Online Data Supplement

Amplified Canonical TGF-β Signaling via Heat Shock Protein 90 in Pulmonary Fibrosis

Zaneta Sibinska*[1,2], Xia Tian*[1,2], Martina Korfei[1,2], Baktybek Kojonazarov[1,2], Janina Susanne Kolb[1,2], Walter Klepetko[5,6], Djuro Kosanovic[1,2], Malgorzata Wygrecka[1,2,3], Hossein Ardeschir Ghofrani[1,2], Norbert Weissmann[1,2], Friedrich Grimminger[1,2], Werner Seeger[1,2,4], Andreas Guenther[1,2,5,7], and Ralph T. Schermuly[1,2]

*These authors contributed equally to this work.

[1]Universities of Giessen and Marburg Lung Center (UGMLC), Giessen, Germany; [2] Member of the German Center for Lung Research (DZL), Germany; [3]Department of Biochemistry, Faculty of Medicine, Universities of Giessen, Giessen, Germany; [4]Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany; and [5]Agaplesion Lung Clinic Waldhof Elgershausen, Greifenstein, Germany; [6]Vienna Medical University, Vienna, Austria; [7]European IPF Network and European IPF Registry, Germany

Corresponding author:

Dr. Ralph Theo Schermuly

Chair for Pulmonary Pharmacotherapy, UGMLC, Justus-Liebig University,

Biomedizinisches Forschungszentrum (BFS), Schubertstr. 81, Giessen, Germany

Tel: +49 641 99 39884

Fax: +49 641 99 42419

E-mail: ralph.schermuly@innere.med.uni-giessen.de

Supplementary Methods

Bleomycin-induced Pulmonary Fibrosis in Mice and Experimental Groups

Adult male 12-week-old C57BL/6 mice (20–25 g body weight) were obtained from Charles River Laboratories (Sulzfeld, Germany). Both the University Animal Care Committee and the Federal Authorities for Animal Research of the Regierungspraesidium Giessen (Hessen, Germany) approved the study protocol (GI20/10, Nr. 59/2012). At day 1, mice were anesthetized with isoflurane (Baxter, Ontario, Canada) followed by orotracheal instillation of bleomycin (Sigma-Aldrich, Munich, Germany) at a dose of 3.5 units/kg or saline alone (sham control), with the noses of the mice pinched. From day 7 to day 21, the mice instilled with bleomycin were treated every 2 days with 10 mg/kg or 25 mg/kg 17-DMAG (LC Laboratories, Woburn, MA) or vehicle, and the initial sham control mice received vehicle. 17-DMAG was prepared freshly in saline at 2 or 5 mg/ml and administered by oral gavage, all in the same manner.

Fluorescence Molecular Tomography (FMT) Combined with Microcomputed Tomography

On day 20, hair from the upper torso of each mouse was removed to avoid auto-fluorescence and 2 nmol of matrix metalloproteinase-responsive fluorescent probe MMPSense 680 (PerkinElmer, Waltham, MA) was injected via the tail vein [1]. Twenty-four hours after the injection, the mice were scanned by microcomputed tomography (SkyScan, Kontich, Belgium) followed immediately by FMT (VisEn Medical, Bedford, MA). During the scan, mice were anesthetized with isoflurane and kept lightly compressed in the imaging cartridge to prevent motion. The collected

fluorescence data were reconstructed by FMT 4000 system software (PerkinElmer, Waltham, MA) for the quantification of the 3D fluorescence signal within the lungs.

Measurement of Lung Function

At day 21, lung function was assessed using the FlexiVent system (SCIREQ Inc., Montreal, Canada). Parameters were calculated by fitting pressure and volume data to the single compartment model and the constant phase model, measuring dynamic compliance with the Snapshot-150 perturbation and tissue damping with the QuickPrime-3 perturbation, respectively. Only measurements with coefficient of determination ≥ 0.9 were used for further analysis [2]. After measurement the right lung lobes were snap-frozen at −80°C, while the left lobe was perfused and fixed with 3.7% formalin.

Bronchoalveolar Lavage Fluid (BALF) Cell Count

After respiratory function measurement, lungs were lavaged three times with 0.3 ml of ice cold saline and all aliquots were pooled for each lung. Further, BAL fluid was centrifuged and cell pellet was resuspended in 1 ml of saline. Cells in a constant volume of 0.2 ml were deposited in a cytospin funnel and transferred to a glass slide with Shandon Cytospin-3® centrifuge at 500 rpm for 5 minutes followed by drying. Slides were stained with May Gruenwald-Giemsa and inflammatory cells were counted with the 40-fold magnification using Leica DM6000 B microscope.

Fibrosis Scoring and Collagen Quantification of Mouse Lung

Formalin-fixed, paraffin-embedded lung tissues were cut into sections with a thickness of 3 µm. Lung sections were stained with hematoxylin (Waldeck, Muenster, Germany) and eosin (Thermo Fisher Scientific, Waltham, MA) according to standard

protocols, and fibrosis scores were quantified using a numerical scale as described by Ashcroft and colleagues [3] with slight modifications: score “0” refers to healthy lungs, whereas score “6” represents the most severe degree of fibrosis. Collagen deposition was assessed by sirius red (Niepoetter, Buerstadt, Germany) staining of lung sections and the area of collagen was measured using Leica image software (Leica Microsystems, Wetzlar, Germany) according to the standard protocol.

Interstitial Lung Fibroblasts (ILFBs) from Human and Mouse Lungs

Human ILFBs were isolated by explant culture of lung tissue from three donors and three patients with IPF as previously described [4], and cultured in MCDB-131 medium (PAN, Aidenbach, Germany), supplemented with 2% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 5 µg/ml insulin (Sigma-Aldrich, Munich, Germany), 2 ng/ml basic fibroblast growth factor, and 0.5 ng/ml human epidermal growth factor (PeproTech EC, Rocky Hill, NJ). Mouse ILFBs were isolated by enzymatic digestion with 0.3 mg/ml type IV collagenase (Sigma-Aldrich, Munich, Germany) and 0.5 mg/ml trypsin (Gibco, Life Technologies, Darmstadt, Germany) in Hanks’ Balanced Salt Solution (HBSSCa^{2+}/Mg^{2+}), (PAN, Aidenbach, Germany). Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium supplemented with 10% FCS (Biochrom, Berlin, Germany).

Protein Isolation from Mouse and Human Lungs

Lung tissues from human or mice were homogenized by Precellys®24 homogenizer (Bertin Technologies, France). Total protein was extracted in RIPA buffer (Santa Cruz Biotechnology, Heidelberg, Germany) according to manufacturer’s instructions.

Epithelial-mesenchymal Transition of A549 Cells

A549 cells were cultured in DMEM/F12 medium, supplemented with 10% FCS, 1% MEM vitamins solution, and 1% MEM nonessential amino acids. To induce epithelial-mesenchymal transition, A549 cells were serum-starved (1% FCS) for 24 hours and then cultured in medium with 5 ng/ml transforming growth factor (TGF)-β1 (R&D Systems, Wiesbaden-Nordenstadt, Germany) and 1% FCS for 72 hours, in the absence or presence of 50 nM 17-AAG (Selleck Chemicals, Munich, Germany).

Co-immunoprecipitation

After serum-starvation for 24 hours, the cells were stimulated with 10 ng/ml human recombinant TGF-β1, in the absence or presence of 50 nM 17-AAG for 24 hours. Total proteins from donor and IPF ILFBs were prepared according to the manufacturer's instructions (Life Technologies, Darmstadt, Germany). Dynabeads Protein G (Life Technologies, Darmstadt, Germany) were incubated with TGF-β receptor II (TGF-βRII) antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 hour at room temperature (RT). Subsequently, Dynabeads-antibody complexes were incubated with 0.5 mg of total proteins overnight at 4°C. The precipitated complexes were immunoblotted with HSP90β and HSP90α antibody (1:1000, Enzo Life Sciences, Loerrach, Germany) and TGF-βRII antibody (1:1000).

Immunoblotting

After 24 hours of serum-starvation, ILFBs were stimulated with recombinant TGF-β1 (10 ng/ml) for 24 hours (western blots), in the absence or presence of 50 nM 17-AAG. For MG-132 experiment, ILFBs were treated with 50 nM 17-AAG in the absence or presence of MG-132 (30 μM) for 5 hours, followed by 1 hour stimulation with TGF-β1. Total proteins extracted with RIPA buffer were separated by 8% or 10%

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% non-fat milk for 1 hour at RT and probed with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour at RT. The blots were developed using an enhanced chemiluminescence kit (Amersham, Pittsburgh, PA) and captured by films. Antibodies are listed as follows: TGF-βRI, TGF-βRII, Smad2 (1:1000, Cell Signaling Technology, Leiden, the Netherlands), fibronectin (1:1000, Sigma-Aldrich, Munich, Germany), collagen type I (1:1000, Meridian Life Science, Memphis, TN), β-actin (1:3000, Abcam, Cambridge, UK), α-smooth muscle actin (1:1000, α-SMA, Cambridge, Abcam), phospho-Smad2 (phospho-Ser465/467) (1:1000, Novus Biologicals, Littleton, CO), E-cadherin (1:1000, Merck Millipore, Darmstadt, Germany), HSP90α, and HSP90β (1:1000, Enzo Life Sciences, Loerrach, Germany).

Immunohistochemistry

Lung sections were stained employing the streptavidin-biotin-alkaline phosphatase (AP) method with use of the ZytoChem-Plus AP Kit (Fast Red, red dye) or the streptavidin-biotin-HRP-method by using the ZytoChem-Plus HRP-DAB Kit (brown dye, both kits from Zytomed Systems, Berlin, Germany), according to a previously published protocol [5]. The stained sections were counterstained with hemalaun (Waldeck, Muenster, Germany) and scanned with NanoZoomer 2.ORS (Hamamatsu, Herrsching am Ammersee, Germany). For staining of human lung tissue sections, the concentrations of primary antibodies were: α-SMA (1:150, Abcam, Cambridge, UK), HSP90α (1:75, Enzo Life Sciences, Loerrach, Germany), and HSP90β (1:100, Abcam, Cambridge, UK). For staining of murine lung tissue sections, the concentrations of primary antibodies were: cytokeratin-5/KRT-5 (1:200, Abcam,

caambridge, UK), proSP-C, 1:750, Millipore, Darmstadt, Germany), α-SMA (1:100, Abcam, Cambridge, UK), TTF1 (1:100, Abcam, Cambridge, UK), HSP90α (1:70, Enzo Life Sciences, Loerrach, Germany), and HSP90β (1:100, Abcam, Cambridge, UK), TGF-β receptor II (TGF-βRII) (1:75, Santa Cruz Biotechnology, Heidelberg, Germany). Double-IHC-stainings were performed on mice lung tissue sections for alpha-SMA (red dye, cytoplasmic staining of smooth muscle cells and myofibroblasts) and TTF1 (brown dye, nuclear staining of alveolar epithelial type-II cells (AECII) and Clara cells.

Immunofluorescence

Cells seeded on 8-well chamber slides were fixed with ice-cold methanol for 10 minutes at 4°C. Then, after blocking in 4% bovine serum albumin for 1 hour at RT, cells were incubated overnight at 4°C with primary antibodies against E-cadherin (1:100, BD Biosciences, Heidelberg, Germany) and fibronectin (1:200, Sigma-Aldrich, Munich, Germany), α-SMA (1:500, Abcam, Cambridge, UK). The next day, DyLight 549-conjugated secondary antibodies (Zytomed, Berlin, Germany) were applied for 1 hour at RT followed by DAPI staining for nuclei. For each antibody, fluorescence microscopy images were captured under the same exposure time and conditions.

Collagen Assay

After serum-starvation for 24 hours, the cells were stimulated with 10 ng/ml human recombinant TGF-β1, in the absence or presence of 50 nM 17-AAG for 72 hours. Soluble collagen content from human ILFBs or mice lung homogenates was assessed by Sircol collagen assay (Biocolor, Carrickfergus, UK) according to the manufacturer's instructions. Briefly, soluble collagen was collected in 0.5 M acetic

acid and stained with sirius red dye, and the absorbance was measured by an enzyme-linked immunosorbent assay (ELISA) reader. The amount of collagen was presented as μg, using a standard curve prepared from the kit at the same time.

In vitro Scratch Assay

Human ILFBs were seeded on 48-well plates. After serum-starvation for 24 hours, scratches were created using a yellow pipette tip. Next, cells were washed twice with PBS and cultured in medium containing 10 ng/ml human recombinant TGF-β1 in the presence or absence of 50 nM 17-AAG. The images were captured by Leica real-time microscope system at the start of the experiment and every one hour until 12 hours later. After live-imaging, cells were immediately fixed with ice-cold methanol followed by DAPI staining and fluorescence images were captured by the same microscope system. The initial two edges of the scratch were marked and migrated cells with DAPI staining were counted by Image Pro Plus software.

Cell Croliferation BrdU Assay

Cell proliferation was assessed by BrdU assay (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, $1x10^4$ human ILFBs were cultured in a 96-well plate. Next day cells were starved overnight followed by stimulation with 10 ng/ml TGFβ-1 (R&D Systems, Wiesbaden-Nordenstadt, Germany) in the presence or absence of 50nM 17-AAG for 12 hours. BrdU was added to cells in the last 4-hour of incubation. Further, being fixed by FixDenat solution, cells were probed with anti-BrdU-POD antibody for 90 minutes followed by substrate development. The reaction product was quantified by measuring the absorbance with an ELISA reader.

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Figure S1. HSP90 expression and localization in lung of donors and IPF patients. (A) Representative immunohistochemical staining of HSP90α, HSP90β, α-SMA and cytokeratin-5 (KRT5) in serial lung sections from patients with IPF. In IPF, α-SMA-positive myofibroblasts in fibroblast foci (FF) as well as overlying abnormal bronchiolar basal cells (indicated by hashmarks and KRT5 expression) revealed robust cytoplasmic overexpression of HSP90α and HSP90β. Overexpression of HSP90α and HSP90β in myofibroblastic foci is indicated by arrows. In addition, HSP90α and HSP90β were robustly overexpressed in bronchial epithelial cells of ′hyperplastic′ bronchioles (BE, bronchioles are indicated by cytoplasmic KRT5-staining of basal cells). Vascular smooth muscle cells (VSMC´s, indicated by hashmark and α-SMA staining) and the smooth muscle present in the wall of bronchioles (indicated by asterisk) indicated nuclear and cytoplasmic expression of HSP90α, but not of HSP90β. **(B)** Representative immunohistochemical staining of HSP90α, HSP90β, and prosurfactant-protein C (proSP-C) in serial lung sections from donors and patients with IPF. In IPF, type-II alveolar epithelial cells (AECII, indicated by proSP-C staining) indicated robust cytoplasmic overexpression of HSP90β (indicated by arrows), whereas HSP90α was not expressed in IPF-AECII (indicated by arrows). In donor lungs, HSP90β-IHC indicated moderate expression in AECII, whereas HSP90α was not expressed in this cell type (see arrows in stainings of donor lungs). Bronchial epithelial cells (BE) in donor lungs indicated basal expression of both HSP90α and HSP90β. **(C)** Representative western blots of HSP90α and HSP90β of lung homogenates from donors and patients with IPF, with densitometric quantification. β-actin served as the loading control. All values are expressed as mean ± SEM of n = 3 per group. $^{**}P < 0.01$, $^{***}P < 0.001$, versus Donor.

Figure S2. HSP90 expression and localization in lungs of sham and bleomycin-challenged mice. (A) Representative immunohistochemical staining of HSP90α, HSP90β, TTF1 (marker for AECII and Clara cells) and α-SMA (marker for myofibroblasts and smooth muscle cells) in serial lung sections from sham and bleomycin-challenged mice. TTF1-IHC (brown dye) and α-SMA-IHC (red dye) were performed in one and the same lung section. AECII in normal lungs of sham mice (AECII are marked by brown TTF1-staining) indicated no significant expression of HSP90α and HSP90β (see arrows). Bronchial epithelial cells (BE) in normal mice lungs of sham mice revealed expression of both HSP90α and HSP90β. In bleomycin-injured lungs (Bleo), myofibroblastic cells in areas of dense fibrotic remodelling (indicated by hashmarks) revealed strong overexpression of HSP90α and HSP90β. TTF1-positive AECII of bleomycin-injured lungs revealed robust overexpression of HSP90β, but not of HSP90α (see arrows in both IHC´s). Bronchial epithelial cells (BE) of bleomycin-treated mice indicated overexpression of both HSP90α and HSP90β, and comprised mainly Clara cells (positive for TTF1). **(B)** Representative western blots of HSP90α and HSP90β of lung homogenates from sham and bleomycin-challenged mice (Bleo), with densitometric quantification. β-actin served as the loading control. All values are expressed as mean ± SEM of n = 3 per group. **P* < 0.05, versus Sham.

Figure S3. 17-AAG dose-dependently inhibits TGF-β1-induced ILFB transdifferentiation and extracellular matrix production. Analysis of protein expression of fibronectin, collagen I and α-SMA by western blot. The experiment was repeated in three donors and three IPF patients; representative blots are shown. After 24 hours of serum-starvation, ILFBs were stimulated with recombinant TGF-β1

(10 ng/ml) for 24 hours in the absence or presence of 17-AAG. Various doses of 17-AAG were used in the experiment (10, 20, 50,100 and 200 nM).

Figure S4. 17-AAG does not affect ILFB proliferation. Cell proliferation measured by BrdU incorporation assay. ILFBs were stimulated with recombinant TGF-β1 (10 ng/ml) for 12 hours in the absence or presence of 17-AAG (50 nM or 100 nM) or 17-AAG alone, following 24 hours of serum-starvation. The experiment was repeated independently three times and all values are given as the mean ± SEM of n= 3 per group.

Figure S5. HSP90α does not co-immunoprecipitate with TGFβRII in ILFBs. Western blots of HSP 90α and TGF-βRII after protein lysate from IPF ILFBs was immunoprecipitated with anti-TGF-βRII antibody or IgG antibody. The experiment was repeated independently three times; representative blots are shown. After 24 hours of serum-starvation, ILFBs were stimulated with recombinant TGF-β1 (10 ng/ml) for 24 hours in the absence or presence of 17-AAG.

Figure S6. TGF-βRII expression is decreased in lungs of bleomycin-challenged mice after 17-DMAG treatment. Representative immunohistochemical staining of α-SMA (marker for myofibroblasts and smooth muscle cells) and TGF-βRII **(A)** as well as proSP-C and (marker for type-II alveolar epithelial cells, AECII) and TGF-βRII **(B)** in serial lung tissue sections of bleomycin-challenged mice, 17-DMAG-treated bleomycin-challenged mice and sham mice. **(A)** In bleomycin-injured lungs, TGF-βRII was expressed in vascular smooth muscle cells (VSMC´s), in α-SMA positive myofibroblasts in areas of dense fibrosis (indicated by asterisks), as well as in

bronchial epithelial cells (BE). Treatment of bleomycin-challenged mice with 10 or 25 mg/kg 17-DMAG at day 7 to day 21 resulted in marked reduction of TGF-βRII expression in the interstitium as well as in bronchial epithelial cells (BE), but not in VSMC´s of lungs from treated mice. Finally, TGF-βRII was generally expressed in VSMC´s and bronchial epithelium (BE) in lungs of sham mice. **(B)** Hyperplastic AECII near areas of dense fibrotic tissue indicated robust overexpression of TGF-βRII (arrows) in comparison to normal AECII of healthy mice lungs (Sham), which did not reveal significant expression of TGF-βRII (see arrows). Of note, hyperplastic AECII in lungs of 17-DMAG-treated bleomycin-challenged mice (with marked reduction of fibrotic tissue) indicated no significant immunostaining for TGF-βRII (indicated by arrows). Strong immunostaining for TGF-βRII was also observed in the ′fibrotic interstitium′ of bleomycin-mice (indicated by asterisks), whereas 17-DMAG-treated bleomycin-challenged mice revealed significant reduction of dense fibrotic tissue.