Online Supplement

Coagulation factor XII may drive idiopathic pulmonary fibrosis by inducing pro-fibrotic changes in lung fibroblasts

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Detailed Experimental Protocols

Patients/healthy donors

Consented IPF patients (n=27), diagnosed at a lung fibrosis multi-disciplinary team (MDT) meeting, provided longitudinal blood samples at time intervals of 3 months (maximum 6 visits total per patient). Of those patients, 12 were on antifibrotic drugs (Nindetanib or Pirfenidone). Four patients received Lebrikuzumab, which was shown not have anti-fibrotic effects, as part of a phase 3 trial. Age and gender-matched healthy controls (n=35) were obtained with consent from Skin and Cancer Foundation (Melbourne, Australia). Lung tissue obtained from IPF patients (n = 8, not on antifibrotic drugs) undergoing transplantation or from deceased organ donors whose lungs were deemed unsuitable for lung transplantation but whose families consented to their use for research (n = 4)(supported by the National Health and Medical Research Council (NHMRC) Centre for Research Excellence in Pulmonary Fibrosis). Disease progression was defined as a decline in FVC% predicted of more than 10% or death over the 12-month study period. Patient demographic information is found in Table 1.

Isolation of HLF and Cell Culture

Human parenchymal fibroblasts were isolated from distal lung tissue. Tissue from distal parenchyma was minced into 1-2 mm^3 pieces and placed into sterile RPMI and centrifuged for 5 minutes at 1000 rpm. After aspiration of the supernatant, the tissue pellet was resuspended in media containing 10% (vol/vol) fetal bovine serum (FBS) with 2% Penicillin-Streptomycin (Invitrogen) and 2.5 µg/mL plasmocin (Invivogen) in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) and plated into tissue culture grade plastic flasks (Nunc). After establishment, cells are maintained in 10% FBS/1% Penicillin-Streptomycin/2.5 µg/mL plasmocin/DMEM. All cultures are routinely tested for mycoplasma contamination using Myco Alert (Lonza).

For all experiments, primary cell cultures at less than 7 passages are used. All cells are cultured in a 37°C incubator with 5% CO_2.

In vitro cell stimulation

Primary non-diseased and IPF fibroblasts (n=6) were plated in 24-well plates or 6-well plates (10^4 cells/cm^2/well) and allowed to reach 80-90% confluency for 3 days. Cells were then quiesced for 24hrs in serum-free media (SFM) media. For antibody inhibition assays, activated FXII (FXIIa-β) at 333 nM (10 µg/mL) was pre-incubated with anti-FXII antibody (CSL312) in SFM for 30 mins at RT. The complex was then added to primary non-fibrotic and IPF lung fibroblasts for 6, 24 or 48 hours.

Immunohistochemistry

From formalin-fixed paraffin-embedded tissue blocks, 4μm thick paraffin sections were immunohistochemically stained for FXII (pan, Abcam, UK). Antigens of interest include IL-6 (Abcam, UK) and α-SMA (Abcam, UK). Antigen retrieval was performed in pH 6 citrate buffer (Sigma, Australia). After incubation with DakoEnVision secondary reagents (Dako, Australia), positive staining was visualized using diaminobenzidine (Dako, Australia). Sections were counterstained in Mayer's hemotoxylin (Sigma, Australia).

Sections were scanned using an Aperio Scanscope AT Turbo (Leica Biosystems, Australia) and images were captured at a resolution of 0.25 μm/pixel.

Enzyme-linked Immunosorbent Assay (ELISA)

Culture supernatant IL-6 levels were determined by Human IL-6 Duoset ELISA Kit, as per manufacturer's instructions (RnD Systems, Minneapolis, MN, USA). IL-6 plasma levels were determined by High sensitivity Human IL-6 ELISA Kit, as per manufacturer's instructions (RnD Systems, Minneapolis, MN, USA). IPF patient or healthy control plasma was collected and FXII levels determined by Human FXII ELISA Kit as per manufacturer's instructions (Abcam, Cambridge, UK).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Cellular RNA and blood cell RNA were isolated using Rneasy Plus Micro Kit and the Preanalytix PAX gene blood RNA Isolation Kit as per manufacturer's instructions (Qiagen, Hilden, Germany). cDNA was reversed transcribed from RNA using iScript Reverse Transcription Supermix for RT-qPCR, as per manufacturer's instructions (Biorad, Hercules, CA, USA). Taqman gene expression assays were performed using the following probes: IL-6 (Hs00164004_m1), GAPDH (Hs99999905_m1), OS9 (Hs00907099_g1) and COPS5 (Hs00272789_m1) and RT-PCR were performed as previously described following Taqman gene expression assays (Thermofisher Scientific, Waltham, MA, USA). Results were analysed using the LinReg software Version 2.1 (Academic Research Centre, Amsterdam, Netherlands) and qBase+ software Version 3.0 (Biogazelle, Zwijnaarde, Belgium). Data are expressed as relative quantities for the experimental gene of interest normalised to the housekeeping genes (GAPDH, OS9 and COPS5) and present as fold change relative to control (serum free media).

Western Blotting

FXII and GAPDH (both Abcam, UK) were immune-detected in lysates of lung tissue. Lysates were subjected to electrophoresis and electro-blotting using the Bio-Rad TurboBlot system. Each blot was probed simultaneously with the primary antibodies of interest, anti-FXII and

GAPDH. Following co-incubation with anti-mouse 790 nm or anti-goat 790 nm and anti-rabbit 680 nm IgG (Abcam, UK), immunoblots were imaged by infrared detection using an Odyssey scanner (Li-Cor, USA). Quantification was performed using Image Studio Version 4 (Li-cor, USA).

Migration Assay

Primary lung fibroblasts were plated in Oris™ 96-well migration plates (Platypus Technologies, WI, USA) at $6x10^3$cells/well (in quadruplicate). Cells were allowed to reach confluency, around the 2 mm silicone stopper (zone of exclusion), at the center of each well, in complete DMEM media at 37°C 5% CO_2. After 3.5 days, cells were quiesced in fresh SFM for 18 hours. Silicon inserts were removed and 333 nM FXIIa-β was added with increasing amount of CSL312 (6, 66, 666 nM) (0.1ml/well). As a positive control, 2% FCS-containing DMEM media was added (0.1ml/well). Cells were allowed to migrate for 72 hours, 37°C, 5% CO_2. Media was then aspirated carefully and washed in warm SFM. Cells were then stained in 2.5 μM Cell Tracker™ Red CMPTX dye (Thermo Fisher Scientific, MA, USA) and 10μg/ml Hoescht 33342 (0.1ml/well) in SFM, 30mins, 37°C, 5%CO_2. Stains were then removed by careful aspiration and re-suspended in warm SFM (0.1ml/well)

Image Analysis and Migration Quantification

Cell migration was imaged on the temperature/CO_2 controlled ImageXpress Micro XLS Widefield High content analysis system (Molecular Devices, CA, USA). Image acquisition was done at one time point (72hrs) on a 4.66 mega pixel scientific CMOS camera coupled with a Nikon Plan Fluor 10X/0.3 objective. 562/40: 624/40 excitation: emission filters were used for Cell Tracker™ Red CMPTX fluorescence (cytoplasm staining). 377/50:477/60 excitation: emission filters were used for Hoechst 33342 fluorescence (nuclei staining). 9 sites per well were imaged with a total of 4.2 x4.2mm image spread. Following image acquisition, images were stitched using the MetaXpress software. Data was then further analysed on Fiji[11], ImageJ based analysis software. Migration into the void area left by the silicone insert was measured by two parameters: 1) nuclei count and 2) area covered. Nuclei count was measured using an ImageJ macro script encompassing isolation of nuclei staining from the background using 'Huang' autothreshold method. Separation of adjoined nuclei is done by watershed algorithm. To exclude the cells outside the original 2mm diameter wound, a circular selection of 2 mm diameter is defined and placed on the wound centre. A particle count of the nuclei confined within the selection is performed using 84.5 – 845 μm^2 size restraint and exclusion of nuclei on the boundary edge.

Area covered was measured using an ImageJ macro script encompassing isolation of cytoplasm staining from background using 'Huang' autothreshold method. To exclude the

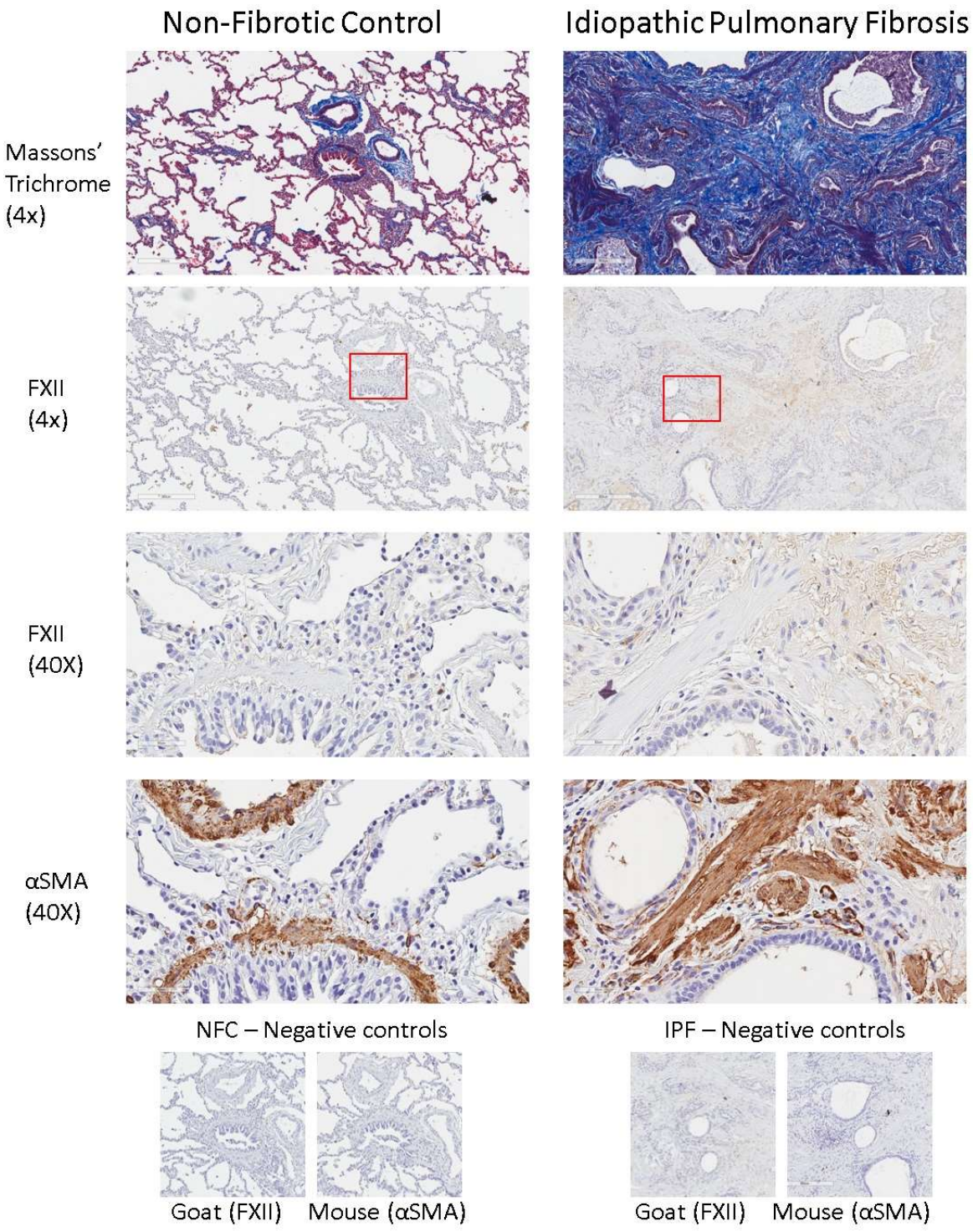
cells outside the original 2 mm diameter wound, a circular selection of 2 mm diameter is defined and placed on the wound centre. To define the area covered within the 2 mm diameter wound, the percentage of pixels above threshold in the selection (area fraction) was measured.

Statistical Analyses

One-way and two-way repeated measures analysis of variance (ANOVA) was used with Dunnetts or Tukey's post test comparisons as appropriate on the Prism Software Version 7 (GraphPad Software Inc., La Jolla, CA, USA). Correlations between basal gene levels in fibroblasts were determined by Spearman's rho (Version 7, GraphPad Software Inc., La Jolla, CA, USA). For plasma measurements, Mann Whitney U-test was performed for statistical analysis with significance noted for $P<0.05$.

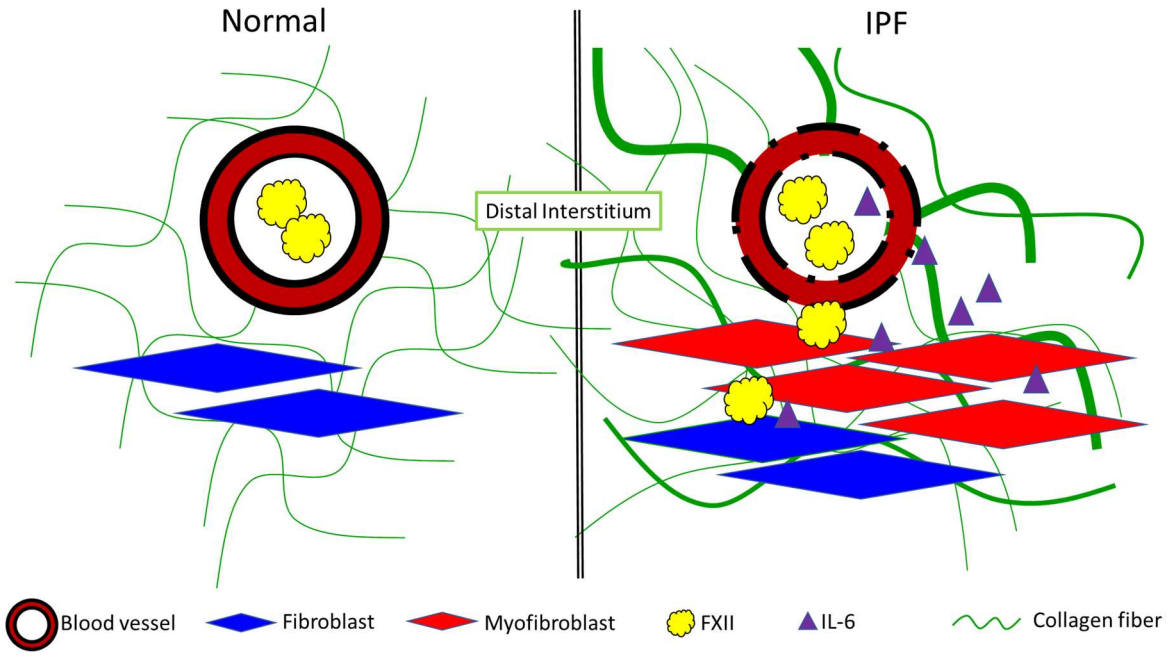
Supplement Table 1. Patient Demographics of patients in this study

Number of patients with IPF in this study = 35	
Age, years (SD)	66 (7.4)
Smoking History, n (%)	19 (54%)
Forced Vital Capacity (FVC), % predicted (SD)	59 (16.0)
Transfer Factor of Carbon Monoxide (TLCO), % predicted (SD)	39 (18.0)
Six Minute Walk Test Distance (6MWT), meters (SD)	452 (128.2)



Supplement Figure 1. FXII localisation in human lung tissue

Representative images of lung tissue obtained from a Non-fibrotic control (NFC, n=3) donor and patient with idiopathic pulmonary fibrosis (IPF, n=3) stained for collagen (blue) using Masson's trichrome, and for FXII (goat antibody), α-smooth muscle actin (αSMA, mouse antibody) (both brown) by immunohistochemistry and counterstained with Meyer's hematoxylin. Red box indicates subsequent areas of higher magnification.



Supplementary Figure 2. Schematic showing the potential role of FXII in IPF

FXII is not normally expressed in the normal lung interstitial tissue. In IPF, circulating FXII "leaks" into the distal lung interstitium due to repeated lung injury and resulting elevated vascular permeability. FXII induces IL-6 production in lung fibroblasts which can drive chronic inflammation and fibrosis.