A Disintegrin and Metalloproteinase Domain-9: A Novel Proteinase Culprit with Multifarious Contributions to Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: ADAMs (proteinases with a disintegrin and a metalloproteinase domain) have not been well studied in chronic obstructive pulmonary disease (COPD).

Objectives: To investigate whether ADAM9 is linked to COPD in humans and mice.

Methods: ADAM9 blood and lung levels were measured in patients with COPD versus control subjects, and in air- versus cigarette smoke (CS)-exposed wild-type mice. Wild-type and *Adam9*^{-/-} mice were exposed to air or CS for 1–6 months, and COPD-like lung pathologies were measured.

Measurements and Main Results: ADAM9 staining was increased in lung epithelial cells and macrophages in smokers and even more so in patients with COPD and correlated directly with pack-year smoking history and inversely with airflow obstruction and/or FEV₁ percent predicted. Bronchial epithelial cell ADAM9 mRNA levels were higher in patients with COPD than control subjects and correlated directly with pack-year smoking history.

Plasma, BAL fluid, and sputum ADAM9 levels were similar in patients with COPD and control subjects. CS exposure increased Adam9 levels in wild-type murine lungs. *Adam9*^{-/-} mice were protected from emphysema development, small-airway fibrosis, and airway mucus metaplasia. CS-exposed *Adam9*^{-/-} mice had reduced lung macrophage counts, alveolar septal cell apoptosis, lung elastin degradation, and shedding of vascular endothelial growth factor receptor-2 and epidermal growth factor receptor in BAL fluid samples. Recombinant ADAM9 sheds epidermal growth factor and vascular endothelial growth factor receptors from epithelial cells to reduce activation of the Akt prosurvival pathway and increase cellular apoptosis.

Conclusions: ADAM9 levels are increased in COPD lungs and linked to key clinical variables. Adam9 promotes emphysema development, and large- and small-airway disease in mice. Inhibition of ADAM9 could be a therapeutic approach for multiple COPD phenotypes.

Keywords: emphysema; small-airway fibrosis; mucus metaplasia; inflammation; growth factor receptor

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At a Glance Commentary

Scientific Knowledge on the

Subject: Matrix metalloproteinases have been strongly implicated in the pathogenesis of emphysema, but less is known about the contributions of proteinases to other key chronic obstructive pulmonary disease (COPD) phenotypes including small-airway fibrosis and mucus hypersecretion. The contributions of ADAMs (proteinases with a disintegrin and a metalloproteinase domain) to the pathogenesis of COPD have not been explored.

What This Study Adds to the

Field: ADAM9 staining is increased in lung epithelial cells and macrophages in smoker lungs, and even more so in COPD lungs, and correlates directly with pack-year smoking history and inversely with airflow obstruction and FEV₁ percent predicted. ADAM9 gene expression is increased in bronchial brushing cells from patients with COPD versus control subjects and correlates directly with pack-year smoking history. In mice exposed to cigarette smoke, Adam9 promotes the development of several key COPD-like phenotypes (emphysema, small-airway fibrosis, and mucus cell metaplasia). Adam9 promotes emphysema development by increasing lung inflammation and inducing alveolar septal cell apoptosis by shedding growth factor receptors from cell surfaces. Thus, ADAM9 is a multifarious culprit in COPD that could be targeted therapeutically to limit disease progression and ameliorate symptoms associated with several COPD phenotypes.

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide (1). The main risk factor for COPD is exposure to cigarette smoke (CS). Pulmonary emphysema develops after CS-induced injury to alveolar septal cells (2) and proteinase-mediated injury to the lung extracellular matrix (3). CS exposure also induces small-airway fibrosis (4) and mucus hypersecretion, and proteinases have been implicated in these processes (3, 5).

Among the proteinase culprits implicated in COPD, most is known about the contributions of serine and cysteine proteinases and matrix metalloproteinases (MMPs) (3). Little is known about the contributions of the ADAM (a disintegrin and a metalloproteinase domain) subfamily of metalloproteinases to the pathogenesis of COPD. ADAMs are type I multidomain transmembrane proteinases that can contain 1) a prodomain that maintains latency of the catalytic domain, 2) a metalloproteinase domain, 3) a disintegrin domain that can bind to integrins, 4) a cysteine-rich domain, 5) an EGF (epidermal growth factor)-like domain, 6) a membrane-spanning domain, and 7) a cytoplasmic tail that may regulate cellular signaling (6). There is only one prior study that measured the expression of an ADAM in COPD samples. This study reported that sputum ADAM8 levels were higher in patients with COPD than in healthy control subjects (7).

ADAM9 is expressed by cells implicated in COPD, including polymorphonuclear leukocytes (PMNs), macrophages, and epithelial cells (8–10), and contains all of the domains listed above. Although ADAM9 promotes injury to the alveolar-capillary barrier during acute lung injury in mice (8), little else is known about its contributions to lung diseases. We tested the hypotheses that 1) ADAM9 levels are increased in blood and/or lung samples from patients with COPD and CS-exposed mice; and 2) ADAM9 promotes the development of COPD-like lung pathologies in CS-exposed mice.

Methods

See the online supplement for details.

Human Subjects

Human studies were approved by institutional review boards in the United States and United Kingdom. Four cohort types were studied (lung immunostaining, sputum, plasma, and bronchoscopy cohorts). Tables 1 and 2, and Tables E1–E3 and E5 in the online supplement, show the demographic and clinical data on these cohorts.

Immunostaining of lung sections for ADAM9. Lung sections from patients with COPD and control subjects were

double-immunostained in red for markers of alveolar macrophages (CD163) or epithelial cells (pancytokeratin; PanCK), and in green for ADAM9. Lung sections were double-immunostained for ADAM9 and EGFR (epidermal growth factor receptor) or VEGFR2 (vascular endothelial growth factor receptor-2).

ADAM9 steady-state mRNA levels in bronchial brushings from the bronchoscopy cohort. See the online supplement.

ADAM9 bronchoalveolar lavage fluid, plasma, and sputum levels. Soluble ADAM9 (sADAM9) levels were measured by ELISA.

Animal and In Vitro Experiments

Studies of mice were approved by the local Institutional Animal Care and Use Committee.

CS exposures. Adult C57BL/6 strain wild-type and Adam9^{−/−} mice were exposed to air or whole-body CS for 2 h/d for 6 d/wk, for 1–6 months (11).

Adam9 levels in wild-type murine lungs. Adam9 levels were measured by quantitative real-time PCR or by ELISA.

Emphysema and small-airway fibrosis. Distal airspace size and fibrosis around small airways were measured in lung sections, using morphometric methods (11).

Airway Muc5ac staining and α-smooth muscle actin. Murine lung sections were immunostained for airway epithelial Muc5ac (mucin 5ac) (12) and α-SMA (α-smooth muscle actin; a myofibroblast marker).

Lung inflammation. BAL leukocytes were counted, and pro- and antiinflammatory mediators and Timp-3 (tissue inhibitor of metalloproteinase-3) protein levels were measured in lung samples, using ELISAs.

Apoptosis of alveolar macrophages. Alveolar macrophages from air- or CS-exposed wild-type and Adam9^{−/−} mice were immunostained for active caspase-3.

Lung elastin degradation. Desmosine (a marker of elastin degradation) was quantified in BAL fluid (BALF) and sera by ELISA.

Alveolar septal cell death. Lung sections were immunostained for TUNEL (terminal deoxynucleotidyltransferase dUTP nick end-labeling)-positive cells.

Active caspase-3 levels in lung epithelial cells and bone marrow-derived macrophages. Wild-type and Adam9^{−/−} alveolar epithelial cells, or bone

Table 1. Demographic and Clinical Characteristics of the Human Immunostaining Cohort

Characteristic	Nonsmokers* (n = 9)	Smokers* (n = 8)	COPD GOLD Stages I–II (n = 6)	COPD GOLD Stages III–IV (n = 8)	P Value†
Males, n (%)	2 (22)	3 (37.5)	4 (67)	5 (62.5)	NS
Age, yr	64 (35–82)	69 (54–79)	65 (52–77)	64 (58–71)	NS†
Pack-years of smoking	0	34 (10–60)	54 (20–86)	42 (25–60)	<0.001‡
Current smokers, n (%)	0 (0)	1 (12.5)	0 (0)	2 (25)	NS
FEV ₁ , % predicted§	97 ± 23	81 ± 16	77 ± 15	28 ± 12	<0.001
FEV ₁ /FVC, % predicted§	76 (71–80)	83 (72–112)	58 (42–66)	44 (22–66)	<0.001¶

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Obstructive Lung Disease; NS = not significant. Shown are the demographic and clinical characteristics of the patients with COPD, smokers without COPD, and nonsmoker control subjects who underwent a lung biopsy procedure, lung volume reduction surgery, or lung transplantation (see the online supplement). Patients with COPD were subdivided according to GOLD criteria.

Data are presented as median (interquartile range) for nonparametric data or as means ± SD for parametric data.

*Nonsmokers were all never-smokers. Smokers were defined as subjects who had at least a 10-pack-year smoking history. Current smokers were defined as active smokers at the time of the biopsy or surgery, or former smokers who had stopped smoking not more than 1 year before the biopsy or surgery.

†Categorical variables were analyzed by χ^2 tests. Statistical analyses included one-way ANOVA tests for continuous variables (age, FEV₁% predicted, FEV₁/FVC, and pack-years) followed by pairwise comparisons by Student's *t* tests for parametric data or by Mann-Whitney *U* tests for nonparametric data.

‡The pack-year smoking histories of the GOLD stage I–II and GOLD stage III–IV COPD groups were not significantly different from those of the smoker group ($P = 0.2$ for both comparisons). The pack-year smoking histories of the GOLD stage I–II and GOLD stage III–IV COPD groups and the smoker group were significantly different from those of the nonsmoker group by design.

§All patients with COPD had an FEV₁/FVC ratio less than 0.7, whereas smokers without COPD and nonsmoker control subjects had an FEV₁/FVC ratio of at least 0.7.

||The FEV₁ of the GOLD stage III–IV COPD group was significantly lower than that of the smoker and nonsmoker groups ($P < 0.001$ for both comparisons). The FEV₁ of the GOLD stage I–II COPD group was not significantly different from that of the smoker and nonsmoker groups ($P = 0.7$ and $P = 0.1$).

¶The FEV₁/FVC ratios of the GOLD stage III–IV COPD group were significantly lower than those of the smoker and nonsmoker groups ($P = 0.002$ and $P = 0.001$, respectively). The FEV₁/FVC ratios of the GOLD stage I–II COPD group were significantly lower than those of the smoker and nonsmoker groups ($P = 0.007$ and $P = 0.002$, respectively).

marrow-derived macrophages (BMDMs), were exposed to 7.5% or 20% cigarette smoke extract (CSE), respectively.

Intracellular active caspase-3 levels were quantified (13) (see the online supplement).

ADAM9-mediated shedding of EGFR and VEGFR2. Soluble EGFR (sEGFR) and sVEGFR2 levels were measured in BALF and sera from mice, using ELISAs. EGFR and VEGFR2 levels on alveolar septal cell surfaces were measured in lung sections by immunostaining. Human bronchial epithelial cells (HBECs) were incubated for 4 hours with or without 10–60 nM active recombinant human (rh)ADAM9, with or without 10 μ M GM6001 (a nonselective metalloproteinase inhibitor). sEGFR and sVEGFR2 levels were measured in supernatants by ELISAs, and EGFR and VEGFR2 levels on HBEC surfaces were quantified by immunostaining.

ADAM9-induced apoptosis of HBECs. HBECs were incubated with or without 60 nM active rhADAM9 with or without 10 μ M GM6001. Intracellular active caspase-3 levels were quantified (13).

Akt phosphorylation in murine lungs. See the online supplement.

Statistics

Data are presented as box plots showing medians and 25th and 75th percentiles and whiskers showing 10th and 90th percentiles (nonparametric data), as means ± SD (parametric data), or as scatter plots.

Human studies. Nonparametric data were analyzed by one-way ANOVA with Dunnett corrections for multiple comparisons. A multivariate regression model, with ADAM9 gene expression as the dependent variable, identified clinical parameters for which to adjust ADAM9 expression in correlation analyses. ADAM9 expression was analyzed by using a Bayesian regression model with informative normal priors on the correcting variables (age and pack-year smoking history). A more restrictive prior distribution was set on age as the multivariate analysis showed that age correlated weakly with ADAM9 expression. Contrasts were performed against the relevant group, and results reported as the posterior probability of no effect.

Murine studies. Data were analyzed by one-way ANOVA followed by pairwise comparisons using Student's *t* tests and Bonferroni corrections (parametric data),

or Kruskal-Wallis one-way ANOVA followed by pairwise comparisons using Mann-Whitney *U* tests with Bonferroni corrections (nonparametric data).

Results

Human Studies

ADAM9 expression is increased in COPD lungs. Table 1 shows the demographic and clinical characteristics of the lung immunostaining cohort. Patients with COPD had greater ADAM9 staining in alveolar (Figures 1A and 1D) and bronchial epithelial cells (Figures 1B and 1E) than smokers. Smokers had greater ADAM9 staining in both cell types than nonsmokers. After correcting correlations for differences in age, sex, pack-year smoking history, and current smoker status, ADAM9 staining in both epithelial cell types correlated directly with pack-year smoking history (Figures E1A and E1B) and inversely with FEV₁/FVC (Figures E1C and E1D). ADAM9 staining in bronchial epithelial cells (but not alveolar epithelial cells) correlated

Table 2. Demographic and Clinical Characteristics of the Bronchoscopy (Bronchial Brushings) Cohort for Microarray Assays

Characteristic	Nonsmokers* (n = 12)	Smokers* (n = 17)	COPD GOLD Stage 0* (n = 18)	COPD GOLD Stage I* (n = 10)	COPD GOLD Stages II–IV* (n = 13)	P Value†
Males, n (%)	6 (50)	7 (41.2)	7 (38.9)	6 (60)	9 (69.2)	NS
Age, yr	54 ± 8	44 ± 10	51 ± 7	59 ± 6	54 ± 7	<0.001‡
Pack-years of smoking	0	33 (10–59)	46 (19–160)	38 (0–53)	60 (30–86)	<0.001§
Current smokers, n (%)	0 (0)	17 (100)	18 (100)	7 (70)	11 (85)	<0.001
FEV ₁ , % predicted	110 (92–120)	105 (88–130)	95 (76–130)	95 (85–100)	67 (25–79)	<0.001¶
FEV ₁ /FVC, % predicted	74 (70–83)	80 (72–90)	76 (70–82)	68 (60–70)	59 (30–69)	<0.001**

Definition of abbreviations: ADAM9 = a disintegrin and metalloproteinase domain-9; COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Obstructive Lung Disease; MUC5AC = mucin 5AC; NS = not significant.

Shown are the demographic and clinical characteristics of the patients with COPD, smokers without COPD, and never-smokers who underwent a bronchoscopy to obtain bronchial brushings and provided endobronchial biopsies for analysis of ADAM9 mRNA levels and MUC5AC staining in epithelial cells, respectively. Data are presented as medians (interquartile range) for nonparametric data or as means ± SD for parametric data.

*Subjects were classified as nonsmoker control subjects, smoker control subjects without COPD, and smokers with COPD. Only those smokers with normal lung function and a high-resolution computed tomography thoracic scan showing no evidence of emphysema were included in the smoker group. The subjects with COPD were further classified according to GOLD criteria into three groups: GOLD stage 0 COPD (having a chronic cough and sputum production but normal spirometry [$n = 18$]); GOLD stage I COPD (with or without cough and sputum production and with FEV₁ ≥ 80% predicted but FEV₁/FVC < 70% [$n = 10$]); and GOLD stage II–IV COPD (with or without cough and sputum production but FEV₁ between 25% and 79% predicted and FEV₁/FVC < 70% [$n = 13$]).

†Categorical variables were analyzed by χ^2 tests. Statistical analyses included one-way ANOVA tests for continuous variables (age, FEV₁, % predicted, FEV₁/FVC, and pack-years) followed by pairwise comparisons by Student's t tests (for parametric data) or Mann-Whitney U tests (for nonparametric data).

‡The ages of the COPD GOLD stages II–IV, GOLD stage I, and GOLD stage 0 groups were not significantly different from those of the nonsmokers. The ages of the COPD GOLD stages II–IV, GOLD stage I, and GOLD stage 0 groups were significantly greater than those of the smokers ($P < 0.05$, $P < 0.001$, and $P < 0.05$, respectively).

§The pack-year smoking histories of the COPD GOLD stage II–IV, GOLD stage I, GOLD stage 0, and smoker groups were significantly higher than those of the nonsmokers by design. The smokers and GOLD 0 and GOLD I patients with COPD were matched for pack-year smoking history. The GOLD stage II–IV patients with COPD had a significantly greater pack-year smoking history than the smoker control subjects.

||The proportions of current smokers in the smoker and COPD GOLD stage 0, I, and II–IV groups were significantly higher than that of the nonsmoker group. However, the proportions of current smokers were similar in the smoker and COPD GOLD stage 0, I, and II–IV groups.

¶The FEV₁ of the COPD GOLD stages II–IV group was significantly lower than that of the smoker and nonsmoker groups ($P < 0.001$ for both comparisons). The FEV₁ of the COPD GOLD stage I group was also significantly lower than that of the smoker and nonsmoker groups ($P < 0.05$ for both comparisons). The FEV₁ of the COPD GOLD stage 0 group was not significantly different from that of the smoker and nonsmoker groups ($P = 0.054$ and $P = 0.098$, respectively).

**The FEV₁/FVC ratio of the COPD GOLD stage II–IV group was significantly lower than that of the smoker and nonsmoker groups ($P < 0.001$ for both comparisons). The FEV₁/FVC ratio of the COPD GOLD stage I group was significantly lower than that of the smoker and nonsmoker groups ($P < 0.001$ and $P < 0.05$, respectively). The FEV₁/FVC ratio of the COPD GOLD stage 0 group was significantly lower than that of the smoker group ($P < 0.05$) but was not significantly different from that of the nonsmoker group ($P = 0.235$).

indirectly with FEV₁ percent predicted (Figures E1E and E1F).

ADAM9 gene expression was quantified in bronchial brushings from a bronchoscopy cohort (most of whom were current smokers [Table 2]), using a microarray assay. ADAM9 transcript levels were higher in bronchial brushings from patients with COPD and symptomatic patients without airflow obstruction (Global Initiative for Obstructive Lung Disease [GOLD] stage 0) than from nonsmokers and smokers but were not related to GOLD stage (Figure 2A). Real-time quantitative RT-PCR (qPCR) analysis confirmed that ADAM9 transcript levels were higher in COPD versus control bronchial brushings (Figure 2B and Table E1). There were significant direct correlations between ADAM9 mRNA levels and MUC5AC staining in endobronchial biopsies, pack-year smoking history, and

quality-of-life scores (Figures 2C–2G); and significant inverse correlations between ADAM9 mRNA levels and FEV₁/FVC, FEV₁ percent predicted, and mean expiratory flow at 25% FVC (MEF₂₅) (Figures 2H–2J). However, after adjusting P values for differences in age and pack-year smoking history between the groups (Table 2), ADAM9 mRNA levels correlated significantly only with pack-year smoking history (Table 3).

Patients with COPD had greater ADAM9 staining in alveolar macrophages (Figures 1C and 1F) than smokers. Smokers had greater ADAM9 staining in alveolar macrophages than nonsmokers. After correcting correlations for differences in age, sex, pack-year smoking history, and current smoker status, ADAM9 staining in alveolar macrophages correlated directly with pack-year smoking history (Figure E2A) and inversely with FEV₁/

FVC and FEV₁ percent predicted (Figures E2B and E2C). ADAM9 staining was detected in PMNs in COPD and smoker lungs (data not shown), but this staining was not quantified as no/few PMNs were detected in nonsmoker lungs. There were no significant differences in plasma, BALF, or sputum sADAM9 levels between patients with COPD and control subjects (Figures E3A–E3C and Tables E2 and E3).

Murine Studies

Adam9 deficiency decreases multiple key COPD-like lung pathologies in CS-exposed mice. Exposing wild-type mice to CS increased both Adam9 transcript and protein lung levels within 1 month, and levels increased further after 2 and 6 months of CS exposure (Figures 3A and 3B). As Timp-3 is the only Timp that inhibits

Figure 1. ADAM9 (a disintegrin and metalloproteinase domain-9) expression is increased in epithelial cells and alveolar macrophages in chronic obstructive pulmonary disease (COPD) lungs. (A–C) Representative lung sections from nonsmoker control subjects, smokers without COPD, and patients with COPD (Global Initiative for Obstructive Lung Disease [GOLD] stages I–II and III–IV) that were double-immunostained with a red fluorophore (left) for markers of airway epithelial cells (pan-cytokeratin; PanCK in A and B) or a marker of macrophages (CD163 in C), and with a green fluorophore for ADAM9 (middle panels in A–C). Nuclei in the sections were counterstained blue using DAPI. The sections were examined by confocal microscopy. Merged images are shown on the right in A–C. The scale bars represent 20, 70, and 10 μm in A–C, respectively. Lung sections from a nonsmoker were stained with a red fluorophore and nonimmune murine (Ms) IgG or nonimmune rat IgG and with a green fluorophore and nonimmune rabbit (Rb) IgG as control subjects (bottom). The images shown are representative of six to nine subjects per group. (D and E) ADAM9-positive cells were quantified in alveolar (D) and bronchial (E) epithelial cells, using MetaMorph software. (F) The percentage of alveolar macrophages that were positively stained for ADAM9 was quantified in 10 randomly acquired microscopic fields. Boxes show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. (D–F) Data were analyzed by one-way ANOVA followed by Dunnett corrections for multiple comparisons. In D–F, * $P < 0.05$ and ** $P < 0.001$ versus nonsmokers or the group indicated ($n = 6$ –9 subjects per group).

Figure 2. Correlations between ADAM9 (a disintegrin and metalloproteinase domain-9) gene expression in human bronchial brushing cells and clinical parameters. (A) ADAM9 mRNA levels were measured by microarray assay in bronchial brushing cells from nonsmoker control subjects ($n = 12$), smoker control subjects ($n = 17$), and patients with chronic obstructive pulmonary disease (COPD) at Global Initiative for Obstructive Lung Disease (GOLD) stage 0 ($n = 18$), GOLD stage I ($n = 10$), and GOLD stage II-IV ($n = 13$). The columns represent mean values ($\pm$ SD). Data were analyzed by Bayesian linear regression adjusting for age and pack-year smoking history. *Posterior probability of no effect (PP) < 0.05 ; **PP < 0.001 versus nonsmoker control subjects or the group indicated. (B) ADAM9 steady-state mRNA levels were measured in bronchial brushing cells from 14 nonsmoker control subjects, 15 smoker control subjects, and 17 GOLD stage 0, eight GOLD stage I, and 13 GOLD stage II-IV patients with COPD in the bronchoscopy cohort, using real-time quantitative RT-PCR. Boxes in the box plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Data were analyzed by Bayesian linear regression adjusting for differences in age and pack-year smoking history. *PP < 0.05 and **PP < 0.001 versus nonsmoker control subjects or the group indicated. (C–J) Correlations between ADAM9 gene expression in human bronchial brushing cells and clinical parameters, including MUC5AC mucin staining in endobronchial biopsies from the same subjects (C; $n = 50$); pack-year smoking history (D; $n = 67$); St. George's Respiratory Questionnaire (SGRQ) quality-of-life total, symptom, and activity scores (E–G; $n = 52$); mean expiratory flow at 25% FVC (MEF₂₅) (H; $n = 62$); FEV₁ percent predicted (I; $n = 62$); and FEV₁/FVC (J; $n = 62$). Data were analyzed by multivariate linear regression with ADAM9 gene expression in human bronchial brushing cells as dependent variable and corresponding clinical parameter as well as relevant correction parameters (pack-year smoking history and age) as independent variables. $P < 0.05$ was considered to be statistically significant.

Table 3. Correlations between *ADAM9* Gene Expression in Bronchial Brushings and Clinical Parameters after Adjusting the *P* Values for Differences in Age and Pack-Years of Smoking

Clinical Parameter	β (SE)*	$\beta^\dagger$	<i>P</i> Value
Pack-years of smoking	0.007 (0.002)	0.438	<0.001
SGRQ symptom score	0.005 (0.003)	0.264	0.10
SGRQ activity score	0.005 (0.003)	0.211	0.18
SGRQ total score	0.005 (0.004)	0.165	0.30
MUC5AC [‡]	−0.005 (0.006)	−0.129	0.43
Pulmonary MEF ₂₅	−0.001 (0.003)	−0.045	0.75
FEV ₁	0.002 (0.003)	0.086	0.55
FEV ₁ /FVC	0.005 (0.006)	0.116	0.39

Definition of abbreviations: *ADAM9* = a disintegrin and metalloproteinase domain-9; MEF₂₅ = mean expiratory flow at 25% FVC; MUC5AC = mucin 5AC; SGRQ = St. George's Respiratory Questionnaire. Bronchoscopies were performed on patients with COPD, smokers without COPD, and never-smokers to obtain bronchial brushing samples for *ADAM9* gene expression analysis and endobronchial biopsies to immunostain airway epithelial cells for MUC5AC as described in the online supplement (*n* = 70 subjects).

Data were analyzed with R v3.4 for Windows (R Foundation for Statistical Computing). To identify the relevant clinical parameters to correct for, a multivariate regression model was run with *ADAM9* gene expression as the dependent variable and all of the clinical parameters. Variable selection was performed by optimization of the Akaike information criterion. The final model retained only six clinical variables of which pack-year smoking history had the highest significance. Thus, subsequent analyses were corrected for differences in pack-year smoking history and also to difference in age.

* β coefficient and SE.

[†] β is the standardized regression coefficient, which evaluates the relative significance of each independent variable in multiple linear analyses.

[‡]MUC5AC immunoperoxidase staining was quantified in the endobronchial biopsies from the same patients with COPD, smokers without COPD, and never-smokers from whom the bronchial brushings were obtained, as described in the online supplement.

Figure 3. Cigarette smoke (CS) exposure induces the expression of Adam9 (a disintegrin and metalloproteinase domain-9) in murine lungs. (A) *Adam9* mRNA levels were measured in lungs from wild-type mice exposed to air (*n* = 20) or CS for 1 month (*n* = 10), 2 months (*n* = 10), or 6 months (*n* = 9), using real-time RT-PCR. (B) Adam9 protein levels were measured in lungs from wild-type mice exposed to air (*n* = 22) or CS for 1 month (*n* = 11), 2 months (*n* = 14), or 6 months (*n* = 9), using an ELISA. (C) Timp-3 protein levels were measured in lungs from wild-type mice exposed to air (*n* = 8) or CS for 1 month (*n* = 8), 2 months (*n* = 9), or 6 months (*n* = 9), using an ELISA. (D) Adam9:Timp-3 protein ratios were calculated in the same lungs from wild-type mice exposed to air (*n* = 8) or CS for 1 month (*n* = 8), 2 months (*n* = 9), or 6 months (*n* = 9). Boxes in the box plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney *U* tests and Bonferroni corrections. **P* < 0.01 and ***P* < 0.001 versus air-exposed control subjects or the group indicated.

Adam9 (14), lung Timp-3 protein levels were measured in wild-type mice. CS exposure increased lung Timp-3 levels, but levels peaked after 1 month of CS exposure (Figure 3C) and Adam9:Timp-3 protein ratios increased after 6 months of CS exposure (Figure 3D).

Unchallenged *Adam9*^{−/−} mice have no abnormal phenotype (15). When exposed to CS for 6 months, *Adam9*^{−/−} mice were protected from emphysema development (Figures 4A and 4B) and small-airway fibrosis (Figures 4C and 4D), and had fewer α -SMA-positive myofibroblasts around their small airways (Figure 4E). *Adam9*^{−/−} mice were also protected from CS-induced airway mucus cell metaplasia (Figures 4F and 4G).

Lung inflammation. Lung inflammation was measured in wild-type and *Adam9*^{−/−} mice as this process promotes emphysema development, small-airway remodeling, and mucus metaplasia (16–20). Compared with wild-type mice, *Adam9*^{−/−} mice had lower BAL total leukocyte and macrophage (Figures 5A and 5B) counts detectable after 1 month of CS exposure, lower PMN counts after 6 months of CS exposure (Figure 5C), but similar BAL lymphocyte counts (Figure 5D). CS-exposed *Adam9*^{−/−} mice had lower macrophage counts in lung sections than wild-type mice (Figures 5E and 5F).

Lung levels of cytokines and chemokines that regulate myeloid leukocyte recruitment, activation, and survival (Ccl-2, Ccl-3, Ccl-5, Cxcl-1, Cxcl-2, Tnf- α , Il-6, Il-1 β , Gm-csf, M-csf, and G-csf), and antiinflammatory mediators (Il-10 and Tgf- β) were measured in wild-type and *Adam9*^{−/−} mice exposed to air or CS for 1 month. Lung levels of Ccl-2, Il-1 β , Il-10, G-csf, and active Tgf- β were similar in CS-exposed wild-type and *Adam9*^{−/−} mice (Figure E4). Lung levels of Gm-csf, M-csf, Ccl-3, and Cxcl-2 were lower in CS-exposed *Adam9*^{−/−} versus wild-type mice (Figures 6A–6D). Thus, *Adam9* deficiency may decrease lung macrophage counts, in part, by reducing lung levels of mediators that promote macrophage recruitment and activation (Ccl-3) or survival (Gm-csf and M-csf).

Surprisingly, lung levels of Tnf- α , Il-6, Ccl-5, and Cxcl-1 (the murine ortholog of IL-8) were higher in CS-exposed *Adam9*^{−/−} than wild-type mice (Figures 6E–6H). However, it is unlikely that the

Figure 4. *Adam9* (a disintegrin and metalloproteinase domain-9) deficiency decreases emphysema development, small-airway fibrosis, and mucus cell metaplasia in cigarette smoke (CS)-exposed mice. (A–G) Adult *Adam9*^{-/-} mice and wild-type (WT) mice were exposed to air or CS 6 days/week for 6 months. (A) Images of Gill's-stained, inflated lung sections from mice. Scale bars, 50 μ m. (B) Box plots of alveolar chord lengths as a measure of airspace enlargement. Boxes show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles, for 6–11 air-exposed mice per group and 10 or 11 CS-exposed mice per group. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney *U* tests and Bonferroni corrections. **P* < 0.017 versus the same genotype exposed to air or the group indicated. (C) Representative images of extracellular matrix (ECM) deposition around small airways (300–699 μ m in diameter) in Masson trichrome-stained lung sections from WT and *Adam9*^{-/-} mice exposed to air or CS for 6 months. Scale bars, 50 μ m. (D) The thickness of the ECM protein layer deposited around small airways was quantified, as described in the online supplement. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles from eight to 10 air-exposed mice per group and from eight or nine CS-exposed mice per group. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney *U* tests with Bonferroni corrections. **P* < 0.017 versus air-exposed mice belonging to the same genotype or the group indicated. (E) Scatter plots of α -SMA (α -smooth muscle actin) staining that was quantified per unit area of airway wall on airways having a luminal diameter of 300–699 μ m, using MetaMorph software (four or five air-exposed mice per group and seven or eight CS-exposed mice per group). Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney *U* tests and Bonferroni corrections. **P* < 0.017 versus air-exposed mice belonging to the same genotype or the group indicated. (F) Images of immunofluorescence staining for bronchial epithelial Muc5ac (stained in green) in the lungs of CS-exposed WT and *Adam9*^{-/-} mice that are representative of five to seven mice per group. A section of lung from a CS-exposed WT mouse stained with a nonimmune murine (Ms) IgG is shown (right panel). Air-exposed WT and *Adam9*^{-/-} mice had minimal staining for Muc5ac in bronchial epithelial cells (data not shown). (G) Muc5ac staining was quantified per unit area of bronchial epithelium, using MetaMorph software. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles for mice exposed to air (*n* = 5 per group) or CS (*n* = 7 per group) for 6 months. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney *U* tests with *post hoc* Bonferroni corrections. **P* < 0.017 versus air-exposed mice belonging to the same genotype or the group indicated. FI = fluorescence intensity.

Figure 5. Lung inflammation is decreased in cigarette smoke (CS)-exposed *Adam9*^{-/-} mice. Wild-type (WT) and *Adam9*^{-/-} mice were exposed to air or CS for 1–6 months. Absolute numbers of (A) all leukocytes, (B) macrophages, (C) polymorphonuclear leukocytes (PMNs), and (D) lymphocytes were counted in BAL samples. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles for eight or nine air-exposed mice per group, 10–12 mice exposed to CS for 1 month per group, 15–22 mice exposed to CS for 2 months per group, five to nine mice exposed to CS for 3 months per group, and five mice exposed to CS for 6 months per group. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney *U* tests with Bonferroni corrections for multiple comparisons. **P* < 0.008 and ***P* < 0.001 versus the group indicated. (E) Images of lung sections from WT and *Adam9*^{-/-} mice that were exposed to air or CS for 6 months and then immunostained with a green fluorophore for CD68 (a marker of macrophages). The images shown are representative of six or seven mice per group. A section of lung from a CS-exposed WT mouse stained with a nonimmune primary murine (Ms) IgG is shown (right panel). Scale bars, 20 μ m. (F) CD68 staining was quantified per unit area of alveolar wall, using MetaMorph software. Columns show the mean ($\pm$ SD) for six mice that were exposed to air per group, and seven mice that were exposed to CS for 6 months per group. Data were analyzed by one-way ANOVA followed by pairwise testing with Student's *t* tests and Bonferroni corrections for multiple comparisons. **P* < 0.017 versus air-exposed mice belonging to the same genotype or the group indicated. *Adam9* = a disintegrin and metalloproteinase domain-9.

Figure 6. Lung levels of cytokines and chemokines in air- versus cigarette smoke (CS)-exposed wild-type (WT) and *Adam9*^{-/-} mice. WT and *Adam9*^{-/-} mice were exposed to air or CS for 1 month, and lung levels of Gm-csf (A; *n* = 6–13 mice per group), M-csf (B; *n* = 8–12 mice per group), Ccl-3 (C; *n* = 13–17 mice per group), Cxcl-2 (D; *n* = 8–12 mice per group), Tnf-α (E; *n* = 10–11 mice per group), Il-6 (F; *n* = 10 or 11 mice per group), Ccl-5 (G; *n* = 8 or 9 mice per group), and Cxcl-1 (H; *n* = 8–10 mice per group) were measured in homogenates of lung samples, using ELISA kits. Data were normalized to total protein levels measured on the same samples. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney *U* tests with Bonferroni corrections for multiple comparisons. **P* < 0.025 and ***P* < 0.001 versus air-exposed mice belonging to the same genotype or the group indicated. *Adam9* = a disintegrin and metalloproteinase domain-9.

Adam9 metalloproteinase domain degrades these mediators in the lung, as active human ADAM9 did not degrade human CCL-5, IL-8, IL-6, or tumor necrosis factor (TNF)-α *in vitro* (Figures E5A–E5E).

To investigate whether *Adam9* deficiency reduces lung macrophage counts by increasing macrophage apoptosis rates, intracellular levels of active caspase-3 were measured in alveolar macrophages isolated from air- versus CS-exposed wild-type and *Adam9*^{-/-} mice. CS-exposed *Adam9*^{-/-} mice had higher rates of alveolar macrophage apoptosis than wild-type mice (Figure 7A). Macrophages isolated from unchallenged wild-type and *Adam9*^{-/-} mice were induced to undergo apoptosis *in vitro* by exposing them to CSE. Intracellular active caspase-3 levels were similar in CSE-treated wild-type and *Adam9*^{-/-} cells (Figure E6). Thus, macrophage-derived *Adam9* is

unlikely to regulate CS-induced macrophage apoptosis.

Proteinase-mediated elastin degradation promotes emphysema development. ADAM9 degrades elastin *in vitro* (8). BALF and serum levels of desmosine (a measure of elastin degradation) were lower in CS-exposed *Adam9*^{-/-} mice versus wild-type mice (Figures 7B and 7C). Thus, *Adam9* may promote emphysema development, in part, by degrading lung elastin.

Alveolar septal cell death. Alveolar septal cell death contributes to emphysema development (2). CS-exposed *Adam9*^{-/-} mice had reduced alveolar septal cell death rates (assessed as fewer TUNEL-positive alveolar septal cells) compared with wild-type mice (Figures 7D and 7E). CSE induced lower rates of apoptosis in lung epithelial cells isolated from naive

Adam9^{-/-} versus wild-type mice (Figure 7F).

Signaling of ligands via the EGFR and the VEGFR2 promotes the survival of alveolar septal cells (21, 22). Blocking VEGFR2 signaling in rats induces alveolar septal cell death and emphysema development (2). To determine whether *Adam9* promotes alveolar septal cell death by shedding their EGFR and/or VEGFR2, BALF sEGFR and sVEGFR2 levels were measured. CS-exposed *Adam9*^{-/-} mice had lower BALF sEGFR (Figure 8A) and sVEGFR2 (Figure 8D) levels than wild-type mice. Residual surface EGFR (Figures 8B and 8C) and VEGFR2 (Figures 8E and 8F) levels were higher on alveolar septal cells in CS-exposed *Adam9*^{-/-} mice versus wild-type lungs.

Incubating HBECs from healthy subjects with active rhADAM9 *in vitro*

Figure 7. Cigarette smoke (CS)-exposed *Adam9*^{-/-} mice have higher alveolar macrophage apoptosis rates but lower lung elastin degradation and alveolar septal cell death rates than CS-exposed wild-type (WT) mice. (A–E) WT and *Adam9*^{-/-} mice were exposed to air or CS for 1–6 months. (A) Alveolar macrophages were isolated from the lungs of WT and *Adam9*^{-/-} mice that had been exposed to air or CS for 1 month, using BAL. Immediately afterward, the alveolar macrophages were fixed and then immunostained for intracellular active caspase-3. Staining was quantified as described in the online supplement ($n = 3$ separate experiments). Data represent means $\pm$ SD. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney U tests and Bonferroni corrections for multiple comparisons. $*P < 0.025$ versus the group indicated. (B) Desmosine levels were measured in BAL fluid (BALF) samples from WT and *Adam9*^{-/-} mice exposed to air ($n = 5$ or 6 mice per group) or to CS for 1 month ($n = 15$ mice per group) or 3 months ($n = 6$ mice per group), using an ELISA. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney U test and Bonferroni corrections for multiple comparisons. $*P < 0.017$ and $**P < 0.001$ versus the group indicated. (C) Desmosine levels were measured in serum samples from WT and *Adam9*^{-/-} mice exposed to air ($n = 5$ mice per group) or CS ($n = 11$ or 12 mice per group) for 6 months, using an ELISA. Data represent means $\pm$ SD. Data were analyzed by one-way ANOVA followed by pairwise testing by Student's t tests and Bonferroni corrections for multiple comparisons. $*P < 0.025$ versus the group indicated. (D) Representative images of terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) staining of formalin-fixed lung sections from WT and *Adam9*^{-/-} mice exposed to air or CS for 6 months. Scale bars, 20 μ m. (E) Alveolar septal cell death was measured as the number of TUNEL-positive alveolar septal cells per unit of alveolar wall area. Data shown represent mean ($\pm$ SD) values from three to six mice per group. Data were analyzed by one-way ANOVA followed by pairwise testing by Student's t tests and Bonferroni corrections for multiple comparisons. $*P < 0.017$ versus air-exposed mice belonging to the same genotype or the group indicated. (F) Type II alveolar epithelial cells were isolated from unchallenged WT and *Adam9*^{-/-} mice, and exposed to 7.5% CS extract (CSE) for up to 48 hours. Intracellular levels of active caspase-3 were measured with a quenched fluorogenic substrate, as described in the online supplement. Data represent means $\pm$ SD from eight separate cell preparations. Data were analyzed by one-way ANOVA followed by pairwise testing by Student's t tests and Bonferroni corrections for multiple comparisons. $*P < 0.01$ and $**P < 0.001$ versus the time points indicated. *Adam9* is a disintegrin and metalloproteinase domain-9; FI = fluorescence intensity.

decreased their surface EGFR (Figures 9A and 9B) and VEGFR2 (Figures 10A and 10B) staining, and increased sEGFR (Figure 9C) and sVEGFR2 (Figure 10C) levels in culture supernatants. A nonselective small-molecule metalloproteinase inhibitor (GM6001) blocked ADAM9-mediated shedding of both receptors from HBECs (Figures 9D and 9E and Figures 10D and 10E). Incubating HBECs with rhADAM9 increased

intracellular active caspase-3 levels, which was rescued by GM6001 (Figure 10F).

HBECs from patients with COPD had greater ADAM9 surface staining, but lower EGFR and VEGFR2 surface staining, than HBECs from control subjects (Figures 11A–11D and Table E4). The greater ADAM9 staining detected in bronchial and alveolar epithelial cells in COPD versus control lung sections was

associated with lower staining for EGFR and VEGFR2 in the same cells (Figure E7 and Table E5).

Signaling through EGFR and VEGFR2 activates (phosphorylates) intracellular phosphoinositide 3-kinase (PI3K) and Akt, inducing downstream activation of prosurvival signaling pathways (23). Incubating HBECs with active rhADAM9 reduced phospho-Ser⁴⁷³ Akt levels. Inhibiting ADAM9 with GM6001 rescued

Figure 8. Adam9 (a disintegrin and metalloproteinase domain-9) sheds EGFR (epidermal growth factor receptor) and VEGFR2 (vascular endothelial growth factor receptor-2) in the lungs of cigarette smoke (CS)-exposed mice. Wild-type (WT) and *Adam9*^{-/-} mice were exposed to air or CS for 1–3 months. Soluble EGFR levels (A; $n = 5$ –12 mice per group) and soluble VEGFR2 levels (D; $n = 5$ –15 mice per group) were quantified in BAL fluid (BALF) samples, using ELISA. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney U tests and Bonferroni corrections for multiple comparisons. * $P < 0.017$ and ** $P < 0.001$ versus the group indicated. (B) Residual EGFR levels expressed on alveolar septal cell

activation (phosphorylation) of Akt (Figures 12A and 12B). HBEs from patients with COPD had lower phospho-Ser⁴⁷³Akt levels than cells from control subjects (Figures 12C and 12D). Total Akt lung levels were similar but phospho-Ser⁴⁷³Akt levels were higher in CS-exposed *Adam9*^{-/-} versus wild-type lungs (Figure 12E and 12F). Thus, ADAM9 proteolytically inactivates EGFR- and VEGFR2-triggered Akt prosurvival signaling in alveolar septal cells to promote emphysema development.

Discussion

This study identifies ADAM9 as a new culprit in COPD, and the first proteinase linked to five key COPD-like pulmonary pathologies (lung inflammation, alveolar septal cell apoptosis, emphysema,

Figure 8. (Continued). surfaces were assessed by immunostaining lung sections from WT and *Adam9*^{-/-} mice exposed to air or CS for 6 months with a green fluorophore for EGFR. Nuclei were counterstained blue with DAPI. As a control, lung sections were stained with a nonimmune rabbit (Rb) IgG (bottom panel). The merged images shown in B are representative of three to five mice per group. Scale bars, 20 μ m. (C) Scatter plots of residual surface EGFR staining that was quantified in alveolar septal cells in mice that were exposed to air (three or four mice per group) or CS (four or five mice per group) for 6 months, using MetaMorph software. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney U tests and Bonferroni corrections for multiple comparisons. * $P < 0.025$ and ** $P < 0.001$ versus the group indicated. (E) Residual VEGFR2 levels expressed on alveolar septal cell surfaces were assessed by immunostaining lung sections from WT and *Adam9*^{-/-} mice that were exposed to air or CS for 6 months with a green fluorophore for VEGFR2. Nuclei were counterstained blue with DAPI. As a control, lung sections were stained with a nonimmune Rb IgG (bottom panel). The merged images shown in E are representative of four to six mice per group. Scale bars, 20 μ m. (F) Scatter plots of residual surface VEGFR2 staining quantified in alveolar septal cells in sections of lungs from mice that had been exposed to air (four or five mice per group) or CS (five or six mice per group) for 6 months. Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney U test and Bonferroni corrections for multiple comparisons. * $P < 0.025$ versus the group indicated.

Figure 9. ADAM9 (a disintegrin and metalloproteinase domain-9) sheds EGFR (epidermal growth factor receptor) from human bronchial epithelial cell (HBEC) surfaces. (A) HBECs from control subjects without chronic obstructive pulmonary disease were grown to confluence on chamber slides and incubated with or without 10–60 nM active recombinant human (rh)ADAM9 for 4 hours at 37°C. HBECs were fixed and immunostained with a green fluorophore for cell surface-associated EGFR. As a control, cells not incubated with rhADAM9 were stained with a nonimmune rabbit (Rb) IgG (right panel). DAPI-counterstained cells were examined with an epifluorescence microscope. The merged images shown in A are representative of four separate experiments. Scale bars, 10 μ m. (B) Residual levels of EGFR on the surface of HBECs were quantified, as described in the online supplement. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles ($n = 4$ separate experiments). Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney U tests. $^*P < 0.029$ and $^{**}P < 0.001$ versus no rhADAM9 control or the group indicated. (C) Soluble EGFR levels in cell-free supernatant samples from the same preparations shown in A and B, measured by ELISA. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles ($n = 5$ separate experiments). Data were analyzed by Kruskal-Wallis one-way ANOVA

small-airway fibrosis, and mucus cell metaplasia) in CS-exposed mice. ADAM9 immunostaining was increased in lung epithelial cells and macrophages in smokers and even more so in patients with COPD. ADAM9 gene expression was increased in bronchial brushings from patients with COPD. ADAM9 expression in the human lung correlated directly with pack-year smoking history and/or inversely with pulmonary function parameters. Our study provides novel mechanistic insights into Adam9 contributions to COPD, as *Adam9*^{-/-} mice have reduced CS-induced lung inflammation, elastin degradation, and alveolar septal cell apoptosis. Adam9 is also the first sheddase of the EGFR and the VEGFR2 identified in a murine model of COPD. Thus, strategies that reduce ADAM9 lung levels may limit the progression of emphysema, small-airway disease, and chronic bronchitis.

Although Adam9 expression is increased in lung macrophages in mice with acute lung injury (8, 24), CS exposure increased lung levels of Adam9 and also Timp-3 (the only Timp family member that inhibits Adam9 [14]) in wild-type murine lungs. However, chronic CS exposure increased lung Adam9:Timp-3 protein ratios, suggesting that active Adam9 is available in the lung to degrade elastin and cleave VEGFR2 and EGFR from alveolar septal cell surfaces.

ADAM9 expression has not been studied previously in patients with COPD or other chronic lung diseases. Increases in ADAM9 expression in lung macrophages and epithelial cells, and bronchial brushings, were related to the presence of COPD but

Figure 9. (Continued). followed by pairwise testing by Mann-Whitney U tests. $^*P < 0.019$ and $^{**}P < 0.001$ versus the group indicated. (D) Images showing residual EGFR staining on the surface of HBECs that were incubated with or without 60 nM rhADAM9 with or without 2 or 10 μ M GM6001 (a nonselective metalloproteinase inhibitor) for 4 hours at 37°C. The right panel shows cells incubated without rhADAM9 that were stained with a nonimmune rabbit (Rb) IgG. Scale bars, 10 μ m. (E) Residual EGFR levels on the surface of HBECs were quantified, as described in the online supplement. Data represent means $\pm$ SD from three separate experiments. Data were analyzed by one-way ANOVA followed by pairwise testing by Student's t tests. $^*P < 0.011$ and $^{**}P < 0.001$ versus the group indicated.

Figure 10. ADAM9 (a disintegrin and metalloproteinase domain-9) sheds VEGFR2 (vascular endothelial growth factor receptor-2) from human bronchial epithelial cell (HBEC) surfaces. (A) HBECs from control subjects without chronic obstructive pulmonary disease were grown to confluence on chamber slides and incubated with or without 10–60 nM active recombinant human (rh)ADAM9 for 4 hours at 37°C. HBECs were fixed and immunostained with a green fluorophore for cell surface-associated VEGFR2. As a control, cells not incubated with rhADAM9 were stained with a nonimmune murine (Ms) IgG (right panel). The merged images shown in A are representative of four separate experiments. Scale bars, 10 μ m. (B) Residual levels of VEGFR2 on the surface of HBECs were quantified, as described in the online supplement. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles ($n = 4$ separate experiments). Data were analyzed by Kruskal-Wallis one-way analysis followed by pairwise testing by Mann-Whitney U tests. $^*P < 0.029$ and $^{**}P < 0.001$ versus no rhADAM9 control or the group indicated. (C) Soluble VEGFR2 levels in cell-free supernatant samples from the same preparations shown in A and B were measured by ELISA. Boxes in the box plots show the median values and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles ($n = 5$ separate experiments). Data were analyzed by Kruskal-Wallis one-way ANOVA followed by pairwise testing by Mann-Whitney U tests. $^*P < 0.011$ and $^{**}P < 0.001$ versus the group indicated. (D) Images of surface VEGFR2 staining on HBECs that were incubated with or without 60 nM rhADAM9 with or without 2 or 10 μ M GM6001 (a nonselective small-molecule metalloproteinase inhibitor) for 4 hours at 37°C. The right panel shows cells incubated without rhADAM9 that were stained with a nonimmune murine (Ms) IgG. Scale bars,

not COPD severity. ADAM9 protein and transcript levels correlated directly with pack-year smoking history. ADAM9 protein levels in macrophages and epithelial cells correlated inversely with airflow obstruction and percent predicted FEV₁, albeit in a small cohort. Gene expression in COPD lungs is generally most strongly correlated with current smoker status (25). However, there was no relationship between current smoker status and ADAM9 expression as 1) smokers (predominantly current smokers) in the bronchial brushings cohort had similar ADAM9 gene expression levels as nonsmokers; and 2) smokers in our lung immunostaining cohort (predominantly former smokers) had higher ADAM9 staining in epithelial cells and macrophages than nonsmokers. Proinflammatory mediators increase ADAM9 expression in cell cultures (8), and proinflammatory signals generated in the lungs of former smokers with and without COPD may drive the increased ADAM9 immunostaining detected in their lungs.

Genetic and/or epigenetic processes may promote the increased ADAM9 expression in COPD lungs. Single-nucleotide polymorphisms in the ADAM9 locus are associated with cone-rod dystrophy (26), but have not been linked to COPD. Although hypermethylation of the ADAM9 locus is linked to malignant transformation of mesenchymal stem cells (27), the methylation status of the ADAM9 locus has not been examined in COPD lungs. MicroRNAs (miRNAs) regulate gene transcription in COPD lungs (28).

Figure 10. (Continued). 10 μ m. (E) Residual VEGFR2 levels on the surface of HBECs were quantified as described in the online supplement. Data represent means $\pm$ SD from three separate experiments. Data were analyzed by one-way ANOVA followed by pairwise testing by Student's t tests. $^*P < 0.012$ and $^{**}P < 0.001$ versus the group indicated. (F) HBECs were grown to confluence on tissue culture plates and incubated with or without 60 nM active rhADAM9 at 37°C for 4 hours in the presence or absence of 10 μ M GM6001. Intracellular levels of active caspase-3 were measured with a quenched fluorogenic substrate, as described in the online supplement. Data represent means $\pm$ SD from four separate cell preparations. Data were analyzed by one-way ANOVA followed by pairwise testing by Student's t tests. $^*P < 0.029$ versus no rhADAM9 control or the group indicated.

Figure 11. Human bronchial epithelial cells (HBECS) from patients with chronic obstructive pulmonary disease (COPD) have higher surface staining for ADAM9 (a disintegrin and metalloproteinase domain-9) and lower surface staining for EGFR (epidermal growth factor receptor) and VEGFR2 (vascular endothelial growth factor receptor-2) than HBECS from control subjects. (A–D) Primary HBECS from three patients with COPD and three control subjects were grown to confluence on chamber slides. HBECS were fixed and immunostained with a green fluorophore for cell surface-associated ADAM9, EGFR, or VEGFR2, and the nuclei were counterstained blue with DAPI. Control cells were also stained with a primary nonimmune rabbit (Rb) IgG or murine (Ms) IgG (right panels). The merged images shown in A are representative of three subjects per group. Scale bars, 10 μ m. Cell surface levels of ADAM9 (B), EGFR (C), and VEGFR2 (D) on HBECS were quantified on cells as described in the online supplement. Data represent means $\pm$ SD and data were analyzed by one-way ANOVA followed by pairwise testing by Student's *t* tests. **P* < 0.05 versus control.

Expression of miR-126 is reduced in COPD lung epithelial cells (29), and ADAM9 is a key target of miR-126 (30). Future studies will determine whether reduced miR-126 expression regulates ADAM9 expression in COPD lungs.

sADAM9 isoforms have not been reported previously in plasma or lung samples, but have been detected in cell cultures (8, 31) and are likely generated by shedding of ADAM9 from leukocytes or epithelial cells (8). The sADAM9 isoforms we detected in COPD plasma, BALF, and sputum samples are unlikely to be a useful

biomarker for COPD development or progression as sADAM9 levels were similar in patients with COPD and control subjects.

Emphysema

Adam9 likely promotes emphysema development in mice by several mechanisms (Figure 13). Adam9 may directly degrade lung elastin to cause loss of the alveolar walls, as human ADAM9 degrades elastin *in vitro* (8), and CS-exposed Adam9^{−/−} mice had reduced lung elastin degradation. Adam9 may also promote emphysema

development by increasing lung macrophage accumulation as 1) lung macrophage counts were lower in CS-exposed Adam9^{−/−} mice than wild-type mice; 2) macrophages are required for emphysema development in CS-exposed mice (16, 17); and 3) lung inflammation contributes to lung injury in patients with COPD (32). Macrophage-derived Adam9 is unlikely to regulate lung macrophage counts in CS-exposed lungs by 1) modulating monocyte *trans*-endothelial migration as Adam9 is not essential for monocyte migration into the lungs in other models (8); or by 2) inhibiting macrophage apoptosis as CSE induced similar rates of apoptosis in wild-type and Adam9^{−/−} macrophages *in vitro*. Adam9 may increase lung macrophage accumulation by 1) increasing lung levels of cytokines that promote monocyte chemotaxis (Ccl-3) and 2) reducing macrophage apoptosis by increasing lung levels of growth factors that promote macrophage survival as Gm-csf and M-csf levels were lower in CS-exposed Adam9^{−/−} lungs.

Adam9 may also promote emphysema development by increasing alveolar septal cell apoptosis. Apoptosis of alveolar epithelial and endothelial cells is driven by loss of signaling via the EGFR and the VEGFR (2, 22). Pharmacologic blockade of VEGF receptors induces alveolar septal cell apoptosis and emphysema in rodents (2), but EGFR inhibitors have not been studied in rodent emphysema models. VEGFR2 levels are decreased in COPD lungs (33), but the mechanisms involved were not identified. Herein, we identify ADAM9 as a VEGFR2 and EGFR sheddase *in vitro* and in CS-exposed mice. It is noteworthy that there was increased ADAM9 staining associated with decreased VEGFR2 and EGFR staining in alveolar septal cells in COPD lungs. EGFR and VEGFR2 signaling promotes cellular survival by activating the PI3K–Akt pathway (34), and increased activation of this pathway was detected in CS-exposed Adam9^{−/−} versus wild-type lungs. Although MMPs cleave EGFR and VEGFR2 in other models (35, 36), ADAM9 is the first proteinase identified that sheds EGFR and VEGFR2 in CS-exposed mice.

Small-Airway Fibrosis

Small-airway fibrosis contributes significantly to airflow obstruction and

Figure 12. ADAM9 (a disintegrin and metalloproteinase domain-9) reduces activation (phosphorylation) of Akt. (A and B) Human bronchial epithelial cells (HBECS) were grown to confluence on tissue culture plates and incubated with or without 10–60 nM active recombinant human (rh)ADAM9 at 37°C for 4 hours in the presence or absence of 10 μM GM6001 (a nonselective metalloproteinase inhibitor). Phosphorylated Akt (p-Akt-Ser⁴⁷³), total Akt (Akt), and a housekeeping control (β-actin) were quantified in cell extracts, using Western blotting and densitometry. The images of Western blots shown in A are representative of five separate experiments. (B) p-Akt-Ser⁴⁷³ signals were normalized to total Akt levels and expressed as a percentage of the no-rhADAM9 control result. Columns show means ± SD (*n* = 5 separate experiments). Data were analyzed by one-way ANOVA followed by pairwise testing by Student's *t* tests. **P* < 0.05 and ***P* < 0.001 versus no rhADAM9 control or the group indicated. (C and D) Primary HBECS from patients with chronic obstructive pulmonary disease (COPD) and control subjects were grown to confluence on tissue culture plates. Phosphorylated Akt (p-Akt-Ser⁴⁷³), total Akt (Akt), and a housekeeping control (β-actin) were quantified in cell extracts, using Western blotting and densitometry. The images of Western blots shown in C are representative of three separate experiments conducted on cells from three different control subjects and three different patients with COPD. (D) p-Akt-Ser⁴⁷³ signals were normalized to total Akt levels and expressed as a percentage of the control result. Columns show means ± SD (three cell preparations per group). Data were analyzed by one-way ANOVA followed by pairwise testing by Student's *t* tests. **P* < 0.05 versus control cells. (E and F) Wild-type (WT) and *Adam9*^{–/–} mice were exposed to air or cigarette smoke (CS) for 3 months. Phosphorylated Akt (p-Akt-Ser⁴⁷³), total Akt (Akt), and a housekeeping control (β-actin) were quantified in homogenates of lung samples, using Western blotting and densitometry. p-Akt-Ser⁴⁷³ signals were normalized to total Akt levels and expressed as a percentage of the values for WT mice exposed to air. The images shown in E are representative of six mice per group. (F) Columns show means ± SD (*n* = 6 mice per group). Data were analyzed by one-way ANOVA followed by pairwise testing by Student's *t* tests and Bonferroni corrections for multiple comparisons. **P* < 0.0125 and ***P* < 0.001 versus air-exposed mice belonging to the same genotype or the group indicated.

FEV₁ decline in patients with COPD (4), and it is noteworthy that bronchial epithelial cell ADAM9 staining correlated inversely with airflow obstruction and percent predicted FEV₁. Activation of growth factors that drive deposition of interstitial collagen by airway myofibroblasts is linked to small-airway fibrosis (37). Although other proteinases proteolytically activate latent Tgf-β (38, 39), CS-exposed *Adam9*^{–/–} mice were protected from small-airway fibrosis in a Tgf-β-independent fashion, as CS-exposed wild-type and *Adam9*^{–/–} mice had similar lung levels of active Tgf-β. Adam9 may promote small-airway fibrosis by activating fibroblasts as ADAM9 is expressed by fibroblasts and regulates fibroblast adhesion (40), and reduced numbers of α-SMA-positive myofibroblasts were

detected around the small airways of CS-exposed *Adam9*^{–/–} mice.

Adam9 may also promote small-airway fibrosis in CS-exposed mice by increasing lung inflammation as macrophage products (including MMPs) promote small-airway fibrosis in CS-exposed rodents (5, 19). Adam9 may also induce M2 polarization of macrophages to promote small-airway fibrosis (41).

Mucus Metaplasia

Mucus metaplasia occurs in the large airways of patients with COPD and contributes to the chronic bronchitis phenotype which is associated with high morbidity, poorer quality-of-life scores, increased rate of decline in FEV₁, more frequent exacerbations, and higher

mortality rates (42). ADAM9 may promote the chronic bronchitis phenotype as 1) CS-exposed *Adam9*^{–/–} mice had reduced mucus cell metaplasia; and 2) ADAM9 expression bronchial brushings was higher in smokers with symptoms of cough and sputum (GOLD stage 0) than asymptomatic smokers. Future studies will measure ADAM9 expression in samples from patients with chronic bronchitis.

Mucin synthesis and goblet cell hyperplasia are induced by EGFR ligands activating this receptor (43). ADAM10 and ADAM17 increase EGFR activation in epithelial cells *in vitro* by shedding (and thereby activating) EGFR ligands (44, 45). Adam9 likely regulates mucin synthesis by an EGFR-independent mechanism as Adam9 sheds the EGFR

Figure 13. Schematic illustrating the potential contributions of ADAM9 (a disintegrin and metalloproteinase domain-9) to various chronic obstructive pulmonary disease phenotypes. 1) Inhaling cigarette smoke increases the expression of ADAM9 in lung macrophages and bronchial and alveolar epithelial cells, either directly or indirectly by generating proinflammatory mediators in the lungs that induce ADAM9 expression by these cells; 2) ADAM9 may directly degrade lung elastin to promote emphysema development; 3) increases in lung macrophage counts increases lung elastin degradation further (by increasing the lung burden of macrophage-derived MMPs) to contribute to emphysema development. Increases in lung macrophage counts may also contribute to lung inflammation and injury by increasing the lung burden of macrophage-derived oxidants (that injure alveolar septal cells) and macrophage-derived proinflammatory mediators that amplify macrophage recruitment and activation (CCL-3) and survival (GM-CSF and M-CSF); 4) ADAM9 expressed by alveolar epithelial cells sheds EGFR and VEGFR2 from lung epithelial cells to promote alveolar septal cell death and emphysema development by reducing activation of the PI3K/Akt prosurvival pathway in alveolar septal cells; 5) Adam9 also promotes small-airway fibrosis, in part, by increasing α -smooth muscle actin-positive fibroblasts in the small airways, or possibly by increasing airway inflammation; 6) Adam9 increases airway mucus cell metaplasia via mechanisms that remain to be elucidated. The scissors indicate processes in which the ADAM9 metalloproteinase domain is likely to be involved. EGFR = epidermal growth factor receptor; GM-CSF = granulocyte-macrophage colony-stimulating factor; M-CSF = macrophage colony-stimulating factor; MMPs = matrix metalloproteinases; PI3K = phosphoinositide 3-kinase; ROS = reactive oxygen species; VEGFR2 = vascular endothelial growth factor receptor-2.

from epithelial cell surfaces. Mucin synthesis is also induced by TNF- α and IL-1 β signaling (46, 47), and other ADAMs and MMPs shed and activate latent pro-TNF- α from macrophage surfaces and activate pro-IL-1 β (48–50). However, lung levels of these cytokines were not reduced in CS-exposed *Adam9*^{-/-} mice. Thus, it is unlikely that Adam9 induces mucin expression by proteolytically activating these mediators.

Limitations of This Study

Small cohorts of patients with COPD and control subjects were studied, and we may not have identified all possible confounding factors. Studies of larger

COPD cohorts enriched for individual COPD phenotypes are needed to confirm our results. ADAM9 activity was not measured in clinical samples, due to the lack of available substrates that are selectively cleaved by ADAM9, and inhibitors that selectively inhibit ADAM9. Our murine studies employed only a loss-of-function approach. Some of our findings could be due to compensatory changes in lung levels of other proteinases in *Adam9*^{-/-} mice, rather than direct effects of Adam9. We did not fully elucidate the mechanisms by which Adam9 regulates small-airway fibrosis and airway mucus cell metaplasia.

Conclusions

Herein, we identify ADAM9 as a novel proteinase culprit in COPD. Unlike other proteinases studied in murine models to date, Adam9 promotes the development of airspace and large- and small-airway disease. Strategies to inhibit ADAM9 activity or reduce its expression in the lung could limit the progression of several key COPD phenotypes that are associated with high morbidity and mortality. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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