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Published in:

American Journal of Physiology - Lung Cellular and Molecular Physiology

DOI:

[10.1152/ajplung.00028.2020](https://doi.org/10.1152/ajplung.00028.2020)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Woldhuis, R. R., de Vries, M., Timens, W., van den Berge, M., Demaria, M., Oliver, B. G. G., Heijink, I. H., & Brandsma, C-A. (2020). Link between increased cellular senescence and extracellular matrix changes in COPD. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 319(1), L48-L60. <https://doi.org/10.1152/ajplung.00028.2020>

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RESEARCH ARTICLE | Senescence in the Lung

Link between increased cellular senescence and extracellular matrix changes in COPD

Roy R. Woldhuis,^{1,4,6,7} Maaike de Vries,^{2,4} Wim Timens,^{1,4} Maarten van den Berge,^{3,4} Marco Demaria,⁵ Brian G. G. Oliver,^{6,7} Irene H. Heijink,^{1,4} and Corry-Anke Brandsma^{1,4}

¹Department of Pathology and Medical Biology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands; ²Department of Epidemiology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands; ³Department of Pulmonary Diseases, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands; ⁴Groningen Research Institute for Asthma and COPD (GRIAC), University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands; ⁵European Research Institute for the Biology of Ageing, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands; ⁶Woolcock Institute of Medical Research, The University of Sydney, Sydney, Australia; and ⁷University of Technology Sydney, Sydney, Australia

Submitted 24 January 2020; accepted in final form 13 May 2020

Woldhuis RR, de Vries M, Timens W, van den Berge M, Demaria M, Oliver BGG, Heijink IH, Brandsma CA. Link between increased cellular senescence and extracellular matrix changes in COPD. *Am J Physiol Lung Cell Mol Physiol* 319: L48–L60, 2020. First published May 27, 2020; doi:10.1152/ajplung.00028.2020.—Chronic obstructive pulmonary disease (COPD) is associated with features of accelerated aging, including cellular senescence, DNA damage, oxidative stress, and extracellular matrix (ECM) changes. We propose that these features are particularly apparent in patients with severe, early-onset (SEO)-COPD. Whether fibroblasts from COPD patients display features of accelerated aging and whether this is also present in relatively young SEO-COPD patients is unknown. Therefore, we aimed to determine markers of aging in (SEO)-COPD-derived lung fibroblasts and investigate the impact on ECM. Aging hallmarks and ECM markers were analyzed in lung fibroblasts from SEO-COPD and older COPD patients and compared with fibroblasts from matched non-COPD groups ($n = 9–11$ per group), both at normal culture conditions and upon Paraquat-induced senescence. COPD-related differences in senescence and ECM expression were validated in lung tissue. Higher levels of cellular senescence, including senescence-associated β -galactosidase (SA- β -gal)-positive cells (19% for COPD vs. 13% for control) and p16 expression, DNA damage (γ -H2A.X-positive nuclei), and oxidative stress (*MGST1*) were detected in COPD compared with control-derived fibroblasts. Most effects were also different in SEO-COPD, with SA- β -gal-positive cells only being significant in SEO-COPD vs. matched controls. Lower decorin expression in COPD-derived fibroblasts correlated with higher p16 expression, and this association was confirmed in lung tissue. Paraquat treatment induced cellular senescence along with clear changes in ECM expression, including decorin. Fibroblasts from COPD patients, including SEO-COPD, display higher levels of cellular senescence, DNA damage, and oxidative stress. The association between cellular senescence and ECM expression changes may suggest a link between accelerated aging and ECM dysregulation in COPD.

aging; COPD; ECM; SEO-COPD; senescence

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory lung disease that causes severe respiratory

symptoms and a poor quality of life. COPD is characterized by airway obstruction and chronic inflammatory processes in the lungs that drives disturbed lung tissue remodeling, including emphysema and chronic bronchitis (23). The pathogenesis of COPD is largely unknown, and as a consequence current treatment strategies mainly act at improving symptoms, without reducing disease progression and mortality. Therefore, novel insights into the pathogenesis of COPD are needed.

Several studies demonstrated features of lung aging in the lungs of COPD patients (29, 30). Hence, COPD has been postulated as a disease of accelerated lung aging (25, 31). Aging is defined as the progressive decline of homeostasis, resulting in increased risk of disease or death (29). Features of lung aging including lung function decline, airspace enlargement, loss of elasticity, increased cellular senescence, genomic instability, and mitochondrial dysfunction are observed in COPD compared with matched healthy controls. All previous studies on lung aging in COPD were mainly focused on lung tissue changes in older COPD patients with mild-moderate COPD (8). However, with respect to accelerated lung aging, COPD patients who develop very severe COPD at an early age [previously defined as age <53 yr (42)] are of particular interest. These severe, early-onset (SEO)-COPD patients often have progressive disease at an early age despite normal alpha-1 antitrypsin levels and relatively few pack-years of smoking (42). Until now, only telomerase mutations and shorter telomeres were linked to SEO-COPD (43), but no further studies have been done to investigate the role of accelerated aging in SEO-COPD patients.

Lung extracellular matrix (ECM) dysregulation has been described as one of the features of lung aging. ECM is important for the function and structure of the lung and plays a major role in tissue repair and remodeling (23, 39). Recently, we showed clear differences in gene expression in lung tissue associated with aging (16). Pathway analyses suggested that age-related differences in ECM composition were more pronounced in COPD patients compared with subjects without COPD. Therefore, we propose that accelerated lung aging contributes to the pathology of COPD by deregulating lung tissue repair and remodeling. Fibroblasts are important struc-

Correspondence: C.-A. Brandsma (e-mail: c.a.brandsma@umcg.nl).

tural cells in the lung controlling ECM homeostasis and, as such, have an essential function in lung repair and remodeling (23, 39). Previous studies have demonstrated alterations in lung fibroblast function and ECM production in patients with COPD (3, 20, 26, 46, 50). In addition, higher levels of markers of cellular senescence were detected in lung tissue and structural lung cells, including lung fibroblasts from patients with COPD (8, 13, 21, 33). However, it is unknown if lung fibroblasts from SEO-COPD patients display an accelerated aging phenotype and if this has functional consequences.

Therefore, in this study we aimed to assess markers of aging in primary lung fibroblasts from SEO-COPD and older, mild-moderate, COPD patients. We focused on SEO-COPD, because accelerated aging may especially play a role in these patients. Moreover, the functional consequences of aging in fibroblasts on ECM regulation were studied and validated in lung tissue using the same patient groups.

METHODS

Subjects. Primary lung fibroblasts and peripheral lung tissue from subjects undergoing lung transplantation or tumor resection surgery were used. Resected lung tissue was isolated distal from the tumor and was macroscopically and histologically normal. Primary parenchymal lung fibroblasts were isolated as described before (35). The following inclusion criteria were used:

- 1) SEO-COPD patients; forced expiratory volume in 1 s/forced vital capacity (FEV1/FVC) < 70% and FEV1 < 30%pred measured at an age < 53 yr [according to (42)] and with age < 56 yr at time of lung transplant surgery;
- 2) non-COPD control subjects (SEO-COPD-matched); FEV1/FVC > 70%, age < 60 yr at time of surgery;
- 3) Older, mild-moderate, COPD patients; FEV1/FVC < 70% and FEV1 30–80%pred, age > 65 yr at time of surgery;
- 4) non-COPD control subjects (older COPD-matched); FEV1/FVC > 70%, age > 65 yr at time of surgery.

None of the COPD patients were alpha-1 antitrypsin deficient. To get sufficient SEO-COPD-matched non-COPD control subjects, we included subjects at an age < 60 yr at the time of surgery, taking into account the age-matching with the SEO-COPD group.

The study protocol was consistent with the Research Code of the University Medical Centre Groningen and national ethical and professional guidelines (“Code of conduct; Dutch federation of biomedical scientific societies”; <https://www.federa.org/>). Lung fibroblasts and lung tissues used in this study were derived from leftover lung material after lung surgery and transplant procedures. This material was not subject to the Medical Research Human Subjects Act in the Netherlands, and, therefore, an ethics waiver was provided by the Medical Ethical Committee of the University Medical Center Groningen. All samples and clinical information were deidentified before experiments were performed.

Primary parenchymal lung fibroblast culture. The fibroblasts were cultured as described before (35). At passage 5, 25,000 fibroblasts were seeded in 12-well plates and after 2 days treated with or without 250 μ M Paraquat dichloride hydrate (PQ) (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 24 h to induce cellular senescence (11). After 24 h, PQ was removed and cells were either harvested immediately for flow cytometry or kept in culture for another 24 h [gene expression analyses, γ -H2A.X staining] or 4 days [gene expression analyses, senescence-associated β -galactosidase (SA- β -gal) staining and secreted proteins] (Supplemental Fig. S1; all supplemental data are available online at <https://doi.org/10.6084/m9.figshare.11661192>). These time-points were carefully chosen based on pilot study results.

SA- β -gal staining. Cellular senescence was assessed with standard SA- β -gal staining as described before (18). Fibroblasts were fixed

with 2% formaldehyde + 0.2% glutaraldehyde in PBS for 5 min. After fixation, cells were incubated with the described staining solution for 16 h (in a dry incubator) at 37°C. After incubation, the staining solution was washed away and cells were covered with 70% glycerol in PBS for storage. Four random images of every well with cells were taken with a Nikon camera on a Leica light microscope at a total magnification of $\times 200$. SA- β -gal-positive cells and total cells were scored blindly to calculate the percentage of SA- β -gal-positive cells.

Immunofluorescence γ -H2A.X staining. DNA damage was assessed using immunofluorescence staining for the DNA damage marker γ -H2A.X. Fibroblasts were cultured on a 16 mm circle glass coverslip (Fisher Scientific, Landsmeer, the Netherlands), and as positive control for the staining, fibroblasts were treated with 500 μ M H₂O₂ for 4 h. Twenty-four hours after PQ removal, fibroblasts were fixed with ice-cold 80% acetone in PBS for 10 min at 4°C. After fixation, nonspecific binding was blocked with 5% BSA in PBS. Fibroblasts were incubated with 2.5 μ g/ml γ -H2A.X conjugated with Alexa Fluor 555 antibody (EMD Millipore, Amsterdam, the Netherlands) in the dark for 1 h at room temperature (RT). After incubation, fibroblasts were counterstained with DAPI for 5 min at RT and mounted on a slide with VECTASHIELD Antifade Mounting Medium (Vector Laboratories, Peterborough, UK). Four random images of every coverslip with cells were taken using a Leica LMD6000 fluorescence microscope at a total magnification of $\times 400$. Positive nuclei and total cells were scored blindly to calculate the percentage of γ -H2A.X-positive cells. Representative examples of the staining are shown in Supplemental Fig. S2.

Analysis of reactive oxygen species. Levels of reactive oxygen species (ROS) were determined by flow cytometry. Directly after 24 h of PQ treatment or untreated, fibroblasts were stained with 2.5 μ g/ml chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; DCF) (Invitrogen, Landsmeer, the Netherlands) in PBS for 1 h in a 5% CO₂ incubator at 37°C. After incubation, fibroblasts were trypsinized and collected in tubes for flow cytometry analyses on a BD LSR-II cytometer (BD Biosciences, Vianen, the Netherlands). The geometric mean fluorescence intensity (gMFI) of DCF in the live cell population was used.

Gene expression analyses. For multiple aging markers and ECM genes (Supplemental Table S1) mRNA expression was measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). RNA was harvested 24 h and 4 days after PQ removal in TRIzol (Invitrogen), and total RNA was isolated according to manufacturer's protocol. RNA of lung tissue was isolated using an RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). RNA concentrations were measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). We used 400 ng of RNA for cDNA synthesis with random primers and Superscript II (Invitrogen) according to manufacturer's protocol. For gene expression analysis, 5 ng of cDNA was used for qRT-PCR with PowerUp SYBR Green Master Mix (Applied Biosystems, Bleiswijk, the Netherlands) using a LightCycler 480 PCR instrument (Roche, Woerden, the Netherlands). For ECM gene expression analyses, TaqMan gene expression assays (Applied Biosystems) were used. *18S rRNA* (*18S*) and *RNA polymerase II* (*RP2*) were used as reference genes. Sequences of used primers are listed in Supplemental Table S2, and TaqMan assay IDs are listed in Supplemental Table S3. Samples including a no template control as negative control were run in triplicate and $2^{(-\Delta\Delta C_p)}$ was calculated for relative mRNA expression levels.

Secreted protein analyses. Cell-free supernatants were harvested 4 days after PQ removal and stored in -80°C before ELISA analysis. Secreted IL-6, IL-8, and decorin levels were measured using Human DuoSet ELISA (R&D Systems, Abingdon, UK). As the numbers of cells were different at the end of culture between COPD and control-derived fibroblasts, and between untreated and PQ-treated (Figs. 1C and 4C), we corrected the secreted protein levels for cell numbers counted at the end of culture.

Statistical analyses. SPSS software was used for the statistical analyses. Mann-Whitney *U* tests were used to test differences between fibroblasts from COPD patients compared with controls. Upon significant difference between COPD and control, subgroup analyses were performed to test differences between SEO-COPD and older COPD compared with their matched control groups, using Mann-Whitney *U* tests. The effect of PQ treatment was analyzed using paired analysis with Wilcoxon signed-rank tests. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics. The characteristics of the 40 lung fibroblast donors are shown in Table 1. The SEO-COPD and older, mild-moderate, COPD groups were similar compared with their matched control groups in terms of age, sex, pack-years, and months of smoking cessation. The male/female ratio was significantly different between the younger groups and the older groups. All SEO-COPD patients suffered from severe emphysema and had a FEV1%pred <30% before the age of 53 yr.

Higher levels of cellular senescence in COPD-derived fibroblasts, including in SEO-COPD-derived fibroblasts. After 7 days of basal cell culture, the percentage of SA- β -gal-positive cells in lung fibroblasts from COPD patients was significantly higher compared with control subjects (Fig. 1, A and B). Subgroup analyses showed a significant difference between SEO-COPD and their matched controls and a trend between older COPD and their matched controls. In line with higher senescence, the total cell numbers at the end of culture were lower in COPD-derived fibroblasts compared with control subjects, which remained only significant between SEO-COPD and their matched controls in the subgroup analyses (Fig. 1C). Gene expression of the senescence marker p16 (*CDKN2A*) was significantly higher in COPD-derived fibroblasts (Fig. 1D) and a similar trend ($P = 0.05$) was observed for the senescence marker p21 (*CDKN1A*) (Fig. 1E). The higher p16 expression was only significant in fibroblasts from older COPD patients compared with their matched control-derived fibroblasts. No differences were observed in the secretion of IL-6 between the groups (Fig. 1F), while lower secretion of IL-8 was observed in fibroblasts from COPD patients compared with controls (Fig.

1G). Levels of secreted cytokines were normalized to cell numbers, but this did not have a big impact on the results (uncorrected data are depicted in Supplemental Fig. S3).

Higher levels of DNA damage and oxidative stress in COPD-derived fibroblasts, including in SEO-COPD-derived fibroblasts. The percentage of γ -H2A.X-positive cells (DNA damage) was higher in lung fibroblasts from COPD patients compared with control subjects (examples of staining in Supplemental Fig. S2), which was only significant between SEO-COPD and their matched controls in the subgroup analyses (Fig. 2A).

Expression of the oxidative stress response gene microsomal glutathione S-transferase 1 (*MGST1*) was higher in fibroblasts from COPD patients compared with control subjects, which was only significant comparing SEO-COPD to matched controls (Fig. 2B). We observed no significant differences in ROS levels between the groups (Fig. 2C). However, a positive correlation was observed between ROS levels and γ -H2A.X-positive cells, and *MGST1* gene expression (Fig. 2D).

No significant differences were observed between the groups in genes involved in DNA repair (*Ku70* and *Ku80*), nutrient sensing (*EIF4B* and *SHC1* gene expression), in mTOR activity (p-S6K1 protein levels) nor in genes or proteins involved in loss of proteostasis (*FOXO3*, *SIRT1*, and *NRF2* gene expression and autophagy markers LC3-II and p62); see Supplemental Fig. S4.

Lower DCN gene expression in COPD-derived fibroblasts is correlated with higher markers of cellular senescence and lower lung function. To assess the impact of the accelerated aging phenotype on lung fibroblast function, we measured gene expression of ECM proteins and alpha smooth muscle actin (*ACTA2*). Decorin (*DCN*) expression was lower in fibroblasts from COPD patients compared with control subjects, both in SEO-COPD and older COPD (Fig. 3A). No differences in gene expression were observed for the other ECM genes nor *ACTA2* (Supplemental Figs. S5 and S6). *DCN* expression was positively correlated with lung function parameters FEV1 and FEV1/FVC (Fig. 3B), and negatively correlated with the cellular senescence marker p16 (Fig. 3C). Similar trends for negative correlation were observed for *DCN* and p21 ($P =$

Table 1. Subject characteristics of fibroblasts

Variable	Control (SEO-COPD-matched)	SEO-COPD	<i>P</i> Value	Control (older COPD-matched)	Older, Mild-Moderate COPD	<i>P</i> Value
<i>n</i>	9	10		10	11	
Age, mean yr (range)	52 (42–59)	50 (44–55)	0.349	70 (65–81)	73 (66–81)	0.176
Men/women, <i>n</i>	1/8	2/8	0.556	8/2	10/1	0.500
Pack-years	32 (28–35)	26 (14–30)	0.673	43 (28–51)	49 (19–53)	0.823
Stop-months	84 (18–168)	78 (63–93)	0.677	186 (81–252)	66 (27–96)	0.421
non-COPD, <i>n</i>	9			10		
COPD, <i>n</i>		10			11	
GOLD 1						
GOLD 2					7	
GOLD 3					4	
GOLD 4		10				
FEV1%pred	87.0 (83.5–92.0)	16.5 (14.3–22.7)	0.000	90.7 (82.2–104.0)	66.7 (43.4–70.5)	0.000
FVC%pred	92.8 (84.6–101.0)	42.6 (37.9–68.1)	0.000	89.5 (76.7–107.5)	83.5 (79.7–98.8)	0.647
FEV1/FVC	75.9 (73.3–79.0)	27.6 (26.0–38.5)	0.000	72.1 (70.3–75.1)	50.0 (41.7–59.0)	0.000

Data are presented as medians with interquartile ranges (IQRs), unless otherwise stated. Significant differences between groups were tested using Mann-Whitney *U* tests or unpaired *t* tests. *P* values are stated and in boldface when significantly different. Gold stage based on FEV1%pred. SEO-COPD, severe, early-onset chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease.

Fig. 1. Higher levels of cellular senescence in chronic obstructive pulmonary disease (COPD)-derived fibroblasts. Examples of senescence-associated β -galactosidase (SA- β -gal) staining of all 4 patient groups (A) and quantification of SA- β -gal-positive cells (B) and total cell numbers (C). Dot plots show mRNA expression (24 h) of p16 (D) and p21 (E) and secretion of IL-6 (F) and IL-8 (G) in cell culture medium (corrected for cell number) of all 4 patient groups. Green, severe, early-onset (SEO)-COPD-matched control; red, SEO-COPD; blue, older COPD-matched control; yellow, older, mild-moderate COPD. Lines represent medians. Significant differences tested with Mann-Whitney U tests. * P value < 0.05 or P value is indicated.

Fig. 2. Higher levels of DNA damage and oxidative stress in chronic obstructive pulmonary disease (COPD)-derived fibroblasts. Dot plots show percentage of γ -H2A.X-positive cells (A) *MGST1* mRNA expression (24 h) (B) and ROS levels as 2',7'-dichlorodihydrofluorescein diacetate (DCF) geometric mean fluorescence intensity (gMFI) (C) of all 4 patient groups. Green, severe, early-onset (SEO)-COPD-matched control; red, SEO-COPD; blue, older COPD-matched control; yellow, older, mild-moderate COPD. Lines represent medians. Significant differences tested with Mann-Whitney *U* tests. **P* value < 0.05 or *P* value is indicated. Dot plots show correlation between ROS levels and γ -H2A.X-positive cells and *MGST1* mRNA expression (24 h) (D). Significant differences tested with Spearman's rank tests. In the plots the Spearman rho and *P* value are indicated and boldfaced when significant.

0.064) and SA- β -gal (*P* = 0.074) (Fig. 3C). To validate differences in *DCN* gene expression, we measured the levels of secreted decorin in the cell culture supernatants. A strong significant positive correlation between *DCN* gene expression and decorin protein secretion was found (Fig. 3E). While the pattern was similar as observed for gene expression, no significant differences were observed for decorin protein secretion between the groups (Fig. 3D).

Paraquat treatment induces cellular senescence in primary lung fibroblasts. To induce cellular senescence in primary lung fibroblasts (see Table 1), we used the herbicide Paraquat (PQ), which causes oxidative stress (11). In occupational exposure, PQ has been documented as risk factor for COPD (10, 45). PQ treatment did not affect cell viability (data not shown). PQ treatment resulted in a significant increase in cellular senescence markers in primary lung fibroblasts, including increased SA- β -gal-positive cells, lower cell numbers, increased p21 gene expression (24 h and 4 days after PQ removal), and IL-6 and IL-8 secretion in fibroblasts from all subject groups (Fig. 4, A–G), whereas a small but significant decrease was observed for p16 after 24 h of PQ removal (Fig. 4D). After 4 days, p16 expression was not different between PQ-treated and untreated, while p21 expression remained significantly increased upon PQ treatment (Supplemental Fig. S7). In addition to senescence induction, PQ treatment induced DNA damage (γ -H2A.X-positive cells, see Supplemental Fig. S2 for example of staining) and ROS levels (Fig. 4, H and I) significantly, with only a trend toward significance for SEO-COPD in γ -H2A.X. None of the effects of PQ treatment were significantly different between COPD and control-derived fibroblasts.

Decreased *DCN* expression and protein secretion upon senescence induction in lung fibroblasts. After PQ induced senescence, *DCN* gene expression and protein secretion were

decreased 4 days after PQ removal (Fig. 5, A and B), which is the time-point when SA- β -gal positivity is increased. At the earlier time-point, 24 h after PQ removal, only a small decrease in *DCN* expression was observed (Supplemental Fig. S8A). Again, we found a clear correlation between *DCN* gene expression and protein secretion in untreated cells and in PQ-treated cells as well (Fig. 5C).

PQ-induced senescence results in altered ECM gene expression in lung fibroblasts. PQ-induced senescence resulted in striking changes in ECM gene expression and *ACTA2* gene expression 4 days after PQ removal. PQ-induced senescence resulted in decreased expression of collagen, type I, alpha 1 (*COL1A1*), fibulin-5 (*FBN1*), elastin (*ELN*), fibronectin (*FNI*), *ACTA2*, and biglycan (*BGN*) and increased expression of versican (*VCAN*) (Fig. 6). The decrease in *COL1A1*, *FNI*, and *BGN* was only significant in control-derived fibroblasts, but not in COPD-derived fibroblasts. *VCAN* expression upon PQ-induced senescence was significantly higher in COPD-derived fibroblasts compared with control-derived fibroblasts. After 24 h of PQ removal, no or only small changes were found in ECM gene expression (Supplemental Fig. S8). *ELN* expression was increased after 24 h, opposite to decreased expression after 4 days.

COPD-derived fibroblasts respond differently to the induction of cellular senescence than control-derived fibroblasts. Next, the response to senescence induction upon PQ treatment was compared between COPD and control-derived fibroblasts. PQ-induced senescence was associated with reduced gene expression of Ku70 (*XRCC6*) in fibroblasts from SEO-COPD patients and Ku80 (*XRCC5*) in fibroblasts from SEO-COPD and older COPD patients, and SEO-COPD-matched control subjects (Fig. 7, A and B). For Ku70, the response on PQ-induced senescence was not different between the groups, whereas Ku80 gene expression was more decreased in older

Fig. 3. Altered *DCN* expression in chronic obstructive pulmonary disease (COPD) fibroblasts is associated with senescence. Dot plot shows mRNA expression (4 days) of *DCN* of all 4 patient groups (A). Green, severe, early-onset (SEO)-COPD-matched control; red, SEO-COPD; blue, older COPD-matched control; yellow, older, mild-moderate COPD. Lines represent medians. Significant differences tested with Mann-Whitney *U* tests. **P* value < 0.05. Dot plots show correlation between *DCN* mRNA expression (4 days) and FEV1% predicted and FEV1/FVC (B), mRNA expression (24 h) of p16 and p21, and senescence-associated β -galactosidase (SA- β -gal)-positive cells (C). Significant differences tested with Spearman's rank tests. In the plots the Spearman ρ and *P* value are indicated and boldfaced when significant. Dot plot shows decorin protein secretion levels (corrected for cell number) of all 4 patient groups (D). Green, SEO-COPD-matched control; red, SEO-COPD; blue, older COPD-matched control; yellow, older, mild-moderate COPD. Lines represent medians. Significant differences tested with Mann-Whitney *U* tests. Dot plot shows correlation between *DCN* mRNA expression (4 days) and decorin protein secretion (E). Significant difference tested with Spearman's rank test. In the plot the Spearman ρ and *P* value are indicated and boldfaced when significant.

COPD-derived fibroblasts compared with their matched control subjects. Additionally, PQ-induced senescence caused lower induction of oxidative stress response genes *MGST1* and *FOXO3* in fibroblasts from COPD patients compared with control subjects, including in fibroblasts from SEO-COPD compared with their matched control subjects (Fig. 7, C and D). Relative expression levels are shown in the online supplement (Supplemental Fig. S9).

Patient characteristics of human lung tissue. To validate the association between senescence and ECM gene expression observed in fibroblasts, we used lung tissue from 59 donors using the same group definitions. The characteristics of the 59 lung tissue donors are depicted in Table 2. The SEO-COPD and their matched control group, and the older mild-moderate COPD and their matched control group were similar in terms of age, sex, pack-years, and months of smoking cessation. The man/woman ratio was significantly different between younger groups and older groups. All SEO-COPD patients suffered from severe emphysema and had a FEV1%pred <30% before the age of 53 yr.

Confirmation of the association between cellular senescence and *DCN* gene expression in human lung tissue. Gene expression of the senescence marker p21, but not p16, was higher in lung tissue from COPD patients (Fig. 8, A and B). In the subgroup analyses this p21 difference was only significant in lung tissue from SEO-COPD patients compared with their matched control subjects (Fig. 8B). No significant differences were observed in *DCN* expression (Fig. 8C). In line with our findings in fibroblasts, a negative correlation was observed between *DCN* and p16 gene expression in lung tissue (Fig. 8D).

DISCUSSION

In this study, we assessed the aging phenotype of lung fibroblasts from COPD patients and the consequences on ECM regulation, with a special focus on SEO-COPD. We observed higher levels of cellular senescence, DNA damage, and markers of oxidative stress in lung fibroblasts from COPD patients

Fig. 4. Induction of cellular senescence in primary lung fibroblasts. Examples of senescence-associated β -galactosidase (SA- β -gal) staining of untreated (basal) and paraquat dichloride hydrate-treated (PQ) fibroblasts (A) and quantification of SA- β -gal-positive cells (B) and total cell numbers (C). Dot plots show p16 (D) and p21 (E) mRNA expression (24 h), secretion of IL-6 (F) and IL-8 (G) in cell culture medium (corrected for cell number), γ -H2A.X-positive cells (H), and ROS levels (I) of untreated (basal) and PQ-treated (PQ) fibroblasts per subgroup. Blue, basal; red, PQ. Significant differences between untreated and PQ treated are tested with Wilcoxon signed-rank tests and are indicated on top. * P value < 0.05 or P value is indicated.

Fig. 5. Decreased *DCN* expression and protein secretion upon senescence induction in primary lung fibroblasts. Dot plots show mRNA expression of *DCN* (A) and decorin protein secretion (B) after 4 days of untreated (basal) and paraquat dichloride hydrate (PQ)-treated fibroblasts per subgroup. Blue, basal; red, PQ. Significant differences between untreated and PQ treated are tested with Wilcoxon signed-rank tests and are indicated on top. * P value < 0.05 . Dot plot shows correlation between *DCN* mRNA expression (4 days) and decorin protein secretion (C) for untreated (Basal) and PQ fibroblasts. Blue, basal; red, PQ. Significant differences tested with Spearman's rank tests. In the plots the Spearman ρ and P value are indicated and boldfaced when significant.

compared with control subjects under normal cell culture conditions. Interestingly, part of these effects was more pronounced in SEO-COPD. With respect to ECM regulation, *DCN* gene expression was lower in COPD compared with control-derived fibroblasts, and lower *DCN* expression was correlated with higher markers of cellular senescence. In addition, PQ-induced senescence resulted in clear alterations in ECM gene expression, including reduced *DCN* expression. Finally, we validated the higher levels of cellular senescence and the correlation between lower *DCN* expression and higher expression of the cellular senescence marker p16, in human lung tissue, despite the presence of different cell types in lung tissue.

With the increasing life expectancy worldwide, interest in the role of aging in health and disease has massively increased (29). Especially for chronic degenerative diseases, including COPD, it has been postulated that acceleration of the normal aging process is involved in disease pathogenesis (25). Up to now, several studies have investigated the role of aging in COPD, and most of these studies found indications for an accelerated aging process to be involved in COPD (8, 25, 30, 31). Our findings in lung fibroblasts on cellular senescence, oxidative stress, and DNA damage are in line with previous studies. However, the uniqueness of our study is that we specifically focused on SEO-COPD patients. These COPD patients develop the most severe form of COPD at an earlier age with relatively low numbers of smoking pack-years compared with the majority of patients with mild-moderate COPD (42). Why these patients are particularly susceptible to developing this progressive and severe form of COPD is a major unsolved question. In particular the fact that they develop COPD at a relatively young age is interesting for studies on the role of accelerated aging. We observed that the higher levels of cellular senescence in fibroblasts and lung tissue, and DNA damage in fibroblasts were most pronounced in SEO-COPD. As these relatively young patients already display this aging phenotype our data support a role for accelerated lung aging in SEO-COPD.

We found more DNA damage and oxidative stress in COPD and SEO-COPD-derived fibroblasts. These results are in line with previous findings in lung tissue and lung epithelial cells from COPD patients (2, 7, 41). DNA damage and oxidative stress can both induce cellular senescence, while oxidative stress can enhance DNA damage (12, 22, 34). The observed correlation between ROS and DNA damage (γ -H2A.X)

suggests that ROS may have contributed to DNA damage in our study. In addition, we observed higher *MGST1* expression in SEO-COPD-derived fibroblasts. *MGST1* has been linked to aging in several studies (27, 40), including in lung aging (16). The positive correlation between *MGST1* and ROS levels in fibroblasts suggests that higher *MGST1* gene expression might be the result of higher ROS levels. In COPD patients, lung fibroblasts are chronically exposed to high levels of oxidative stress resulting from chronic inflammation, tissue damage, and also directly from oxidative stress exposure. These exposures may explain the higher levels of cellular senescence, DNA damage and oxidative stress in COPD-derived fibroblasts.

Although a link between aging and ECM dysregulation was proposed previously (8, 17, 30), to our knowledge no studies investigated the link between an accelerated aging phenotype and fibroblast dysfunction in COPD yet. We observed a correlation between higher levels of cellular senescence and lower *DCN* gene expression in both COPD and SEO-COPD-derived fibroblasts and confirmed this association in our PQ-induced senescence model and in lung tissue as well. Decorin is a proteoglycan that binds many growth factors and their receptors, including transforming growth factor (TGF)- β , thereby inhibiting their activity (14, 37, 48). TGF- β is known to be consistently upregulated in COPD (15). Stimulation with TGF- β in vitro induces ECM protein production via the SMAD pathway, while in contrast TGF- β inhibits the production of decorin (35, 50). Decorin also binds to collagen fibrils, providing structural support for the ECM. In emphysema lower gene expression and protein levels of proteoglycans, including lower decorin, have been detected (35, 47), and lower decorin levels have been linked to skin aging as well (28, 36). It has been proposed that lower *DCN* expression in the small airway contributes to loss of fiber organization in the airway walls contributing to airway obstruction (1, 6, 47). Thus, lower *DCN* expression in senescent lung fibroblasts as observed in our study may affect the ECM structure in the peripheral lung and contribute to lung tissue remodeling and small airway obstruction in COPD. In addition to *DCN*, we also measured gene expression of multiple other ECM proteins. However, we did not find significant differences between COPD and non-COPD control-derived fibroblasts at both time-points (Supplemental Figs. S5, S6). So, the observed

Fig. 6. Altered extracellular matrix (ECM) gene expression upon senescence induction in primary lung fibroblasts. Dot plots show mRNA expression of *COL1A1* (A), *FBN5* (B), *ELN* (C), *FN1* (D), and *ACTA2* (E), *BGN* (F), and *VCAN* (G) after 4 days of untreated (basal) and paraquat dichloride hydrate (PQ)-treated fibroblasts per groups and per subgroup. Blue, basal; red, PQ. Significant differences between untreated and PQ treated are tested with Wilcoxon signed-rank tests and are indicated on top. **P* value < 0.05 or *P* value is indicated.

decrease in decorin is likely not preceded by changes in gene expression of the other ECM proteins. We observed a significant correlation between *DCN* gene expression and protein secretion, but we could not confirm the differences between COPD- and control-derived fibroblasts on decorin protein secretion. This may be due to overcorrection for the differences in cell number, since we assessed the cell numbers at the end of culture, while secreted proteins were accumulated over 4 days. The decorin secretion levels were indeed significantly lower in COPD-derived cultures when we did not correct for cell numbers (Supplemental Fig. S3).

The fact that we confirmed the correlation between lower *DCN* expression and higher p16 in lung tissue further supports the link between cellular senescence and ECM dysregulation in vivo.

Most ECM gene expression changes upon PQ-induced senescence were observed after 4 days, when the cells are senescent (SA- β -gal positive). Since, p21 expression was increased after 24 h, it is likely that the ECM gene expression changes develop as a result of the senescence induction and not as a direct effect of the PQ treatment. Opposite to the decreased expression of the majority of ECM genes upon PQ-induced

Fig. 7. Chronic obstructive pulmonary disease (COPD)-derived fibroblasts respond differently to induction of cellular senescence than control-derived fibroblasts. Dot plots show fold changes of mRNA expression after paraquat dichloride hydrate (PQ) treatment (24 h) compared with untreated (basal) fibroblast expression of Ku70 (A), Ku80 (B), *MGST1* (C), and *FOXO3* (D) of all 4 patient groups. Lines represent medians. Significant differences between untreated and PQ-treated per group are tested with Wilcoxon signed-rank tests and if significant are indicated above the x-axis with #. Significant differences in fold changes are tested with Mann-Whitney *U* tests and indicated at the right graph. **P* value < 0.05 or *P* value is indicated.

senescence, we observed increased *VCAN* gene expression. Interestingly, most of these ECM gene expression changes are in the same direction as changes in lung tissue from COPD patients (1, 6), including higher versican protein levels (20). Versican inhibits the synthesis and regeneration of elastic fibers and is believed to be a negative regulator of elastin (20, 32). Indeed, together with increased *VCAN* expression upon PQ-induced senescence, we observed decreased *ELN* expression after 4 days. Elastin dysregulation plays an important role in COPD and lower elastin protein levels have been shown in small airway walls from COPD patients (19). Together, our findings support the notion that cellular senescence of lung fibroblasts can lead to ECM dysregulation in COPD and contribute to aberrant tissue remodeling.

Remarkably, previous studies using senescent fibroblasts from idiopathic pulmonary fibrosis patients have shown higher ECM gene expression, including *ACTA2* and collagen (38, 49), which is different from our findings in senescent fibroblasts from COPD patients. These differences suggest that the effect of cellular senescence on fibroblasts and their function is context dependent and can be different between cell origin and diseases.

Another interesting finding of our study is that we found differences in the response toward senescence induction between COPD and control-derived fibroblasts. The reduction in DNA damage repair markers Ku70 and Ku80 upon PQ-induced senescence was more pronounced in fibroblasts from COPD patients, which is in line with previous findings

Table 2. Subject characteristics of lung tissue

Variable	Control (SEO-COPD-matched)	SEO-COPD	<i>P</i> Value	Control (older COPD-matched)	Older, Mild-Moderate COPD	<i>P</i> Value
Number	14	14		15	16	
Age, mean yr (range)	52 (42–60)	52 (47–55)	0.763	71 (65–82)	71 (65–79)	0.971
Men/women, <i>n</i>	4/10	4/10	1.000	12/3	12/4	0.749
Pack-years	28 (20–35)	30 (22–40)	0.476	43 (25–52)	44 (21–50)	0.657
Stop-months	72 (15–252)	66 (51–93)	0.412	186 (54–288)	54 (15–123)	0.222
non-COPD, <i>n</i>	14			15		
COPD, <i>n</i>		14			16	
GOLD 1						
GOLD 2					11	
GOLD 3					5	
GOLD 4		14				
FEV1%pred	90.1 (86.6–99.78)	17.6 (15.2–23.9)	0.000	87.0 (79.1–101.2)	64.5 (45.8–67.6)	0.000
FVC%pred	97.3 (92.8–112.5)	46.7 (41.8–63.7)	0.000	86.5 (74.6–103.7)	83.9 (69.9–90.5)	0.426
FEV1/FVC	75.2 (73.0–78.9)	27.3 (25.9–39.8)	0.000	72.7 (70.7–76.7)	54.2 (43.1–62.3)	0.000

Data are presented as medians with IQRs, unless otherwise stated. Significant differences between groups were tested using Mann-Whitney *U* tests or unpaired *t* tests. *P* values are stated and in boldface when significantly different. GOLD stage based on FEV1%pred. FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

Fig. 8. Association between senescence and *DCN* gene expression confirmed in human lung tissue. Dot plots show mRNA expression of p16 (A), p21 (B), and *DCN* (C) of all 4 patient groups. Green, severe, early-onset chronic obstructive pulmonary disease (SEO-COPD)-matched control; red, SEO-COPD; blue, older COPD-matched control; yellow, older, mild-moderate COPD. One of the dots of the SEO-COPD group contains the average of 2 lung samples from the same patient. Lines represent medians. Significant differences tested with Mann-Whitney *U* tests. **P* value < 0.05. Dot plot shows correlation between p16 mRNA expression and *DCN* mRNA expression (D). Significant difference tested with Spearman's rank test. In the plot the Spearman ρ and *P* value are indicated and boldfaced when significant.

of lower protein levels of Ku80 in lung tissue from COPD patients (9). In addition, expression of the oxidative response genes *FOXO3* and *MGST1* upon PQ-induced senescence was more induced in fibroblasts from control subjects than from COPD patients. *FOXO3* is a well-known antiaging and antioxidant protein, and protein levels were shown to be lower in lung tissue from smokers and COPD patients previously (24). Together, these results suggest that COPD-derived fibroblasts are less capable of responding to aging-related damage.

One apparent different result between lung fibroblasts and lung tissue from COPD patients was higher p16 expression in fibroblasts, while p21 expression was higher in lung tissue. Both are markers of cellular senescence and important cell cycle inhibitors. Oxidative stress and DNA damage can induce p53, which activates p21 downstream, while p16 can be activated by multiple stressors (4, 34). In addition, p21 has been implicated in the early stage of senescence and p16 in the latter stage of senescence (44). The differences between fibroblasts and lung tissue can be explained by differences in cell composition and cell responses, or potential effects of prolonged cell culture of fibroblasts being outside of their diseased microenvironment, since in lung tissue oxidative stress may still be present. Importantly, because we observed higher levels of cellular senescence in lung fibroblasts and lung tissue from COPD patients, our data indicate an accumulation of senescent cells in lungs of COPD patients. This accumulation may contribute to impaired tissue function in lungs (5, 34).

In conclusion, this is the first study showing a link between cellular senescence and deregulated ECM gene expression in

COPD, including SEO-COPD. Future studies on the functional consequences of senescent lung fibroblasts may lead to a better understanding of the pathogenesis of accelerated aging in COPD with respect to lung tissue remodeling. Ultimately, this knowledge might lead to novel therapeutic targets for COPD patients, including SEO-COPD patients, which is important since no treatment is available to cure the disease or to stop or delay the progression of the disease.

ACKNOWLEDGMENTS

We thank Simone Brandenburg (European Research Institute for the Biology of Ageing, University of Groningen, University Medical Centre Groningen) for help with setting up the SA- β -gal staining in our laboratory. We thank Wierd Kooistra and Marjan Reinders-Luinge (Department of Pathology and Medical Biology, University of Groningen, University Medical Centre Groningen) for isolation of the primary parenchymal lung fibroblasts from lung tissue from patients and subjects.

GRANTS

National Health and Medical Research Council (NHMRC), Australia; Noordelijke CARA Stichting (NCS), Groningen, the Netherlands.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

M.d.V., W.T., M.v.d.B., M.D., B.G.G.O., I.H.H., and C.-A.B. conceived and designed research; R.R.W. performed experiments; R.R.W., M.d.V., I.H.H., and C.-A.B. analyzed data; R.R.W., M.d.V., W.T., M.v.d.B., B.G.G.O., I.H.H., and C.-A.B. interpreted results of experiments; R.R.W. prepared figures; R.R.W., M.d.V., I.H.H., and C.-A.B. drafted manuscript; R.R.W., M.d.V., I.H.H., and C.-A.B. edited and revised manuscript; R.R.W., M.d.V., W.T., M.v.d.B., M.D., B.G.G.O., I.H.H., and C.-A.B. approved final version of manuscript.

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