The transcription factor ets-2 plays an important role in the pathogenesis of pulmonary fibrosis

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ONLINE DATA SUPPLEMENT

METHODS

Materials: The active TGFβ ELISA kit and recombinant proteins were purchased from R&D Systems (Minneapolis, MN); bleomycin through the Ohio State University Hospitals Pharmacy via Gensia Sicor Pharmaceuticals (Irvine, CA); reverse transcription and real time PCR reagents from Applied Biosystems (Foster City, CA); type-I collagen and αSMA antibodies from Abcam (Cambridge, MA); Sircol Collagen Assay from Accurate Chemical (Westbury, NY); phosphorylated-ets-2 antibody from Michael Ostrowski, PhD (The Ohio State University); Nuance FX Multispectral Imaging System from Cambridge Research and Instrumentation (Woburn, MA); detection reagents for immunohistochemistry kindly provided by Kathleen Sergott and Christopher Roberts.

Real Time PCR: Total RNA was extracted from lung tissue by freeze fracture and Trizol extractions. Reverse transcription was performed on 2 μg of isolated RNA. The resulting cDNA was analyzed and used for real time PCR with SYBR Green. Primer sequences: mouse GAPDH: 5′-GCACAGTCAAGGCCGAGAAT-3′ (For); 5′-GCCTTCTCCATGGTGGTGAA-3′ (Rev); mouse type I Collagen: 5′-ATGGATTCCCGTTCGAGTACG-3′ (For); 5′-TCAGCTGGATAGCGACATCG-3′ (Rev); mouse type III Collagen: 5′-CACCCTTCTTCATCCCACTCTTA-3′ (For); 5′-ACCAAGGTGGCTGCATCC-3′ (Rev); mouse α-smooth muscle actin: 5'-CTGACAGAGGCACCACTGAA-3 (For); 5'-CATCTCCAGAGTCCAGCACA-3' (Rev); mouse CTGF: 5'-AAAGTGCATCCGGACACCTAA-3' (For); 5'-TGCAGCCAGAAAGCTCAAACT-3' (Rev). Real time PCR analysis was done using an ABI 7700 machine with GAPDH as an internal loading control, using the ΔΔCt method to express relative changes in expression levels between comparison groups.

Sircol collagen assay: Assay was done according to the instructions provided by the manufacturer (Accurate Chemical and Scientific Corp., Westbury, NY) and as previously described [1]. Data was expressed as [(μg/ml)/kg body weight] to standardize for any lung volume loss and protein increase in the lungs due to the bleomycin regimen and resulting lung injury.

Bronchoalveolar lavage (BAL) analysis: BAL fluid was centrifuged to pellet the

cells, red blood cells were lysed, and supernatant (BALF) was collected and stored for ELISA analyses. Cells were resuspended in 100 μl of PBS, cytospun on slides, stained, and analyzed under a microscope for cell differentials.

Cell proliferation assay: Primary lung fibroblasts were isolated by mincing the lungs into small pieces and plating the pieces in a T-75 flask containing DMEM medium supplemented with heat inactivated FBS. After 3 hours, non-adherent cells were removed and the flask was washed gently with fresh media while maintaining the lung pieces. After 3 days, the lung pieces were removed, and the cells were allowed to expand for 10-14 days in DMEM medium supplemented with heat inactivated FBS. For the cell proliferation assay, we used the XTT Cell Proliferation Kit (Roche Applied Science, Indianapolis, IN) and followed the protocol as described.

TITLES AND LEGENDS TO THE FIGURES

Table E1: Inflammatory cell profiles in the lungs of ets-2 (WT/WT) and ets-2 (A72/A72) mice following bleomycin administration.

Figure E1: Levels of total active TGFβ in BALF from ets-2 (WT/WT) and ets-2 (A72/A72) following bleomycin treatment at 33 days do not significantly differ. Following bleomycin administration (33 day regimen), ets-2 (WT/WT) and ets-2 (A72/A72) mice were sacrificed, and 1.0 ml of BALF was collected. 100 μl of the BALF was separated, and 20 μl of 1.0 N HCl was added to activate any latent TGFβ and incubated at room temperature for 10 minutes. The sample was then neutralized by addition of 13 μL of 1.2 N NaOH/0.5 M HEPES and subjected to ELISA analysis for active TGFβ. n=3 mice per group. Statistical analysis gave a p-value of 0.22.

Figure E2: Ets-2 (WT/WT) mice do not express elevated levels of phosphorylated ets-2 at 11 days and 22 days following bleomycin treatment. Mice were treated with bleomycin as described. At 11 days (top panels) or 22 days (bottom panels), mice were sacrificed, the lungs were removed, sectioned and stained for phosphorylated ets-2 as described. **Left panels** show the RGB image, with the 11 days having a red stain for positive phosphorylated ets-2, and the 22 days having a brown stain for phosphorylated ets-2, which was primarily background staining (not nuclear). These images were then converted to fluorescence (**right panels**) using the Nuance imaging system, as described. Appearance of a red signal in the right panels indicates positive nuclear staining for phosphorylated ets-2.

Figure E3: Ets-2 (A72/A72) mice express significantly less CCL12. A. BMMs were derived from ets-2 (WT/WT) and ets-2 (A72/A72) mice. Cells were serum-starved overnight and stimulated with 100 ng/ml of M-CSF for 36 hours. The cell supernatants were collected and analyzed for CCL12/ expression via ELISA. Data is adjusted for stimulated value minus non-

stimulated value to correct for background expression. Data is represented as mean ± S.E.M. of three independent experiments. Ets-2 (WT/WT) and ets-2 (A72/A72) mice were treated with bleomycin as described for 33 days. **B.** BAL fluid was analyzed for CCL12 expression via ELISA. Data shown are the values of TGFβ-stimulated samples corrected for the basal secretion (non-stimulated), with equivalent cell numbers in each set. Data is representative of at least 3 mice per condition and is expressed as mean ± S.E.M. **C.** mRNA expression of CCL12 was determined via real time PCR. Data is representative of at least 3 mice per condition and is expressed as mean ± S.E.M.

Figure E4: Primary fibroblasts from ets-2 (A72/A72) mice have an increased proliferation rate compared to primary fibroblasts from ets-2 (WT/WT) mice. Primary lung fibroblasts were also isolated from ets-2 (WT/WT) and ets-2 (A72/A72) mice as described. Cells were plated in varying densities in DMEM supplemented with 10% FBS. An XTT assay was then performed per the manufacturer's instructions. Data is represented as mean ± S.E.M and cells from at least six mice [three ets-2 (WT/WT) mice and three ets-2 (A72/A72) mice]. * $p < 0.05$ vs ets-2 (WT/WT) for each indicated cell number.

Reference List

1. **Baran, C. P., J. M. Opalek, S. McMaken, C. A. Newland, J. M. O'brien, Jr., M. G. Hunter, B. D. Bringardner, M. M. Monick, D. R. Brigstock, P. C. Stromberg, G. W. Hunninghake, and C. B. Marsh. 2007. Important Roles for Macrophage Colony-stimulating Factor, CC Chemokine Ligand 2, and Mononuclear Phagocytes in the Pathogenesis of Pulmonary Fibrosis. *Am.J.Respir.Crit Care Med.* 176:78-89.**