Cardiovascular, Pulmonary, and Renal Pathology

Matrix Metalloproteinase 3 Is a Mediator of Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) may be triggered by epithelial injury that results in aberrant production of growth factors, cytokines, and proteinases, leading to proliferation of myofibroblasts, excess deposition of collagen, and destruction of the lung architecture. The precise mechanisms and key signaling mediators responsible for this aberrant repair process remain unclear. We assessed the importance of matrix metalloproteinase-3 (MMP-3) in the pathogenesis of IPF through i) determination of MMP-3 expression in patients with IPF, ii) *in vivo* experiments examining the relevance of MMP-3 in experimental models of fibrosis, and iii) *in vitro* experiments to elucidate possible mechanisms of action. Gene expression analysis, quantitative RT-PCR, and Western blot analysis of explanted human lungs revealed enhanced expression of MMP-3 in IPF, compared with control. Transient adenoviral vector-mediated expression of recombinant MMP-3 in rat lung resulted in accumulation of myofibroblasts and pulmonary fibrosis. Conversely, MMP-3-null mice were protected against bleomycin-induced pulmonary fibrosis.

***In vitro* treatment of cultured lung epithelial cells with purified MMP-3 resulted in activation of the β -catenin signaling pathway, via cleavage of E-cadherin, and induction of epithelial-mesenchymal transition. These processes were inhibited in bleomycin-treated MMP-3-null mice, as assessed by cytosolic translocation of β -catenin and cyclin D1 expression. These observations support a novel role for MMP-3 in the pathogenesis of IPF, through activation of β -catenin signaling and induction of epithelial-mesenchymal transition. (Am J Pathol 2011; 179:1733–1745; DOI: 10.1016/j.ajpath.2011.06.041)**

Idiopathic pulmonary fibrosis (IPF) is a relentlessly progressive and ultimately fatal lung disease with a median survival of 3 to 5 years from the time of diagnosis.¹ Histologically, IPF is characterized by dispersed myofibroblast proliferation and deposition of collagen and other extracellular matrix proteins within alveolar walls resulting in diminished lung compliance and ultimately leading to respiratory failure and death.² Despite advances in the understanding of the basic molecular pathways that drive this uncontrolled fibrotic process, no effective therapy is currently available.³

A broad spectrum of mediators have been implicated in the fibrotic process, including growth factors, cytokines, chemokines, and proteolytic enzymes such as matrix metalloproteinases (MMPs). The precise interrelationships among these various mediators, their upstream and downstream effects, and their respective mechanisms of action are under intensive investigation.⁴ An improved

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understanding of these molecular signaling pathways may ultimately lead to the development of novel therapeutic approaches for IPF.

The MMPs include a family of 24 structurally related zinc-dependent endopeptidases that degrade a diverse range of substrates, including components of the extracellular matrix. The degradative functions of MMPs are critical in tissue remodeling, most notably in wound repair.⁵ In addition to their enzymatic cleavage of matrix proteins, MMPs are able to activate cytokines, growth factors, and cell surface receptors, the last by limited proteolytic processing.⁶ Based on their multiple biological activities, MMPs are thought to participate in a diverse range of pathological processes, including rheumatoid arthritis and fibrosis of the liver, kidneys, heart, and lungs.^{7–11} Notably, MMP-3 has been directly implicated in the epithelial-mesenchymal transformation (EMT) program, a process that is central to the pathogenesis of neoplasia¹² and pulmonary fibrosis.¹³

Here, we provide novel evidence for a primary role of MMP-3 in the pathogenesis of pulmonary fibrosis in humans and in animal models. In explanted lungs from IPF patients, we demonstrate an increase in MMP-3 mRNA and protein expression, compared with control. Further, transient adenoviral vector-mediated expression of recombinant MMP-3 in the lungs of rats induces pulmonary myofibroblast accumulation and fibrosis; in contrast, MMP-3-null mice are largely protected from bleomycin-induced pulmonary fibrosis. Our studies in cultured cells and in animal models indicate that MMP-3 mediates fibrotic responses in part through activation of β -catenin signaling via cleavage of E-cadherin in lung epithelial cells, possibly resulting in an induction of the EMT program.

Materials and Methods

Human Lung Microarray Analysis

Lung tissue samples for microarray analysis were obtained through the University of Pittsburgh Health Sciences Tissue Bank.⁹ Twenty-three samples were obtained from surgical remnants of biopsies or lungs explanted from patients with IPF who underwent lung transplantation. In addition, 15 samples of control normal lung tissues were obtained from disease-free margins with normal histology of lung cancer resection specimens.¹¹ Total RNA from snap-frozen lung tissue was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and 500 ng was used as a template for cDNA synthesis and generation of Cy-3-labeled cRNA. Labeled cRNA was hybridized to Agilent 4x44K whole human genome microarrays and scanned with an Agilent scanner (Agilent Technologies, Santa Clara, CA).¹⁴ Scanned images were then processed with Agilent Feature Extraction version 9.5.3 software. The data are available at GSE-10667 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10667>, last accessed August 26, 2011.). MMP-3 gene expression was further assessed by real-time quantitative PCR as described previously.¹⁴ Total protein from the lung tissue was extracted with

Pierce T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. Western blot analysis of human tissue specimens for MMP-3 was performed as described previously.¹¹

Adenoviral Constructs

Adenoviral vectors were constructed with human MMP-3 cDNA.¹⁵ Briefly, human cDNA for MMP-3 in the pSP6 plasmid was excised and ligated into the shuttle vector, pDC316 (Microbix Biosystems, Mississauga, ON, Canada) and used to transform DH5 α competent cells (Invitrogen, Burlington, ON, Canada). HEK293 cells were cotransfected with the shuttle plasmid, pMMP-3, as well as the adenoviral genomic plasmid pBHGlox Δ E1,3Cre and monitored for plaque formation over 14 days. Plaques from the monolayer were selected and stored at -70°C . Ten microliters of DNA was digested with HindIII, and the fragments were separated by agarose gel electrophoresis. Viruses that displayed the correct restriction digest pattern were prepared in large scale and purified using CsCl-ethidium bromide gradient centrifugation. The human lung carcinoma cell line A549 (ATCC, Manassas, VA) was used to confirm expression and activity of the hMMP-3 transgene. Cells were infected and expression of the hMMP-3 transgene was confirmed by casein zymography.¹⁶ Briefly, cell supernatant was loaded onto a 12% SDS-PAGE gel containing 2 mg/mL of β -casein (Sigma-Aldrich, Burlington, ON), run at a constant voltage of 100 V for 1 hour, and then washed in 2.5% Triton X-100 for 30 minutes. Gels were incubated in activation buffer (50 mmol/L Tris-HCl, pH 8, 10 mmol/L CaCl_2 , 5 $\mu\text{mol/L}$ ZnSO_4 , 150 mmol/L NaCl) for 72 hours at 37°C . After incubation, enzymatic activity was visualized by staining gels with Coomassie Blue.

Rat Model of Pulmonary Fibrosis

Adult female Sprague-Dawley rats received 5×10^8 plaque-forming units of recombinant adenovirus, AdDL (empty vector virus), or AdMMP-3 (MMP-3 viral vector) by direct tracheal instillation. Rats were euthanized on day 14 or 21, and the lungs were harvested and inflated with 10% neutral buffered formalin and processed for routine histology. Lungs were stained with H&E and Masson's trichrome. Immunohistochemistry was performed to localize α -smooth muscle actin (α -SMA; Sigma-Aldrich).

Murine Model of Pulmonary Fibrosis

Wild-type C57Bl/6 and MMP-3-null mice (Taconic Farms, Hudson, NY) were treated by intratracheal instillation of 3 U/kg of bleomycin (6 to 12 animals per group). Briefly, an oral gavage feeding tube was inserted transorally and 3 U/kg of pharmaceutical-grade bleomycin (Bedford Laboratories, Bedford, OH) in 50 μL saline was instilled over 1 minute. After recovery, treated animals were returned to their housing and subsequently euthanized at selected time points up to 21 days after bleomycin instillation. Lung compliance was measured using a FlexiVent

small animal ventilator (Scireq Scientific Respiratory Equipment, Montreal, QC, Canada).

Western Blot Analysis of Lung Collagen Content

The pulmonary vasculature was perfused with PBS, and the lungs were excised and snap-frozen in liquid nitrogen and stored at -80°C until analysis. Sections of lung tissue were cut from the frozen lung and homogenized in buffer (15 mmol/L Tris, 2 mmol/L EDTA, 20% glycerol, pH 7.5 containing 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ aprotinin, 1 mmol/L NaF, 1 mmol/L dithiothreitol, 1 mmol/L sodium orthovanadate, and 0.5 mmol/L 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride) at 4°C using a Bullet Blender homogenizer (Next Advance, Averill Park, NY). NP-40 and phenylmethylsulfonyl fluoride were added to a final concentration of 0.1% and 1 mmol/L respectively and the homogenates were sonicated. Proteins in the supernatant were separated by SDS-PAGE on an 8% gel and transferred to nitrocellulose. Membranes were probed for type 1 collagen (rabbit polyclonal anti-mouse type 1 collagen; Millipore, Billerica, MA) and GAPDH (Abcam, Cambridge, MA) using enhanced chemiluminescence diluted 1:100. Densitometry was performed on protein bands using ImageJ analysis software (NIH, Bethesda, MD). Collagen content was normalized to GAPDH. Lung collagen content was also measured biochemically using Sircol (Biocolor, Carrickfergus, UK) according to the manufacturers instructions.

Lung Histology and Immunohistochemistry

Human lung sections were deparaffinized in HistoChoice tissue fixative (AMRESCO, Solon, OH), hydrated with graded ethanol solutions, and equilibrated to water. Antigen retrieval was performed by boiling slides in 10 mmol/L sodium citrate buffer, pH 6.0. Sections were incubated in 3% hydrogen peroxide for 30 minutes, blocked in 5% goat serum in Tris buffered saline-Tween 20, and then incubated with rabbit polyclonal anti-MMP3 (Abcam) diluted 1:100 in 5% goat serum with Tris buffered saline-Tween 20. Slides were washed, and bound antibody was detected using a Vectastain kit (Vector Laboratories, Burlingame, CA). 3,3'-Diaminobenzidine was diluted 1:5 and exposed for 15 seconds. Sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls included use of an irrelevant (nonimmune) primary antibody and secondary antibody alone. Positive controls for MMP-3 included rat lung in which recombinant MMP-3 was expressed using adenoviral vector-mediated gene transfer. Digital images were acquired using an Olympus DC70 microscope and saved in TIFF format.

For murine lung sections, four randomly selected lungs from each experimental group of mice were embedded, sectioned, and stained with H&E, Picrosirius Red, and pentachrome (Movat's stain). Picrosirius Red stained sections were visualized using transmitted and polarized light. Digital images were acquired using an Olympus microscope and saved in TIFF format. For quantification of the extent of fibrosis, computer-assisted image analy-

sis was conducted using MetaMorph software version 7.7 (Perkin Elmer, Waltham, MA) in a blinded manner as described previously.¹⁷ Cyclin D1 immunohistochemistry and β -catenin immunofluorescence staining (both from Cell Signaling Technology, Danvers, MA) were performed on paraffin-embedded lung tissue.^{18,19} Briefly, lung tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling slides in sodium citrate buffer (pH 6.0) for 10 minutes. For immunohistochemistry, slides were blocked with 0.03% peroxide solution to inhibit endogenous peroxidases (Envision+ system; Dako, Glostrup, Denmark) for 5 minutes. Anti-cyclin D1 antibody (Cell Signaling diluted 1:25) was applied for 1 hour at room temperature, and subsequently a labeled polymer-horseradish peroxidase anti-rabbit secondary antibody diluted 1:100 was applied for 1 hour. 3,3'-Diaminobenzidine-positive chromogen was applied for 5 minutes and slides were then washed, counterstained with hematoxylin, dehydrated, and mounted. For immunofluorescence staining, slides were blocked with 1% goat serum for 1 hour before application of anti- β -catenin antibody diluted 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Lung sections were then incubated for 1 hour at room temperature with Alexa Fluor 488 goat anti-rabbit IgG diluted 1:100 (Invitrogen) and stained with DAPI for 15 minutes. Slides were then washed with PBS and mounted with ProLong Gold antifade reagent (Invitrogen). Image analysis was performed with an inverted Zeiss 200M microscope (Carl Zeiss, Thornwood, NY), and digitally deconvoluted using SlideBook version 4.2 software (Intelligent Imaging Innovations, Denver, CO). Quantitative analysis of cyclin D1 expression was performed using computer-assisted image analysis (MetaMorph version 7.7) on 20 high-power fields selected by random stratified sampling from three mice per group (~ 750 epithelial cells in all).

Measurement of MMP Protein Levels and Activity in Bronchoalveolar Lavage Fluid

The levels of MMP-2 and MMP-9 were measured in bronchoalveolar lavage fluid (BALF) from wild-type and MMP-3-null mice using a matrix metalloproteinase antibody array (RayBiotech, Norcross, GA). Briefly, BALF diluted 3:1 in blocking buffer was applied to antibody-impregnated membranes. Membranes were then exposed to biotin antibodies, followed by horseradish peroxidase-conjugated streptavidin, and were developed by chemiluminescence. Detected proteins were quantified by ImageJ software version 1.44. Assessment of the activity of MMP-2 and MMP-9 in BALF was conducted using zymography, with gelatin as the substrate, as described previously.²⁰

Transfection and β -Catenin Reporter Assays

Murine lung epithelial cells (MLE-12) were transfected with a TCF/LEF-TOPflash reporter plasmid (Upstate-Millipore, Billerica, MA) to determine the ability of purified MMP-3 to induce β -catenin activation. Cotransfection

with a constitutively expressed Renilla luciferase reporter plasmid was used to correct for transfection efficiency. FOPflash (mutated TCF/LEF binding sites) was used as a negative control (Upstate-Millipore). Cells were grown in Dulbecco's modified Eagle's medium F-12 (HyClone, Logan, UT), supplemented with insulin (5 μ g/mL), transferrin (0.1 mg/mL), hydrocortisone (10 nmol/L), β -estradiol (1 nmol/L), L-glutamine (2 mmol/L), sodium selenite (30 nmol/L), fetal bovine serum (2%), and penicillin/streptomycin. Cells were plated in six-well plastic culture plates at 50% to 80% confluence and incubated for 18 hours at 37°C in 5% CO₂. Cells were cotransfected with TCF/LEF-TOPflash or FOPflash reporter plasmids and control Renilla reporter plasmid for 24 hours. Cells were subsequently cultured in serum-free medium for 24 hours before treatment with recombinant murine MMP-3 (R&D Systems, Minneapolis, MN). MMP-3 was activated with 4-aminophenylmercuric acetate for 60 minutes at 37°C and was subsequently purified using desalting spin columns (Thermo Scientific, Rockford, IL) to remove the 4-aminophenylmercuric acetate before use in cell culture. Activated MMP-3 was administered to cells for 15, 30, and 60 minutes, and cell lysates were subsequently harvested for determination of luciferase activity using a dual-luciferase reporter assay kit (Promega, Madison, WI). Western blot analysis of cell lysates was performed with anti-cyclin D1 antibody diluted 1:200 (Cell Signaling Technology).

Immunofluorescence

MLE-12 cells were plated on collagen-coated glass chamber slides. Cells were treated with purified activated recombinant murine MMP-3 or control medium for 24 hours. Cells were washed and fixed with -20°C methanol for 4 minutes. Cells were then washed, blocked with 0.2% bovine serum albumin in PBS for 15 minutes, and incubated with either E-cadherin antibody (Invitrogen, Carlsbad, CA) or monoclonal anti-vimentin antibody (1:200; Sigma-Aldrich) in 0.2% bovine serum albumin for 1 hour at 37°C. Cells were washed and then incubated with a secondary goat-anti-mouse Alexa Fluor 488 antibody (Invitrogen) for 1 hour at 4°C. Cells were washed with PBS and mounted. Images were acquired using an inverted Zeiss 200M long working distance microscope using SlideBook version 4.2 software (Intelligent Imaging Innovations). Image analysis was performed using ImageJ software.

MMP-3 Cleavage of E-Cadherin

Human CALU-3 lung epithelial cells were plated and grown to confluence in six-well tissue culture dishes. Human activated recombinant MMP-3 (400 ng/mL) or control medium was added to the cells. Medium was removed at prespecified time points and was concentrated using centrifugal filter devices (Centricon, Temecula, CA). Immunoprecipitation of E-cadherin was conducted as described previously,²¹ using a primary anti-E-cadherin (extracellular domain) antibody diluted 1:100 (Cell Signaling Technology) and protein G aga-

rose. The beads were boiled in Laemmli buffer, and protein from the immunoprecipitates was analyzed using SDS-PAGE and Western blotting as described previously¹¹ with antibodies to the extracellular domain of E-cadherin (Millipore, Billerica, MA).

Analysis of Gene and Protein Expression in Cultured Lung Epithelial Cells

At various time points after MMP-3 treatment, RNA was extracted from the epithelial cells, reverse-transcribed into cDNA, and analyzed by real-time PCR using individual primers optimized for each gene. Real-time PCR was performed for 40 cycles on a CFX96 system (Bio-Rad, Hercules, CA) using iQ SYBR Green super mix (Bio-Rad). Relative mRNA expression levels were calculated using the 2^{- $\Delta\Delta$ Ct} method.²² For experiments using the MMP-3 inhibitor, cells were preincubated with MMP-3 inhibitor I (Calbiochem-EMD Chemicals, La Jolla, CA) for 30 minutes before addition of recombinant MMP-3. Cells were exposed to human activated recombinant MMP-3, harvested at 48 hours using SDS-containing buffer, and analyzed using SDS-PAGE and Western blot analysis with antibodies to cyclin D1 as described above.

Statistical Analysis

Processed signals extracted using Agilent Feature Extraction software²³ were cyclic-LOESS normalized using the R language for statistical computing²⁴ and the Bioconductor package.²⁵ SAM software (SAM: Significance Analysis of Microarrays) was used for statistical analysis²⁶ with a 5% false discovery rate. In murine experiments, we used continuous variable analysis for comparison of lung compliance curves, using an exponential decay regression; groups were compared using the coefficient of decay and the intercept. Grouped analyses were conducted using two-way analysis of variance with Bonferroni's post hoc test comparison. Single variable analyses were conducted with one-way analysis of variance with Dunnett's post hoc test. For quantification of lung collagen content using Western blot analysis, statistical significance was determined using one-way analysis of variance with Tukey's post hoc test.

Results

Increased MMP-3 Expression in the Lungs of Humans with IPF

To assess the potential importance of MMP-3 in IPF, RNA was extracted from explanted lung specimens of patients with IPF and control subjects and was subjected to microarray analysis. A graphical representation of the gene expression profile in IPF patients relative to control subjects is shown in Figure 1A. Using gene microarray analysis, we found a highly significant increase of MMP-3 mRNA expression in IPF lung samples, compared with non-IPF control subjects ($q = 0$). The levels of expression of several other MMPs (MMP-1, -7, -9, -10, -11, and -13)

Figure 1. MMP-3 mRNA and protein levels are increased in lungs from humans with IPF. **A:** Gene expression array analysis of human IPF/UIP lung tissue samples demonstrates a significant increase in MMP-3 mRNA expression ($q = 0$) compared with non-IPF control samples. Relative expression of other MMPs (ie, MMP-1, -7, -9, -10, -11, and -13) are shown for comparison. Genes overexpressed in IPF patients, compared with control subjects, are represented by circles plotted above the diagonal axis; genes underexpressed relative to control are represented below the diagonal. The distance of a given gene from the diagonal axis is proportional to the difference in its expression relative to control. **B:** Quantitative RT-PCR analysis of explanted surgical lung specimens demonstrates a greater than fourfold increase in MMP-3 mRNA levels in IPF versus non-IPF lung specimens ($*P < 0.05$ IPF versus control). **C:** Western blot analysis of homogenized lung tissue specimens from four non-IPF control subjects (HB300, HB303, HB305, and HB313) and four IPF patients (H075, H134, F273 and E712) demonstrates an increase in MMP-3 protein levels in IPF, compared with control lungs. **D:** Immunohistochemical analysis of human control (top) and IPF (bottom) lung sections to demonstrate cellular expression of MMP-3 (original magnification, $\times 40$). The control sections are from explanted lungs from brain-dead organ donors. Representative sections from control lung demonstrate that MMP-3 staining (brown) is largely confined to alveolar macrophages (solid arrow). By contrast, in sections from IPF lung, there is staining of alveolar macrophages (solid arrowhead, bottom left), alveolar epithelial cells (open arrowheads, bottom left and right), airway epithelial cells (solid arrowheads, bottom right), and intravascular leukocytes (solid arrow, bottom right). Scale bars = 100 μ m.

are included for comparison. Quantitative RT-PCR analysis of explanted surgical specimens confirmed the microarray results, demonstrating a greater than fourfold increase in MMP-3 mRNA levels in the lungs of IPF patients, compared with control (Figure 1B). To determine whether alterations in the mRNA levels were reflected in alterations in the levels of MMP-3 protein, Western blot analysis of tissue extracts was conducted in which the levels of MMP-3 protein (normalized to β -actin) in the explanted lungs of four IPF patients were compared with those of four non-IPF control subjects. This analysis revealed a significant increase in MMP-3 protein levels in all IPF specimens, compared with control (Figure 1C). Immunohistochemical analysis of lung sections from IPF patients revealed that MMP-3 was expressed by diverse cell types in the lung, including bronchial and alveolar epithelial cells, alveolar macrophages, and fibroblasts within the interstitium, as well as by leukocytes within the vasculature (Figure 1D). By comparison, in control non-IPF lungs, MMP-3 expression was faint and was largely confined to alveolar macrophages and intravascular leukocytes (Figure 1D).

Intratracheal Delivery of Adeno-MMP-3 Vector Induces Transient Pulmonary Fibrosis in Rats

To determine whether MMP-3 expression is sufficient to induce pulmonary fibrosis, we used a rodent model in which recombinant MMP-3 was expressed in rat lungs using adenoviral vector-mediated gene transfer. An adenoviral vector using the pDC316 backbone was constructed to enable infected cells to constitutively express MMP-3 (AdMMP-3) (Figure 2A). Preliminary *in vitro* experiments demonstrated an increase in active MMP-3 in the supernatants of human lung epithelial cells 24 hours after infection with AdMMP-3 at a multiplicity of infection (MOI) of 50:1 (Figure 2, B and C), confirming the functional integrity of the vector. Subsequently, AdMMP-3 or empty viral vector was delivered intratracheally to Sprague-Dawley rats (5×10^8 plaque-forming units per animal). Animals receiving empty viral vector demonstrated only a mild neutrophilic infiltration, which was resolved by day 14 (Figure 2D), consistent with previous reports.²⁷ On day 14 after adenoviral vector administration, animals receiving intratracheal AdMMP-3 demonstrated an in-

Figure 2. MMP-3 expression in rat lungs induces myfibroblast accumulation and fibrosis. **A:** AdMMP-3 vector constructed using human MMP-3 cDNA cloned into the shuttle vector pDC316. **B:** Western blot analysis of supernatant harvested from nontransduced A549 cells or cells transduced with empty (AdDL) vector or AdMMP-3 vector demonstrating an increase in MMP-3 secretion. The MOI from these studies was 50:1. The last lane represents an MMP-3 protein standard. **C:** Casein zymography of supernatants harvested from transfected A549 cells transfected with AdDL vector or AdMMP-3 at MOI₁₀ and MOI₅₀ confirms presence of a functionally active form of MMP-3. **D:** Lung histology (H&E, trichrome, anti- α -SMA; original magnification, $\times 5$) at day 14 from rats treated with AdDL control or AdMMP-3 vector demonstrates an increase in α -SMA staining and increased collagen deposition in rats receiving AdMMP-3 vector, compared with AdDL control vector. Scale bars = 100 μ m.

crease in collagen deposition, compared with empty vector control, as determined by trichrome staining (Figure 2D). Further, there was an increase in numbers of focal patches of α -SMA staining consistent with myfibroblast accumulation in the lung in the animals treated with AdMMP-3, but not with empty vector. The fibrosis was maximal by day 14, and by day 21 there was substantial resolution of fibrosis as assessed by trichrome staining (data not shown).

Role of MMP-3 in a Murine Model of Bleomycin-Induced Pulmonary Fibrosis

To seek additional evidence for the importance of MMP-3 in pathogenesis of pulmonary fibrosis, we used a well-characterized murine model of pulmonary fibrosis induced by intratracheal administration of bleomycin (3 U/kg). In this model, levels of MMP-3 were significantly elevated in BALF from bleomycin-treated wild-type C57BL/6 mice, compared with saline-treated controls, 48 hours after bleomycin treatment (Figure 3A). To provide more direct evidence of the importance of MMP-3 in pulmonary fibrosis, we compared the degree of pulmo-

nary fibrosis in bleomycin-treated MMP-3-null and wild-type C57BL/6 mice 21 days after bleomycin treatment. Compared with wild-type C57BL/6 controls, in MMP-3-null mice the pulmonary fibrosis was largely attenuated, as assessed by several parameters including physiological impairment (lung compliance) and biochemical and histological assessment of collagen content and of the lungs. Specifically, at day 21 after bleomycin administration, wild-type mice demonstrated a significant reduction in pulmonary compliance, compared with MMP-3-null mice, as demonstrated by pressure-volume curves and measurement of lung compliance (Figure 3, B–D). Furthermore, although wild-type mice developed a significant increase in lung collagen content 21 days after treatment with bleomycin as assessed by Western blot analysis of lung homogenates with an anti-collagen antibody, the collagen content of the lungs of bleomycin treated MMP-3-null mice was not significantly different from controls (Figure 3E). This finding was confirmed biochemically using the Sircol assay to assess the collagen content of lungs from bleomycin-treated wild-type and MMP-3-null mice (Figure 3F) and histologically using Picrosirius Red staining of lung tissue and quantitative image analysis (Figure 3G). Notably, in otherwise unchallenged MMP-3-null mice, no abnormalities in lung histology, pulmonary compliance, or pulmonary collagen content were observed.

To assess whether there are compensatory changes in the levels of other MMPs in the MMP-3-null mice, we measured the protein levels and activity of MMP-9 and MMP-2 in BALF at 2 and 7 days after instillation of saline or bleomycin. There were no significant differences in the protein levels of either MMP-2 or MMP-9 in BALF in wild-type mice, compared with MMP-3-null mice (Figure 4A). With respect to enzymatic activity, although there was a trend toward slightly increased activity of MMP-2 and -9 in the BALF of wild-type mice, compared with MMP-3-null mice, at 2 days after bleomycin instillation, by day 7 the levels of MMP-9 trended higher in the MMP-3-null mice, whereas the levels of MMP-2 were similar in wild-type and MMP-3-null mice (Figure 4, B and C). None of these differences were statistically significant.

Given that TGF- β is a central mediator of pulmonary fibrosis,^{28,29} we assessed the levels of this growth factor in BALF from bleomycin-treated wild-type and MMP-3-null mice. This analysis revealed no difference in the levels of TGF- β in BALF samples collected from wild-type and MMP-3-null mice at early (48 hours) or at late (21 days; Figure 4D) time points after bleomycin administration.

MMP-3 Induces Activation of β -Catenin-Dependent Signaling

Having demonstrated the importance of MMP-3 in pulmonary fibrosis, we next investigated the mechanisms by which MMP-3 participates in the fibrotic response. We focused on the β -catenin signaling cascade, which has been recently implicated in the pathogenesis of pulmonary fibrosis^{18,30,31} and also in the EMT program.^{12,13} For

Figure 3. Importance of MMP-3 in a murine model of bleomycin-induced pulmonary fibrosis. **A:** Wild-type C57/BL6 mice demonstrate a significant increase in BALF MMP-3 concentration 48 hours after the intratracheal delivery of bleomycin, compared with mice receiving saline control (* $P < 0.05$). **B–D:** Pressure-volume curve of the lungs of wild-type mice treated with saline (WT NS), MMP-3-null mice treated with saline (MMP-3 Null NS), wild-type mice treated with bleomycin (WT BLM), and MMP-3-null mice treated with bleomycin (MMP-3 Null BLM) are plotted at day 21 after saline or bleomycin administration. There is a downward shift in WT BLM, compared with WT NS indicating a reduction in lung compliance 21 days after the administration of bleomycin. The effect was markedly attenuated in the MMP-3-null mice; there was no significant change in lung compliance at day 21 after bleomycin administration (* $P < 0.05$ WT NS versus WT BLM). **E:** Western blot analysis of lung homogenates of wild-type and MMP-3-null mice 21 days after treatment with saline or bleomycin. **F:** Sircol assay of lung collagen content illustrating that bleomycin-treated MMP-3-null mice have significantly less collagen than bleomycin-treated wild-type controls (* $P < 0.05$ WT BLM versus MMP-3 Null BLM). **G:** Representative lung sections (**top**) from wild-type (WT) and MMP-3-null mice treated with bleomycin (original magnification, $\times 4$ and $\times 40$ as indicated), stained with Picrosirius Red (PS) and Fast Green FCF (Fisher Chemical, Waltham, MA) counterstain or with H&E demonstrating an increase in collagen (red staining) deposition in WT, compared with MMP-3-null mice. Quantitative image analysis (**bottom**) confirmed an increase in collagen deposition in WT mice, compared with MMP-3-null mice treated with bleomycin (* $P < 0.05$ WT BLM versus MMP-3 Null BLM).

these experiments, cultured lung epithelial cells (MLE-12) were transiently transfected with the TOPflash luciferase reporter plasmid to monitor activation of β -catenin-dependent transcriptional events. In this system, nuclear translocation of β -catenin results in transcriptional activation at TCF/LEF binding sites driving expression of luciferase in transfected cells. In response to treatment with activated MMP-3, the lung epithelial cells demonstrated a significant increase in luciferase reporter activity, compared with control medium (Figure 5A). Under these con-

ditions, no activation of the negative control FOPflash reporter was observed after MMP-3 treatment (data not shown). To provide independent evidence of the capacity of MMP-3 to activate β -catenin signaling pathways, we assessed whether MMP-3 is able to induce expression of cyclin D1, a known β -catenin-dependent gene, using Western blot analysis of cell lysates of lung epithelial cells at selected times after treatment with recombinant MMP-3. Under these conditions, MMP-3 treatment triggered a time-dependent increase in cyclin D1 expres-

Figure 4. Analysis of levels of levels of MMP-2 and -9 and TGF- β in BALF from wild-type and MMP-3-null mice. **A:** The levels of MMP-2 and -9 protein in BALF from wild-type and MMP-3-null mice treated with either saline or 3 U/kg bleomycin were assessed using an MMP antibody array (RayBiotech). The array shown is representative of $n = 3$ independent experiments done in duplicate. **B:** Enzymatic activity of MMP-2 and -9 in BALF was assessed by zymography using gelatin as the substrate. **C:** Results of zymography experiments assessing the enzymatic activity of MMP-2 and -9 in BALF. Data are reported as means $\pm$ SEM of three experiments. **D:** Analysis of levels of active TGF- β by ELISA in BALF from wild-type and MMP-3-null mice 21 days after treatment with bleomycin. Data are reported as means $\pm$ SEM of three experiments.

sion, peaking at 48 hours (Figure 5B). In contrast, there was minimal change in the expression of cyclin D1 in cells cultured in serum-free culture medium alone. To verify that the catalytic activity of MMP-3 is required for this response, cells were pretreated with MMP-3 inhibitor 1 before addition of MMP-3. This treatment abrogated the increase in cyclin D1 expression (Figure 5C), thus confirming the importance of MMP-3 catalytic activity for induction of gene expression.

MMP-3 Cleaves E-Cadherin in Lung Epithelial Cells

The β -catenin signaling pathway can be activated by various mechanisms, including the canonical Wnt-dependent pathway involving frizzled (FZD) and low-density lipoprotein receptor related protein (LRP) receptors, as well as by noncanonical pathways such as release of β -catenin bound to E-cadherin in interepithelial adherens junctions.³² Here, we focused on the potential role of proteolytic cleavage of E-cadherin by MMP-3 that can result in release of β -catenin bound to E-cadherin and subsequent nuclear translocation and activation of target gene transcription.^{21,32–34} To investigate the involvement of this alternative pathway, we assessed the ability of MMP-3 to cleave the extracellular domain of E-cadherin in cultured lung epithelial cells. Human CALU-3 lung epithelial cells were treated with activated MMP-3 and the supernatant collected at selected times and concentrated. E-cadherin cleavage products were isolated by immunoprecipitation with antibodies to the extracellular (N-terminal) domain. The immunoprecipitates were ana-

lyzed by SDS-PAGE and Western blotting with antibodies to the extracellular domain of E-cadherin. These experiments revealed that MMP-3 cleaved epithelial E-cadherin, releasing an 80-kDa proteolytic fragment of the extracellular domain into the supernatant (Figure 5D).

MMP-3-Null Mice Exhibit a Reduction in Cytosolic Translocation of β -Catenin and Cyclin D1 Expression

To determine whether there was evidence of activation of the β -catenin signaling pathway *in vivo* in our murine model of bleomycin-induced pulmonary fibrosis, we assessed translocation of β -catenin and expression of cyclin D1 in lung epithelial cells in mice treated with bleomycin using immunofluorescence for β -catenin and immunohistochemistry for cyclin D1 (Figure 6A). In saline-treated control mice, β -catenin was localized predominantly to the interepithelial junctions. In bleomycin-treated wild-type mice, there was a reduction in membrane-associated β -catenin and a concomitant increase in cytosolic β -catenin. In contrast, cytosolic translocation of β -catenin was considerably attenuated in lung epithelial cells of bleomycin-treated MMP-3-null mice. Furthermore, immunohistochemical staining for cyclin D1 (CCND1), a downstream target gene of β -catenin, demonstrated a significant increase in nuclear staining in epithelial cells in bleomycin-treated wild-type mice, compared with MMP-3-null mice (Figure 6A). There was minimal cyclin D1 staining in lung epithelial cells from control mice treated with saline. Quantitative morphometric analysis confirmed a signif-

Figure 5. MMP-3 induces activation of β -catenin in lung epithelial cells. **A:** Murine lung epithelial (MLE-12) cells transfected with LEF/TCF-TOPflash reporter plasmid demonstrate a significant increase in luciferase activity when cultured in the presence of MMP-3, indicating an increase in transcriptional activity of β -catenin ($P < 0.05$ versus control). **B:** Western blot analysis of lysates from lung epithelial cells stimulated with recombinant murine MMP-3 demonstrates a time-dependent increase in cyclin D1 protein expression. GAPDH is shown as a control for protein loading. **C:** Epithelial cells were pretreated with MMP-3 inhibitor 1 or vehicle control before addition of MMP-3. Expression of cyclin D1 in cell lysates at 24 hours was assessed by Western blot analysis. **D:** Purified activated MMP-3 was added to human lung epithelial cells and the supernatant collected at selected times, concentrated, and E-cadherin purified by immunoprecipitation. The immunoprecipitates were analyzed by SDS-PAGE and Western blot with antibodies to the extracellular domain of E-cadherin. There is an increase in the amount of the 80-kDa cleavage product of the extracellular domain (ectodomain) of E-cadherin after MMP-3 treatment. The heavy chain of IgG was used as a loading control. The density of the E-cadherin ectodomain band was normalized to the density of the IgG heavy chain band and is illustrated numerically as relative densitometry. Representative results of three independent experiments are shown.

icant decrease in the number of epithelial cells that stained positively for cyclin D1 in MMP-3-null, compared with wild-type mice, 21 days after bleomycin administration (Figure 6B).

MMP-3 Induces EMT in Lung Epithelial Cells

One of the key processes believed to participate in fibrotic responses in the lung is EMT: the transition (transformation) of epithelial cells to a mesenchymal (myofibroblast) phenotype.³⁵ To determine the effects of MMP-3 on EMT, lung epithelial cells were cultured in the presence of MMP-3 for up to 72 hours. By 24 hours, the lung epithelial cells treated with MMP-3 displayed a distinctive change in cell morphology, with loss of cell-to-cell contacts and with cell spreading, unlike the morphology of control cells (Figure 7A). Immunofluorescence analysis at this time revealed a decrease in E-cadherin (epithelial cell marker) and an increase in vimentin expression (mesenchymal marker) in MMP-3 treated lung epithelial cells, compared with control cells. As an independent assessment, we used Western blot analysis to confirm that there was a reduction in E-cadherin and an increase in vimentin protein levels in a time-dependent fashion (Figure 7B). Quantitative real-time PCR analysis in cultured lung epithelial cells treated with MMP-3 confirmed a decrease in E-cadherin mRNA expression at 24 hours and an increase in vimentin mRNA expression at 72 hours (Figure 7C). Furthermore, this analysis also revealed an increase in expression of WNT1 inducible signaling pathway protein 1 (*WISP1*), a target gene of the Wnt/ β -catenin pathway, at 72 hours (Figure 7C) providing independent evidence of activation of the β -catenin signaling pathway (compare Figure 5).

Discussion

Our main finding is that MMP-3 may be involved in the pathogenesis of pulmonary fibrosis in humans and in animal models. In human IPF lungs, there was increased expression of MMP-3 mRNA and protein. Expression of recombinant MMP-3 in the lungs of rats induced a fibrotic response; conversely, mice genetically deficient in MMP-3 were protected against bleomycin-induced pulmonary fibrosis, as measured by changes in pulmonary physiology and lung collagen content and by histological analysis. The responses observed in these animal models were supported mechanistically by *in vitro* studies demonstrating that MMP-3 induced activation of the β -catenin signaling pathway, possibly via cleavage of E-cadherin, in addition to its ability to induce EMT in lung epithelial cells. In turn, these observations were supported by evidence that bleomycin-induced activation of the β -catenin pathway in lung epithelial cells was attenuated in MMP-3-null mice.

There is increasing evidence that MMPs have pleiotropic effects in the context of tissue remodeling and repair that extend beyond their well-recognized roles in matrix degradation. In this regard, there is emerging evidence to suggest that MMPs, including MMP-3, have the ability to regulate tissue repair by altering the activity of other nonmatrix proteins, including cytokines and membrane receptors,^{33,36} by facilitating cellular signaling through the byproducts of tissue degradation, and by activating cellular signaling pathways leading to EMT.¹² This phe-

Figure 6. MMP-3-null mice demonstrate diminished translocation of β -catenin and expression of cyclin D1 in lung epithelial cells in response to bleomycin, compared with wild-type mice. **A:** Immunofluorescence staining for β -catenin (top; original magnification, $\times 100$; scale bar = 20 μ m) and immunohistochemical staining for cyclin D1 (bottom; original magnification, $\times 40$; scale bars = 100 μ m) in wild-type mice treated with saline (WT Saline), wild-type mice treated with bleomycin (WT BLM), and MMP-3-null mice treated with bleomycin (MMP-3 Null BLM). **B:** Quantitative analysis demonstrates a reduction in small airway alveolar epithelial cells staining positive for cyclin D1 in MMP-3-null versus WT mice treated with bleomycin.

nomenon, whereby a class of tissue-degrading enzymes can paradoxically promote the deposition of excess tissue matrix (ie, collagen), may be attributable to these nondegradative signaling functions.

The ability of MMP-3 to mediate fibrogenic responses has been previously demonstrated in extrapulmonary organs. For example, transgenic expression of MMP-3 in murine mammary epithelial cells leads to collagen deposition during pregnancy and lactation.³⁷ Similarly, elevated expression of MMP-3 in canine renal tissues was associated with tissue destruction and fibrotic remodeling in Alport's syndrome.³⁸ Indirect evidence has also implicated a pathological role for MMP-3 among other MMPs in the

pathogenesis of hepatic fibrosis in the context of cirrhosis.³⁹ Our observations in rat lungs, in which overexpression of recombinant MMP-3 induced accumulation of myofibroblasts and accumulation of excess collagen, provide additional direct support for the role of MMP-3 in tissue fibrosis. However, the fact that the pulmonary fibrosis induced by overexpression of MMP-3 was transient suggests that additional factors may contribute to the sustained and progressive fibrosis observed in other experimental models and in humans with IPF.

Although the profibrotic effects of MMP-3 have not been previously studied in the context of pulmonary fibrosis, other MMPs (including MMP-2, -7, and -9) have

Figure 7. MMP-3 induces epithelial mesenchymal transition in cultured lung epithelial cells. **A:** Differential interference contrast (DIC) and immunofluorescence images of murine lung epithelial (MLE) cells taken 24 hours after exposure to activated MMP-3 or serum-free control medium. Note cell spreading (bottom left; scale bar = 200 μ m), decreased E-cadherin expression (bottom middle; scale bar = 20 μ m), and increased vimentin expression (bottom right; scale bar = 50 μ m) after treatment with MMP-3. **B:** Western blot analysis of lung epithelial cells treated with activated MMP-3 demonstrates a significant reduction in E-cadherin levels, compared with control at 12 and 24 hours after the addition of MMP-3 (* $P < 0.05$ versus control) and an increase in vimentin levels at 72 hours. **C:** Assessment of mRNA expression in lung epithelial cells at selected times up to 72 hours after exposure to activated MMP-3 or control medium using quantitative real-time PCR analysis. Note the reduction in E-cadherin mRNA levels at 24 hours and the increase in vimentin and WISP-1 levels at 72 hours after exposure to MMP-3 compared with control.

been implicated in this disease process.⁴⁰ Using a similar experimental approach to that of the present study, Zuo et al¹¹ demonstrated enhanced expression of MMP-7 (matrilysin) mRNA in the lungs of patients with IPF and showed that that genetic deficiency of MMP-7 in mice conferred protection against bleomycin-induced pulmonary fibrosis. Thus, although the precise mechanism or mechanisms by which MMPs are involved in pulmonary fibrosis remain to be clarified, these studies provide support for the notion that MMPs play a crucial role in the pathobiology of IPF. The present study supports and extends these observations by providing evidence for a critical role of MMPs in pathogenesis of pulmonary fibrosis. Our data also provide additional mechanistic insights into the signaling pathways by which MMPs potentially contribute to fibrotic responses, specifically via activation of β -catenin and promotion of EMT.

We provide evidence in support of the notion that MMP-3 contributes to the fibrotic response through both activation of the β -catenin signaling pathway and through an ability to induce the EMT program, processes that are closely related. Recent evidence has identified β -catenin, a powerful regulator of cell proliferation, as a key effector in fibrosis of both pulmonary and extrapulmonary organs.⁴¹ Chilosi et al¹⁸ reported evidence of aberrant Wnt/ β -catenin activation in fibroproliferative bronchiolar lesions and also nuclear β -catenin accumulation in the fibroblastic foci in lung samples from patients with IPF. More recently, Konigshoff et al³⁰ reported that *WISP1*, a target gene of the Wnt/ β -catenin pathway, is a key regulator of pulmonary fibrosis through its ability to induce collagen matrix deposition by fibroblasts and EMT. As discussed above, MMP-7, known to be transcriptionally regulated by nuclear β -catenin, has been identified as an important regulator of pulmonary fibrosis in both humans and mice.¹¹ In the present study, we demonstrate an increase in the nuclear translocation of β -catenin in epithelial cells transfected with a TCF/LEF luciferase reporter when cultured in the presence of MMP-3. Furthermore, we show that the cytosolic translocation of β -catenin and subsequent production of cyclin D1 is attenuated in MMP-3-null mice, compared with wild-type mice, when treated with bleomycin in an *in vivo* model. There are several potential mechanisms by which MMPs such as MMP-3 could activate β -catenin signaling pathways (discussed in greater detail below).

In addition to proliferation and differentiation of resident pulmonary fibroblasts into myofibroblasts and recruitment of circulating fibrocytes to the lung,⁴² the induction of epithelial mesenchymal transition (EMT) represents a potential key mechanism that could contribute to accumulation of fibroblasts within the lung interstitium. Epithelial cells acquire phenotypic mesenchymal (fibroblast) markers when exposed to TGF- β .⁴³ With respect to the importance of MMPs in EMT, Illman et al⁴⁴ reported that epilysin (MMP-28) can induce EMT, leading to cell invasion in lung adenocarcinoma cells through a TGF- β -dependent mechanism. Additionally, there is strong evidence that MMP-3 can induce EMT in mouse mammary epithelial cells through alteration in Rac1b, a splice variant of Rac1, leading to increases in reactive

oxygen species resulting in EMT.¹² Although the specific signaling mechanisms have not been determined, this phenomenon was also demonstrated in response to MMP-3 exposure in a human lung epithelial cell line.⁴⁵ Our observations in cultured lung epithelial cells (Figure 7) support this concept.

The mechanisms by which MMP-3 activates β -catenin remain incompletely understood. β -catenin can be regulated by various pathways, the best characterized of which is the canonical Wnt/ β -catenin pathway that is triggered by binding of soluble Wnt ligands to FZD and LRP receptors.³² On ligand binding, FZD and LRP5/6 are activated, resulting in inhibition of β -catenin phosphorylation, thus stabilizing the protein, which then translocates to the nucleus, where it regulates gene transcription.⁴⁶ In addition, the subcellular distribution of β -catenin is dynamically regulated by reversible binding to E-cadherin, a component of adherens junctions, and β -catenin-dependent nuclear transcriptional events are influenced by this binding.^{32,47,48} Thus, induction of EMT by MMP-3 may be mediated in part via cleavage of surface bound E-cadherin (Figure 5D), with subsequent liberation of the E-cadherin bound pool of β -catenin leading to its nuclear translocation and possible induction of gene transcription.^{32,33}

Although multiple complex signaling pathways are likely to contribute to end-stage tissue fibrosis, a prominent role of TGF- β in both experimental animal and human studies has been established.^{28,29,49,50} Among its various properties, TGF- β has been shown to induce myofibroblast differentiation, EMT in pulmonary epithelial cells, and extracellular matrix gene transcription promoting collagen and fibronectin deposition. As noted above, however, there was no difference in the TGF- β levels in BALF between wild-type and MMP-3-null mice at early or later time points after bleomycin administration, despite considerable differences in physiological and histological measures of fibrosis. These observations suggest that MMP-3 may exert its profibrotic responses through a TGF- β -independent signaling pathway, or it may be that the effects of MMP-3 are downstream of TGF- β .

In conclusion, our data indicate that MMP-3 is pivotal in the pathogenesis of pulmonary fibrosis, based on observations of enhanced expression of MMP-3 in lungs from patients with IPF in conjunction with animal models providing more direct evidence for the role of MMP-3 in pulmonary fibrosis. Furthermore, in addition to its well-known degradative effects, MMP-3 triggers activation of the β -catenin signaling pathway leading to EMT of lung epithelial cells. Taken together, our data indicate that MMP-3 is a novel mediator in the pathogenesis of pulmonary fibrosis. The selective targeting of MMP-3 may represent a potential target for future therapeutic strategies.

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