Online Supplementary Data

Overexpression of Fibroblast Growth Factor-10 during Both Inflammatory and Fibrotic Phases Attenuates Bleomycin-induced Pulmonary Fibrosis in Mice

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Methods

Mice

The *SP-C-rtTA* transgenic mouse line serves as the driver line, in which the *rtTA* activator is under the control of the human 3.7 kb *SP-C* promoter. On being crossed with the *tet(O)Fgf10* responder line ectopic *Fgf10* expression is induced in the alveolar epithelial type II cells (AEC2) of double transgenic offsprings fed with doxycycline (DOX) in the diet (625 mg/kg from Harlan Teklad, WI, USA). These double transgenic mice were maintained in the C57BL/6 background and were obtained from Dr. Jeffrey Whitsett (E1). All animal studies were carried out using the protocols and guidelines of the institutional animal care and use committee (IACUC). Double transgenic *rtTA; tet(O)Fgf10* mice that overexpress *Fgf10* under the control of rtTA expressed from the ubiquitous *Rosa26* promoter (E2) were used for the isolation of AEC2 cells.

Experimental scheme and bleomycin treatment for induction of fibrosis

A micro-osmotic pump containing bleomycin sulfate (0.1 mg/g mouse body wt dissolved in saline) was surgically inserted into the mid back region of 8-week-old female *SP-C-rtTA; tet(O)Fgf10* mice (E3). The mice of this experimental group were then treated with doxycycline for a week and then returned to a normal diet for next 3 weeks (BLM/DOX+ group). Control mice consisted of three groups, the first group comprising *SP-C-rtTA; tet(O)Fgf10* mice treated with bleomycin for one week but being fed on a normal rodent diet (BLM/DOX-) throughout the 4 week period. The negative control groups were treated with saline and either fed on a DOX diet (SAL/DOX+) or a normal diet (SAL/DOX-). All surgeries were performed in accordance with the IACUC and the mice

were monitored for stress and pain from the surgeries. At 3, 7, 14 and 28 days after the surgery *SP-C-rtTA; tet(O)Fgf10* mice were anesthetized with pentobarbital and the lungs were removed for immediate RNA extraction, protein analysis, measurements of collagen content or other histological examination. Similar experiments were carried out with double transgenic mice treated with bleomycin in the first week and given a doxycycline diet either in week 2 (BLM/ Week 2 DOX+) or in week 3 (BLM/ Week 3 DOX+) after pump implantation. These mice were sacrificed at 28 days and their lungs were analyzed for collagen content and fibrosis scoring.

Histological analysis and Immunohistochemistry

Mouse lungs were perfused at a constant pressure of 25 cm H_2O with 4% paraformaldehyde and fixed overnight. They were dehydrated and embedded in paraffin. 5 μm thick lung sections were stained either with haematoxylin-eosin or for collagen using Masson's trichrome staining procedure in order to determine the extent of fibrosis. The sections were analyzed for overall lung architecture and extracellular matrix deposition. Further, to detect cell type-specific protein expression, primary antibodies to surfactant protein C from Seven Hills Bioreagents (Cincinnati, OH) were used. Immunostaining was visualized using Alexa fluor labeled secondary antibodies (Invitrogen). Slides were mounted in a solution containing DAPI for visualization of nuclei (Vector labs). Proliferation was analyzed using the PCNA staining kit from Zymed. Sections were stained according to the manufacturer's instructions. Similarly, apoptosis studies (TUNEL) were carried out using the In Situ Cell Death Detection Kit, POD from Roche. Quantification of immunostainings was done by counting the number

of positive cells and the number of total cells in 5 fields in lung sections at a magnification of 40X and expressing these as percentage of cells in each case. αVβ6 immunostaining was performed using a chimeric version of the anti- αVβ6 primary antibody 6.2G2 (E4) in which the mouse constant region was replaced with a human IgG1 sequence, following the instructions of the Vectastain ABC kit from Vetor Labs. In order to count the SP-C/TUNEL double positive cells, TUNEL and SP-C IHC were carried out on serial sections (5 μm apart) in 28 days BLM/DOX+ vs. BLM/DOX- (DOX was delivered week 1). The 28-day time point was chosen since maximum differences with regard to proliferation and apoptosis were observed at this time point between the saline and bleomycin groups. Also, at this time point, there is a significant difference in the number of SP-C positive cells in BLM/DOX+ vs. BLM/DOX- lung specimen. Topologic adjustments were done using high-resolution pictures at low (10X) and high (40X) magnification. For precise positioning, pictures were taken in areas exhibiting specific histological hallmarks such as bronchi, arteries, edges of the lung, etc. Cells considered for counting exhibited common nuclei (stained with DAPI) in both SP-C and TUNEL stained adjacent sections. SP-C and TUNEL positive cells were counted in 10 fields for each marker (40X magnification, n=3). The percentage of SP-C and TUNEL double positive cells compared to the total number of TUNEL positive cells as well as the percentage of SP-C and TUNEL double positive cells compared to the total number of SP-C positive cells were determined. A total of 300 TUNEL positive cells and 2200 SP-C positive cells were counted.

Fibrosis score

Morphological fibrotic changes were quantified using a numerical scale as described previously in Ashcroft et al., 1998 (E5). Briefly, 5μm thick haematoxylin-eosin stained lung sections were coded and examined without knowledge of the experimental conditions on a Leica microscope by 3 observers in a blinded manner. The grade of lung fibrosis was scored on a scale from 0 to 8. Ten randomly examined fields (200X magnification) of the total lung area were analyzed from each of the experimental and control groups in order to evaluate and score pulmonary lesions in these mice. The criteria for grading lung fibrosis were in accordance with the method reported by Ashcroft et al.: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to the lung architecture; grade 5, increased fibrosis with definite damage to the lung architecture and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of architecture and large fibrous area; and grade 8, total fibrous obliteration of the field. The severity of fibrotic changes in each lung section was assessed as the mean score of severity from the observed microscopic fields. After examining the entire section, the mean of the scores from all the fields was considered as the fibrotic score. A total of three mice were studied in each group and the average of these scores was the score of the experimental or control group being studied.

Collagen quantification

Total soluble collagen was assessed for 3 lungs in each group by Sircol™ Collagen Assay kit (Biocolor Ltd, Ireland) according to the manufacturer's instructions. Each right lung was homogenized in 1 ml of PBS, centrifuged and filtered through a 1.2 µm syringe filter. 50 µl of sample was mixed with 50 µl of 0.5 M acetic acid. The samples were mixed by gentle inversion, and 1 ml of Sirius Red reagent was added to 100 µl of test sample and mixed for 30 min at room temperature. The collagen-dye complex was precipitated by centrifugation at 5000 *g* for 10 min and mixed with 1.0 ml of Sircol™ alkali reagent; the absorbance was measured at 540 nm using a microtiter plate reader. A calibration curve was set up based on the collagen standard provided by the manufacturer. All assays were performed in triplicates.

Bronchoalveolar lavage fluid collection for inflammatory cell counts

Bronchoalveolar lavage fluid (BALF) was collected as previously described (E6) in order to assess the total number of macrophages and TGFß1 content at 3, 7 and 14-day time points. Briefly, the lungs of each mouse were lavaged thrice with a total of 1.8 ml 0.9% saline by inserting an 18G plastic cannula into the trachea. The BALF recovery rate in all cases was greater than 90%. The BALF collected was then centrifuged at 500 *g* (4°C), and the supernatant used for the measurement of TGFß1 was flash frozen and stored at -80°C. The cell pellets were re-suspended in 1.0 ml of red blood cell lysis buffer. The differential cell counts in BALF were obtained by counting 300 cyto-spun cells stained with Wright's Giemsa for each sample.

Flow Cytometry on BALF samples

Mini pumps containing bleomycin were surgically implanted *in SP-C-rtTA; tet(O)Fgf10* mice. At 7 days post pump implantation, bronchoalveolar lavage was collected from bleomycin treated mice and the lavage cells were stained with antibodies for CD4-FITC, CD25-APC and Foxp3-PE and the appropriate isotype controls (from Ebioscience). The concentration of the antibodies used was according to the manufacturer's instructions. Flow cytometry was done and populations of $CD4^+CD25^+Foxp3^+$ cells were analyzed in individual BAL samples. Data acquisition and analysis was performed on a four color FACSCalibur, equipped with CellQuest Pro software (BD biosciences). Treg frequency is reported as percentage of CD4+ cells.

Determination of TGFß1 activity in BALF

For the assessment of TGFß1 bioactivity, murine lung epithelial (MLE12) cells stably transfected with the *Plasminogen Activator Inhibitor (PAI-1) promoter* driving luciferase expression were used (E7). Briefly, the *PAI-1/luciferase* transfected MLE12 cells were maintained at 37°C/ 5% CO_2 in DMEM medium containing 10% FCS and G418. Standards or samples containing TGFß were added to the 48 well plate in triplicates 3 hours after plating the cells and the actual TGFß1 activity was calculated on the basis of serial standard dilutions. After an overnight incubation, the cells lysed with 100 μl of 1x lysis buffer per well and stored at -20°C until analyzed. 80 μl of the lysate from each well was analyzed for luciferase activity using the BD Monolight™ system according to the manufacturer's instructions.

Real Time PCR Analysis

The *SP-C-rtTA; tet(O)Fgf10* mice were treated with doxycycline or regular diet (control)for a period of 7 days in order to induce *Fgf10* expression. Total RNA was extracted from individual *SP-C-rtTA; tet(O)Fgf10* mice (n=3 for each group) at 7 and 28 days post bleomycin injury using the RNeasy kit (Qiagen) according to the manufacturer's instructions. DNA contaminations were removed using Turbo DNAse (Ambion). Total RNA was reverse-transcribed using the Superscript-III first strand super mix (Invitrogen) following the manufacturer's recommendations. 5 µg of the total RNA was used to prepare cDNA from the isolated total RNA using oligo dT primers. For real-time PCR, 25 pg cDNA was used for each of the PCR reactions using the primers and probes for *Tgfß1* and *Smad3* designed by the online Roche software: Probe finder version 2.20. All real-time PCR reactions were performed with Roche: FastStart TaqMan Probe Master kits, according to the manufacturer's instructions in Roche Light Cycler 1.5 Real-Time PCR machine. 18S ribosomal RNA was used as an internal control for all analysis.

Reverse Transcriptase PCR analysis

RNA isolation and cDNA preparation was performed as described for the Real time PCR. 1 µg cDNA and 10 pg of primers were used in every PCR reaction. Mouse *Fgf10* Forward: *CTG GAG ATA ACA TCA GTG GAA ATC G* and mouse *Fgf10* reverse: *GAG CAG AGG TGT TTT TCC TTC TT* primers were used for total *Fgf10*. The mouse exogenous *Fgf10* Forward: *GAC GCC ATC CAC GCT GTT TTG ACC* and mouse exogenous *Fgf10* reverse: *ATT TGC CTG CCA TTG TGC TGC CAG* primers were used

to detect the exogenous *Fgf10*. GAPDH was used for housekeeping gene. Mouse *GAPDH* Forward: *ATC ACT GCC ACC CAG AAG ACT* and Mouse *GAPDH* Reverse: *CAT GCC AGT GAG CTT CCC GTT*. Each RT-PCR was performed for 40 cycles. Gels were documented using a Biorad gel imager and Biorad QuantityOne software.

Western blot analysis

Seven days after the onset of bleomycin or saline application, the right lung of *SP-C-rtTA; tet(O)Fgf10* was homogenized in lysis buffer 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM Na_3VO_4, 100 µM NaF, 1% Triton X-100). Protein concentration was measured in the total tissue lysate using Biorad protein quantification assay. 10 µg of total lysate was loaded onto a 4-15% SDS PAGE gel, electrophoresed and blotted onto Immobilion membranes (Invitrogen). Blocking was done in 5% BSA and the membrane was incubated overnight with rabbit anti P-SMAD3 (from Cell Signaling) or with β Actin (Cell Signaling). Antibody binding was carried out according to the manufacturer's instructions. Chemiluminescence reactions were carried out using ECL kits for horseradish peroxidase from Amersham. The signal was visualized and recorded using Amersham Hyperfilm ECL. Quantification of the signals was done using the ImageJ software (from the NIH).

Measurement of MMP-2 activity by SDS-PAGE zymography.

Equivalent volumes of BALF were collected from saline or bleomycin treated double transgenic *SP-C-rtTA; tet(O)Fgf10* mouse lungs. After centrifugation at 300 *g* for 10 min

at 4°C to pellet cells, the cell-free supernatants were concentrated 2-fold using Centricon 10 concentration units (Millipore) according to the manufacturer's instructions. 20 µl of BAL concentrates were electrophoresed through commercially prepared 10% Tris-Glycine zymogram gels (Invitrogen) under non-reducing conditions with 0.1% gelatin as the substrate. Conditioned medium from NIH/3T3 cells, which contained both inactive and active MMP-2, was included as control. The gels were renatured in Novex renaturing buffer and developed using Novex developer according to the manufacturer's instructions (30 min at room temperature, then overnight at 37°C). The gels were then stained for 30 min in 0.5% Coomassie brilliant blue R, destained, and dried at room temperature. Areas of protease activity showed up as clear bands in the gel against a blue background.

Isolation of alveolar type II (AEC2) cells

Double transgenic *Rosa26*^{rtTA}*; tet(O)Fgf10* mice to be used for AEC2 isolation were put on a doxycycline diet (DOX+) 2 days prior to isolation for induction of transgene expression. Alternatively, those mice that were fed a normal diet prior to cell isolation were termed DOX-. To isolate murine AEC2, mice were first anesthetized by I.P. injection of pentobarbital. Following excision of the left kidney, exsanguination was achieved via injection of sterile saline into the right ventricle of the heart with subsequent flow out through the left renal vein. De-blooded lungs were perfused *in situ* with dispase (Gibco) followed by molten, 1% agarose, both in sterile saline and both delivered *via* a 20G I.V. catheter inserted into the trachea. Lungs were then removed *en bloc* and incubated in dispase solution with gentle agitation for 45 minutes at room

temperature. Digested lung parenchyma was teased away from airway and connective tissue, then minced in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO) supplemented with DNase (Sigma). The resulting cellular suspension was filtered sequentially through 100 μm, 70 μm and 25 μm mesh to achieve a single cell suspension. Differential adherence on murine IgG-coated plates was used to eliminate white blood cell contaminants from the preparation. Briefly, 100 mm Optilux dishes (Falcon, Franklin Lakes, NJ) were pre-coated with monoclonal antibodies CD45 and CD32/16 (Pharmingen, San Diego, CA) at 4.4 and 1.6 μg/ml respectively in 50 mM Tris pH9.5. Plates were washed thoroughly with Phosphate Buffered Saline (PBS) before the cellular suspension was added. Plates were incubated for 1 hour at 37^{o}C. Non-adherent cells were gently panned and recovered. Cells were pelleted at 800 X g for 8 minutes, then used freshly isolated or suspended in DMEM with 10% FBS supplemented with antibiotics and plated at 2 x 10^5 cells/cm^2 in 6 well plates or chamber slides coated with fibronectin (Becton-Dickinson, Bedford, MA). The cells thus obtained had a purity of >95% in each isolation. Doxycycline was added in the DMEM medium for AEC2 cells isolated from *rtTA; tet(O)Fgf10/* DOX+ mice at a concentration of 10 ng/ml. Cultures were maintained at 37^{o}C in a humidified atmosphere supplemented with 5% CO_2 for 40-48 hours prior to analysis.

AEC2 Immunohistochemical Analysis.

AEC2 cells cultured for 40 hours in chamber slides were fixed in acetone- methanol 1:1, washed with PBS, then blocked with CAS-Block (Zymed, San Francisco, CA). To

detect cell type-specific protein expression, monoclonal antibodies to cytokeratin or vimentin (Pharmingen, San Diego, CA) were used at a concentration of 1:5000. Immunostaining was visualized using Cy3 labeled secondary antibody at 1:500 (Molecular Probes, Eugene, OR). Slides were mounted in a solution containing DAPI for visualization of nuclei (Molecular Probes). For detection of SP-C expression, a rabbit anti SP-C primary antibody from Seven Hills Bioreagents (Cincinnati, OH) was used at 1:500 on cells fixed and blocked as described. For SP-C analysis, Cy3 labeled secondary was used to detect AEC2 specific expression. 1% Bovine Serum Albumin (BSA) in PBS was used as a diluent for all antibodies. Results were observed under a Leica DMA microscope at 16X.

Data presentation and statistical analysis

Data are expressed as mean +/- SD unless otherwise stated. Statistical analyses were performed on the data through two factor ANOVA between more than two groups and with Student's t-test for comparisons of two groups using Microsoft Excel. P-values <0.05 were considered as significant.

References

E1. Clark JC, Tichelaar JW, Wert SE, Itoh N, Perl AK, Stahlman MT, Whitsett JA. Fgf-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L705-L715.

E2. Parsa S, Ramasamy SK, De Langhe S, Gupte VV, Haigh JJ, Medina D, Bellusci S. Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev Biol* 2008;317:121-131.

E3. Harrison JH, Jr., Lazo JS. High dose continuous infusion of bleomycin in mice: A new model for drug-induced pulmonary fibrosis. *J Pharmacol ExpTher* 1987;243:1185-1194.

E4. Weinreb PH, Simon KJ, Rayhorn P, Yang WJ, Leone DR, Dolinski BM, Pearse BR, Yokota Y, Kawakatsu H, Atakilit A, Sheppard D, Violette SM. Function-blocking integrin alphavbeta6 monoclonal antibodies: Distinct ligand-mimetic and nonligand-mimetic classes. *J Biol Chem* 2004;279:17875-17887.

E5. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 1988;41:467-470.