

## **Online supplemental Materials and Methods**

### *Human samples*

All sera and tissue were collected with patient consent in compliance with the Research Ethics Board of St. Joseph's Healthcare Hamilton. Hamilton Integrated Research Ethics Board (HIREB #00-1839) approval was obtained prior to beginning the study. Aged matched healthy volunteers were used as controls. Peripheral blood was collected from healthy volunteers or confirmed IPF patients and sera were aliquoted and stored at -80°C. The biopsies analysed in this study revealed a usual interstitial pneumonia (UIP) pattern on histopathology. Non-fibrotic human lungs were used as control. Following biopsy, all tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Patients were classified as “moderate” or “advanced” based on pulmonary function tests and a FVC threshold of 50–55% predicted was used to separate moderate patients from those with severe disease as previously described [1].

### *Antibodies and reagents*

Antibodies are  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (ab7817, Abcam), pSmad3 (ab51451, Abcam), Smad3 (ab40854, Abcam), VEGF (ab1316, Abcam), Cleaved caspase-3 (#9661, cell signaling), ET-1 (ab117757, Abcam), CD31 (sc-1506, Santacruz biotech.), ETRA (ab117521, Abcam), ETRB (ab117529, Abcam), GAPDH (#5174, Cell signaling technology). Anti-rabbit HRP linked IgG (#7074, Cell Signaling Technology), Anti-mouse IgG HRP-linked Antibody (#7076, Cell Signaling Technology). For fluorescence microscopy, we used goat or donkey secondary antibody conjugated with Alexa Fluor-488 and Alexa Fluor-555 (Abcam). Human rET-1 (100-21, PerproTech) and human rTGF- $\beta$ 1 (240-B, R&D systems).

### *Cell culture*

Human derived normal primary pulmonary artery smooth muscle cells (ATCC, PCS-100-023) were grown in Vascular Cell Basal Medium (ATCC, PCS-100-030) with Vascular Smooth Muscle Cell Growth Kit components (ATCC, PCS-100-042). Human derived normal primary pulmonary artery endothelial cells (ATCC, PCS-100-022) were grown in Vascular Cell Basal Medium (ATCC, PCS-100-030) with the Endothelial Cell Growth Kit-BBE (ATCC, PCS-100-040). Fibroblast cells were obtained from humans during surgical biopsy (control and IPF) and grown in RPMI medium (ATCC, 30-2001) supplemented with 10% FBS and 1% pen-strep (ATCC). All cells were incubated at 37°C, 5% CO<sup>2</sup> and grown in T75 Falcon flasks. All cells were used at passages between P2 and P8.

### *Animal Experiments*

Pulmonary fibrosis was induced by an adenoviral gene vector encoding biologically active TGF- $\beta$ 1 (AdTGF- $\beta$ 1). Female Sprague-Dawley rats (225–250 g; Charles River, Wilmington, MA) received  $5.0 \times 10^8$  PFU of AdTGF- $\beta$ 1 by single intratracheal instillation under isoflurane anesthesia at D0. Control animals received an empty vector construct (AdDL). Rats received either macitentan (Actelion pharmaceuticals Ltd., Switzerland), pirfenidone (Chemcia Scientific, USA) either a combination of both (n=6 animal per group). Pirfenidone (0.5% food admix, ad libitum), macitentan (daily gavage 100 mg/kg/d) and corresponding vehicles (Gelatin 7.5% in water) were given from day 14 to day 28. Rats were sacrificed at day 14, 21 or 28 and bronchoalveolar lavage (BALF), blood and lung tissue was harvested. Before sacrifice, rats were anesthetised with ketamine/xylazine (Xylazine (Bayer Healthcare, 10 mg/kg) and ketamine (150 mg/kg)) and a plastic catheter (PE tubing, SP0109, ADInstruments Inc, USA) was introduced in the jugular vein of the rats up to the right ventricle of the heart. The catheter was then pushed further into the pulmonary artery. The catheter was linked to a pressure transducer (MLT844, ADInstruments Inc, USA) and an analysis system (PowerLab 4/35, LabChart Pro, ADInstruments Inc, USA) in order to record the mean pulmonary artery pressure (PAP). Rats were left untouched for at least 2 minutes before the measurements in order to record a stable value of PAP. After the measurement, an incision was made in the femoral artery and lungs were harvested and either fixed in 10% formalin for histology or flash frozen in liquid nitrogen for protein and RNA analysis.

All animal work was conducted under the guidelines from the Canadian Council on Animal Care and approved by the Animal Research Ethics Board of McMaster University under protocol #13-12-48.

### *CT scans imaging*

Rats were sedated via an intraperitoneal injection of Xylazine (Bayer Healthcare, 10 mg/kg) and ketamine (150 mg/kg). After exposing a section of the anterior side of the neck a 16-gauge needle was inserted into the jugular vein and contrast agent (Isovue-300 (iopamidol injection), 0270-1315-25, Bracco Diagnostics Canada Inc., Mississauga, Ont. Canada) was perfused for at least 10 minutes (0.2 mm/minutes). An incision was made in the femoral artery to bleed the animal. Once contrast agent had perfused the entire vascular of the animal, rats were placed in the CT scan machine and imaged (n=3). All imaging work was completed at the McMaster Centre for Preclinical and Translational Imaging (MCPTI) at McMaster

University (Hamilton, ON, Canada). The CT scan was acquired on an X-SPECT system (Gamma Medica, Northridge, CA, USA) and consisted of 1024 X-ray projections with x-ray tube characteristics of 75 kVp and 355  $\mu$ A. The projection images were reconstructed using a Feldkamp cone beam backprojection algorithm in COBRA (Exxim Software, Pleasanton, CA, USA) into 512 $\times$ 512 $\times$ 512 arrays (0.1 mm isotropic voxels). Each CT image was converted to Hounsfield Unit (HU) scaling using empty airspace within the field of view and a water-filled tube included in each scan. Images were analysed with the AMIRA software (FEI Visualization Sciences Group, USA). The 3D reconstruction of the pulmonary arterial tree was achieved with multiple steps. The total chest space volume, excluding the heart, was selected using a combination of manual segmentation and semi-automated contouring. Threshold segmentation identified the vascular tree and was optimized for each image. Due to voxel size accurate segmentation and subsequent quantification could only be performed on vessels larger than 75  $\mu$ m. Data provided segment volume and density.

#### *Western blotting*

Crushed lungs were homogenized in cell lysis buffer (Hepes 50 mM pH7.4, NaCl 150 mM, EDTA 5 mM, Triton X-100 0.5%) using a mechanical homogenizer (Omni International, Waterbury CT), and the collected supernatant was used for western blotting. 40  $\mu$ g of total protein from lung homogenate or cells were separated on a 10% SDS Polyacrylamide Electrophoresis gels. Proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, 1620177, Hercules, CA) using a wet transfer apparatus and blocked at room temperature for 1 hour using 8% skim milk. Western blotting assay was used to detect  $\alpha$ -SMA, pSmad3, Smad3, VEGF, ET-1, ETRA and ETRB. GAPDH was used as loading control. Protein detection was performed using the SuperSignal West Pico chemiluminescent system (Thermo Fischer Scientific, 34580) and read in a ChemiDoc XRS Imaging System (Bio-Rad Laboratories). Densitometry measurements were performed with ChemiDoc XRS Imaging System Software and were normalized to a control sample when studies required more than one blot.

#### *Hydroxyproline assay*

Hydroxyproline content in rat lung tissue was measured by a colorimetric assay as described previously [2]. Briefly, lung lobes were turned into a finely ground up powder and immediately homogenized in RIPA buffer. The total pellet formed from the centrifugation of the RIPA homogenized lung tissues was resuspended in PBS and allowed to freeze at -80°C.

The pellet was then subsequently lyophilized for at least 24 hours using a freezer dryer apparatus (Modulyod Freezer Dryer, Thermo Electron Corporation). Following the addition of 10% TCA solution and subsequent centrifugation, 6ml of 6N HCL were added into each tube for pellet hydrolysis at 110°C in dry bath incubator. Samples were later brought to a pH of 7 by the addition of NaOH and were incubated for 20 minutes after the addition of 0.05M Chloramine T reagent. Chloramine T reagent was destroyed by the adding 70% perchloric acid and samples were ultimately incubated for 20 minutes in a 55-65°C water bath shortly after adding Ehrlich's reagent solution. The final reaction absorbance was read at 550nm and samples concentrations were determined from the hydroxyproline standard curve. Hydroxyproline concentrations were finally calculated and expressed as microgram of hydroxyproline per ml of solution.

#### *Ashcroft score*

Pulmonary fibrosis of Masson Trichrome stained lung sections was graded from 0 (normal lung) to 8 (completely fibrotic lung), using a modified Ashcroft score [3].

#### *Isolation of mRNA and gene expression*

Total RNA was extracted from frozen lung tissue with TRIzol® reagent (Thermo fisher scientific, 15596026). Two µg of total RNA was reverse transcribed using qScript cDNA Super Mix (Quanta Bioscience, 95048-025, Gaithersburg, MD). The cDNA was amplified using a Fast 7500 real-time PCR system (AB Applied Biosystems) using TaqMan® Universal PCR Master Mix and predesigned primer pairs (Life Technologies, 4304437, Burlington, ON, Canada) for Collagen1A (Hs00164004\_m1), TGFβR1 (Hs00610320\_m1), ACTA2 (Hs00426835\_g1) and 18S (Hs03003631\_g1).

#### *Histology and immunohistochemistry*

Lungs were fixed by intratracheal instillation of 10% neutral-buffered formalin at a pressure of 20 cm H<sub>2</sub>O. Paraffin sections were cut at 4 µm and processed in-house at the core histology facility at McMaster (Hamilton, ON, Canada). Tissue slides were generated and subsequent staining was performed with Masson Trichrome (MT) or Picrosirius Red (PSR). Picture acquisition of PSR and MT staining were performed using an Automatic slide scanner microscope (Olympus VS 120-L). PSR quantification was performed on whole lung sections using the ImageJ software (NIH, USA). Endothelial Diameter (ED) was defined as distance between external elastic laminae, while Medial Wall Thickness (MWT) was determined as

distance between external and internal elastic laminae. Vessels were categorized as follows: Small:  $ED < 50 \mu m$  and large:  $ED > 50 \mu m$ . MWT was calculated using the following formula:  $MWT (\%) = (2 \times MT/ED) \times 100\%$ . The number of vessels was evaluated in histological sections using ImageJ. Briefly, lung slides pictures were converted in TIFF and 20 random fields were chosen per pictures and the number of small and large vessels were manually evaluated using ImageJ in each field (using a straight line of  $50\mu m$  in imageJ to determine the size of the vessels  $>$  or  $<$  to  $50\mu m$ ).

This automatic slide scanner can digitalize whole slides at 20X magnifications using polarized detection. Immunohistochemical staining was performed to characterize the localization and expression of VEGF (ab1316, Abcam). Images were captured using an automatic slide scanner microscope (Olympus VS 120-L). All sections were digitalized at 20X from 4 transverse sections and quantitated by ImageJ automatic analysis, excluding the main bronchus and larger airways. Images were analysed using an internally developed macro on ImageJ. The macro was created to threshold and quantify the amount of staining and could subsequently be used to determine total tissue area within a region of interest (ROI). Olympus vsi. files were extracted using the BIOP plugin on ImageJ and converted to tiff. files. The images were then edited in Adobe Photoshop to remove any debris surrounding the tissue sample as to minimize extraneous detection of undesired particulates. Next, the macro was run and set to threshold and display a specific hue (H), saturation (S), and brightness (B) range (specific values were then determined for ETRA, ETRB and VEGF staining) using the Colour Threshold plugin. Once thresholding was applied to only display the desired H, S, and B ranges, images were converted into 8-bit images. The analyse particle function was then used to determine the total area of the stained regions. Specific H, S, and B values to quantify total tissue area were then used. Finding the total stained area, and the total tissue area, a proportion of the sample which was stained could be determined.

The fibrotic area on lung slices was evaluated using an in-house macro for ImageJ. Briefly, the macro opened a .tiff image in ImageJ and allowed successive manual drawing of total lung area and fibrotic area. The macro automatically calculated total lung area and fibrotic area and results were expressed as % of fibrotic area compared with total lung area.

### *Immunofluorescence*

Immunostaining of VEGF and  $\alpha$ -SMA was performed on formalin fixed rat lung tissues sections. Briefly, following deparaffinization and saturation of nonspecific sites with BSA (5%, 30 min), cells were incubated with primary antibodies overnight in a humidified

chamber at 4°C. Conjugated secondary antibodies were used at a dilution of 1:2000. Slides were mounted in Prolong-gold with DAPI (ProLong® Gold antifade reagent with DAPI, Life technologies, P36931). Pictures were taken were performed using an Automatic slide scanner microscope (Olympus VS 120-L).

### *ELISA*

The levels of active TGF-β1, VEGF and ET-1 in rat and human BALF supernatants and sera were measured using a rat TGF-β1-specific ELISA kit (MB100B , R&D Systems), a rat VEGF ELISA kit (abcam, ab100786), a rat ET-1 ELISA kit (E-EL-R0167, Elabscience) and a human ET-1 ELISA kit (R&D Systems, DET100) respectively, according to the manufacturer's recommendations.

### *Contraction assay*

Collagen gel solution was made at the bottom of a 24 well following the manufacturer's recommendations (CBA-201, Cell BioLabs, Inc.).

Human derived pulmonary fibroblasts or pulmonary artery smooth muscle cells ( $5 \times 10^6$  cells/mL) were mixed with the collagen gel solution and seeded onto the 24 well plate. This mix was then incubated for 1-hour at 37°C to allow it to set. After 1-hour 1mL of respective medium was carefully added onto the now set gel solution and then incubated overnight at 37°C, 5% CO<sup>2</sup>. The following day the medium was changed and the cells in the gel were treated with vehicle (DMSO), rTGF-β1 (5ng/ml, PerproTech, 100-21) or rET-1 (10 μM, Abcam, ab158332) and Macitentan (100μM), pirfenidone (100μM) or both. A control well was treated with rTGF-β1 (5ng/ml, PerproTech, 100-21) and a contraction inhibitor provided by the manufacturer. Cells were incubated at 37°C, 5% CO<sup>2</sup> for 24 more hours to allow for stress to develop. Gels were released from the slides of the wells to allow contraction to occur. This was accomplished by running a scalpel along the perimeter of the wells. Gels were measured prior to release, and every 1 hour after release for 6 hours. Measurements were then repeated every 12 hours for the next 48 hours.

### *Flow cytometry*

Endothelial cell apoptosis has been assessed via Annexin-V/PI staining using a Annexin V-FITC Apoptosis Detection Kit (ab14085, Abcam) according to manufacturer recommendation. Analysis has been perform using a FACS CANTO flow cytometer (BD Biosciences) and FlowJo software.

### *Statistical Analysis*

All data were expressed as median with interquartile range. Statistical analysis between two groups was performed using a non-parametric Mann-Whitney test. Statistical analysis between multiple groups with one control group was performed by Kruskal-Wallis test, with Dunn comparison test (post hoc). Analysis was performed with GraphPad Prism 6.0 (GraphPad Software Inc.). A p-value less than 0.05 was considered significant.

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2. Ask K, Bonniaud P, Maass K, Eickelberg O, Margetts PJ, Warburton D, Groffen J, Gauldie J, Kolb M. Progressive pulmonary fibrosis is mediated by TGF-beta isoform 1 but not TGF-beta3. *The international journal of biochemistry & cell biology* 2008; 40(3): 484-495.
3. Hubner RH, Gitter W, El Mokhtari NE, Mathiak M, Both M, Bolte H, Freitag-Wolf S, Bewig B. Standardized quantification of pulmonary fibrosis in histological samples. *BioTechniques* 2008; 44(4): 507-511, 514-507.