ORIGINAL RESEARCH

Upregulation and Nuclear Location of MMP28 in Alveolar Epithelium of Idiopathic Pulmonary Fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive aging-associated disease of unknown etiology. A growing body of evidence indicates that aberrant activated alveolar epithelial cells induce the expansion and activation of the fibroblast population, leading to the destruction of the lung architecture. Some matrix metalloproteinases (MMPs) are upregulated in IPF, indicating that they may be important in the pathogenesis and/or progression of IPF. In the present study, we examined the expression of MMP28 in this disease and evaluated its functional effects in two alveolar epithelial cell lines and in human primary bronchial epithelial cells. We found that the enzyme is expressed in bronchial (apical and cytoplasmic localization) and alveolar (cytoplasmic and nuclear localization) epithelial cells in two different groups of patients with IPF. *In vitro* MMP28 epithelial silencing decreased the proliferation rate and delayed wound closing, whereas overexpression showed opposite effects, protecting from apoptosis and enhanced epithelial–mesenchymal transition. Our findings demonstrate that MMP28 is upregulated in epithelial cells from IPF lungs, where it may play a role in increasing the proliferative and migratory phenotype in a catalysis-dependent manner.

Keywords: matrix metalloproteinase; lung epithelial cells; idiopathic pulmonary fibrosis

Clinical Relevance

Idiopathic pulmonary fibrosis is a progressive and lethal disease of unknown etiology and uncertain pathogenesis. In this study, we discovered that matrix metalloproteinase 28 is upregulated in idiopathic pulmonary fibrosis lungs, is located in the nucleus of alveolar epithelial cells, and may play a profibrotic role.

Idiopathic pulmonary fibrosis (IPF) is a progressive, aging-related, fibrosing interstitial pneumonia of unknown etiology (1–4). IPF is a complex, epithelium-driven disorder in which genetic, epigenetic, and environmental factors interact, triggering the aberrant activation of lung epithelium. In turn, hyperactivated alveolar epithelial cells (AECs) produce numerous mediators, which result in an increase of the fibroblast/myofibroblast population, excessive accumulation of extracellular matrix (ECM), and the destruction of lung architecture (1, 2, 5, 6).

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Deregulation in the expression of numerous matrix metalloproteinases (MMPs) has been consistently reported in the complex pathways likely involved in the pathogenesis of IPF, suggesting that MMPs may be important in the pathogenesis and/or progression of IPF (7). MMPs are a family of zinc-dependent endopeptidases with 23 members in humans. These enzymes cleave not only ECM components but also cytokines, growth factors, receptors, and other bioactive molecules participating in cellular processes such as differentiation, proliferation, and angiogenesis (8).

In 2001, the last member of the human MMP family was discovered simultaneously by two research groups (9, 10). MMP28 (epilysin) is expressed during development and regeneration of the nervous system before myelination, and in these circumstances, three substrates, still the only ones described for MMP28 so far, have been suggested: neural cell adhesion molecule, Nogo-A, and neuregulin (11). MMP28 mRNA has been found in many human epithelia; therefore, a role in homeostasis has been suggested (9). MMP28 has also been revealed in some pathological conditions, including osteoarthritis and gastric carcinoma (12, 13). In murine lung, it has been demonstrated that MMP28 is expressed by club cells (14), promoting epithelial cell survival *in vitro* and in a model of influenza infection (15). Loss of Mmp28 in mice results in reduced macrophage polarization to the M2 phenotype and attenuation of bleomycin-induced lung fibrosis (16). More recently, it was found that Mmp28-deficient mice are also protected from tobacco smoke–induced inflammation and emphysema, suggesting a role in chronic obstructive pulmonary disease (17).

However, this enzyme has not been studied in human fibrotic lung disorders. In this context, the aims of this study were to evaluate the expression and localization of MMP28 in IPF and to analyze its functional effects in alveolar and airway epithelial cells *in vitro*.

Methods

Human Samples

The human samples were obtained under protocols approved by the local ethics committees, and all participants gave written informed consent. Samples from 17 Mexican patients were obtained at the Instituto Nacional de Enfermedades Respiratorias (INER). Samples from nine German patients were kindly provided by the CPC-M bioarchives at the Comprehensive Pneumology Center. Some demographic characteristics of the patients are reported in Table E1 in the data supplement. Four liquid nitrogen–frozen tissues from patients with IPF and four tissues from healthy donors were homogenized using a Mikro-Dismembrator (Sartorius) and lysed with radioimmunoprecipitation assay buffer for protein isolation and Western blot analysis, as described previously (18).

Cell Culture

Human alveolar epithelial cell line A549 (human AEC) and rat alveolar epithelial cell line RLE-6TN (rat AEC) were purchased from the American Type Culture Collection. Human primary type II AECs (19) (kindly donated by Melanie Königshoff) and human primary basal bronchial epithelial cells (BECs) had been isolated from a histologically normal tumor-free region of a lung tumor resection and were provided by the CPC-M bioarchives at the Comprehensive Pneumology Center. All cells were cultured in an incubator (5% CO_2 and 95% air) at 37°C. Cells were lysed with radioimmunoprecipitation assay buffer, or cytoplasmic and nuclear enriched fractions were obtained with the NE-PER Kit (78835; Thermo Scientific).

Silencing and Overexpression

Silencing of MMP28 in AECs was performed using lentiviral particles (Santa Cruz Biotechnology) with scrambled control shRNA (sc-108080) or shMMP28 (sc-62278-V). For silencing of MMP28 in BECs, reverse transfection was achieved with scrambled siRNA (AM4611; Ambion) or siMMP28 (s35627; Ambion). For overexpression, AECs and rat AECs were transfected with empty vector pCMV6-Entry (Mock, PS100001; OriGene), fusion MMP28-DDK (MMP28, RC215325; OriGene), catalytically inactive MMP28-DDK (EA), or KQ-mutated MMP28-DDK (KQ). For imaging and Western blot analysis results, *see* the Supplementary Methods section of the data supplement.

Functional Effects

Functional effects were evaluated according to the supplier's recommendations for each test. Growth rate was studied with WST-1

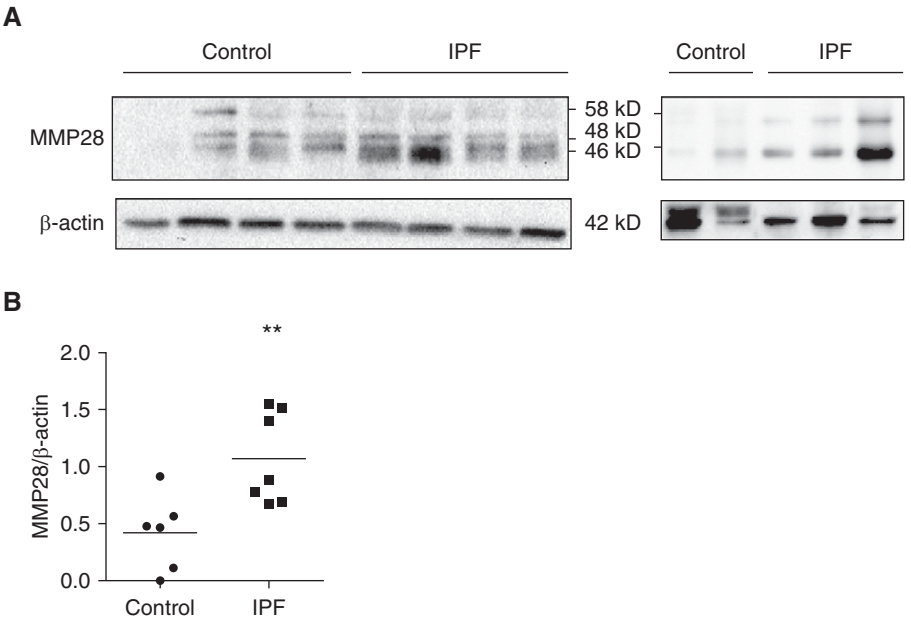

Figure 1. Matrix metalloproteinase 28 (MMP28) is increased in idiopathic pulmonary fibrosis (IPF). (*A*) The expression of the MMP28 protein was analyzed by Western blotting in seven IPF and six control lungs from German (left) or Mexican (right) patients. (*B*) Densitometric analysis showing 58, 48, and 46 kD bands. $^{**}P < 0.01$.

reagent (Roche). Proliferation rate was measured with CyQUANT reagent (Thermo Fischer Scientific). Early apoptosis was determined with annexin V by flow cytometry through the externalization of phosphatidylserine. A wound-healing assay was performed as previously reported (20). Transmigration was evaluated with type I collagen–coated 8-μm transwells using 50 ng/ml of epidermal growth factor as a chemoattractant (QCM Haptotaxis Cell Migration Assay–Collagen 1, Colorimetric

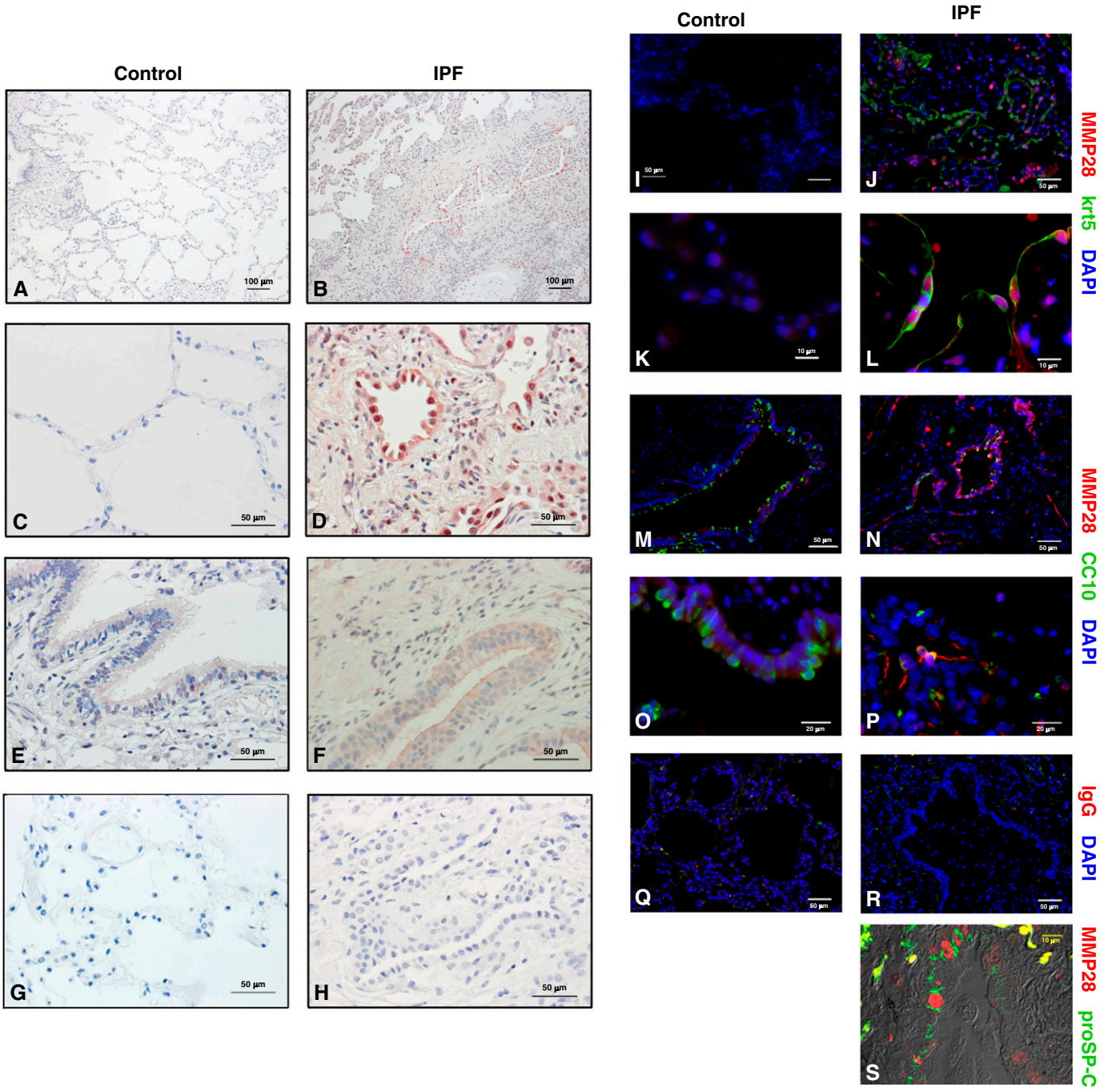

Figure 2. MMP28 is expressed by alveolar and airway epithelial cells in IPF. Left panels: Immunohistochemical localization of MMP28 in control lungs (*A*, *C*, and *E*) and in IPF lungs (*B*, *D*, and *F*). Right panels: MMP28 localization visualized by immunofluorescence in control lungs (*I*, *K*, *M*, and *O*) and IPF lungs (*J*, *L*, *N*, and *P*). For confocal microscopy, krt5 (keratin 5) was used as a marker of epithelial cells, CC10 (club cell 10 kD protein) as a marker of club cells, and DAPI for nuclei. Immunohistochemistry-negative control cells were incubated with no primary antibody (*G* and *H*). Rabbit IgG was used as a negative control in immunofluorescence experiments (*Q* and *R*). (*S*) Cells coexpressing prosurfactant protein C (proSP-C) and MMP28 in a merged image with transmitted light differential interference contrast. Immunohistochemistry experiments: IPF, $n = 8$; Control, $n = 5$. Immunofluorescence experiments: IPF, $n = 5$; Control, $n = 3$. The numbers represent total number of used lung samples. Scale bars: 10 μm (*K*, *L*, and *S*), 20 μm (*O* and *P*), 50 μm (*C*–*J*, *M*, *N*, *Q*, and *R*) , and 100 μm (*A* and *B*)

ECM582; EMD Millipore). *See* the Supplementary Methods section for details.

Murine Pulmonary Fibrosis Model

The generation of the mice is described elsewhere (14). The mice were housed in specific-pathogen-free conditions. All experiments were approved by the ethics committee at INER. Pulmonary fibrosis was induced by a single intratracheal instillation of 10 mg of bleomycin sulfate (Cayman Chemical) in 50 μl of saline solution. Control groups received only the vehicle. Mice were killed at 14 days after instillation. Left lungs were frozen and consigned for hydroxyproline determination. Right lungs were fixed with 4% paraformaldehyde for immunohistochemistry (IHC) and staining with hematoxylin and eosin and Masson's trichrome stains.

Statistics

All experiments were performed three times in triplicate, unless stated otherwise. Results are given as mean ± SD if not mentioned otherwise. ANOVA with Tukey's or Dunnett's adjustment for multiple comparisons was applied. $P < 0.05$ was considered significant. Results were analyzed with Prism version 5 software (GraphPad Software).

Results

MMP28 Is Upregulated in IPF Lungs and Expressed by Epithelial Cells

A dataset derived from previous research suggested that *MMP28* gene expression was increased in IPF lungs compared with control lungs (21). To validate this finding at the gene and protein levels, lung tissue samples from IPF ($n = 7$) and control donors ($n = 6$) were analyzed by qRT-PCR and Western blotting. As illustrated in Figure 1, MMP28 showing bands of approximately 58 kD (proenzyme), approximately 48 kD, and approximately 46 kD, representative of the molecular weight of the active enzyme, was significantly increased in IPF ($P < 0.01$). No significant differences were found by qRT-PCR (data not shown). Cellular localization of MMP28 in IPF and control lungs was examined by performing IHC and immunofluorescence. MMP28 was mostly expressed by AECs in IPF (Figures 2B, 2D, 2J, and 2L), and it was weakly expressed by the normal alveolar epithelium (Figures 2A, 2C, 2I, and 2K).

In addition to a cytoplasmic localization, notable nuclear staining was found in AECs (Figures 2D and 2L). MMP28 was also observed in airway epithelial cells (Figures 2F, 2N, and 2P), which were identified by CC10 (club cell 10 kD protein) expression. In this experiment, the staining was mainly apical and sometimes cytoplasmic.

To confirm the nuclear location of MMP28 in AECs, Z-stacks were obtained using confocal microscopy, which demonstrated that the staining of MMP28 was inside the nucleus in some cells of IPF lungs (Figures E1A and E1B), whereas in others, it was perinuclear (Figure E1C). MMP28 was also observed to be coexpressed with krt5 (keratin 5) in some areas (Figures 2J and 2L). Although krt5 has been described mainly in progenitor airway epithelial cells (22), recent single-cell research performed in IPF lungs has demonstrated that AECs show an aberrant phenotype, displaying both airway and alveolar proteins (23). As shown in Figure E1E, human A549 AECs also express krt5. To further corroborate the nuclear expression by AECs *in vivo*, IPF lungs were costained with SP-C (surfactant protein C) and MMP28

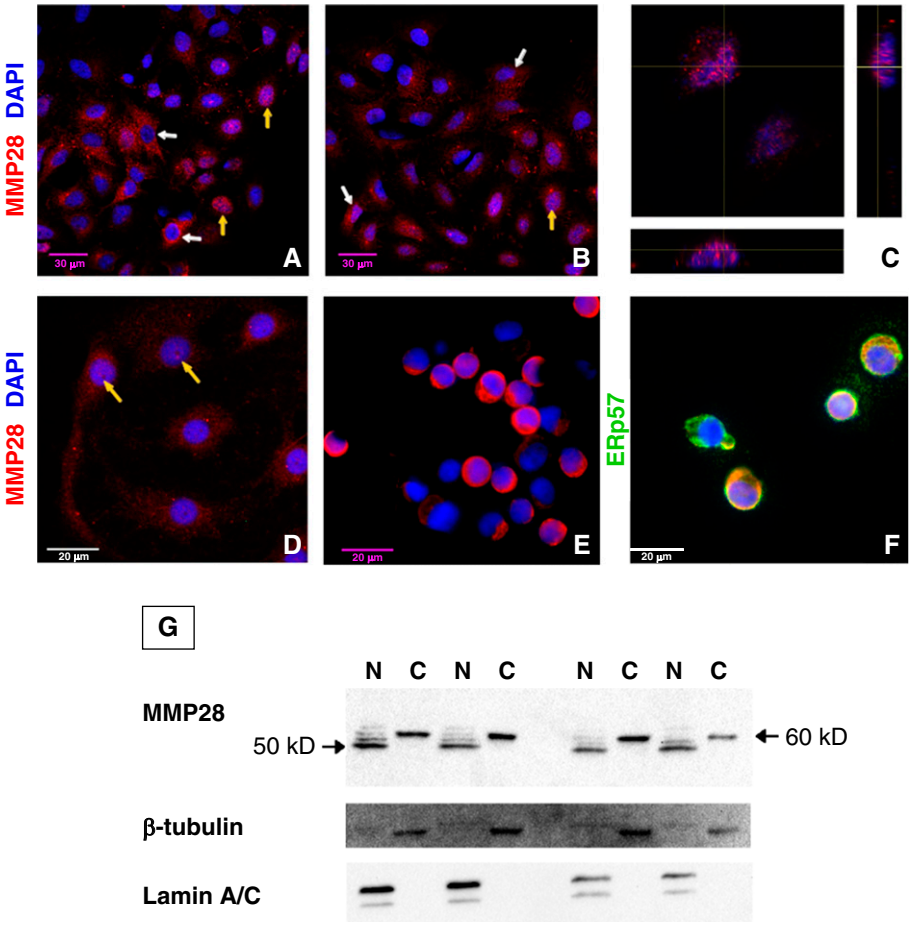

Figure 3. MMP28 localization by immunofluorescence in epithelial cells *in vitro*. Native human alveolar epithelial cells (A549; *A*, *B*, and *C* represent five independent experiments performed in triplicate), primary human alveolar epithelial cells (*D*; two independent experiments), and primary human airway epithelial cells differentiated *in vitro* (*E* and *F*; two independent experiments in duplicate). (*C*) Cytoplasmic localization is indicated by white arrows, and nuclear localization is indicated by yellow arrows. XZ and YZ images from Z-stacks of alveolar epithelial cells validate the nuclear localization of MMP28. (*F*) MMP28 colocalizes with ERp57. (*G*) Western blot of nuclear (N) and cytoplasmic (C) fractions from A549 cells. β-Tubulin and lamin A/C were used as markers of cytoplasmic and nuclear fractions, respectively. These are the results of two independent experiments performed in duplicate. Scale bars: 20 μm (*D*, *E*, and *F*) and 30 μm (*A* and *B*).

and examined by performing immunofluorescence (Figure 2S).

Intracellular Localization of MMP28 in AECs and BECs *In Vitro*

To evaluate whether MMP28 is present in the nucleus of lung epithelial cells *in vitro*, we performed immunofluorescence of fixed A549 and primary AECs as well as in BECs. In the case of the A549 cell line, many of them showed cytoplasmic signaling, but in some cells, clear nuclear localization was observed (Figures 3A and 3B), which was confirmed by the Z-stacks (Figure 3C). Nuclear localization was also observed in some primary human AECs (Figure 3D). In primary human differentiated BECs, MMP28 was detected only in the cytoplasm, colocalizing with the endoplasmic reticulum protein ERp57 (Figures 3E and 3F). This finding correlates with our observations *in vivo* (IHC and immunofluorescence).

To verify the intracellular MMP28 localization in AECs, nuclear and cytoplasmic fractions of A549 native cells were obtained. As shown in Figure 3G, the presence of the enzyme in the nuclear fraction was confirmed. An approximately 48–50 kD band likely representing the active form of the enzyme was revealed in the nuclear compartment, whereas in the cytoplasmic portion, an approximately 60–62 kD band, probably representing the proform, was observed.

Looking for a possible MMP28 nuclear translocation mechanism, we performed an *in silico* analysis using two different software tools (*see* Supplementary Methods section). Both revealed the same likely nuclear localization signal (NLS): 112-RHRTKMRRKKR-122 (prediction cutoff, 0.7). To find out whether the mechanism of entering the nucleus depends on this putative NLS, we performed site-directed mutagenesis to change lysines to glutamines in the NLS (K116Q, K120Q, and K121Q, polar amino acids with similar molecular weight and no charge). A549 cells were transfected either with DDK-tagged MMP28 (WT-MMP28) or with DDK-tagged MMP28 mutant (KQ-MMP28) or empty vector (Mock, PS100001; OriGene).

Cells were analyzed by imaging flow cytometry with an anti-DDK antibody. Figure E2A shows that about 24% of the cells transfected with the KQ-MMP28 presented a nuclear location of the protein with no significant differences from the WT-MMP28–transfected cells (~29%). Likewise, analyzing Mock, WT-MMP28, and KQ-MMP28 cells with anti-MMP28, which binds to the transfected protein as well as to the endogenous protein, we found that around 30% of Mock cells lacking transfected protein showed a nuclear localization of MMP28 that increased to 45% in the transfected cells. Figure E2B illustrates a representative image of Mock, WT-MMP28, and KQ-MMP28 cells with nuclear or cytoplasmic localization of the enzyme.

Effects of Overexpression and Silencing of MMP28 *In Vitro*

To analyze the possible role of MMP28 in AECs and BECs, human A549 AEC, rat AEC, and primary human BECs were used. AECs (A549 and RLE-6TN) were transfected with human WT-MMP28 DDK tag (*MMP28*) (Figures E3A, E3B, E3E, and E3F). Whereas intracellular MMP28 levels were moderately increased, extracellular MMP28 was drastically increased. In addition, MMP28 was silenced in A549 cells using shRNA (*shMMP28*), achieving 80% efficiency at the RNA level and 70% at the protein level in one of the clones (Figures E3C and E3D). Also, MMP28 expression in primary human BECs was transiently decreased with siRNA (*siMMP28*), reaching around 70% of knockdown efficiency from 24 to 72 hours of culture (Figures E3G and E3H).

MMP28 Increases Proliferation and Growth Rate

Overexpression of MMP28 in AECs significantly increased their growth and proliferation rates compared with their controls ($P < 0.001$). By contrast, when

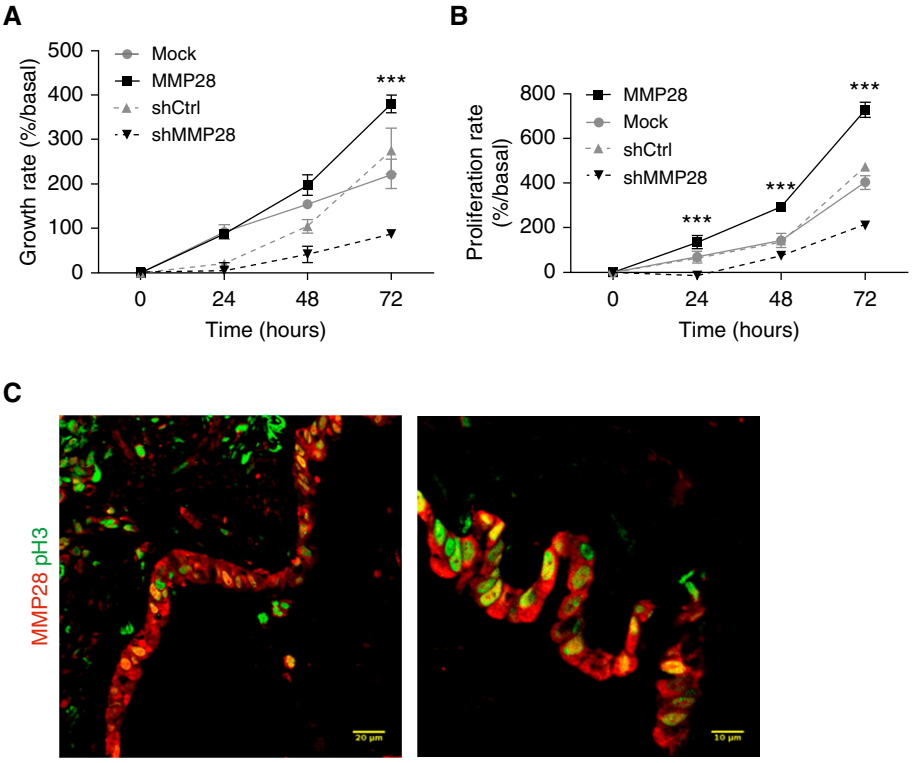

Figure 4. MMP28 induces proliferation *in vitro* and colocalizes with a proliferation biomarker in idiopathic pulmonary fibrosis lungs, protects from apoptosis, and enhances epithelial–mesenchymal transition. MMP28-transfected or MMP28-silenced A549 alveolar epithelial cells were evaluated for (*A*) growth rate and (*B*) proliferation. *A* and *B* show one representative experiment of four independent experiments performed in triplicate (data are presented as mean ± SD; ***$P < 0.001$). (*C*) Epithelial cells coexpressing anti–phospho-histone H3, a proliferation marker, and MMP28. $n = 2$ represent lung samples from two patients. Scale bars: 20 μm and 10 μm. shCtrl = scrambled control shRNA; shMMp28 = MMP-28 silenced A549 alevolar epithelial cells.

MMP28 was silenced, growth and proliferation rates significantly decreased ($P < 0.001$) (Figures 4A and 4B). Similarly, the transfection of human MMP28 to rat AECs significantly increased their growth and proliferation rates compared with Mock ($P < 0.001$) (Figures E4A and E4B). Regarding primary human BECs, when MMP28 was silenced, a significant decrease in growth and proliferation rates was also observed ($P < 0.001$) (Figures E4C and E4D).

We examined whether nuclear MMP28 colocalized with the proliferation biomarker phospho-histone H3 (Ser10, pH3). As illustrated in Figures 4C and 4D, some IPF epithelial cells showed nuclear colocalization of MMP28 and pH3.

MMP28 Protects Epithelial Cells from Apoptosis, Promotes Migration, and Enhances Epithelial–Mesenchymal Transition

Given that the growth rate includes cell proliferation and cell death, the effect of MMP28 on apoptosis was also evaluated using bleomycin as an apoptotic stimulus. As shown in Figure 5A, MMP28 overexpression significantly protected AECs from apoptosis ($P < 0.05$), whereas silencing the enzyme induced an increase in apoptosis ($P < 0.01$).

Cell migration was evaluated by the scratch wound–healing assay. As shown in Figure 5B, at 48 hours, in A549 cells transfected with MMP28, the wound closed 94.5 ± 4%, whereas healing was only 70.7 ± 3% using Mock cells. By contrast, MMP28-silenced cells delayed this process with 52 ± 6.8% of wound extension at 48 hours. There was no significant difference between Mock and shCtrl cells. Likewise, overexpression of MMP28 in rat AECs accelerated wound closing 88.56 ± 3.6% versus 55.15 ± 14.46% in Mock cells at 12 hours (Figure E5A), whereas silencing of MMP28 in BECs delayed wound closing at 18 hours to 40 ± 16% versus 74.7 ± 6.5% obtained with the scrambled RNA–treated cells (Figure E5B). Transmigration of AECs performed in Boyden chambers covered with type I collagen revealed that overexpression of MMP28 increased transmigration in 42 ± 4% ($P < 0.001$), whereas silencing of MMP28 decreased it in 26 ± 10.7% ($P < 0.01$) (Figure 5C).

There have been contradictory results regarding a putative role of MMP28 in epithelial–mesenchymal transition (EMT) (15, 24). We tested this effect on A549 AECs. We observed that the expression of E-cadherin (as an epithelial marker) in MMP28-transfected cells was remarkably decreased compared with Mock-transfected cells under basal conditions, and when cells were stimulated with transforming growth factor (TGF)-β1 for 2 days, this effect was amplified (Figure 5D). The presence of fibronectin as a mesenchymal marker was observed until the fourth day of TGF-β1 stimulation, and with the overexpression of MMP28, the intensity of the fibronectin band increased (Figure 5E).

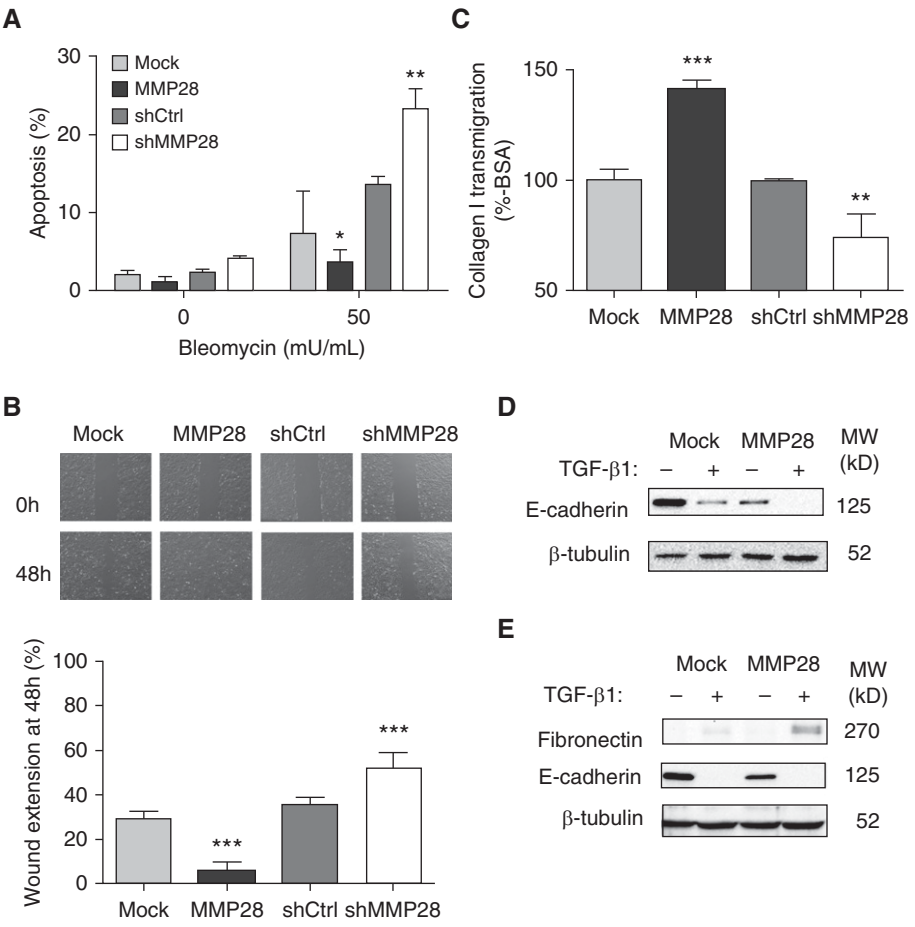

Figure 5. MMP28 protects from apoptosis, promotes migration, and enhances epithelial–mesenchymal transition of A549 alveolar epithelial cells. (*A*) Percentage of apoptosis induced by bleomycin. Results are expressed as mean ± SEM of three independent experiments (*$P < 0.05$; **$P < 0.01$). (*B*) Scratch wound-healing assays with MMP28-transfected or MMP28-silenced A549 cells. Magnification, ×10. The figure shows one representative experiment from four experiments performed in quadruplicate. ***$P < 0.001$. (*C*) Transmigration over type I collagen in Boyden chambers. One representative experiment of two independent experiments performed in triplicate is shown. (*D* and *E*) Mock- and MMP28-transfected cells were stimulated with transforming growth factor (TGF)-β1 for (*D*) 2 days and (*E*) 4 days. E-cadherin was used as a marker of epithelial cells, and fibronectin was used as a marker of mesenchymal cells. Western blots are representative of three independent experiments. MW = molecular weight.

Effects of MMP28 on Migration and Proliferation Are Catalysis Dependent

To test if the functional effects observed were due to the catalytic activity of MMP28, site-directed mutagenesis was performed in accordance with previous reports (15, 24) to change the glutamate in the catalytic site for an alanine (E241A) (*see* the Methods section above). Human AECs and rat AECs were transfected with the EA mutant MMP28, and the experiments evaluating

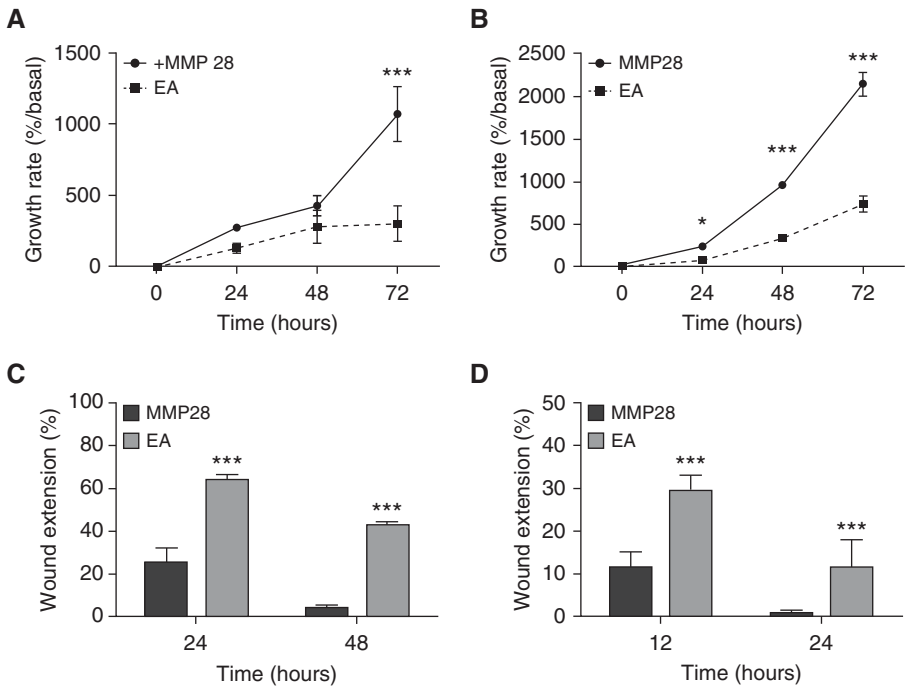

Figure 6. MMP28 effects on migration and proliferation are catalysis dependent. Cells were transfected with wild-type MMP28 (MMP28) or catalytically inactive MMP28 (EA), and proliferation (*A* and *B*) and wound closure (*C* and *D*) were measured. Each figure is representative of three experiments performed in triplicate. Data are presented as mean ± SD; $^{*}P < 0.05$; $^{***}P < 0.001$.

functional effects were repeated. As shown in Figures 6A–6D, the increase in growth rate and the accelerated wound closure observed in cells transfected with WT-MMP28 are not preserved in cells transfected with the EA mutant MMP28. Consequently, these are catalysis-dependent effects of MMP28.

Analysis of Mmp28 in the Murine Model of Pulmonary Fibrosis

As reported before (14), in healthy murine lungs, Mmp28 is expressed by airway epithelial cells but also by some AECs. At 14 days after bleomycin injury, immunoreactive Mmp28 expression was observed in addition to epithelium in intraalveolar macrophages (Figure 7A). After 14 days of bleomycin instillation, we observed a significant increase of Mmp28 at the protein level (Figure 7B).

It has previously been reported that *Mmp*28-deficient mice showed a modest reduction of collagen accumulation at Days 14 and 21 after low-dose bleomycin injury (16). With the usual dose that we used, we corroborated that *Mmp*28$^{-/-}$ mice develop less fibrosis at Day 14 of the fibrotic phase of the model. Figure 7C shows representative images of Masson's trichrome stain, and Figure 7D demonstrates a significantly lower hydroxyproline content in lungs of *Mmp*28-deficient mice.

To confirm our results with epithelial cell lines, we isolated primary lung epithelial cells from WT and *Mmp*28$^{-/-}$ mice and performed transmigration assays in Boyden chambers covered with type I collagen. As shown in Figure 7E, cells from *Mmp28*-deficient mice demonstrated significantly less migration than cells from WT mice.

Discussion

IPF is a complex disorder in which the interaction of aging and multiple genetic and environmental risk factors results in the hyperactivation of the alveolar epithelium, which triggers an aberrant wound-healing response to repetitive lung microinjuries. In this pathological process, a variety of mediators, such as growth factors, cytokines, chemokines, and coagulation factor receptors, are involved (1, 2). In this context, a growing body of evidence supports a critical role of MMPs either releasing or activating growth factors such as TGF-β or actively participating in lung-remodeling processes. Most of them are expressed by the aberrantly activated alveolar epithelium, and they may play profibrotic (e.g., MMP7) or antifibrotic (e.g., MMP19) roles (25, 26).

In this study, we focused on MMP28, the last revealed member of the MMP family. This enzyme has the typical structural domains of the MMPs consisting of a signal peptide at the N-terminus that leads it through the secretory pathway, a prodomain that keeps it as zymogen, a catalytic domain with a Zinc ion, and a hinge region that is adjacent to the four hemopexin-like domains in the C-terminus. Between the prodomain and the catalytic domain, it has the RKKR motif classically recognized by proprotein convertases; however, MMP28 is identified by furin not at this site but at an upstream YGYL motif (specifically at the Y45L46) in the prodomain (27).

In the present study, we examined the expression, localization, and possible functions of MMP28 in IPF, a usually progressive and irreversible epithelium-driven fibrosis. We found that the enzyme is expressed mainly by the lung epithelium, specifically by AECs and BECs. Interestingly, immunohistochemical detection with four different antibodies revealed that, in some AECs, MMP28 localized in the nuclei, a finding that was confirmed with Z-stacks from human tissues and cultured cells. The enzyme was also observed in the nuclei of krt5^{+} (likely) progenitor cells that have recently been found in fibrotic areas of IPF lungs (22), as well as in the nuclei of pro-SP-C–positive cells, a biomarker of type II AECs. By contrast, in BECs, MMP28 staining was mostly apical. It is important to emphasize that in IPF, many lung epithelial cells acquire aberrant, multilineage-like states, and some of them share characteristics of both conducting airway epithelial cells and AECs (23). Moreover, the A549 cell line that was used in this study also expressed krt5. Notably, whereas Mexican IPF biopsies were obtained from patients at the time of diagnosis, German IPF samples were explants obtained during lung transplant, thus representing end-stage disease, indicating that the epithelial

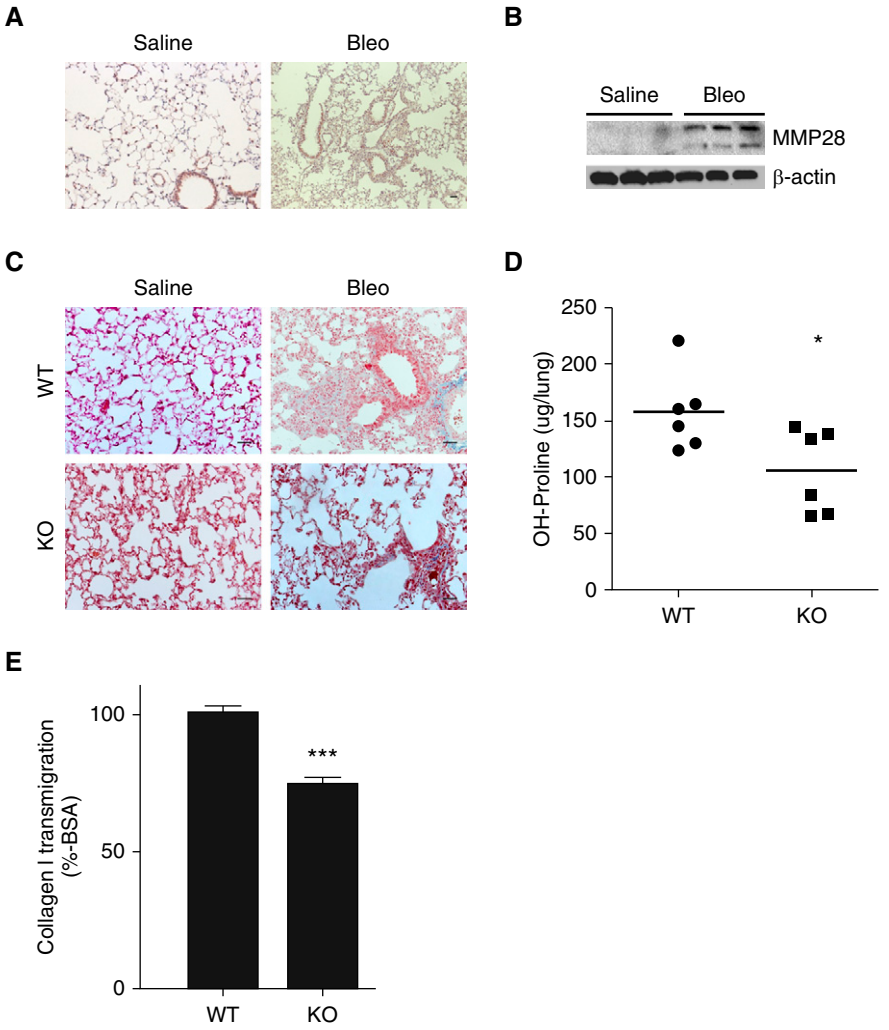

Figure 7. Analysis of Mmp28 in a murine pulmonary fibrosis model. Wild-type (WT) mice after 14 days of saline or bleomycin instillation: (*A*) Mmp28 immunohistochemistry; (*B*) Mmp28 Western blot. WT or Mmp28-deficient mice (KO) after 14 days of saline or bleomycin instillation: (*C*) Masson's trichrome stain; (*D*) OH-proline quantification; (*E*) transmigration of lung epithelial enriched fraction over type I collagen in Boyden chambers. Two independent experiments with 6 animals in each experiment (*$P < 0.05$; ***$P < 0.001$). Scale bars: 50 μm (*A* and *C*). Bleo = bleomycin.

expression of this enzyme is maintained during the progression of the disease.

The roles of MMPs have been described mostly in the extracellular environment; however, some of them have previously been found in intracellular compartments, such as nuclei and mitochondria (20, 28–34). Specifically, nuclear localization has been shown for MMP2, MMP3, MMP12, and MMP14, where these proteins have been proposed to have certain roles as transcriptional (co) factors or as apoptosis-related proteases (35–41). Nevertheless, a nuclear localization of MMP28 has not been reported before.

There are some studies showing that nuclear translocation of MMPs may be dependent on new NLSs found in their sequences (35, 36, 40). In the case of MMP28, a putative NLS was found between the prodomain and the catalytic domain. Nevertheless, changing the lysines for glutamines in the putative NLS did not modify the localization of the enzyme, suggesting that this sequence is not a real NLS; therefore, MMP28 nuclear translocation deserves further study.

The putative role of epithelial expression of MMP28 in IPF is currently unclear. By conducting gain-and-loss experiments, we found that the enzyme increases epithelial growth rate, proliferation, and migration. Interestingly, we found that, in IPF lungs, epithelial nuclear MMP28 colocalizes with the marker of proliferation phospho-histone H3, suggesting that it might be related to a proliferative phenotype, one of the features described in a subset of IPF epithelial cells. In addition, we confirmed that MMP28 confers resistance to apoptosis, as previously reported (15).

These functions were lost when the catalytic domain was affected by site-directed mutagenesis changing the glutamate in the catalytic site for an alanine. This finding supports the hypothesis that the catalytic activity of MMP28 promotes an invasive phenotype in AECs, as suggested previously (24). In fact, in gastric carcinoma and in several gastric cancer cell lines, MMP28 is increased and correlates with a more invasive phenotype (13). Regarding the role of MMP28 in EMT, contradictory results have been documented (15, 24). Our results indicate that this enzyme enhanced this process, and interestingly, we found that whereas the epithelial marker E-cadherin was modified earlier, the mesenchymal marker fibronectin appeared only after 4 days of TGF-β stimulation in the MMP28-transfected cells.

The finding that the increase in proliferation and wound closure occurred not only in AECs but also in BECs suggests that these effects are not related only to nuclear MMP28. Future approaches are necessary to identify protein-binding partners and/or target DNA sequences and should include high-throughput proteomics to analyze substrates as well as the signaling pathways of the effects we have described.

Taken together, our findings indicate that MMP28 is expressed in IPF lungs mainly by epithelial cells enhancing survival, proliferation/migration, and EMT. Some of these processes characterize the aberrantly activated epithelial cells in IPF. However, the pathological result of these effects *in vivo* is currently unclear.

MMP28 has been described as profibrotic in the bleomycin-induced lung injury model (16). Recently, MMP28 has also been implicated in the pathogenesis of tobacco smoke–induced emphysema (17). In both experimental murine models, Mmp28 was expressed primarily by lung macrophages. We found that Mmp28 was also expressed by AECs and corroborated

the profibrotic role in the model. Moreover, we found that Mmp28$^{-/-}$ lung epithelial cells displayed a less migratory phenotype than WT cells.

In conclusion, our study demonstrates the upregulation of MMP28 in IPF lungs, where it is expressed in BECs (apical and cytoplasmic localization) and AECs (cytoplasmic and nuclear localization) in two different IPF groups of patients. *In vitro* MMP28 protects lung epithelial cells from apoptosis, increases proliferation and migration, and enhances EMT in a catalysis-dependent manner. ■

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