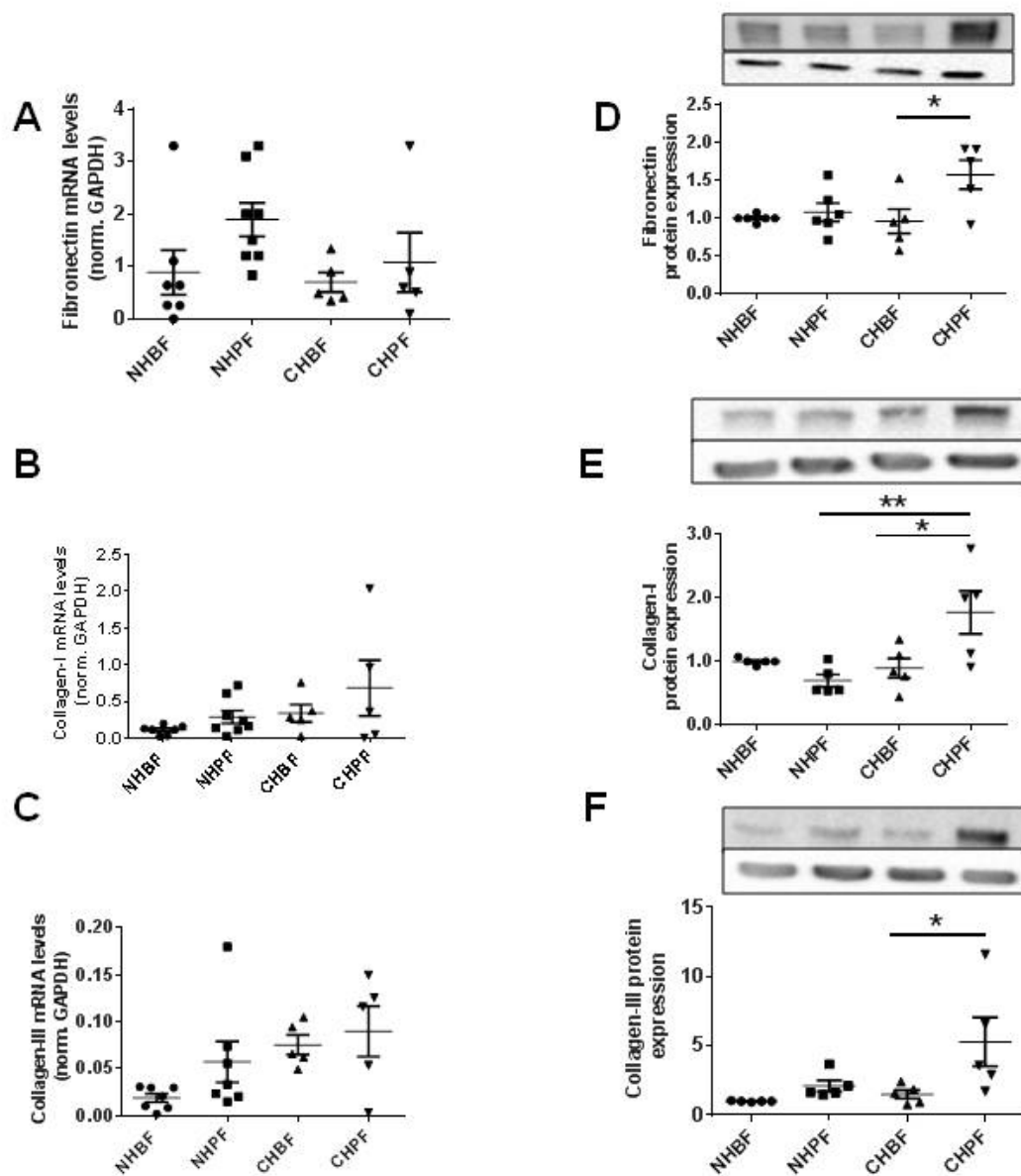


Figure 2: Basal ECM expression in NHBF, NHPF, CHBF and CHPF

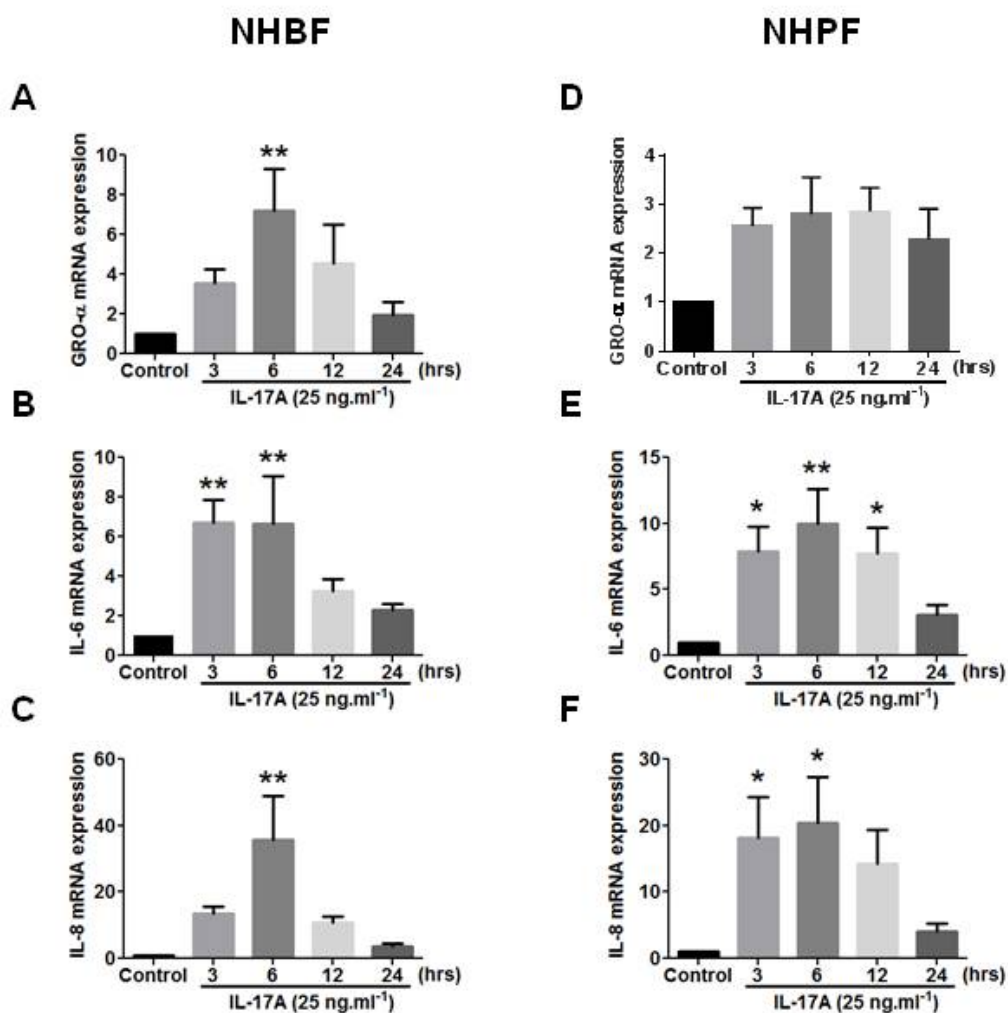


Figure 3. Interleukin-17A increases mRNA expression of cytokines in NHBF and NHPF.

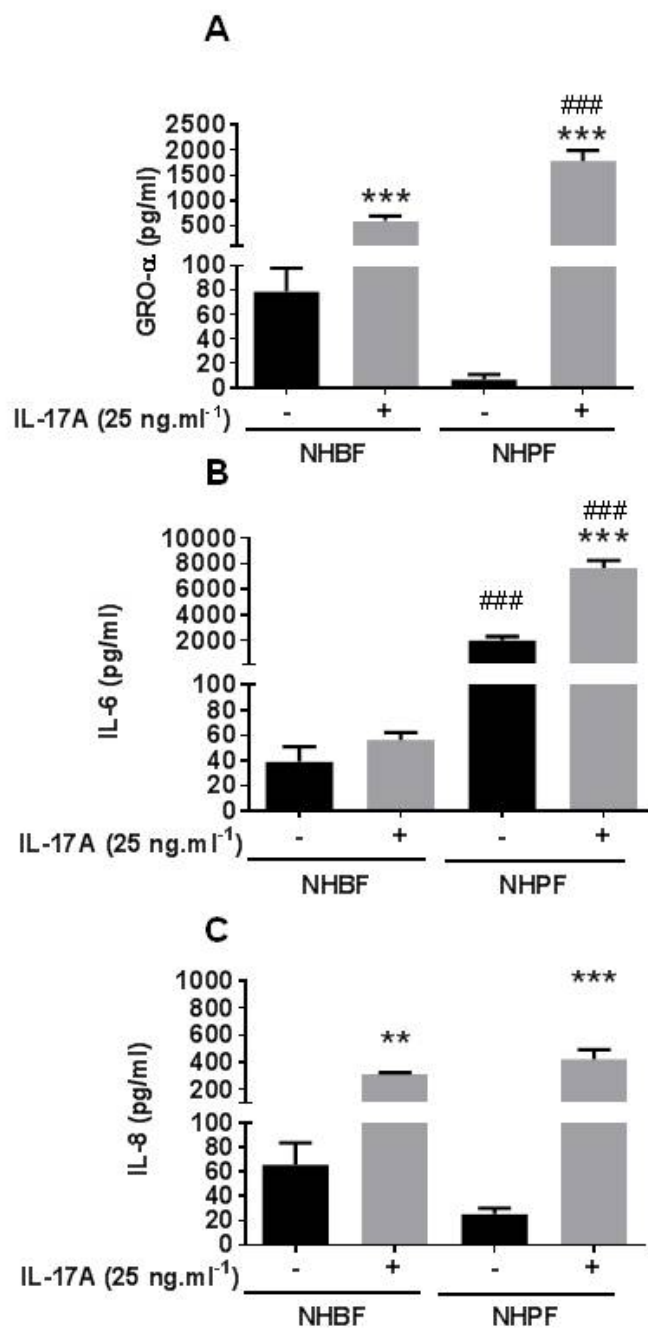


Figure 4. Differences in fibroblast cytokine expression due to IL-17A treatment, and/or to lung origin.

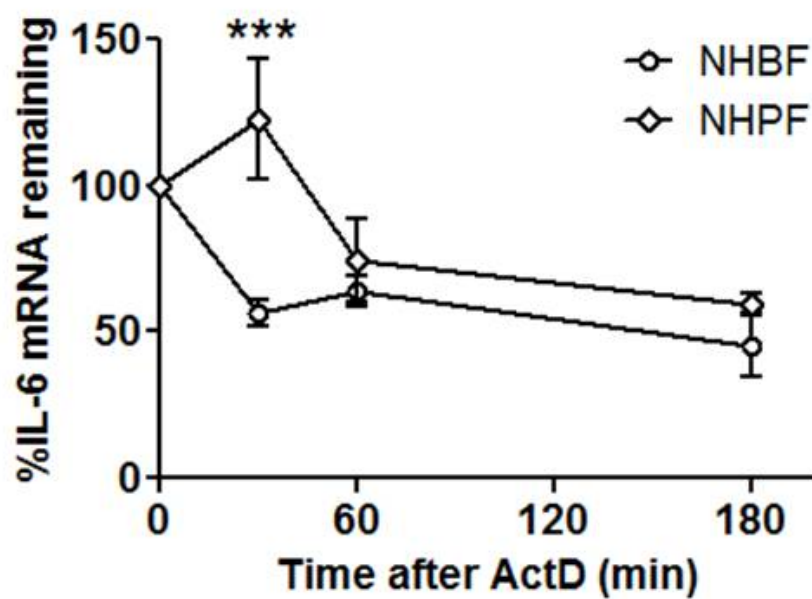


Figure 5. IL-17A modulates IL-6 mRNA stability in NHBF and NHPF

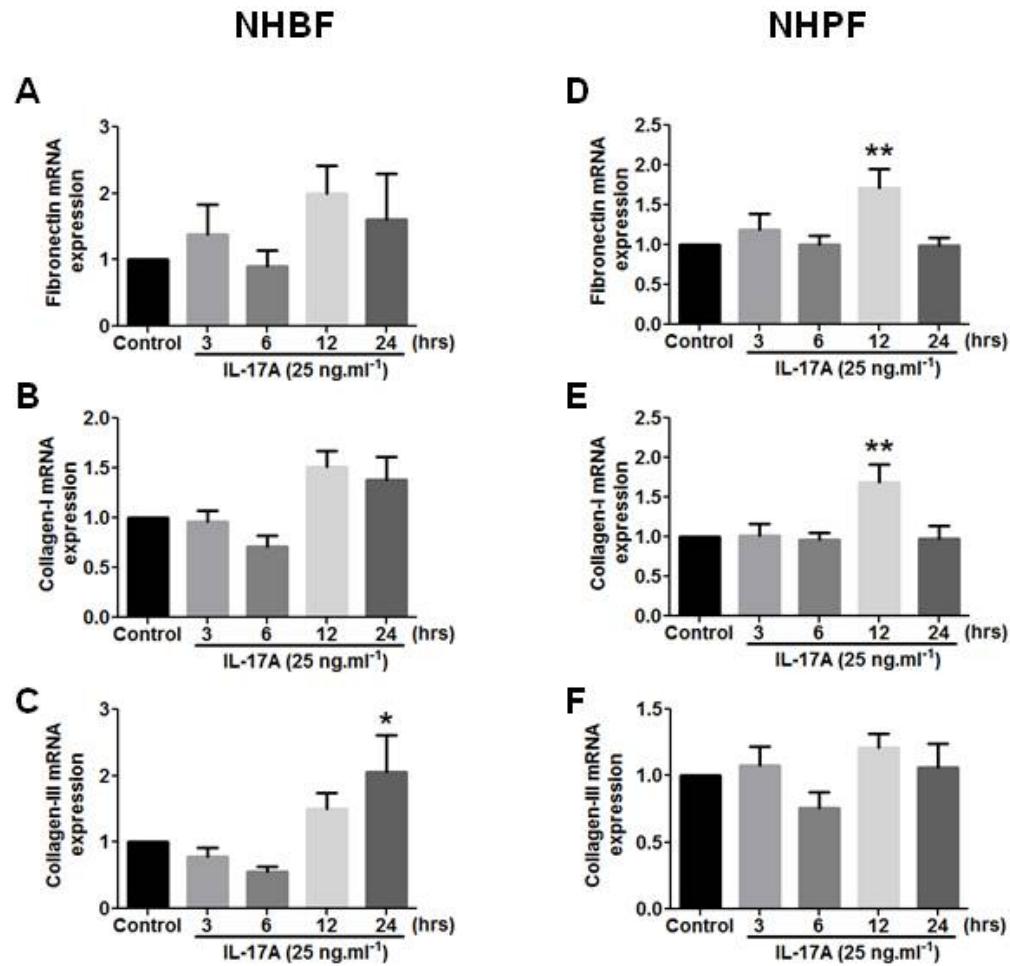


Figure 6. Interleukin-17A modulates mRNA expression of ECM in NHBF and NHPF.

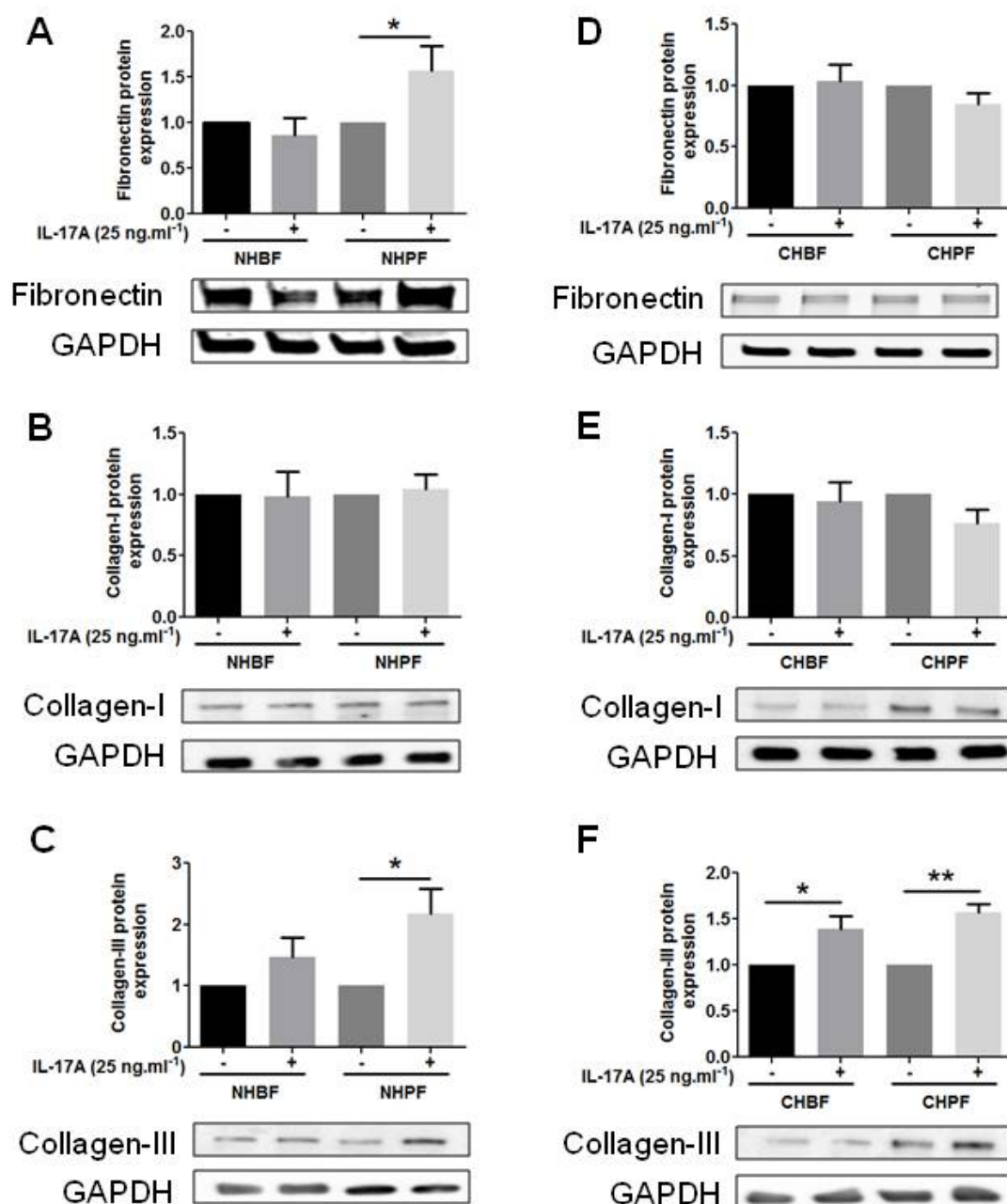


Figure 7. Interleukin-17A modulates ECM protein expression in NHBF, NHPF, CHBF and CHPF.