

# **Membrane-anchored serine protease matriptase is a trigger of pulmonary fibrogenesis**

## **Supplementary information**

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## **Supplemental methods**

### **Human Tissues**

Lung tissue was obtained from 61 patients with IPF (8 females, 53 males; mean age  $57.6 \pm 7.5$  years), and 43 control subjects (patients undergoing lung surgery for removal of a primary lung tumor; mean age  $62.5 \pm 13.8$ , 17 females, 26 males). Control tissues were derived from normal lung sample, obtained from a noninvolved segment, remote from the solitary tumor lesion, as described previously. Normalcy of control lungs was verified histologically (1, 2). Primary fibroblasts were isolated as described in (3). The study protocol was approved by the institutional Ethics committee (comité d'éthique du CEERB Paris Nord, biobank registration number DC 2009-940).

### **Isolation of primary IPF human alveolar epithelial cells (phATII) and IPF fibroblasts (German cohort)**

Isolation of primary human alveolar epithelial cells was conducted as previously described (4) with some modifications. In brief, tissue specimens were manually minced and digested with a dispase/collagenase mixture (Roche) for 2h at 37°C. Samples were subsequently filtered through nylon meshes and the single cell suspension was centrifuged at 400 g, 4°C for 10 minutes. The cell pellet was resuspended in DMEM/F12 Medium and then layered onto a discontinuous Percoll density gradient (1.04– 1.09 g/ml) and centrifuged at 300 g for 20 minutes. Cells in the interphase representing macrophages and alveolar epithelial cells were recovered and depleted for Macrophages using CD45 specific magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Collected cell suspension was centrifuged and the cell pellet was snapped frozen in liquid nitrogen.

### **Isolation and culture of primary human lung fibroblasts (phFB)**

Isolation of primary human lung fibroblasts was performed as previously described in (5). In brief, human lung tissue was digested using 1mg/ml Collagenase I (Biochrom, Cambridge, UK) for 2 hours at 37 °C. Samples were filtered through nylon meshes to obtain a single cell suspension, which was subsequently centrifuged at 400 g, 4°C for 5 minutes. Cell pellets were resuspended in DMEM/F-12 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (Pan Biotech, Aidenbach, Germany) and

Penicillin/Streptomycin (Life Technologies). Cells were cultured on 10 cm cell-culture dishes with a media change at every other day.

### **Antibodies and Reagents**

The following antibodies were used in this study: PAR-2 (SAM11), Matriptase (sc-25234), CD-45 (sc-1178, all from Santa Cruz Technology, Santa Cruz, CA, USA), Matriptase (MAB-3946, R&D Systems, Minneapolis, MN), Fibronectin (ab2413, Abcam, Cambridge, UK),  $\alpha$ -SMA (091m4832, Sigma-Aldrich, St. Louis, MO), Collagen type I (1310-01, Southern Biotech, Birmingham, AL),  $\beta$ -tubulin (ab6046, Abcam, Cambridge, UK), GAPDH (MAB90009, Covalab, Cambridge, UK), ABCA3 (WMAB-ABCA3-13, Seven Hills Bioreagents, Cincinnati, Ohio, USA), phospho-ERK1/2, phospho-Akt and phospho-Smad 2 (#9106s, #9271s and #3101s respectively), all from Cell Signaling Technology (Beverly, MA). The PAR-2 antagonist GB-88 was provided by provided by the University of Queensland (Brisbane, Australia). Camostat mesilate (#3193) was purchased from Tocris (Bristol, UK), and human recombinant protein Matriptase (3946-SE-010) from R&D Systems. Thrombin (T7009;  $\geq 1000$  NIH Units/mg) was from Sigma (Sigma-Aldrich, St. Louis, MO). The different approaches to inhibit matriptase/PAR-2 interactions are summarized in supplemental figure E2.

### **RNA isolation and quantitative reverse transcription polymerase chain reaction.**

Total RNA was extracted using NucleoSpin1 RNA II extraction kit (Macherey Nagel, Hoerd, France) according to the manufacturer's protocol, and cDNAs were generated by reverse transcription using SuperScript II (Invitrogen). To quantify mRNA expression of the indicated genes, quantitative PCR was performed using fluorogenic SYBR Green with specific primers from GenScript and the Sequence Detection System 7700 (Applied Biosystems). Transcripts of Ubiquitin (UBC) for human and Beta2-Microglobulin (B2M) for mouse were used as reference genes in all qRT-PCR reactions. Results were expressed in fold changes compared to control, subsequently to normalization to the reference gene

### **Western Blot**

Samples (cells washed three times with ice cold PBS, or pulverized lungs) were lysed in Laemmli lysis buffer, incubated for 5 minutes at 95°C, and whole lysates were separated by 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to an immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were incubated overnight at 4°C with the indicated primary antibodies. All secondary antibodies were horseradish peroxidase-conjugated from DakoCytomation (Glostrup, Denmark). Blots were imaged using Clarity substrate from Roche (Basel, Switzerland) on an imager Pxi (Syngene, Cambridge, UK). For quantification, densitometry was performed using ImageJ software. Briefly, raw volumes corresponding to the histogram function of the band corresponding to the protein of interest were corrected for those of the loading control. Data are expressed as mean  $\pm$  SEM from (at least) three independent experiments.

### **Immunohistochemistry**

For immunohistochemistry, 4- $\mu$ m (murine) or 5- $\mu$ m (human) sections were first deparaffinized and rehydrated. Heat-induced epitope antigen retrieval was performed using 10 mmol/L citrate buffer pH 6.0, for 40 minutes at 98°C for human matriptase staining. Subsequently, for all stainings, endogenous peroxidase activity was quenched with 0.3% peroxidase blocking reagent (S2023, Dako Denmark, Glostrup, Denmark). anti-aSMA-1 clone 1A4 antibody at 1:4200 (1 hour at room temperature, Sigma-Aldrich, St. Louis, MO), with Histofin mouse stain kit (414322F, Nichirei Biosciences Inc, Tokyo, Japan), using diaminobenzidine staining (K3468, Dako North America Inc, Carpinteria, CA, USA). For human matriptase staining, normal horse serum (S-2000, Vector Laboratories Inc, Burlingame, CA, USA) and BSA 3% were used to block phosphatase activity. Human anti-matriptase primary antibody (Santacruz biotechnology) was used at 1:80 dilution (o/n 4°C), and anti-goat secondary antibody (Vector Laboratories Inc) was applied at 1:300 (30 minutes at room temperature). Phosphatase alkaline stain with Alkaline Phosphatase Standard (AK-5000, Vector Laboratories Inc) and Dako Liquid Permanent Red (K0640, Dako North America Inc) were used to reveal human matriptase. For the isotype control, similar procedures were applied, only the first antibody was omitted. Slides were photographed on a Leica DM4000B with a Leica DFC420 camera (Leica Microsystems GmbH, Wetzlar, Germany).

### **Fluorogenic substrate cleavage assay.**

Matriptase and thrombin activities were evaluated following the hydrolysis of the fluorogenic peptide substrates Boc-QAR-AMC and Boc-VPR-MCA (both R&D systems). Briefly, 10 nM enzyme was mixed with 100  $\mu$ l of peptide substrate (100 $\mu$ M) in a reaction buffer according to the manufacturer's recommendations. The release of fluorescence resulting from hydrolysis of the peptide substrate was monitored on a microplate reader (Varioscan, Thermo Fisher Scientific Inc, Waltham, MA, USA) for 300 min at 37°C with excitation at 360 nm and emission at 480 nm.

### **Cell Culture**

Human pulmonary epithelial cells A549 (CCL-185) were purchased from American Type Culture Collection (ATCC), Rockville, MD. Normal human lung fibroblast (NHLF, CC-2512) were obtained from Lonza (Rockland, ME, USA). Cells were maintained in Dulbecco's modified Eagle's medium or RPMI (A549 cells) supplemented with 10% fetal calf serum and antibiotics, and passed according to routine procedures. Primary pulmonary fibroblasts were used between passage 3-7. Unless stated otherwise, cells were washed twice with PBS, serum-starved 18h and subsequently stimulated as described.

### **Proliferation Assay**

Cells seeded at a density of  $10^4/\text{cm}^2$  in 96-well plates in 100  $\mu$ l DMEM supplemented with 1% FCS, were (if indicated) pretreated with CM (5  $\mu$ mol/L), or PAR-2 antagonist GB-88 (10  $\mu$ mol/L), and subsequently incubated with matriptase, or PBS as a control. Cell proliferation was determined at the indicated intervals using a 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) assay (Takara, Shiga, Japan), as described in (6, 7).

### **Cell migration**

Cell migration was assayed using a modified Boyden chamber technique (Transwell analysis). 1% serum-containing medium containing PBS as a negative control, 1 nM matriptase, and (if indicated) CM (5  $\mu$ mol/L), or PAR-2 antagonist GB-88 (10  $\mu$ mol/L), was added into the lower chamber as a chemoattractant, and cells ( $5 \times 10^4$ ) were plated in the upper chamber and allowed to migrate for 24 h through 8 $\mu$ m pore filters (Thin Certs-TC inserts, Greiner Bio-one; Brockville, ON). Non-migrating cells were removed from the upper chamber with a cotton swab, filters were stained with Diff-Quik stain, and migrating cells adherent to the underside

of the filter were enumerated using an ocular micrometer and the total number of cells migrated was counted. Data are presented as relative migration compared to control, and represent mean $\pm$ SEM of 4 different experiments.

## **Animals**

C57BL/6N mice were purchased from Janvier Labs (Le Genest Saint Isle, France). When indicated, 0.5 mg camostat mesilate (CM) treatment was initiated 7 days after bleomycin challenge, and administrated intranasally every other day from day 7 until day 14. St14 hypomorphic mice were generated by Pr Thomas Bugge and have been described previously(8-10). Briefly, these mice possess one null allele and one allele in which a reporter gene trap is inserted into the matriptase locus and disrupts gene expression. A low level of alternative splicing in the gene trap allele results in low level synthesis of full length matriptase, which is sufficient to enable mouse survival (9). All experiments performed with St14 hypomorphic mice were littermate controlled from mice generated from heterozygous crosses (8). For all experiments, adult mice (8-10 week-old) received intratracheally bleomycin hydrochloride as previously described (11). Mice referred to as controls received the same volume of sterile phosphate-buffered saline. Mice were sacrificed at the indicated time points after bleomycin challenge. Lung tissues excision for histological analyses, bronchoalveolar lavage (BAL) and BAL cell count was performed as previously described (11). All experiments were performed in compliance with the Institutional Standards for Use of Laboratory Animals.

## **Histopathological Assessment of Pulmonary Fibrosis**

Following fixation, entire mouse lungs were embedded in paraffin. Four-micron thick sections were stained with H&E,  $\alpha$ -actin and Masson's trichrome, according to routine procedures. Different systems were used to assess fibrosis. To prevent observer bias, all histological specimens were randomly numbered and interpreted in a blinded fashion. For each scoring, between 5 and 10 fields in lung lobes (depending on the size and the homogeneity of the histological changes) were examined using light microscopy ( $\times 200$  magnification). Fields were examined to cover each entire lobe and were discarded if nonrepresentative areas or if they included large airways and vessels as described in (12). The mean score for each lobe

was expressed as the average of scores determined in each field. The severity of the lesions was determined by using the Ashcroft scoring system(13). Briefly, each field examined was assessed individually for the severity of fibrotic changes and allotted a score from 0 (normal) to 8 (total fibrosis) using a predetermined scale of severity (numerical fibrotic scale). After examination of the whole section, the mean of the scores from all fields was taken as the fibrotic score. Semi quantitative assessment of  $\alpha$ -actin and Masson's trichrome was performed by grading the stained slides in a blinded fashion as follows:  $\alpha$ -actin was graded on a 0 to 4 scale as follows : 0 = absent staining, 1 = weak staining with focal distribution, 2 = moderate with focal distribution, 3 = strong with focal distribution or weak and diffuse, and 4 = strong and diffuse(14). Masson's trichrome staining was graded on a 0 to 3 as in (15): grade 0, normal lung; grade 1, minimal lesion (lesion area <20%); grade 2, moderate lesion (lesion area, 20–50%); or grade 3, severe lesion (lesion area >50%).

### **Quantification of secreted collagen**

For quantification of total secreted collagen, the Sircol assay was performed according to manufacturer's instructions (Biocolor, Carrickfergus, UK). The Sircol assay was used without incorporating the recent modified method with a pepsin digestion step described by Lareu and colleagues (16). More specifically, according to the manufacturer's recommendation for soft tissue handling, to minimize background due to the interference of serum proteins - especially albumin in cell culture medium- with the Sircol reagent, the lungs were flushed before excision, then lung samples were further diced and washed in PBS to clear blood

## Supplemental Figure Legends

### **Figure E1: Expression and activity of matriptase in the bronchoalveolar lavage (BAL) of controls and IPF**

(A) Immunoblot analysis of Matriptase expression in (BAL) of controls (lanes 1-4) or patients (IPF, lanes 5-8). (B) Relative proteolytic activity of Matriptase determined in IPF (n=9, black bars) and control (n=7, white bars), as determined by the cleavage of the Matriptase highly selective fluorogenic substrate t-butyloxycarbonyl Boc-Gln-Ala-Arg-MCA (Boc-QAR-AMC) at 300 minutes. Results presented are expressed as percentage of fluorescence in control BALs, and represent the mean  $\pm$  SEM of two independent experiments. \*P<0.05.

### **Figure E2: Summary of the approaches used in the present study to inhibit Matriptase/PAR2 interactions in vitro and/or in vivo.**

**Figure E3: Effect of camostat mesilate on the enzymatic activity of matriptase and thrombin.** (A) Recombinant human Matriptase (10nM) was incubated with 0-10  $\mu$ M camostat mesilate (CM). Subsequently, activity as determined by the cleavage of the fluorogenic substrate Boc-QAR-AMC was measured as described in the Supplemental methods section. Values are representative of an experiment, which was repeated three times. Values are expressed as mean  $\pm$ SEM fold increase over vehicle. Shown is a. \*p<0.05 vs vehicle. Cleavage of the fluorogenic substrates Boc-VPR-AMC (B) and Boc-QAR-AMC (C) by matriptase, or thrombin, in the absence (vehicle, white bars) or in the presence (black bars) of CM. Datas are expressed as mean  $\pm$ SEM RFUs of independent experiments (n $\geq$ 3). \*\*p<0.005, \*\*\*p<0.001 versus Matriptase+vehicle.



## Supplemental References

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