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Title: A microRNA-21-mediated SATB1/S100A9/NF-κB axis promotes chronic obstructive pulmonary disease pathogenesis

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OVERLINE: LUNG DISEASE

One Sentence Summary: Inhibition of cigarette smoke-induced microRNA-21 suppresses chronic obstructive pulmonary disease through effects on a SATB1/S100A9/NF-κB axis.

Editor's summary:

Abstract: Chronic obstructive pulmonary disease (COPD) is the third leading cause of morbidity and death worldwide. Inhalation of cigarette smoke (CS) is the major cause in developed countries. Current therapies have limited efficacy in controlling disease or halting its progression. Aberrant expression of microRNAs (miRNAs) is associated with lung disease, including COPD. We performed miRNA microarray analyses of the lungs of mice with CS-induced experimental COPD. miR-21 was the second highest upregulated miRNA, particularly in airway epithelium and lung macrophages. Its expression in human lung tissue correlated with reduced lung function in COPD. Prophylactic and therapeutic treatment with a specific miR-21 inhibitor (Ant-21) inhibited CS-induced lung miR-21 expression in mice, suppressed airway macrophages, neutrophils and lymphocytes and improved lung function as evidenced by decreased lung hysteresis, transpulmonary resistance, and tissue damping, in mouse models of COPD. In silico analyses identified a potential miR-21/special AT-rich sequence binding protein (SATB)1/S100 calcium binding protein A9 (S100A9)/nuclear factor-kappa B (NF-κB) axis, which was further investigated. CS exposure reduced lung SATB1 in a mouse model of COPD, whereas Ant-21 treatment restored SATB1 and reduced S100A9 expression and NF-κB activity. The beneficial effects of Ant-21 in mice were reversed by treatment with SATB1-targeting small-interfering RNA. We have identified a pathogenic role for a miR-21/SATB1/S100A9/NF-κB axis in COPD and defined miR-21 as a therapeutic target for this disease.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is now the 3rd most common cause of death in the world (*1*). Common symptoms range from chronic bronchitis with a productive cough to impaired lung function and severe breathing difficulties. These symptoms result from the pathologies of chronic airway inflammation, airway remodeling and emphysema (*2-5*). Tobacco smoking and second-hand exposure to cigarette smoke (CS), are the major causes of COPD in developed countries, but air pollution and other exposures are also important (*6*). COPD is a progressive disease that typically worsens over time, and its pathophysiological features persist despite smoking cessation. There is no cure for COPD and current treatments, including physiotherapeutic and pharmacologic interventions, aim to mitigate symptoms and acute exacerbations. However, they do not modify the long-term decline in lung function in COPD patients. Development of therapies is hampered by the lack of understanding of the mechanisms that drive disease pathogenesis and progression.

Roles for microRNAs (miRNAs) in health and disease are established and increasing clinical and experimental evidence implicates their dysregulated expression in the pathogenesis of chronic respiratory diseases including COPD (*7-13*). They exert their effects post-transcriptionally by fine-tuning the expression of multiple target genes (*8*). miR-21-5p/miR-21 is a broadly conserved miRNA that is implicated in a range of inflammatory diseases and cancers (*14, 15*). In experimental severe asthma, we previously showed that respiratory infection-induced miR-21 promotes steroid-insensitive airway inflammation and airway hyper-responsiveness by downregulating phosphatase and tensin homolog (PTEN), which increases phosphoinositide-3-kinase activity leading to reduced histone deacetylase-2 responses (*11*). miR-21 expression is increased in serum of patients with mild to moderate COPD and asymptomatic smokers, compared

to healthy controls, suggesting its involvement in early pathogenesis (*16*). Elevated miR-21 expression is also associated with either increased or decreased pro-inflammatory nuclear factor-kappa B (NF-κB) activity, dependent on type of stimulus, cellular source, and target genes affected (*17, 18*). NF-κB activity is substantially elevated in sputum and bronchial biopsies of COPD patients (*19-21*), and is associated with the induction of CS-induced experimental COPD in mice (*22*). NF-κB can bind to miR-21 promoters and induces its expression (*23*) suggesting that miR-21-dependent, NF-κB-mediated inflammatory responses may self-perpetuate. Pathogen-associated molecular patterns and endogenous damage-associated molecular patterns (DAMPs) are the major inducers of NF-κB activity (*24, 25*).

Calgranulins are a group of 22 low molecular weight (10-13kDa) calcium-binding proteins that are primarily expressed in myeloid cells but also occur in epithelial cells, vascular endothelial cells and fibroblasts (*26, 27*). Under normal conditions, calgranulins modulate cellular calcium homeostasis, growth and differentiation, and immune responses (*28, 29*). The calgranulins S100A8 (calgranulin-A, myeloid-related protein-8, MRP8), S100A9 (calgranulin-B or MRP14) and their heterodimeric product, calprotectin, make up 45% of the cytosolic content of granulocytes and 1% of monocytes (*29, 30*). Cell damage or necrosis at sites of tissue injury induce their release into the extracellular environment where they function as DAMPs and induce innate immune responses and the production of pro-inflammatory cytokines (*31*). Calgranulins are potent neutrophil chemo-attractants (*32*) and are implicated in the development of cancers, and cardiovascular, autoimmune and chronic respiratory diseases, including COPD (*27, 28, 31*). Calprotectin responses are substantially elevated in the serum of patients with stable and exacerbating COPD and inversely correlate with lung function (forced expiratory volume in 1 sec [FEV_1] % predicted) suggesting that S100A8 and S100A9 have roles in disease pathogenesis (*33, 34*).

Substantial clinical and experimental evidence shows that the transcriptional repressor, special AT-rich sequence binding protein (SATB)1, negatively regulates S100A8 and S100A9 expression (*35, 36*). Notably, SATB1 is a validated miR-21 target (*37, 38*), and both S100A8 and S100A9 responses induce, and are dependent on, NF-κB signaling (*39, 40*). Here, we assessed the roles of miR-21, SATB1, S100A8 and S100A9 and the potential for therapeutic targeting in COPD.

RESULTS

CS exposure increases miR-21 expression in mouse models of COPD

Wild-type (WT) BALB/c mice were exposed to CS via the nose only, or normal air, for 2, 4, 6 or 8 weeks to induce experimental COPD (Fig. 1A) (*12, 22, 41-48*). Lungs were excised, RNA was isolated and miRNA expression microarray profiling was performed. miR-21 was the second highest upregulated at week 8 when chronic disease features emerge (table S1). qPCR validation confirmed that miR-21 was the second-most highly up-regulated miRNA at 4, 6 and 8 weeks of CS exposure, compared to air-exposed controls (Fig. 1B and fig. S1, A to D). Since the hallmark features of human COPD are observed at 8 weeks of CS exposure in our model (*22, 41-43, 49*), we assessed miR-21 expression in blunt-dissected airway and lung parenchymal tissue at this time point. CS exposure significantly increased lung miR-21 expression in both compartments ($P < 0.001$) (Fig. 1, C and D). miR-21 expression was increased in primary lung macrophages (Fig. 1E) as well as in $CD45^-$ mesenchymal primary lung cells ($CD45^-Sca\text{-}1^+$; $P < 0.001$) (fig. S1E). There was a trend toward increased miR-21 expression in epithelial ($CD45^-CD326^+$; $P = 0.099$), endothelial ($CD45^-CD31^+$; $P = 0.127$), and $CD45^+$ mesenchymal ($CD45^+Sca\text{-}1^+$; $P = 0.084$), but not immune ($CD45^+$), primary lung cells isolated from mice exposed to CS for 2 weeks compared

to air-exposed controls (fig. S1E). CS also increased lung miR-21 expression in C57BL/6 mice (Fig. 1, F and G) at 4 and 24 weeks of whole-body CS exposure, indicating that this CS-induced effect is not mouse strain- or model-specific. CS-induced miR-21 expression in the lung occurred in the airway epithelium of CS-exposed, compared to normal air-exposed, groups in this latter model (Fig. 1H). Thus, acute and chronic CS exposure increased lung miR-21 expression in the airway epithelium, lung macrophages and other cells in experimental COPD.

Increased lung miR-21 expression is associated with increased disease severity and reduced lung function in patients with COPD

We next validated our findings in human clinical samples. We measured the expression of hsa-miR-21-5p in bronchial biopsies of 5 healthy subjects (never smokers) and 10 patients with COPD without lung cancer (table S2). In biopsies from patients with COPD, the expression of hsa-miR-21-5p was significantly increased compared with never smokers ($P < 0.01$) (Fig. 2A). We then assessed hsa-miR-21-5p expression in resected lung tissue of never smokers, asymptomatic smokers, or patients with mild (GOLD II) or moderate-to-severe (GOLD III-IV) COPD (table S3). Hsa-miR-21-5p expression was significantly increased in mild COPD GOLD II samples compared to never smoker samples, and expression further increased in severe COPD GOLD III-IV patients compared to all other groups ($P < 0.05$) (Fig. 2B). Lung hsa-miR-21-5p expression was widespread and enriched in the airway epithelium of COPD GOLD IV patients, compared to asymptomatic smokers without COPD, as assessed by fluorescent and conventional brightfield *in situ* hybridization (Fig. 2C and fig. S2). Hsa-miR-21-5p expression negatively correlated with post-bronchodilator FEV_1 % predicted [r=-0.393 (P=0.0027)], FEV_1/forced vital capacity (FVC) ratio [r=-0.441 (P=0.0008)], diffusing capacity of the lungs for carbon monoxide [DL_{CO} % predicted;

r=-0.433 (*P*=0.0019)] and diffusion coefficient [K_{CO} % predicted; *r*=-0.357 (P=0.0119)] (Fig. 2, D to G). This cohort had a difference in sex ratios between groups (table S3), however, there was no correlation between hsa-miR-21-5p expression and gender. Furthermore, linear regression analyses showed that there was no effect of gender on hsa-miR-21-5p expression (table S3). Post-bronchodilator FEV_1 % predicted was significantly associated with hsa-miR-21-5p expression independent of gender, age, pack-years and use of inhaled corticosteroids ($P < 0.05$) (table S3). These data showed that exaggerated lung expression of miR-21 occurs in patients with COPD and correlates with the severity of airway obstruction. Furthermore, a regression analysis on this subset of patients with lung cancer (*n*=43; table S4) revealed that hsa-miR-21-5p expression is increased in the lung tissue of patients with COPD GOLD stage II, which persisted after adjusting for age and sex (model 1), after adjusting for age, sex and Tumor – Node – Metastasis (TNM) classification (model 2), after adjusting for age, sex and cancer stage (model 3), and after adjusting for age, sex and cancer stage and type (model 4) (table S5). Moreover, DL_{CO} (% predicted) was significantly associated with hsa-miR-21-5p expression independently of age, sex and cancer stage and type ($P = 0.026$, table S5). Regression analysis shows that hsa-miR-21-5p expression is increased in the lung tissue of COPD patients (both GOLD stage II and GOLD stage III-IV; highest coefficients observed in the GOLD stage III-IV group), which persists after adjusting for age and sex (model 1) and age, sex, smoking status and use of inhaled corticosteroids (model 2) (table S6). In addition, post-bronchodilator FEV_1 (% predicted) and DL_{CO} (% predicted) are associated with hsa-miR-21-5p expression independent of gender, age, smoking status and use of inhalation corticosteroids (table S6).

Inhibition of CS-induced miR-21 suppresses chronic airway inflammation in an experimental mouse model of COPD

We next assessed the role of CS-induced lung miR-21 expression in experimental COPD in mice. BALB/c mice were exposed to CS for 8 weeks to induce experimental COPD and were treated with scrambled (Scr) control or miR-21-specific antagomir (Ant-21) throughout (Fig. 3A). CS-exposed, Scr-treated (Smk+Scr) mice had increased lung miR-21 expression compared to air-exposed, Scr-treated controls (Air+Scr; Fig. 3B) after 8 weeks. Ant-21 treatment (Smk+Ant-21) inhibited CS-induced lung miR-21 expression to concentrations observed in air-exposed, Scr-treated controls. CS-induced miR-21 expression in the lung occurred in both the airway epithelium and parenchyma of CS-exposed, compared to normal air-exposed, groups (Fig. 3C). Ant-21 treatment decreased CS-induced miR-21 expression in both of these tissue compartments. We next assessed the role and potential for therapeutic targeting of increased lung miR-21 expression in experimental COPD. Treatment with Ant-21 substantially suppressed CS-induced influx of all inflammatory cells into the airways and specifically the numbers of macrophages, neutrophils and lymphocytes in the experimental COPD mouse model (Smk+Ant-21 versus Smk+Scr; Fig. 3, D to G). Treatment also inhibited CS-induced lung cytokine production, specifically tumor necrosis factor (TNF)α and chemokine (C-X-C motif) ligand 1 (CXCL1) proteins, back to the concentrations observed in air-exposed, Scr-treated controls (Fig. 3, H and I). Ant-21 treatment had no effect on CS-induced interleukin (*IL*)-*1β*, macrophage inflammatory protein (*MIP*)-*1α*, *MIP-1β*, regulated on activation, normal T cell expressed and secreted (*RANTES*), *IL-6*, and monocyte chemoattractant protein (*MCP*)-*1* mRNA expression in the lungs (fig. S3, A to F). Neither CS exposure nor Ant-21 treatment had effects on lung *IL-17A* mRNA expression (fig. S3G). We previously showed that lung macrophages played central roles in the pathogenesis of

CS-induced experimental COPD (*41*). In the current experiments, CS exposure decreased inducible nitric oxide synthase (*INOS*) and increased resistin-like molecule alpha-1 (*FIZZ-1*) and *YM-1* mRNA expression in lung tissue in experimental COPD (fig. S4, A to D) suggesting a potential role for alternatively activated lung macrophages in pathogenesis. However, Ant-21 treatment had no effect on the CS-induced expression of these factors in lung tissues. These data showed that CS-induced lung miR-21 expression plays a role in promoting airway inflammation in experimental COPD.

CS-induced lung miR-21 expression promotes small airway fibrosis and reduces lung function in experimental COPD

We next examined the effects of targeting CS-induced lung miR-21 expression on small airway remodeling, emphysema-like alveolar enlargement, and lung function in experimental COPD in BALB/c mice. CS-exposed, Scr-treated (Smk+Scr) mice had marked airway remodeling with collagen deposition around the small airways compared to normal air-exposed, Scr-treated (Air+Scr) controls at 8 weeks (Fig. 4, A and B). Ant-21 treatment inhibited CS-induced small airway collagen deposition to the baseline observed in air-exposed, Scr-treated (Air+Scr) controls. CS-exposed, Scr-treated mice also had increased mean alveolar diameter (Fig. 4, C and D) and tissue destructive index (Fig. 4E). Treatment with Ant-21 had no effect on these CS-induced emphysema-like changes. CS-exposed, Scr-treated mice had increased lung hysteresis (Fig. 4F) and transpulmonary resistance (Fig. 4G), with decreased transpulmonary compliance (Fig. 4H), and increased tissue damping (Fig. 4I). Treatment with Ant-21 protected against all of these CS exposure-induced changes in lung function, with values similar to those observed in air-exposed, Scr-treated (Air+Scr) controls. These data demonstrated that CS exposure induces small airway

remodeling and reduces lung function in experimental COPD through a miR-21-dependent mechanism that can be inhibited for therapeutic effect.

Inhibition of CS-induced miR-21 suppresses mRNA but not protein of canonical targets in experimental COPD

To further examine the role of CS-induced miR-21 in the pathogenesis of experimental COPD in BALB/c mice, we evaluated the mRNA expression of some known miR-21 targets. We observed decreased mRNA expression of sprouty RTK signaling antagonist (*SPRY*)*1*, *SPRY2*, *PDCD4* and reversion-inducing-cysteine-rich protein with kazal motifs (*RECK*) in CS-exposed, Scr-treated (Smk+Scr) mice compared to air-exposed, Scr-treated controls (Air+Scr; fig. S5, A to D) after 8 weeks. The mRNA expression of other canonical targets, such as tissue inhibitor of metalloproteinase (*TIMP*)*3* and *PTEN*, were not affected by CS exposure (fig. S5, E and F). Treatment with Ant-21 had no effect on *SPRY1*, *SPRY2* and *PDCD4* mRNA expression in CS-exposed, Scr-treated mice (Smk+Scr; fig. S5, A to C), and resulted in reduced *RECK*, *TIMP3* and *PTEN* mRNA expression (fig. S5, D to F). MiRNAs can suppress the translation of their targets without altering their mRNA expression, therefore we measured lung proteins of all of these factors using immunoblot. Ant-21 treatment had no effect on the lung proteins of these factors in experimental COPD (fig. S5, G to L). These data suggested that CS-induced miR-21 responses drive airway inflammation, small airway fibrosis, and impaired lung function, in experimental COPD through other target genes.

CS-induced lung miR-21 targets the transcriptional repressor SATB1 in experimental COPD

SATB1 is an anti-inflammatory factor and a recently defined mRNA target of miR-21 that possesses a miR-21 target sequence that is highly conserved between human and mouse genomes (*38*). Direct regulation of *SATB1* mRNA by miR-21 has recently been demonstrated using a luciferase reporter assay in primary human and murine keratinocytes (*38*). We therefore investigated whether CS-induced miR-21 exerted its effects in COPD by inhibiting SATB1. In BALB/c mice, CS-exposure significantly reduced lung *SATB1* mRNA expression at 4 and 6 weeks compared to normal air-exposed mice ($P < 0.001$) (Smk versus Air; Fig. 5, A and B), and in CS-exposed, Scr-treated mice (Smk+Scr) compared to normal air-exposed, Scr-treated controls (Air+Scr) at 8 weeks ($P < 0.05$) (Fig. 5C). Treatment with Ant-21 during CS exposure (Smk+Ant-21) restored *SATB1* mRNA expression to that observed in normal air-exposed, Scr-treated controls. These CS- and Ant-21-induced effects on SATB1 were confirmed in whole lung protein homogenates by immunoblot analyses (Fig. 5, D and E). We next assessed the tissue localization of SATB1 protein in mouse lung histology sections by immunofluorescence. In normal air-exposed, Scr-treated controls, SATB1 was primarily localized to the airway epithelium with some positive staining in the parenchyma (Fig. 5, F and G). CS exposure reduced SATB1 in both the airways and parenchyma. Treatment with Ant-21 during CS exposure increased SATB1 responses in both of these tissue compartments (Smk+Ant-21 versus Smk+Scr). These data showed that the CS-induced increase in lung miR-21 expression is associated with reduced SATB1 expression, and that inhibiting miR-21 during experimental COPD restores SATB1 expression in both the airways and parenchyma.

Inhibiting SATB1 abrogates the beneficial effect of targeting miR-21 in experimental COPD

We next assessed the roles of SATB1 downstream of CS-induced miR-21 responses in experimental COPD in BALB/c mice (Fig. 5H). We administered SATB1-targeting small interfering (si)RNA during CS exposure in the absence or presence of Ant-21 treatment. Ant-21 treatment, similar to our initial experiment (Fig. 3), substantially suppressed CS-induced influx of inflammatory cells into the airways and reversed lung function changes (Smk+Ant-21+Scr-siRNA versus Smk+Scr+Scr-siRNA; Fig. 5, I to K). Treatment with SATB1-siRNA during CS exposure abrogated the protective effects of Ant-21 treatment by increasing the numbers of total leukocytes, macrophages (a trend) and neutrophils in experimental COPD (Smk+Ant-21+SATB1-siRNA versus Smk+Ant-21+Scr-siRNA; Fig. 5K). Furthermore, treatment with SATB1-siRNA reversed the protective effects of Ant-21 treatment on CS-induced changes in lung function in terms of hysteresis (Fig. 5L), transpulmonary resistance (Fig. 5M) and compliance (Fig. 5N), and tissue damping (Fig. 5O). Inhibition of SATB1 almost completely reversed the protective effects of Ant-21 treatment. These data demonstrated that CS-induced miR-21 responses drive experimental COPD pathogenesis through the suppression of SATB1 responses.

CS-induced lung miR-21 increases S100A9 expression and NF-κB activity in experimental COPD

SATB1 can negatively regulate S100A8 and S100A9 expression (*35, 36*). S100A8 and S100A9 are pro-inflammatory mediators, potent chemoattractants of neutrophils, inducers of NF-κB signaling (*39, 40*) and implicated in the pathogenesis of several chronic inflammatory diseases, including COPD (*32, 50-53*). Thus, we next assessed the lung mRNA expression of *S100A8* and *S100A9* in experimental COPD in BALB/c mice. CS exposure significantly increased the expression of *S100A9* ($P < 0.05$), but not *S100A8*, in the airways and parenchyma at 8 weeks (Smk

versus Air; Fig. 6, A to D). Treatment with Ant-21 suppressed CS-induced lung *S100A9* mRNA expression (Fig. 6E). This effect was associated with inhibition of CS-induced NF-κB activity (Fig. 6F). We also examined the relationship between CS-induced miR-21 responses and pro-inflammatory activation of NF-κB responses in vitro using an NF-κB luciferase reporter assay (Fig. 6G). Exposure of HEK293 cells to cigarette smoke extract (CSE) increased luciferase activity. Co-transfection with Ant-21 almost completely suppressed CSE-induced increases in NF-κB luciferase activity. These data showed that CS-induced miR-21 increases lung S100A9 expression and NF-κB activity in experimental COPD.

Reduced lung function is associated with decreased SATB1 and increased S100A9 responses in patients with COPD

We next assessed SATB1 and S100A9 responses in resected lung tissues of never smokers, asymptomatic smokers, and patients with mild (GOLD II) or moderate-to-severe (GOLD III-IV) COPD (table S7). Patients with severe COPD GOLD III-IV had a trend toward lower lung SATB1 protein compared to never smokers (P = 0.06; Fig. 7A), and substantially and significantly increased S100A9 protein compared to all other groups ($P < 0.05$) (Fig. 7B). Lung SATB1 protein, similar to miR-21 expression, was increased in the bronchial epithelium of a never smoker, and its presence was progressively diminished with increasing COPD severity (Fig. 7C). SATB1 protein positively correlated with lung function, including higher post-bronchodilator FEV_1 % predicted [r=0.233 (P = 0.047)] and DL_{CO} % predicted [r=0.300 (P = 0.0198)] (Fig. 7, D and E). In contrast, increased S100A9 protein negatively correlated with post-bronchodilator FEV_1 % predicted [r=-0.240 (P = 0.0335)] (Fig. 7F). To assess the impact of SATB1 and S100A9 on pulmonary function in patients with COPD, we performed sub-analyses on patients with COPD only, from this cohort.

We found that SATB1 protein positively correlated with higher post-bronchodilator FEV_1 % predicted [r=0.322 (P = 0.049)] (Fig. 7G) and a trend toward a positive correlation with DL_{CO} % predicted [r=0.325 (P = 0.070)] (Fig. 7H). We also found that increased S100A9 protein had a trend toward negative correlation with post-bronchodilator FEV_1 % predicted [r=-0.283 (P = 0.066)] (Fig. 7I). These data showed that reduced lung SATB1 and elevated S100A9 responses correlate with impaired lung function and increased disease severity in human COPD. We also measured miR-21, SATB1, S100A8 and S100A9 expression in primary broncho-epithelial cells (pBECs) from a small but different cohort of patients with COPD without lung cancer (Fig. 7, J to M). pBEC miR-21 expression was increased and Ant-21 reduced miR-21 expression to below the baseline in cells from healthy subjects and patients with COPD, and increased *SATB1* expression above the baseline in healthy cells.

Therapeutic targeting of CS-induced miR-21 reverses key features of disease progression in experimental COPD

We previously showed that the severity of disease features in mice with experimental COPD (8 weeks CS exposure) increased with an additional 4 weeks of CS exposure, which accurately modeled COPD progression in humans (*3, 41*). We next assessed the potential therapeutic benefit of targeting miR-21 in established disease to suppress or halt disease progression. To do this we administered Ant-21 or Scr control from 9 to 12 weeks of CS exposure in BALB/c mice (Fig. 8A). CS-exposed, Scr-treated (Smk+Scr) mice had increased lung miR-21 expression compared to air-exposed, Scr-treated controls (Air+Scr; Fig. 8B) after 12 weeks. Therapeutic Ant-21 treatment (Smk+Ant-21) inhibited CS-induced lung miR-21 expression (Fig. 8B) and substantially suppressed CS-induced influx of all inflammatory cells into the airways (Smk+Ant-21 versus

Smk+Scr; Fig. 8, C to E). CS-exposed, Scr-treated mice had increased lung hysteresis (Fig. 8F) and transpulmonary resistance (Fig. 8G), with decreased transpulmonary compliance (Fig. 8H) and increased tissue damping (Fig. 8I). Therapeutic Ant-21 treatment reversed these CS-induced changes in lung function. Collectively, these data demonstrate that targeted inhibition of CS-induced miR-21 responses after the development of experimental COPD suppresses airway inflammation and improves lung function.

DISCUSSION

Here we discovered a CS-induced lung miR-21/SATB1/S100A9/NF-κB axis that promotes experimental COPD and miR-21 expression correlated with disease severity in human COPD. Increased miR-21 expression is linked with several inflammatory diseases, including COPD but has not been widely investigated (*16*). The role of miR-21 in immunity is complex, due to its range of potential targets, expression in different cell types and its ability to induce pro- and anti-inflammatory responses. We demonstrated that CS exposure induces persistent increases in lung miR-21 expression throughout the induction of experimental COPD. This is consistent with a study showing that COPD patients and asymptomatic smokers had substantially higher serum miR-21 expression than healthy controls (*16*). This suggests that chronic CS-induced increases in miR-21 expression have a role in COPD pathogenesis. Furthermore, our data suggests that CS-induced miR-21 responses as well as other ancillary pathways and stimuli drive NF-κB-mediated effects on target genes in several cell types to produce the observed phenotype. However, other target genes were unaffected, which may be because NF-κB-mediated effects are determined by the type of stimulus and cellular source.

Roles for aberrant miR-21 responses are known in fibrotic lung diseases such as idiopathic pulmonary fibrosis (*54*). It promotes cardiac and hepatic fibrosis by negatively regulating anti-fibrogenic SPRY1 (*55*), SPRY2 (*56*), and PDCD4 (*57*). We demonstrated that CS-induced miR-21 expression promotes collagen deposition around small airways, however, Ant-21 treatment had no restorative effects on the expression of these anti-fibrogenic factors or other canonical targets including RECK, TIMP3 and PTEN. These findings suggest that these factors are not regulated by CS-induced miR-21 responses and are controlled by other pathways. We next assessed the effects of inhibiting CS-induced lung miR-21 on lung function parameters in experimental COPD. Ant-

21 treatment improved lung function in mice by decreasing lung hysteresis and transpulmonary resistance, and tissue damping back to air-exposed responses. Changes in lung physiology are a balance between the effects of airway remodeling (fibrosis) and emphysema. The timepoint assessed was during the earlier stages of relatively mild experimental COPD, and the degree of emphysema may not be sufficient to cause increases in lung compliance. We also showed that Ant-21 treatment reverses CS-induced small airway fibrosis and increases lung compliance without having a significant effect on emphysema, suggesting that the change in compliance may be due to the reversal of CS-induced airway fibrosis and chronic airway inflammation. CS can also induce interstitial fibrosis in humans and therefore the observed impact on compliance may also be produced by a therapeutic effect on interstitial and not necessarily only on airway fibrosis. Furthermore, the physiological defect in bronchitic COPD is airway remodeling manifested by a change in resistance, and -induced interstitial fibrosis represents a distinct pathological entity.

We showed that CS exposure increases lung miR-21 expression from 4 weeks with concomitant reductions in lung SATB1 expression in experimental COPD, and that lung epithelial SATB1 responses progressively diminish with increasing disease severity. SATB1 is an anti-inflammatory and recently defined miR-21 target (*37, 38*) and its direct regulation by miR-21 has been demonstrated using luciferase assays in human and murine keratinocytes. The human *SATB1* 3' UTR contains two highly and one less conserved miR-21-5p binding sites, and there is a single highly conserved binding site in the murine *Satb1* 3' UTR (table S8). This suggests that targeting miR-21 responses may have more pronounced effects in human COPD. Our findings are consistent with previous reports showing increased miR-21 and reduced SATB1 expression (*58, 59*) suggesting that miR-21-dependent suppression of SATB1 may play important roles in COPD pathogenesis. SATB1 can regulate numerous immune functions. SATB1-deficient thymocytes

exhibit de-repression of some proto-oncogenes, and cytokine, cytokine receptor and apoptosis-related genes (*60*). SATB1 can acetylate histone H3 (*61*), and chronic histone modifications are implicated in COPD pathogenesis (*62*), providing further support that miR-21-dependent suppression of lung SATB1 may have important roles. The beneficial effects of Ant-21 treatment were associated with increases in lung SATB1 responses and were reversed following combined treatment with SATB1-targeting siRNA. Recently, liver fibrosis in rats was reduced by overexpressing SATB1 (*63*), suggesting that SATB1 has anti-fibrogenic effects and its downregulation in the lungs may result in small airway fibrosis. Our data indicates that miR-21 exerts bronchitic inflammatory and fibrogenic, but not emphysematous, effects through SATB1. We consider that targeting key upstream factors such as miR-21 is likely to be more effective than targeting single downstream mediators or effectors. To our knowledge, there are no commercially available SATB1-specific activators or S100A9-specific inhibitors. One other possibility would be to administer recombinant mouse SATB1 directly to the lungs, however, these proteins are N- and C-terminal modified in commercially available formulations and it is unclear if they would permeate into relevant cells.

SATB1 activity can negatively regulate S100A8 and S100A9 responses (*36*), and SATB1-null mice exhibit increased S100A8 and S100A9 expression (*64, 65*). Furthermore, S100A9 is a potential biomarker for several inflammatory diseases such as rheumatoid arthritis (*50*), severe neutrophilic asthma (*51*) and myocardial infarction (*66*). Thus, we assessed lung S100A8 and S100A9 expression in CS-induced experimental COPD. CS-induced increases in lung miR-21 expression were associated with a concomitant decrease in SATB1 and increased S100A9 responses in the airways and parenchyma in experimental COPD. S100A9 responses are elevated in broncho-epithelial cells (*52*) and bronchoalveolar lavage fluid (BALF) of COPD patients (*53*),

implicating increased S100A9 responses in pathogenesis. In our study CS exposure had no effect on S100A8 expression, suggesting that S100A8 and S100A9 are unlikely to be co-induced. This is supported by similar observations (*67*) where stimulation of murine macrophages with LPS, IFNγ or TNF did not co-induce S100A9 and S100A8 expression. We also showed that targeted inhibition of lung miR-21 expression during experimental COPD increased SATB1 responses and decreased S100A9 expression. It is possible to target S100A9 directly using paquinimod, an orally active quinoline derivative for treating autoimmune diseases and exhibits some inhibitory activity on S100A9:TLR4 binding (*68, 69*). However, oral administration of paquinimod would complicate the examination of CS-induced, lung local miR-21/SATB1/S100A9 responses by introducing TLR4 responses as an experimental variable. We showed that lung SATB1 and S100A9 responses correlate with impaired lung function and increased severity of COPD in humans, and that increased lung miR-21 expression occurs in an independent COPD patient cohort without lung cancer.

We, and others, have demonstrated that CS exposure induces NF-κB activation (*22, 70*). Elevated S100A9 responses can induce NF-κB activation in a TLR4- and RAGE-dependent manner and promote pro-inflammatory responses (*39, 71, 72*). NF-κB responses can also induce miR-21 (*23*) and S100A9 (*40*) expression suggesting that a bi-directional miR-21/SATB1/S100A9/NF-κB axis exists (fig. S6) that could self-perpetuate. We showed that depleting CS-induced lung miR-21 expression restores SATB1 expression and decreases S100A9 expression and NF-κB activity that play key roles in CS-induced airway inflammation and remodeling, impaired lung function and are drivers of COPD pathogenesis and disease progression.

Limitations of our studies are that we have not specifically identified the targets of miR-21 inhibition that suppress CS-induced collagen deposition or the specific anti-fibrogenic factors that may be controlled by other pathways. There are also likely other miR-21 targets that mediate or regulate NF-κB activation. A rigorous investigation of these factors is beyond the scope of this study and should be the focus of future investigations.

We also showed that multiple tissues and cells, primarily airway epithelium and macrophages, are major sources of CS-induced miR-21 expression. Specifically, we found CS-induced increases in miR-21 expression in mesenchymal cells ($CD45^{-}Sca1^{+}$), which contain broncho-alveolar stem cells. These cells have major roles in airway regeneration and are considered a site of damage in COPD (*73, 74*). There were also CS-induced trends toward increased miR-21 expression in epithelial and endothelial cells early, and increases later in monocyte/macrophages. In humans, $CD16^{+}$ monocytes are highly pro-inflammatory and contribute to inflammation and COPD, and $CD14^{+}$ marks them as classical subsets, whereas other subsets are anti-inflammatory and promote wound repair (*75*). A more rigorous examination of the relationship between CS-induced miR-21 and the regulation of its downstream effectors, their biological and immunological consequences, and interplay between airway epithelium and macrophages, in COPD is also warranted. Such studies might also examine the effects of over-expression of lung miR-21 in the absence of CS exposure to further delineate its contributions to lung pathology. Indeed, miR-21 is elevated in a variety of cancers and substantial evidence identifies it as a bona fide onco-miR (*76*). miR-21 over-expression in vivo using mimetics or lentiviral vectors is possible, however, they are complicated by strong sequence homology between human and mouse genomes imparting ethics and safety issues. Nevertheless, here we provide strong evidence that CS-induced miR-21 in airway epithelial cells and macrophages

modulates SATB1 responses that result in increased NF-κB responses (fig. S6). Additional functional studies with infectious exacerbations and microbiome/systemic effects would be interesting (*6, 77-80*).

In summary, we demonstrate that CS-induced lung miR-21 expression promotes airway inflammation and fibrosis, and reduces lung function in COPD through a pathogenic signaling pathway involving SATB1/S100A9/NF-κB. Specific targeting of CS-induced lung miR-21 may be a treatment approach in COPD and potentially a more attractive strategy for suppressing excessive miR-21/SATB1/S100A9-induced NF-κB responses than total inhibition of NF-κB activity.

MATERIALS AND METHODS

Study design. Studies were designed to characterize the functional role, effects, and potential for therapeutic targeting of increased miRNA and specifically miR-21 responses in COPD. A range of in vivo, ex vivo and in vitro experiments were performed. Sample sizes are listed below and patient characteristics provided in the Supplementary Materials. Statistical tests used are detailed below and in the figure legends. All patients gave written informed consent. For in vivo studies, sex- and age-matched animals were randomly assigned to exposure, treatment or vehicle treatment groups. Animal numbers for each study type were determined by the investigators based on previous experience with the disease models or from pilot studies. Mice were humanely euthanized at defined study endpoints, and all experimental procedures were performed in accordance with ethics approvals (see Ethics statement).

Ethics statement. The mouse studies were performed in accordance with the recommendations in the Australian code of practise for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All procedures were performed with approval from The University of Newcastle, Sydney Local Health District, and Ghent University animal ethics committees. The human studies were approved by the medical ethics committee of the Ghent University Hospital (2011/14), the University Hospital Gasthuisberg (S51577), Hunter New England Health (05/08/10/3.09) and The University of Newcastle Human Ethics committees (H-163-1205). All subjects provided written informed consent.

Induction of experimental COPD. Female wild-type (WT) BALB/c mice were chronically exposed to CS from 3R4F reference cigarettes (University of Kentucky), or normal air, via the nose-only for 75 minutes at a time, twice per day, 5 days per week for 8 or 12 weeks to induce experimental COPD, as described previously (*12, 22, 41-47*). In other studies, male C57BL/6 mice were exposed (whole-body exposure) to the smoke of five 3R4F reference cigarettes, 4 times a day, with 30 minute smoke-free intervals. Mice were exposed for 5 days per week, for 4 weeks (acute exposure) or 24 weeks (chronic exposure) (*81, 82*). Controls were exposed to normal air.

Human study populations. For miR-21 expression studies in bronchial biopsies: Bronchial biopsies from 15 donors, consisting of 5 healthy subjects (never smokers) and 10 patients with COPD, were obtained during bronchoscopy (John Hunter Hospital, Newcastle, Australia). None of the patients were diagnosed with lung cancer. For miR-21 expression studies in resected lung specimens: Resected lung specimens were obtained from a cohort of 56 patients, of which 43 were obtained from surgery for solitary pulmonary tumors (harvested by a pathologist at maximum

distance from the tumor, Ghent University Hospital, Ghent, Belgium) and 13 were from explant lungs of end-stage COPD patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium). The cohort of 56 patients was divided into 4 subgroups: 10 never smokers, 15 smokers without airflow limitation, 18 patients with GOLD stage II and 13 with GOLD stage III-IV COPD (table S3). For classification we used the TNM (Tumor – Node – Metastasis) staging of cancer, where T1 to T4 describes the size of the original (primary) tumor and whether it has invaded nearby tissue; N1 to N3 describes nearby (regional) lymph nodes that are involved; and M0 to M1 describes distant metastasis (spread of cancer from one part of the body to another). We have also grouped the TNM combinations into four less-detailed stages; Stage I, II and III: the higher the number, the larger the tumor and the more it has spread into nearby tissues; Stage IV: the cancer has spread to distant parts of the body (table S4). Regression analysis on this subset of patients have been added to the online supplement.

For SATB1 and S100A9 enzyme-linked immunosorbent assay (ELISA): Similarly, lung homogenates were prepared from a cohort of 80 patients, of which 66 were obtained from surgery for solitary pulmonary tumors (harvested by a pathologist at maximum distance from the tumor, Ghent University Hospital, Ghent, Belgium) and 14 were from explant lungs from end-stage COPD patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium). The cohort of 80 patients was divided into 4 subgroups: 19 never smokers, 17 smokers without airflow limitation, 30 patients with GOLD stage II and 14 with GOLD stage III-IV COPD (table S7). All patients with COPD had stable disease and patients with exacerbations within 2 months of the commencement of the study were excluded. Other exclusion criteria were chemotherapy or radiotherapy in the last six months, diagnosis of mesothelioma or asthma and infection of the upper or lower respiratory tract in the preceding 4 weeks.

Treatment with antagomirs and small-interfering (si)RNA. CS-induced lung miR-21 expression was inhibited with a miR-21-specific inhibitor (antagomir, Ant). The sequence for miR-21 was obtained from miRBase and a complementary sequence/antagomir for miR-21 (Ant-21) was designed. A scrambled sequence RNA VIII matched against the mouse genome was used as a control to test the specificity of Ant-21. Antagomirs were synthesized by Sigma-Aldrich, supplied in lyophilized form, and resuspended with nuclease free water. WT BALB/c mice were administered Ant-21 intranasally (i.n.) once a week (2.5mg/kg) under isoflurane anesthesia. Lung SATB1 expression was inhibited with custom in vivo ready (HPLC purified) mouse SATB1-targeting siRNA (*Satb1*$_{siRNA}$; ON-TARGETplus™) synthesized by Dharmacon. ON-TARGETplus™ Non-Targeting Control siRNA (Scr$_{siRNA}$) was used as a control. *Satb1*$_{siRNA}$ and Scr$_{siRNA}$ were supplied in lyophilized form, and resuspended with nuclease free water. WT BALB/c mice were administered *Satb1*$_{siRNA}$ or Scr$_{siRNA}$ i.n. three times a week (2.5mg/kg) under isoflurane anesthesia. Mice were sacrificed after 8 or 12 weeks of CS exposure and the features of experimental COPD assessed.

Quantification of miRNA and mRNA expression by real-time qPCR. Total RNA was isolated from homogenized lung tissue with TRIzol® Reagent (Invitrogen).

For quantification of miR-21 (Figs. 1B, D and E, 3B, 8B) and miRs-135b, -146b, -9, and -122 (fig. S1) in mouse lung tissue: Expression of miRs was assessed by real-time qPCR, as described previously (*10*). Briefly, multiplex reverse transcriptions were performed on DNase I-treated total RNA using a combination of reverse primers specific for mature mmu-miR-21 and the endogenous controls small nuclear (sn)RNA U6 and small nucleolar (sno)RNA U49, to a final

concentration of 40 nM each. The relative expression or abundance of miRs was calculated against the geometric mean of U6 and U49. For primer sequences refer to table S9. All reactions were performed using BioScript™ reverse transcriptase in 1x first-strand buffer according to the manufacturer's instructions (Bioline). Real-time qPCR assays were performed with SYBR Green Supermix (KAPA Biosystems) and a Mastercycler® ep realplex2 system (Eppendorf South Pacific). For quantification of miR-21 in mouse lung tissue (Fig. 1F): RNA was reverse transcribed using a miRNA reverse transcription kit (Applied Biosystems, Life Technologies). RT-qPCR was then performed according to the TaqMan miRNA Assay PCR protocol (Applied Biosystems, Life Technologies, Assay ID: 000397). The expression of miR-21-5p was normalized using the geometric mean of three control RNAs (snoRNA 135, snoRNA 202, and snoRNA 292). For quantification of miR-21 in human bronchial biopsies and lung tissue: RNA was reverse transcribed using a miScript II RT kit (Qiagen). RT-qPCR was then performed according to the miScript PCR protocol (Qiagen, Assay ID: MS0009079). The expression of miR-21-5p was normalized using the geometric mean of three control RNAs (SNORD68, SNORD95 and SNORD96A, or SNORD61, SNORD68 and SNORD95).

For quantification of mRNAs in mouse lung tissue: Random-primed reverse transcriptions were performed followed by real-time qPCRs. Gene expression was normalized to the expression of the transcript of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (*Hprt*) (*83*). Primer sequences are provided in table S9. All reactions were performed using BioScript™ reverse transcriptase in 1x first-strand buffer according to manufacturer's instructions (Bioline). Real-time qPCR assays were performed with SYBR Green Supermix (KAPA Biosystems) and a Mastercycler® ep realplex2 system (Eppendorf South Pacific).

Airway inflammation. Airway inflammation was assessed in cytospin preparations of cells from BALF collected at 8 weeks (Fig. 3D-G and 5I-K) and 12 weeks (Fig. 8C-E) of CS exposure (*84, 85*). These samples were collected by 2x 0.5mL lung lavages (left hand side single lung lobe) with Hank's Balanced Salt Solution (Life Technologies) via a cannula inserted into the trachea. BALF was centrifuged (300x*g*, 10 min, 4°C), red blood cells lysed with lysis buffer (200µL, Tris-buffered NH_4Cl) and remaining cells pelleted before total leukocyte numbers were determined using a hemocytometer. Cells were cytocentrifuged and stained with May-Grunwald-Giemsa. Differential leukocyte counts were enumerated according to morphological criteria (≈175 cells by light microscopy [40x magnification]) (*85, 86*). All samples were coded and counts were performed in a blinded fashion.

Assessment of pulmonary fibrosis. Pulmonary fibrosis was assessed by staining longitudinal lung histological sections with Verhoff-Van Gieson stain, which develops collagen into a visually pink color. Image J software was used to quantify the area (μm^2) of fibrosis around small airways, which was normalized by dividing the area by the total perimeter (µm) of the airway basement membrane (*42, 46*).

Assessment of alveolar enlargement. Lungs were perfused with 0.9% NaCl, fixed in formalin, and embedded in paraffin. 5µm thick longitudinal histological sections were stained with hematoxylin and eosin (H&E). Emphysema-like alveolar enlargement was assessed by measuring the average alveolar diameter in the first 10 viable images (40x magnification) using the mean linear intercept method, and also as a percentage of destroyed space using the destructive index method, as described previously (*22, 41-43*).

Assessment of lung function. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, Troy Laboratories) and their tracheas cannulated (tracheostomy with ligation) (*84, 85, 87, 88*). FlexiVent™ apparatus (FX2 System; SCIREQ) with a Forced Expiration Extension (FEV) was used to assess lung function parameters (tidal volume of 8 mL/kg at a respiratory rate of 450 breaths/min) (*41*). This combination of anesthesia and ventilation is commonly used and recommended by the manufacturer (*41, 85*). All maneuvers were performed at least three times and the average was calculated.

Human pBEC cultures and quantification of miRNA and mRNA expression by real-time qPCR. Human pBECs were obtained by endobronchial brushing and cultured in Bronchial Epithelial Cell Growth Medium (BEGM™; Lonza) as described previously (*43, 44, 78, 89*). Patient characteristics are described in table S10. Ant-21 or Scr control were transiently transfected into pBECs for 24 hours using siPORT™ *NeoFX*™ Transfection Agent (Ambion) according to the manufacturer's instructions. For quantification of miR-21, SATB1, S100A8 and S100A9 from human pBECs: Transfected pBECs were lysed and total RNA isolated using a miRNeasy Mini Kit (Qiagen) and QIAcube apparatus (Qiagen). RNA (200 ng) was reverse transcribed using a miScript II RT kit and HiFlex buffer (Qiagen). For SATB1, S100A8, S100A9, and reference 18s, qPCR was performed using Taqman Assays (Applied Biosystems; SATB1-Hs00962580_m1; S100A8-Hs00374264_g1; S100A9-Hs00610058_m1; 18s-Hs99999901_s1). The expression of miR-21-5p was normalized to RNU6B, and the expression of SATB1, S100A8 and S100A9 was normalized to 18S (*44*).

Statistical analyses. Comparisons between two groups were made using unpaired *t*-tests or a non-parametric equivalent where appropriate. Comparisons between multiple groups were made using a one-way analysis of variance (ANOVA) and an appropriate post hoc test, or a non-parametric equivalent where appropriate, as detailed in the figure legends. For murine data, biological outliers were removed using Grubb's outlier testing. Regression analyses of resected lung tissue data were made using Spearman's rank correlation. Analyses were performed using GraphPad Prism Software. All data shown are representative of individual mice or human patients. No data has been pooled unless otherwise indicated.

List of Supplementary Materials

Materials and Methods

Fig. S1 to S6.

Table S1 to S10.

Data file S1

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Figure legends

Fig. 1. Cigarette smoke (CS) exposure increases lung miR-21 expression in experimental chronic obstructive pulmonary disease (COPD). (**A**) Wild type (WT) BALB/c mice were exposed to CS (Smk) via the nose only, or normal air (Air), for 2, 4, 6 and 8 weeks. (**B**) miR-21 expression in whole lungs was normalized to the geometric mean of the housekeeping controls small nuclear (sn)RNA U6 and small nucleolar (sno)RNA U49. In other mice, lungs were blunt dissected to determine miR-21 expression in (**C**) airways and (**D**) parenchyma after 8 weeks of CS exposure. miR-21 expression is expressed as fold change from Air controls (n=4-8 mice per group). Data are presented as means ± s.e.m. *P<0.05; ***P<0.001; ****P<0.0001. (**E**) miR-21 expression in mouse lung macrophages isolated at 8 weeks of CS exposure normalized to the geometric mean of the housekeeping controls snRNA U6 and snoRNA U49 (n=4 mice per group). (**F**) WT C57BL/6 mice were exposed to CS via whole-body exposure (Smk), or normal air (Air), for 4 or 24 weeks, and (**G**) miR-21 expression in whole lung tissue normalized to the geometric mean of the housekeeping controls snoRNA 135, snoRNA 202 and snoRNA 292 (n=8 mice per group). Data are expressed as relative quantities ± s.e.m. *P<0.05; **P<0.01; ***P<0.001. (**H**) miR-21 fluorescent *in situ* hybridization in lung histological sections (scale bar, 50μm). miR-21-positive signal (red) is visible in the airway epithelium. miR-21-positive signal is not evident with the use of scrambled control LNA miR probe. Sections were counterstained with DAPI. Data (**B**, **C**, **D**, **E** and **G**) were analyzed by two-tailed unpaired student's t test.

Fig. 2. Increased miR-21 expression correlates with disease severity and impaired lung function in chronic obstructive pulmonary disease (COPD). (**A**) Hsa-miR-21 expression in lung biopsies from healthy controls and patients with COPD without lung cancer, normalized to the geometric mean of SNORD68, SNORD95 and SNORD96A. Data are presented as means ± s.e.m (*n*=5-10 per group; see *Materials and Methods* and table S2). ***P*<0.01. Data analyzed by two-tailed unpaired student's *t* test. (**B**) Hsa-miR-21 expression in resected lung tissue from never smokers, smokers without COPD and patients with mild (GOLD II) or moderate-to-severe (GOLD III-IV) COPD, normalized to the geometric mean of the housekeeping controls SNORD61, SNORD68 and SNORD95. Data are presented as means ± s.e.m (*n*=10-18 per group; see *Materials and Methods* and table S3). **P*<0.05; ***P*<0.01. Data analyzed by one-way ANOVA with Welch's correction. (**C**) Hsa-miR-21 fluorescent *in situ* hybridization in lung histological sections (scale bar, 50µm). Hsa-miR-21 expression in resected lung tissues from a cohort of 56 patients (see *Materials and Methods* and table S3) was correlated with (**D**) post-bronchodilator forced expiratory volume in 1 second (FEV_1) % predicted, (**E**) FEV_1/forced vital capacity (FVC) ratio, (**F**) diffusing capacity of the lungs for carbon monoxide (DL_{CO}) % predicted, and (**G**) the diffusion coefficient (K_{CO}) % predicted. Associations for each comparison (**D** to **G**) are expressed as Spearman's rank correlation coefficient (Spearman's rho; *r*). **P*<0.05; ***P*<0.01; ****P*<0.001.

Fig. 3. Inhibition of cigarette smoke (CS)-induced lung miR-21 suppresses airway inflammation and inhibits cytokine/chemokine production in experimental chronic obstructive pulmonary disease (COPD). (**A**) Wild-type (WT) BALB/c mice were exposed to CS via the nose only (Smk), or to normal air (Air), for 8 weeks to induce experimental COPD and treated intranasally with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir once weekly throughout. (**B**) miR-21 expression normalized to the geometric mean of the housekeeping controls small nuclear (sn)RNA U6 and small nucleolar (sno)RNA U49, and expressed as fold change from air exposed, Scr-treated (Air+Scr) controls (*n*=4-6 mice per group). (**C**) Representative photomicrographs (scale bar, 60μm) showing tissue localization of CS-induced miR-21 in lung histological sections collected at 8 weeks. miR-21 was detected using *in situ* hybridization analyses with a miR-21-specific locked nucleic acid (LNA™) probe (miR-21-positive signal [blue]). Sections were counterstained with Nuclear Fast Red™. (**D**) Total leukocytes, (**E**) macrophages, (**F**) neutrophils, and (**G**) lymphocytes were enumerated in bronchoalveolar lavage fluid (BALF) at 8 weeks (*n*=5-6 mice per group). (**H**) Tumor necrosis factor (TNF)α and (**I**) chemokine (C-X-C motif) ligand (CXCL)1 proteins in whole lung homogenates at 8 weeks (*n*=3-4 mice per group). Data are presented as means ± s.e.m. *n.s* = not significant. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data were analyzed with (**B**) one-way ANOVA with Sidak's post hoc test, (**D** to **G**) one-way ANOVA with Fisher's least-significant difference post hoc test, and (**H** and **I**) one-way ANOVA with Tukey's post hoc test.

Fig. 4. Inhibition of cigarette smoke (CS)-induced lung miR-21 inhibits small airway remodeling and prevents the impairment of lung function in experimental chronic obstructive pulmonary disease (COPD). Wild-type (WT) BALB/c mice were exposed to CS (Smk) via the nose only, or normal air (Air), for 8 weeks to induce experimental COPD and treated intranasally with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir once weekly throughout (see Fig. 3A for protocol). (**A**) Representative photomicrographs (scale bar, 60μm) of Verhoff-Van Gieson-stained lung sections showing collagen deposition around small airways (pink). (**B**) Quantification of collagen deposition (μm^2) around small airways normalized to per μm of the airway basement membrane perimeter. (**C**) Representative photomicrographs (scale bar, 60μm) of hematoxylin and eosin-stained lung sections. (**D**) Mean alveolar diameter, and (**E**) Destructive Index (%) (*n*=4-6 mice per group). Lung function assessed in terms of (**F**) lung hysteresis, transpulmonary (**G**) resistance and (**H**) compliance, and (**I**) tissue damping (*n*=7-8 mice per group). Data are presented as means ± s.e.m. *n.s* = not significant. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data (**B**, **D** to **I**) were analyzed with one-way ANOVA with Tukey's post hoc test.

Fig. 5. Cigarette smoke (CS)-induced lung miR-21 reduces SATB1 responses, resulting in experimental chronic obstructive pulmonary disease (COPD). (**A**) Wild-type (WT) BALB/c mice were exposed to CS (Smk) via the nose only, or normal air (Air), for 4 and 6 weeks (see Fig. 1A for protocol). (**A** and **B**) Whole lung *Satb1* mRNA expression normalized to the housekeeping control hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and expressed as fold change from Air controls (*n*=5-6 mice per group). WT BALB/c mice were exposed to CS via the nose only, or to normal air, for 8 weeks to induce experimental COPD and treated intranasally with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir once weekly throughout (see Fig. 3A for protocol). (**C**) Whole lung *Satb1* mRNA expression normalized to *Hprt* and expressed as fold change from air-exposed, Scr-treated (Air+Scr) controls (*n*=5-6 mice per group). (**D** and **E**) Whole lung protein of SATB1 determined by immunoblot and densitometry (*n*=11-12 mice per group from two independent experiments). Representative photomicrographs (scale bar, 60μm) of SATB1 immunofluorescence (Alexa Fluor® 594 with Hoechst 33342 nuclear counterstain) in (**F**) airways, and (**G**) parenchyma of lung histology sections at 8 weeks. (**H**) WT BALB/c mice were exposed to CS via the nose only, or normal air, for 8 weeks to induce experimental COPD and treated intranasally with Ant-21 or Scr antagomir once weekly throughout in the absence or presence of SATB1-targeting small-interfering (si)RNA administered thrice weekly. (**I**) Total leukocytes, (**J**) macrophages, and (**K**) neutrophils were enumerated in bronchoalveolar lavage fluid (BALF) at 8 weeks (*n*=8 mice per group). Lung function assessed in terms of (**L**) lung hysteresis, transpulmonary (**M**) resistance and (**N**) compliance, and (**O**) tissue damping (*n*=7-8 mice per group). Data are presented as means ± s.e.m. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 compared to normal air-exposed controls. Data in (**A** and **B**) were analyzed with

two-tailed unpaired student's *t* test. Data in (**C**, **E**, and **I** to **O**) were analyzed with one-way ANOVA with Sidak's post hoc test.

Fig. 6. Cigarette smoke (CS)-induced miR-21 promotes lung S100A9 expression and NF-κB activity, which are reversed by miR-21 inhibition in experimental chronic obstructive pulmonary disease (COPD). Wild-type (WT) BALB/c mice were exposed to CS (Smk) via the nose only, or normal air (Air), for 8 weeks to induce experimental COPD (see Fig. 1A for protocol). Blunt dissected (**A**) airways and (**B**) parenchyma *S100A8* mRNA expression, and (**C**) airways and (**D**) parenchyma *S100A9* mRNA expression, normalized to the housekeeping control hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and expressed as fold change from normal air-exposed (Air) controls (n=7-8 mice per group). *P<0.05; ***P<0.001. WT BALB/c mice were exposed to CS via the nose-only, or to normal air, for 8 weeks to induce experimental COPD and treated intranasally with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir once weekly throughout (see Fig. 3A for protocol). (**E**) Whole lung *S100A9* mRNA expression normalized to *Hprt* and expressed as fold change from air-exposed, Scr-treated (Air+Scr) controls (n=6 mice per group). (**F**) NF-κB activity determined by measuring DNA-bound NF-κB p65 subunit (n=11-12 mice per group from two independent experiments). (**G**) NF-κB luciferase reporter assay in HEK293 cells exposed to CS extract (CSE; 1%) and co-transfected with Ant-21 or Scr for 6 hours (n=5 per condition). NF-κB luciferase reporter activity expressed as relative luminometer units (RLU; NF-κB-Response Element [RE] Luc/Control Luc). Data are presented as means ± s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Data in (**A** to **D**) were analyzed with two-tailed unpaired student's t test. Data in (**E**) were analyzed with one-way ANOVA with Tukey's post hoc test, (F) one-way ANOVA with Bonferroni's post hoc test, and (**G**) one-way ANOVA with Sidak's post hoc test.

Fig. 7. Decreased SATB1 and increased S100A9 responses correlate with impaired lung function in chronic obstructive pulmonary disease (COPD). (**A**) SATB1 and (**B**) S100A9 proteins in resected lung tissue from never smokers, smokers without COPD and patients with mild (GOLD II) or moderate-to-severe (GOLD III-IV) COPD. Data are presented as means ± s.e.m. **P*<0.05; ***P*<0.01. (**C**) SATB1 immunohistochemistry in lung histological sections from a never smoker, smoker, and patients with GOLD II, GOLD III and GOLD IV COPD (scale bar, 100μm). In resected lung tissues from a cohort of 80 patients (see *Materials and Methods* and table S7), SATB1 protein was correlated with (**D**) post-bronchodilator forced expiratory volume in 1 second (FEV_1) % predicted, and (**E**) diffusing capacity of the lungs for carbon monoxide (DL_{CO}) % predicted, and S100A9 protein was correlated with (**F**) FEV_1 % predicted. In a sub-analysis of only COPD patients (from cohort detailed in table S7), SATB1 protein was correlated with (**G**) FEV_1 % predicted, and (**H**) DL_{CO} % predicted, and S100A9 protein was correlated with (**I**) FEV_1 % predicted. Associations for each comparison are expressed as Spearman's rank correlation coefficient (Spearman's rho; *r*). Human primary bronchial epithelial cells (pBECs) from healthy subjects and patients with COPD (without lung cancer) were transfected with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir, and (**J**) hsa-miR-21, (**K**) SATB1, (**L**) S100A8, and (**M**) S100A9 expression determined at 24 hours post-transfection (*n*=6 per group; see *Materials and Methods* and table S10). Expression of hsa-miR-21 was normalized to RNU6B, and the expression of SATB1, S100A8 and S100A9 was normalized to 18S rRNA. hsa-miR-21, SATB1, S100A8 and S100A9 mRNA are expressed as fold change from Scr-treated, healthy control pBECs. Data are presented as means ± s.e.m. **P*<0.05; ***P*<0.01. Data in (**A**, **B**, and **J** to **M**) were analyzed with two-tailed unpaired student's *t* test.

Fig. 8. Inhibition of cigarette smoke (CS)-induced lung miR-21 improves features of disease progression in experimental chronic obstructive pulmonary disease (COPD). (**A**) Wild-type (WT) BALB/c mice were exposed to CS via the nose only (Smk), or to normal air (Air), for 8 weeks to induce experimental COPD, and were treated intranasally with miR-21-specific (Ant-21) or scrambled (Scr) antagomir once weekly from 9 to 12 weeks. (**B**) miR-21 expression was normalized to the geometric mean of the housekeeping controls small nuclear (sn)RNA U6 and small nucleolar (sno)RNA U49, and expressed as fold change from air exposed, Scr-treated (Air+Scr) controls (n=8 mice per group). (**C**) Total leukocytes, (**D**) macrophages, and (**E**) neutrophils were enumerated in bronchoalveolar lavage fluid (BALF) at 12 weeks (n=10 mice per group). Lung function was assessed in terms of (**F**) lung hysteresis, transpulmonary (**G**) resistance and (**H**) compliance, and (**I**) tissue damping (n=7-8 mice per group). Data are presented as means ± s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Data in (**B** to **I**) were analyzed with one-way ANOVA with Tukey's post hoc test.