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SHORT REPORT

FAM13A regulates cellular senescence marker p21 and mitochondrial reactive oxygen species production in airway epithelial cells

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Abstract

Inhalation of noxious gasses induces oxidative stress in airway epithelial cells (AECs), which may lead to cellular senescence and contribute to the development of chronic obstructive pulmonary disease (COPD). FAM13A, a well-known COPD susceptibility gene, is highly expressed in airway epithelium. We studied whether its expression is associated with aging and cellular senescence and affects airway epithelial responses to paraquat, a cellular senescence inducer. The association between age and *FAM13A* expression was investigated in two datasets of human lung tissue and bronchial brushings from current/ex-smokers with/without COPD. Protein levels of FAM13A and cellular senescence marker p21 were investigated using immunohistochemistry in lung tissue from patients with COPD. In vitro, *FAM13A* and *P21* expression was assessed using qPCR in air-liquid-interface (ALI)-differentiated AECs in absence/presence of paraquat. In addition, FAM13A was overexpressed in human bronchial epithelial 16HBE cells and the effect on *P21* expression (qPCR) and mitochondrial reactive oxygen species (ROS) production (MitoSOX staining) was assessed. Lower *FAM13A* expression was significantly associated with increasing age in lung tissue and bronchial epithelium. In airway epithelium of patients with COPD, we found a negative correlation between FAM13A and p21 protein levels. In ALI-differentiated AECs, the paraquat-induced decrease in *FAM13A* expression was accompanied by increased *P21* expression. In 16HBE cells, the overexpression of FAM13A significantly reduced paraquat-induced *P21* expression and mitochondrial ROS production. Our data suggest that *FAM13A* expression decreases with aging, resulting in higher *P21* expression and mitochondrial ROS production in the airway epithelium, thus facilitating cellular senescence and as such potentially contributing to accelerated lung aging in COPD.

NEW & NOTEWORTHY To our knowledge, this is the first study investigating the role of the COPD susceptibility gene FAM13A in aging and cellular senescence. We found that FAM13A negatively regulates the expression of the cellular senescence marker P21 and mitochondrial ROS production in the airway epithelium. In this way, the lower expression of FAM13A observed upon aging may facilitate cellular senescence and potentially contribute to accelerated lung aging in COPD.

airway epithelium; COPD; FAM13A; lung aging; paraquat

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a prevalent lung disease that predominantly affects the elderly (1). It is characterized by lung function decline as a consequence of airway inflammation and remodeling (chronic bronchitis) and/or destruction of the alveoli (emphysema) (1, 2). Although lung function generally declines with increasing age, this process is accelerated in patients with COPD. Moreover, COPD shares various features of the aging lung, including the loss of elastic recoil, airspace enlargement, and loss of alveolar septa (3). In addition, several hallmarks of aging have been observed in cells from patients with COPD, including cellular senescence, telomere shortening, mitochondrial dysfunction, and low-grade inflammation (4). Thus, aging has been proposed to contribute to the development of COPD.

The main risk factor for COPD is the inhalation of noxious particles, including cigarette smoke and herbicides such as paraquat, in combination with genetic predisposition. Inhaled cigarette smoke first encounters the airway epithelial barrier, where its radicals induce mitochondrial damage, resulting in oxidative stress and generation of reactive oxygen species (ROS) (5). Oxidative stress has been recognized as a key driver in the process of accelerated aging due to its

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harmful cellular effects, inducing mitochondrial dysfunction, loss of proteostasis, and DNA damage (5, 6). The increased burden of oxidative stress upon aging and with COPD may thus cause cellular damage. Once the damage is beyond the self-repair capacity, cells become senescent. This includes activation of cyclin-dependent kinase inhibitor p21, thereby inducing an irreversible cell cycle arrest (7). Of interest, higher levels of cellular senescence markers, including *p21*, have been observed in small airway epithelial cells (8) and alveolar type II cells of patients with COPD compared with nonsmokers and asymptomatic smokers (9, 10).

Not all smokers suffer from accelerated lung function decline and develop COPD, highlighting the role of genetics in the susceptibility to develop features of cellular senescence and COPD. Previously, we and others showed that Family with Sequence Similarity 13 A (*FAM13A*) is associated with lung function (11). In addition, we observed that FAM13A protein expression is lower in the airway epithelium of patients with COPD and plays a protective role against cigarette smoke-induced damage and proinflammatory responses (12). In addition, *FAM13A* is known to be involved in fatty acid oxidation, thus potentially affecting mitochondrial function and ROS production (13). Whether *FAM13A* expression decreases upon aging, is linked to markers of cellular senescence and/or is a regulator of mitochondrial ROS production is currently unknown. We hypothesized that expression of *FAM13A* is lower upon aging, which leads to cellular senescence. This affects the susceptibility of the airway epithelial cells (AECs) to cellular senescence through regulating ROS production. Hence, we investigated whether *FAM13A* expression is associated with aging and the cellular senescence marker p21, known to be increased in the lungs of patients with COPD. Furthermore, we studied the effect of the senescence inducer paraquat on *FAM13A* expression in air-liquid interface (ALI)-differentiated AECs (14). Finally, we studied the effect of FAM13A overexpression on *P21* gene expression and mitochondrial ROS production in the human bronchial epithelial cell line 16HBE.

MATERIALS AND METHODS

Association between Age and FAM13A in Lung Tissue and Bronchial Brushings

To investigate the association between age and *FAM13A* in lung tissue, we extracted the results of *FAM13A* from our previously published analysis of a gene expression signature for the aging lung containing lung tissue from 1,197 current/ex-smokers with/without COPD. Lung tissue for this study was taken from tissue databanks of three academic centers: Laval University (Quebec, Canada), University of British Columbia (Vancouver, Canada), and University Medical Center Groningen (Groningen, The Netherlands. Lung tissue within these databanks was obtained from patients undergoing lung surgery for various lung diseases, and all samples were histologically checked for abnormalities. If surgery was conducted for tumor removal, macroscopically normal tissue was taken far distant from the tumor (15).

To investigate the association between age and *FAM13A* in bronchial brushes, we performed linear regression analysis in the Groningen cohort (16) data set, containing gene expression data from 147 never/current/ex-smokers without respiratory symptoms and no history of respiratory diseases. All included subjects had a normal lung function, defined as the ratio between postbronchodilator forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) above the lower limit of normal, no bronchial hyperresponsiveness, and reversibility of FEV_1.

Immunohistochemical Staining of p21 and FAM13A in Lung Tissue

Human lung tissue was derived from 15 ex-smoking patients with GOLD stage II–IV COPD as part of the HOLLAND (HistopathOLogy of Lung Aging aNd COPD) project. Of these, 8 were female, the average age was 53 and the average predicted FEV_1% was 51.4. The study protocol was consistent with the Research Code of the University Medical Center Groningen (Research Code UMCG) and national ethical and professional guidelines (code of conduct; Dutch federation of biomedical scientific societies).

The intensity of FAM13A and p21 protein was determined in the lung tissue sections using immunohistochemical staining and analyzed according to the protocol previously described (17). Primary antibodies against FAM13A (55401-1-AP; 1:300; Proteintech, Manchester, UK) and p21 (OP64, 1:100; EMD Millipore Corp., Temecula, CA); secondary Rabbit-Anti-Mouse Immunoglobulins/HRP (P0260; 1:100; Dako, Amsterdam, The Netherlands) and Goat-Anti-Rabbit Immunoglobulins/HRP (P0448; 1:100; Dako); and tertiary Streptavidin/HRP (P0397; 1:300 for FAM13A staining; 1:100 for p21 staining; Dako) antibodies were used.

Air-Liquid Interface Culture and Treatment of Primary Airway Epithelial Cells

AECs were isolated from de-identified leftover tracheobronchial tissue of three nondiseased transplant donor lungs from which no subject information was available. The materials have been obtained according to the standard and ethical code of the UMCG as mentioned above, and pseudonymization was used for the coding. The cells were isolated using enzymatic treatment, cryopreserved until thawed for ALI culture in *passage 3* following the previous published protocol with minor modifications (18). In brief, cells were seeded in transwell inserts (Corning, Corning, NY) coated with BSA/collagen/fibronectin at a density of 75,000 cells/insert, cultured in Airway Epithelial Growth Medium (AEGM; Promocell, Heidelberg, Germany) to confluence, and air-exposed for 28 days. On air exposure, the medium was replaced with ALI medium, consisting of 1:1 DMEM (Lonza) and Airway Epithelial Basal Medium (AEBM; Promocell) supplemented with an AEGM growth supplement mix (Promocell), 1% P/S and 1.5 μg/mL BSA. Furthermore, the medium was supplemented with 15 ng/mL retinoic acid (Sigma-Aldrich, Darmstadt, Germany) just before use and refreshed every 2–3 days.

On *day 28*, cells were hormonally deprived overnight, treated with 200 μM paraquat or medium control from apical side for 6 h, washed, and cultured for another 24 or 72 h on air exposure before harvested with TRIzol (MRC, Steinbach, Germany) for RNA isolation. Successful differentiation was confirmed by the expression of mucus (visually) and the increase in transepithelial resistance measured using an $EVOM^2$-voltohmmeter (World Precision Instruments, FL).

Cell Culture and Treatment

The human bronchial epithelial cell line 16HBE was generously offered by D.C. Gruenert (University of California, San Francisco, CA). Cells were cultured in EMEM (Lonza) supplemented with GlutaMax (LifeTechnologies, Paisley, UK), 1% P/S, and 10% FCS (Sigma-Aldrich) in collagen/BSA-coated flasks as previously mentioned (19). When ~90% confluent, the cells were passaged or seeded for experiments.

A FAM13A overexpression plasmid was created by GenScript (Nanjing, China) and plasmid pCMV6-6XL was used as empty vector control. 16HBE cells were seeded in duplicates into 24-well plates at a density of 125,000 cells/well. The next day, cells were transfected with 250 ng/well plasmid using 1 μL/well Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA) in 500 μL Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific) for 4 h. The next day, cells were serum-deprived overnight, treated with 200 μM paraquat or medium control, incubated for another 24 h before stained with the fluorescent dye MitoSOX for detection of mitochondrial ROS, and harvested for total RNA isolation.

RNA Isolation and Real-Time PCR

Total RNA was isolated following the user guide of TRIzol solution and quantified using a Nanodrop-1000 (NanoDrop Technologies, Wilmington, DE). One microgram of total RNA per sample was reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). qPCR was performed in duplicate with GoTaq(R) Probe qPCR Master Mix kits (Promega Benelux, Leiden) using QuantStudio (Thermo Fisher Scientific). Gene expression was normalized to housekeeping genes *B2M* and *PPIA* and expressed as $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$. Taqman probes B2M (Hs99999907_m1), PPIA (Hs99999904_m1), FAM13A (Hs00208453_m1), and p21 (Hs00355782_m1) were purchased from Thermo Fisher Scientific.

MitoSOX Assay

Cells were stained with 5 μM MitoSOX in PBS with Ca^{2+} and Mg^{2+} ($PBS^{+/+}$) at 37°C for 10 min and washed with HBSS, trypsinized, centrifuged, and resuspended with PBS complemented with 1% FCS. The percentage of MitoSOX-positive cells was determined using NovoCyte Quanteon flow cytometer (Agilent, Amstelveen, the Netherlands) at PE channel as an indication for mitochondrial ROS production. As positive control, 80 μM Antimycin A (Thermo Fisher Scientific) was used for the induction of mitochondrial ROS 15 min before staining with MitoSOX.

Statistics

The association between age and *FAM13A* expression in lung tissue was tested using linear regression analysis adjusted for the potential confounders sex, smoking status, disease, and technical variation in the three cohorts separately and followed by a meta-analysis to combine the three cohorts. The association between age and *FAM13A* expression in bronchial brushes was tested using linear regression analysis adjusted for sex, smoking status and technical variation. The correlation between expression of FAM13A and p21 was tested using Spearman's correlation test. To test for differences between conditions, we applied the Wilcoxon signed-rank test. $P < 0.05$ was considered significant.

RESULTS

FAM13A Is Lower Expressed with Increasing Age in Lung Tissue and Bronchial Epithelial Cells

First, we studied whether the expression of *FAM13A* is associated with age in lung tissue from current/ex-smokers with/without COPD (LUNG cohort) and in bronchial brushings from never/current smokers with normal lung function (Groningen cohort). In both cohorts, expression of *FAM13A* was significantly lower with increasing age (Fig. 1).

FAM13A Expression Is Negatively Correlated with p21 Expression in the Airway Epithelium of Patients with COPD

Using immunohistochemistry, we assessed whether the lower protein expression of FAM13A, as previously observed in lung tissue of patients with COPD (12), was accompanied by higher expression of senescence marker p21. In lung tissue of patients with COPD, FAM13A protein was most strongly expressed in the airway epithelium, immune cells, and stromal cells (Fig. 2*A*). P21 protein was expressed less abundantly, mainly nuclear and only strongly expressed in occasional airway epithelial and immune cells (Fig. 2*A*). As shown in Fig. 2*B*, we observed a negative correlation between FAM13A and p21 in the extracted airway epithelial layer from the lung tissue sections.

The Paraquat-Induced Decrease in FAM13A Expression Is Accompanied by Increased P21 Expression in Mucociliary-Differentiated AECs

Next, we studied in vitro whether induction of cellular senescence leads to reduced *FAM13A* expression accompanied by an increase in *p21* expression in AECs cultured at air-liquid interface (ALI). As shown in Fig. 3, *A* and *B*, paraquat exposure decreased *FAM13A* expression 24 and 72 h after the treatment, which was accompanied by increased expression of *P21* at mRNA level (Fig. 3, *A* and *B*). Of interest, despite

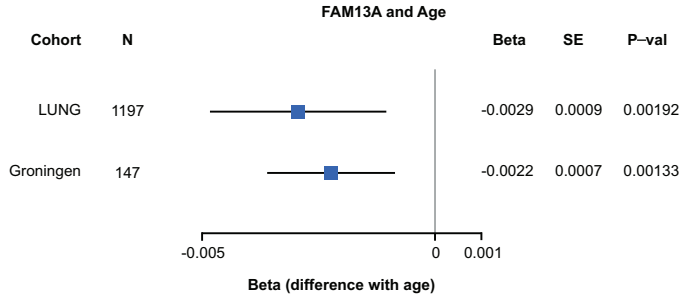

Figure 1. Association of *FAM13A* with age in human lung tissue and bronchial epithelial brushings. Forest plot of the association between *FAM13A* mRNA expression and age in lung tissue from patients undergoing lung surgery (LUNG) and in bronchial epithelial cells from individuals with normal lung function (Groningen). Beta indicates the effect estimate, i.e., the difference in expression upon increasing age. A negative value indicates a negative association. SE, standard error.

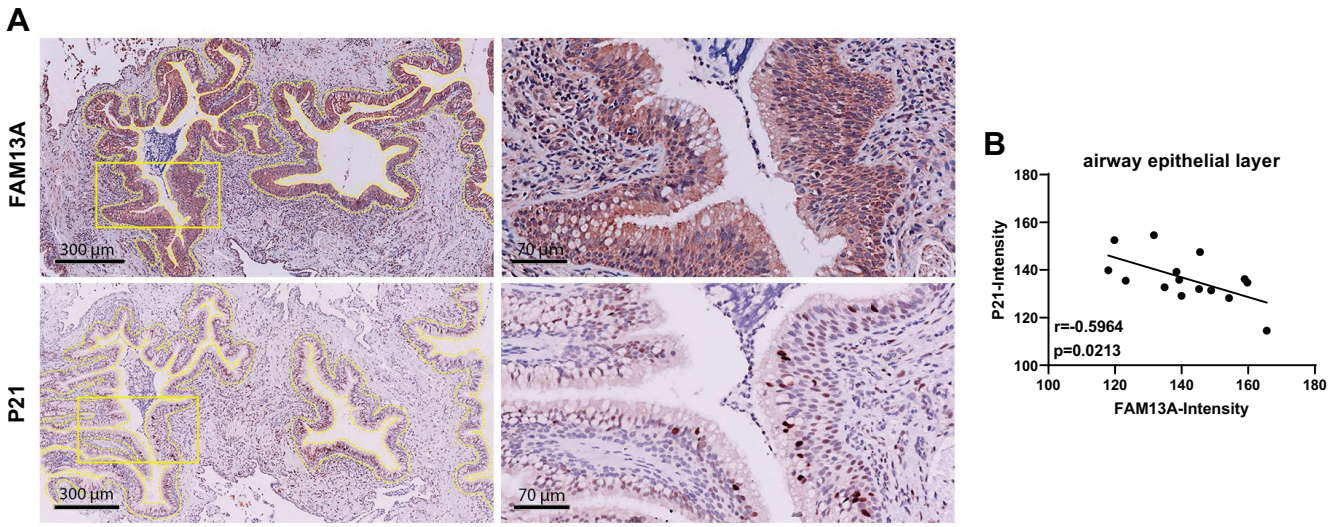

Figure 2. FAM13A expression is negatively correlated with p21 expression in the airway epithelium. FAM13A and p21 protein expression was assessed in the airway epithelial layer in the same airway in lung tissue from 15 patients with COPD by immunohistochemical staining. *A*: representative images of lung tissue (COPD) stained for FAM13A and p21. An overview is shown in *left*, with the analyzed airway area marked by a dotted line. *Right*: zoom in on the airway epithelial layer. *B*: the correlation between FAM13A and p21 expression in the airway epithelial layer was tested using the Spearman's correlation test. Each dot represents the average intensity of all airways from one individual. COPD, chronic obstructive pulmonary disease.

the relatively low sample size, the expression of *FAM13A* and *p21* was significantly negatively correlated (Fig. 3*C*).

Paraquat-Induced P21 Expression Is Attenuated by Overexpression of FAM13A in 16HBE Cells

Given the difficulties with transfection of primary AECs cultured at ALI (18), we introduced human bronchial epithelial 16HBE cells to study the function of FAM13A by overexpression. As shown in Fig. 4*A*, similar to the effect observed in ALI cultures, paraquat exposure for 24 h significantly increased the expression of *P21*, confirming the suitability of 16HBE as cell model for our functional studies. Next, the overexpression of FAM13A in 16HBE cells was confirmed at mRNA level as depicted in Fig. 4*B*.

As shown in Fig. 4*C*, overexpression of FAM13A did not affect the baseline expression of *P21*. However, on paraquat treatment, we observed a small but significant reduction of *P21* in cells overexpressing FAM13A. To study whether this suppressive effect of FAM13A on *P21* expression was accompanied by alterations in mitochondrial ROS production, which induces cellular senescence and *p21* expression, we assessed whether FAM13A could inhibit mitochondrial ROS production. Paraquat treatment for 24 h significantly increased the percentage of cells positive for mitochondrial ROS as detected by MitoSOX (Fig. 4*G*). Overexpression of FAM13A did not affect the number of mitochondrial ROS-positive cells (Fig. 4*H*). Interestingly, the paraquat-induced increase in mitochondrial ROS-positive cells was significantly reduced upon overexpression of FAM13A compared with cells transfected with empty vector (Fig. 4*H*).

DISCUSSION

In this study, we investigated the association between age and *FAM13A* in human lungs and the association between

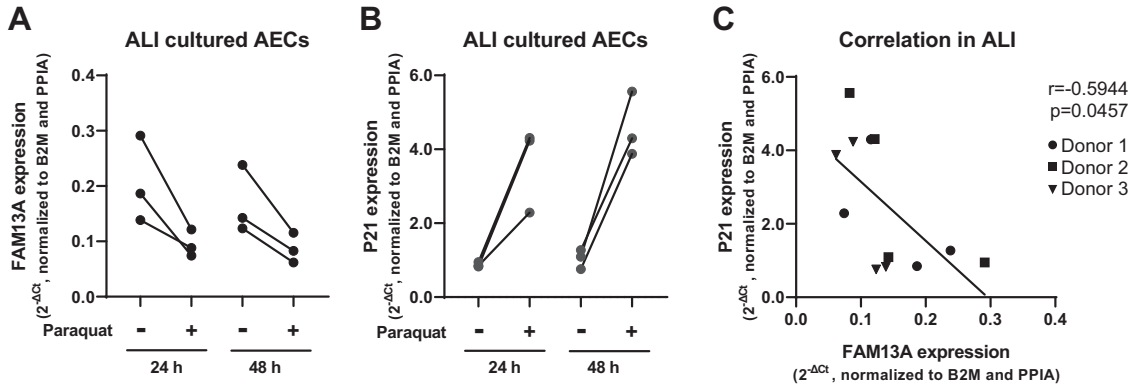

Figure 3. Paraquat treatment decreased *FAM13A* and induced *P21* expression in mucociliary-differentiated AECs. Primary AECs from three donors were differentiated at air-liquid interface (ALI) and treated with/without 200 µM paraquat. mRNA expressions of *FAM13A* (*A*) and *P21* (*B*) were assessed using qPCR and expressed as $2^{-\Delta Ct}$. *C*: Pearson's test was used to test the correlation between *FAM13A* and *p21* expression. Since we could not assume normal distribution and the nonparametric Wilcoxon signed-rank test requires a minimum of six independent measurements, statistical testing was not performed for *A* and *B*.

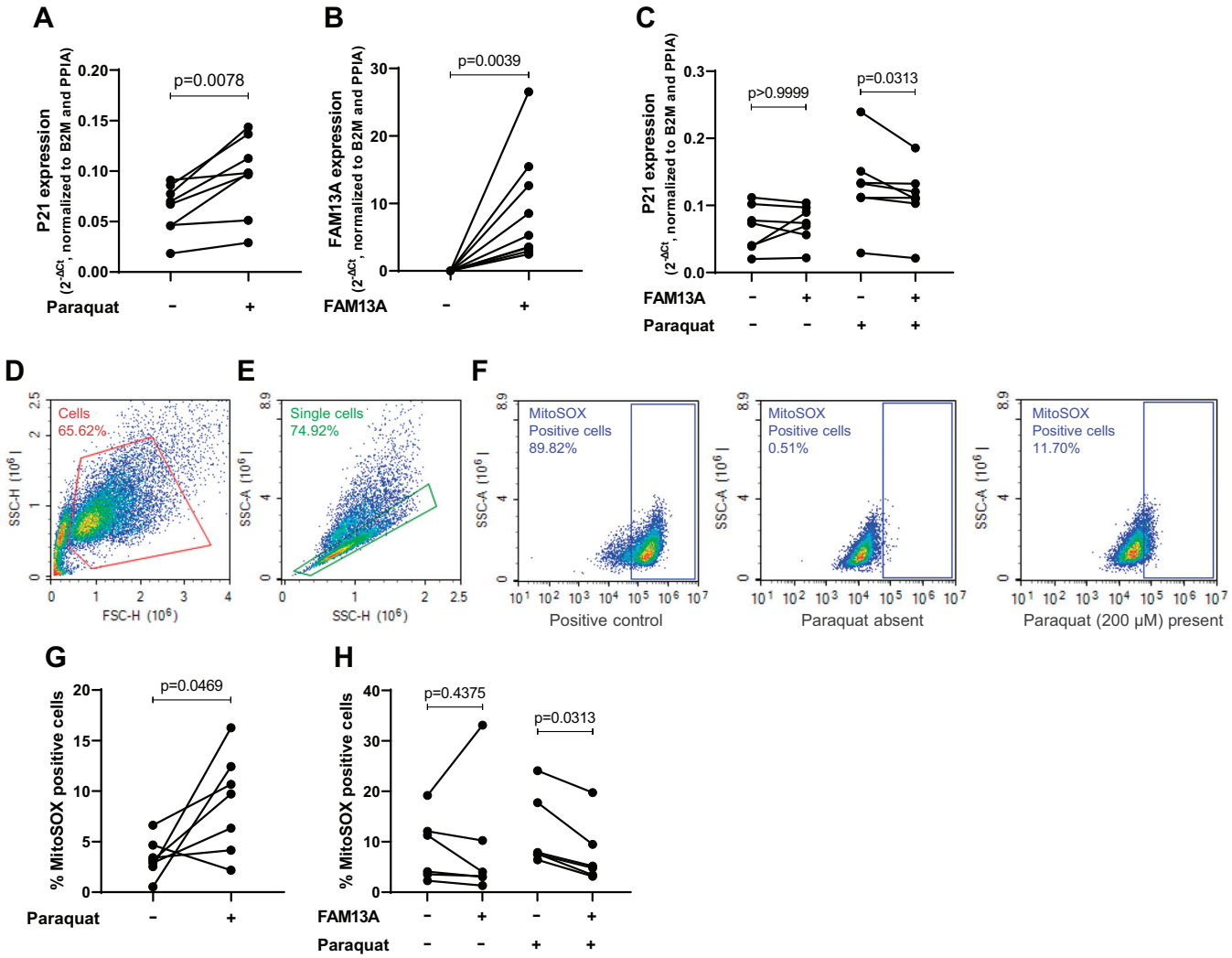

Figure 4. FAM13A reduces *P21* expression and mitochondrial ROS production in 16HBE cells. 16HBE cells were transfected with empty vector (−) or FAM13A (+) plasmid as indicated and treated with/without 200 μM paraquat for 24 h before harvested for RNA isolation ($n = 7$) or for MitoSOX staining followed by flow cytometry analysis ($n = 6$ or 7). *P21* mRNA expression (*A* and *C*) and *FAM13A* mRNA expression (*B*) in 16HBE cells were assessed using qPCR and expressed as $2^{-\Delta Ct}$. *D*: gating strategy of MitoSOX: live cells were selected based on cell size as indicated by forward scatter height and area (FSC-H and FSC-A) and sideward scatter area (SSC-A). *E*: single cells were selected on basis of similar size in the forward and sideward scatter. *F*: in the PE channel, MitoSOX-positive cells were gated on treatment with antimycin A (positive control), the same gating was used in all samples. *G*: the percentage of cells positive for MitoSOX in cells with/without paraquat. *H*: percentage of MitoSOX-positive cells with/without paraquat treatment in cells transfected with empty vector or FAM13A. ROS, reactive oxygen species.

FAM13A and cellular senescence marker p21 and mitochondrial ROS production in human airway epithelial cells in vitro. We observed lower *FAM13A* gene expression with increasing age in lung tissue and bronchial epithelial cells. In lung tissue from patients with COPD, the lower protein expression of FAM13A was associated with higher protein expression of p21 in the airway epithelial layer. In line, we observed the same association in paraquat-treated mucociliary-differentiated AECs. In addition, FAM13A overexpression reduced paraquat-induced *P21* expression and mitochondrial ROS production in human bronchial 16HBE cells. Overall, our data suggest a protective role of FAM13A against oxidative stress-induced *P21* expression in AECs, which may reduce cellular senescence in COPD.

Although genetic variants in *FAM13A* have been associated with lower FEV_1/FVC and/or increased risk for COPD in adults in multiple GWA studies (20), these variants have also been associated with higher FEV_1 and FVC during childhood (21) and lower risk of lung fibrosis (22), suggesting that FAM13A may play a protective role in the lungs. These protective effects may, at least in part, be mediated by the beneficial effects of FAM13A on the epithelial barrier (23). Corvol et al. (23) found that downregulation of FAM13A by siRNA decreases E-cadherin expression in A549 cells. In line, we observed protective effects of FAM13A on epithelial barrier function (12), whereas our current findings suggest that FAM13A may protect against harmful effects of oxidative stressors such as paraquat. Given the relatively small induction of mitochondrial ROS production by paraquat, it will be of interest in future studies to use an additional inducer of senescence, such as cigarette smoke. ROS-induced oxidative stress has been shown to cause disruption of epithelial barrier function by inducing phosphorylation and subsequent delocalization of junctional proteins involved in the barrier

function, including E-cadherin (24). As FAM13A overexpression was able to reduce mitochondrial ROS production, loss of FAM13A may thus lead to increased vulnerability of the airway epithelial layer and barrier dysfunction in the presence of high levels of oxidative stress, as observed with aging and COPD. This may be accompanied by increased susceptibility to cellular senescence. The lower *FAM13A* expression with aging may also help explain our previous finding on lower epithelial barrier-related gene expression with aging (16).

Instead of being a consequence of increased ROS generation, impaired epithelial barrier function may also be causative to increased vulnerability toward inhaled toxicants and higher ROS production. In addition to junctional stabilization (12), another possible mechanism by which FAM13A may modulate oxidant-induced ROS production involves the supportive effect of FAM13A on fatty acid oxidation (FAO). Increased FAO has been observed as an adaptive response to cellular damage and disruption of homeostatic energy metabolism (25). FAO is associated with cellular survival and resistance to metabolic stress, increasing the redox ability to counteract oxidative stress and reduce ROS production (26). As FAM13A has been shown to enhance FAO (13), in this way FAM13A may reduce oxidative stress.

In addition to being a consequence of mitochondrial damage, increased ROS levels are known to enhance mitochondrial dysfunction and induce the expression of *p21*, resulting in cellular senescence, a hallmark of aging (2). P21 has been found higher expressed in various types of senescent cells in COPD lungs compared with asymptomatic smokers, including airway epithelial cells (14, 27). Our result that FAM13A overexpression leads to decreased paraquat-induced *p21* levels suggests that FAM13A may play an antisenescence role through regulating p21. Of note, given the lack of downregulatory effect of *FAM13A* on *p21* at baseline, we do not consider it likely that FAM13A directly regulates p21 transcription. This effect may rather be a consequence of the protection from oxidative stress, as discussed above, although the effect of paraquat was not completely blocked and also FAM13A insensitive effects are involved.

In conclusion, our results indicate that FAM13A has a protective effect in oxidative stress and aging-related processes. Its lower expression with aging may thus facilitate cellular senescence and might contribute to accelerated lung aging, increasing the susceptibility for COPD.

DATA AVAILABILITY

Data will be made available upon reasonable request.

ACKNOWLEDGMENTS

The stainings of FAM13A and P21 on lung tissue performed in this manuscript were conducted as part of the HOLLAND (HistopathOLogy of Lung Aging aNd COPD) project. The HOLLAND project was initiated and supervised by Corry-Anke Brandsma, Wim Timens, and Janette Burgess; technical support was provided by Marjan Reinders-Luinge, Anja Bakker, and Theo Borghuis; and image analyses pipelines were developed by Theo Borghuis, Maunick Lefin Koloko Ngassie, and Nicolaas J. Bekker. We thank them for their support on the immunohistochemistry data. Graphical abstract was created with a licensed version of BioRender.com.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Q.C., M.d.V., and I.H.H. conceived and designed research; Q.C., G.F.V., K.O.N., and N.J.B. performed experiments; Q.C., N.J.B., and M.d.V. analyzed data; Q.C., M.d.V., and I.H.H. interpreted results of experiments; Q.C. and M.d.V. prepared figures; Q.C. drafted manuscript; Q.C., G.F.V., K.O.N., N.J.B., M.v.d.B., C.B., M.d.V., and I.H.H. edited and revised manuscript; Q.C., G.F.V., K.O.N., N.J.B., M.v.d.B., C.B., M.d.V., and I.H.H. approved final version of manuscript.

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