ORIGINAL RESEARCH

Intracellular Heat Shock Protein 70 Deficiency in Pulmonary Fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) pathogenesis has been postulated to involve a variety of mechanisms associated with the aging process, including loss of protein homeostasis (proteostasis). Heat shock proteins are cellular chaperones that serve a number of vital maintenance and repair functions, including the regulation of proteostasis. Previously published data have implicated heat shock protein 70 (Hsp70) in the development of pulmonary fibrosis in animal models. We sought to identify alterations in Hsp70 expression in IPF lung. Hsp70 mRNA and protein were decreased in primary fibroblasts cultured from IPF versus normal donor lung tissue. In addition to cultured fibroblasts, Hsp70 expression was decreased in intact IPF lung, a stressed environment in which upregulation of protective heat shock proteins would be anticipated. In support of a mechanistic association between decreased Hsp70 and fibrosis, cultured primary lung fibroblasts deficient in Hsp70 secreted increased extracellular matrix proteins. Treatment of primary normal human lung fibroblasts *in vitro* with either of the profibrotic molecules IGFBP5 (insulin-like growth factor–binding protein 5) or transforming growth factor-β1 downregulated Hsp70, suggesting Hsp70 is a downstream target in the fibrotic cascade. Hsp70-knockout mice subjected to an inhalational bleomycin model of pulmonary fibrosis demonstrated accelerated fibrosis versus wild-type control animals. We therefore conclude that reduced Hsp70 protein contributes to fibrosis and that interventions aimed at restoring normal expression of Hsp70 represent a novel therapeutic strategy for pulmonary fibrosis.

Keywords: heat shock protein 70; proteostasis; transforming growth factor-β; idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is the most common and severe idiopathic interstitial lung disease (1). It has an estimated annual incidence in the United States of over 30,000 (2) and carries a poor prognosis, with average 5-year mortality estimated between 50% and 70% (3, 4). It is characterized by fibroblast proliferation and extracellular matrix (ECM) accumulation in the lung. Although the basic mechanisms of fibrogenesis evolved as a conserved survival mechanism to repair damaged tissue, a comprehensive understanding of the altered regulation of the ECM responsible for the initiation and perpetuation of pathologic fibrosis remains elusive (reviewed in [5]).

Loss of protein homeostasis (proteostasis) is one of the hallmarks of aging (6). All cells have control mechanisms to preserve the stability and functionality of their proteomes. Heat shock proteins are the most important cellular proteins involved as molecular chaperones to stabilize proteostasis (6). They have important housekeeping functions under

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nonstress conditions and are induced in response to various stressful stimuli either to mediate repair of damaged proteins and promote cell survival or, alternatively, to commit an irreparably damaged cell to death (7, 8). In the process of aging, most of these chaperones reduce their function and their capacities to balance the proteome (9).

The Hsp70 (heat shock protein 70) family of heat shock proteins is particularly important. This family is highly conserved and in humans comprises at least eight unique proteins (10, 11), of which some of the best studied are the major constitutive isoform Hsc70 (heat shock cognate 71 kD protein, encoded in humans by the *HspA8* [heat shock 70 kD protein 8] gene) and a major inducible isoform Hsp72 (encoded in humans by the *HspA1A* [heat shock 70 kD protein 1A] gene). Observations in animal models have suggested an association between Hsp70 deficiency and lung fibrosis. Mouse models of bleomycin-induced pulmonary fibrosis suggest that increased expression of Hsp70 is protective against lung injury and fibrosis (12) and that the suppression of expression of Hsp70 contributes to more significant lung fibrosis (13). However, studies of Hsp70 in human IPF are scarce. Circulating autoantibodies to Hsp70 have been reported in association with worse outcome among individuals with IPF (14), and analysis of Hsp70 gene polymorphisms in a cohort of Mexican patients with IPF demonstrated that some of these polymorphisms are associated with a decreased risk of IPF (15). These are intriguing observations; however, these studies did not specifically investigate the role of Hsp70 in the pathogenesis of human IPF.

In this study, we demonstrated reduced expression of both the major inducible (Hsp72) and constitutive (Hsc70) isoforms of Hsp70 in both cultured fibroblasts and intact fibrotic areas of lungs explanted from individuals undergoing transplant for IPF. We have identified alteration of the heat shock response, specifically impaired Hsp72 expression, as one mechanism through which the profibrotic mediators transforming growth factor-β1 (TGF-β1) and IGFBP5 (insulin-like growth factor–binding protein 5) affect the homeostatic balance of fibroblasts, the cells responsible for ECM production and therefore development of fibrosis. Furthermore, we demonstrated increased susceptibility of Hsp70.1/3 (Hsp72 ortholog)–knockout mice to fibrosis in an inhalational bleomycin model. Modulation of the heat shock response represents a novel and promising therapeutic strategy for pulmonary fibrosis, a family of fatal diseases for which there is no cure other than lung transplant.

Methods

Reagents

Dulbecco's modified Eagle medium (DMEM) was obtained from Mediatech. Cell culture dishes with 35-mm wells were purchased from Costar. FBS and anti–β-actin antibodies were obtained from Sigma-Aldrich. Antifibronectin, anti–type I collagen α1-chain, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology. Anti-Hsp70, anti-Hsp72, and anti-Hsc70 antibodies were obtained from Santa Cruz Biotechnology or StressGen/Enzo Life Sciences. Anti–platelet-derived growth factor (anti-PDGF) receptor-β antibodies were purchased from Abcam. Anti–pan-cytokeratin antibodies were obtained from Abcam. Chemiluminescence reagents were purchased from PerkinElmer Life Sciences, Inc. Penicillin, streptomycin, antimycotic agent, TRIzol reagent, oligo(dT) (10, 11, 16, 17) primer, random hexamer primer, and SuperScript II reverse transcriptase were obtained from Life Technologies. The TGF-β BMP Signaling Pathway Array (RT2 Profiler PCR Array PAMM-035Z) was purchased from Qiagen. Recombinant proteins (TGF-β1, PDGF, IL-4, IL-13) were obtained from R&D Systems, Inc. Bleomycin was purchased from Enzo Life Sciences. All other chemicals were obtained from Sigma-Aldrich unless otherwise noted.

Histopathology, Immunohistochemistry, and Immunofluorescence

Paraffin embedding and preparation of tissue sections was performed by the University of Pittsburgh Research Histology Service. Hematoxylin and eosin and Masson's trichrome staining was performed using a commercially available kit (IMEB Inc.). Immunohistochemistry was performed by our clinical pathology core laboratory using validated antibodies. Images were taken on an Olympus BX46 microscope with a calibrated Olympus DP25 camera using identical settings and postcapture processing. Immunofluorescence analysis was performed using validated antibodies, and images were taken with an Olympus FluoView FV1000 confocal microscope.

Primary Fibroblast Culture

Under a protocol approved by the University of Pittsburgh Institutional Review Board, human primary lung fibroblasts were cultured from the explanted normal lungs of organ donors or from the lungs of patients with IPF undergoing lung transplant surgery as we previously described (18). Mouse primary lung fibroblasts were cultured from lung tissues of Hsp70.1/3-knockout or B6129SF1 wild-type control mice. All primary lung fibroblasts were maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic as previously described and were used in passages 3 to 6 for all experiments (18).

Detection of mRNA in Cultured Primary Lung Fibroblasts

Hsp72, Hsc70, IGFBP5, and collagen Iα1 mRNA expression in cultured human and mouse lung fibroblasts was examined using semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). Total RNA from cultured lung fibroblasts was extracted using TRIzol reagent per the manufacturer's protocol for adherent cells. First-strand cDNA was synthesized using oligo(dT) (10, 11, 16, 17) primer and SuperScript II reverse transcriptase. Hsp72, Hsc70, collagen Iα1, IGFBP5, and β-actin mRNAs were detected by PCR using cDNA (50 ng of total RNA equivalent) as a template. Primer sets for human samples were forward 5′-AAGCGGTCCGGATAACGG and reverse 5′-ATCACCTGGAAAGGCCAGTG to amplify Hsp72 (511 bp); forward 5′-CCCGAGGTGTTCCTCAGATT and reverse 5′-CTCCTGCACTCTGGTACAGC to amplify Hsc70 (444 bp); forward 5′-GCAGAGTGTTGTCTCTCCCC and reverse 5′-CACGTGGGGAATCACTGTCA to amplify IGFBP5 (612 bp); and forward 5′-ATGTTTGAGACCTTCAACAC-3′ and reverse 5′-CACGTCACACTTCATGATGG to amplify β-actin (494 bp). Primer sets used for mouse samples were forward 5′-GAGCGGAGAGTACTGGATCG and reverse 5′-GTTCGGGCTGATGTACCAGT to amplify collagen Iα1 (142 bp); forward

5′-AGACCCTCTTCCGACCATGA and reverse 5′-TCGACGAGACCTCTTTCCCT to amplify IGFBP5 (269 bp); and forward 5′-CGAGCGGTTCCGATGCCCTG and reverse 5′-ACGCAGCTCAGTAA CAGTCCGC to amplify β-actin (394 bp). PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. One-step qRT-PCR was performed with p21 primer from Life Technologies and normalized using GAPDPH.

Fibroblast Stimulation

Actively growing primary human lung fibroblasts in early passage were plated at a density of 2×10^5 cells per well in 6-well tissue culture plates. After 24 hours, cells were serum starved in DMEM with 1% antibiotic-antimycotic for 12–16 hours before stimulation with human recombinant TGF-β1 (10 ng/ml), PDGF (20 ng/ml), IL-4 (20 ng/ml), or IL-13 (20 ng/ml). Control wells were treated with the appropriate vehicle for each recombinant protein (4 mM hydrochloric acid or PBS). IGFBP5 protein was expressed using replication-deficient adenoviral expression vectors to infect primary human normal lung fibroblasts, as previously described (18). Conditioned media and cell lysates were harvested at the indicated time points and evaluated for expression of Hsp72 and Hsc70 by Western blot analysis as previously described (19). Because bleomycin induces cellular senescence (20), we also stimulated lung fibroblasts with bleomycin (25 ng/ml) during 3 days.

Hsp72 Knockdown

To knock down Hsp72 expression, RNA interference experiments were performed using human HSP70 siRNA targeting the coding region 245–265 (accession no. NM_021979; 5′-AAGAAC- CAGGTGGC CATGAA-3′) of the HSP70 sequence designed with Dharmacon software and a control nonspecific siRNA from Dharmacon according to the manufacturer's instructions. Furthermore, nontargeting scrambled siRNA was used as a negative control.

Mice and Bleomycin Administration

All animal experiments were performed according to our University of Pittsburgh Institutional Animal Care and Use Committee–approved protocol in an Association for Assessment and Accreditation of Laboratory Animal Care International–certified animal facility. Hsp70.1/3 (Hsp72 ortholog)–knockout mice (B6;129S7-*Hspa1a/Hspa1b*^{tm1Dix}/Mmcd) generated by Dr. David Dix (21–23) were obtained from the Mutant Mouse Regional Resource Center at the University of California, Davis. These mice do not express detectable mRNA or protein for the major inducible isoform of the Hsp70 family. Wild-type control mice of the same genetic background (B6129SvF1) were obtained from The Jackson Laboratory. For all experiments, age- and sex-matched (8–10-wk-old male and 22–24-wk-old male) mice were anesthetized with 2% isoflurane vapor in an anesthesia chamber and were administered either PBS (vehicle control) or 2 mg/kg bleomycin (in PBS) in a 30-μl volume via the inhalational route by slowly beading the solution onto the nares. The health of the mice was monitored including serial weights after treatment. Mice were killed on Day 14 after treatment. Lung tissues were flash frozen and stored at −80°C or were inflation fixed at 20 cm H_2O with 10% neutral buffered formalin, then embedded in paraffin for subsequent histologic analysis.

Hydroxyproline Assay

Hydroxyproline content of flash-frozen left mouse lungs was measured using a recently described modification (24) of the technique of Woessner and colleagues (25). Briefly, lungs were minced and digested for 12–16 hours at 110°C in 1 ml of 6N hydrochloric acid. After neutralization with 6N sodium hydroxide, the pH was adjusted within a range of 6.0–9.0. Samples (100 μl) were mixed with 1 ml of chloramine T solution (1.4% chloramine T, 10% isopropanol, 0.5 M sodium acetate, pH 6.0) and incubated for 20 minutes at room temperature. Next was added 1 ml of Ehrlich's solution (14.9% *p*-dimethylaminobenzaldehyde, 70% isopropanol, 20% perchloric acid), and samples were incubated for 15 minutes at 65°C. Aliquots (200 μl) were transferred to 96-well plates, and absorbance at 570 nm was measured. Standard curves for the experiment were generated using known concentrations of *cis*-4-hydroxy-L-proline.

Fibrosis Scoring

Ashcroft scoring was performed as described previously (26). All lobes were sectioned on a single slide, and 20 nonoverlapping 10× fields were scored per sample. The average score of all fields indicates the Ashcroft score of a single sample. The scoring scale was as follows: 0 = no abnormalities; 1 = slight thickening of alveolar membranes; 2 = small areas of fibrosis (<10%); 3 = 10–20% fibrotic area; 4 = 20–40% fibrotic area; 5 = 40–60% fibrosis; 6 = 60–80% fibrosis; 7 = greater than 80% fibrosis; and 8 = complete fibrosis.

TGF-β/Bone Morphogenetic Protein Signaling Pathway Array

Using TRIzol reagent according to the manufacturer's protocol for adherent cells, total RNA was extracted from cultured primary mouse lung fibroblasts from Hsp70.1/3-knockout, heterozygous, and wild-type mice. Genes related to TGF-β/bone morphogenetic protein (BMP)–mediated signal transduction were evaluated by the SABiosciences Service Core for Gene Expression and Genomic Analysis, using the Qiagen Mouse TGFβ/BMP Signaling Pathway PCR Array.

Densitometric and Statistical Analysis

Densitometric analysis of RT-PCR and Western blot results were performed on 8-bit grayscale scanned images of the original films using NIH ImageJ open-access software or on digital images acquired using the ProteinSimple FluorChem workstation using the AlphaView software package (ProteinSimple, Inc.). Statistical comparisons were performed using Student's *t* test. Values are expressed as average (mean) ± SD. *P* values less than 0.05 are considered to be statistically significant.

Results

Hsp70 Expression Is Reduced in IPF Fibroblasts

Fibroblasts isolated from IPF versus normal human lung tissue demonstrate increased ECM production (27), and we hypothesized that Hsp70 expression would also be altered. Primary human lung fibroblasts were cultured from normal (nondiseased) and IPF lungs. Total RNA and protein were prepared from fibroblasts cultured from five independent IPF (average age, 66 ± 9 yr) or normal donors (average age, 51 ± 17 yr), and concentrations of the major inducible (*HspA1A* gene, Hsp72 protein) and

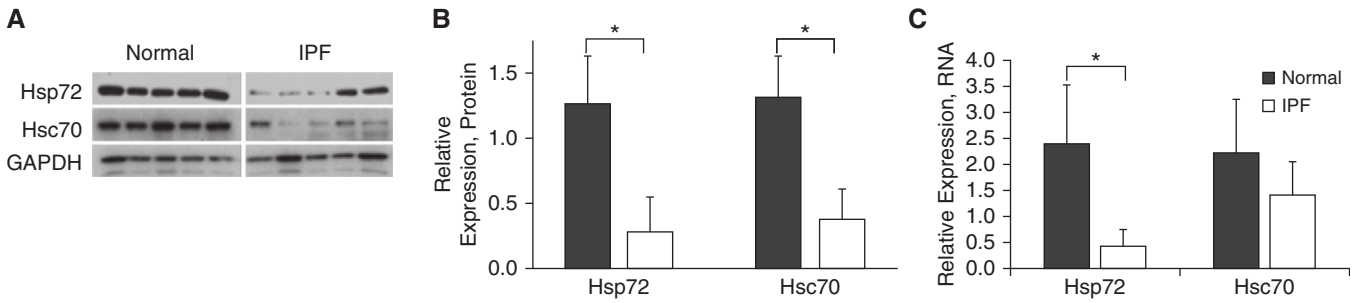

Figure 1. Inducible and constitutive heat shock protein 70 (Hsp70) expression is reduced in idiopathic pulmonary fibrosis (IPF) fibroblasts. (*A–C*) Total RNA and whole-cell protein lysates were prepared from primary fibroblasts cultured from four independent normal (average age, 51 ± 17 yr) and IPF (average age, 66 ± 9 yr) donor lungs and analyzed for relative expression of inducible (Hsp72, HspA1A gene) and constitutive (Hsc70 [heat shock cognate 71 kD protein], HspA8 gene) Hsp70 isoforms by Western blotting (*A* and *B*) and semiquantitative RT-PCR (*C*). In IPF fibroblasts, inducible Hsp70 message is significantly less abundant. At the protein level, both inducible and constitutive isoforms are significantly reduced (*$P < 0.05$).

constitutive (*HspA8* gene, Hsc70 protein) isoforms of Hsp70 were evaluated by semiquantitative RT-PCR and Western blot analysis (Figure 1). mRNA for the inducible isoform was decreased over fivefold in IPF versus normal lung fibroblasts. At the protein level, both Hsp72 and Hsc70 were significantly diminished in IPF versus normal lung fibroblasts.

Hsp70 Expression Is Reduced in IPF and Aging Lung

We next tested the hypothesis that impaired Hsp70 expression is also seen *in vivo* in fibrotic lung tissue and is not an artifact of *in vitro* culture conditions. We examined the distribution of Hsp70 expression in lung tissue from individuals with IPF versus adult control donors (Figure 2). In the normal lung, there was prominent Hsp70 immunostaining (Figure 2C) that at higher magnification was demonstrated to be primarily a nuclear staining pattern of the alveolar cells (Figure 2D). In contrast, in the IPF lung (Figures 2A and 2B), there was no Hsp70 immunostaining of either alveolar epithelial cells or fibroblasts in areas with fibroblast foci. Consistent with recently published observations (14), we observed nuclear and cytoplasmic Hsp70 staining in the airway epithelial cells of both IPF and normal lung (data not shown).

Using immunofluorescence, we assessed the expression of Hsp70 and confirmed it was highly expressed in lungs from adult donors but was not detectable in IPF lung tissue, confirming the significant reduction of Hsp70 expression in IPF lung (Figures 3A and 3B). Interestingly, in adult donors, we observed colocalization of Hsp70 and pan-cytokeratin antibodies, suggesting that the expression of Hsp70 in alveolar regions is mainly in epithelial cells (*see* Figure E1 in the data supplement).

Reduced Fibroblast Hsp72 Results in a Fibrotic Phenotype

On the basis of our human lung and fibroblast data and published observations that induction of Hsp70 may repress TGF-β1 signaling in epithelial cell lines (28–30), we hypothesized that reduction of Hsp72 in fibroblasts would be associated with a fibrotic phenotype even in the absence of exogenous TGF-β1 or IGFBP5. In fibroblasts isolated from young Hsp70.1/3-knockout (23) and wild-type littermate control mice, genes related to TGF-β/BMP–mediated signal transduction were evaluated using the Qiagen Mouse TGFβ/BMP Signaling Pathway PCR Array.

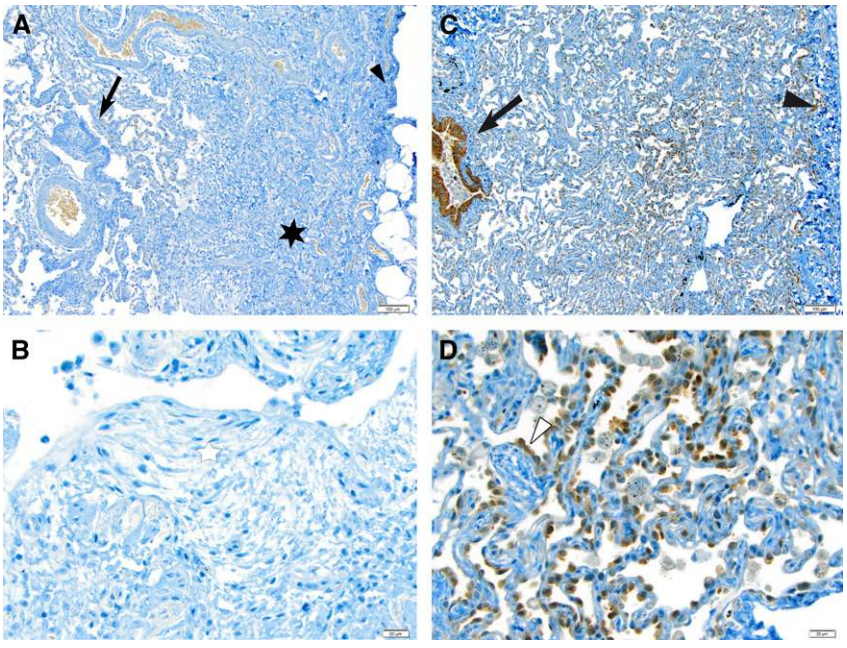

Figure 2. Total Hsp70 expression is reduced in IPF lung. Secondary lobules with central bronchiole (arrows) and pleura (black arrowheads) showing (*A*) lack of Hsp70 expression in IPF with fibrosis (black star) compared with (*C*) normal lung from an adult donor (aged 53 yr). At higher magnification, (*B*) no Hsp70 expression is seen in fibroblastic foci (white star) of IPF, whereas (*D*) Hsp70 can be demonstrated in nuclei and cytoplasm of pneumocytes in the normal lung from a patient (white arrowhead). Immunohistochemical stain for Hsp70. Scale bars: 100 μm (*A* and *C*) and 20 μm (*B* and *D*).

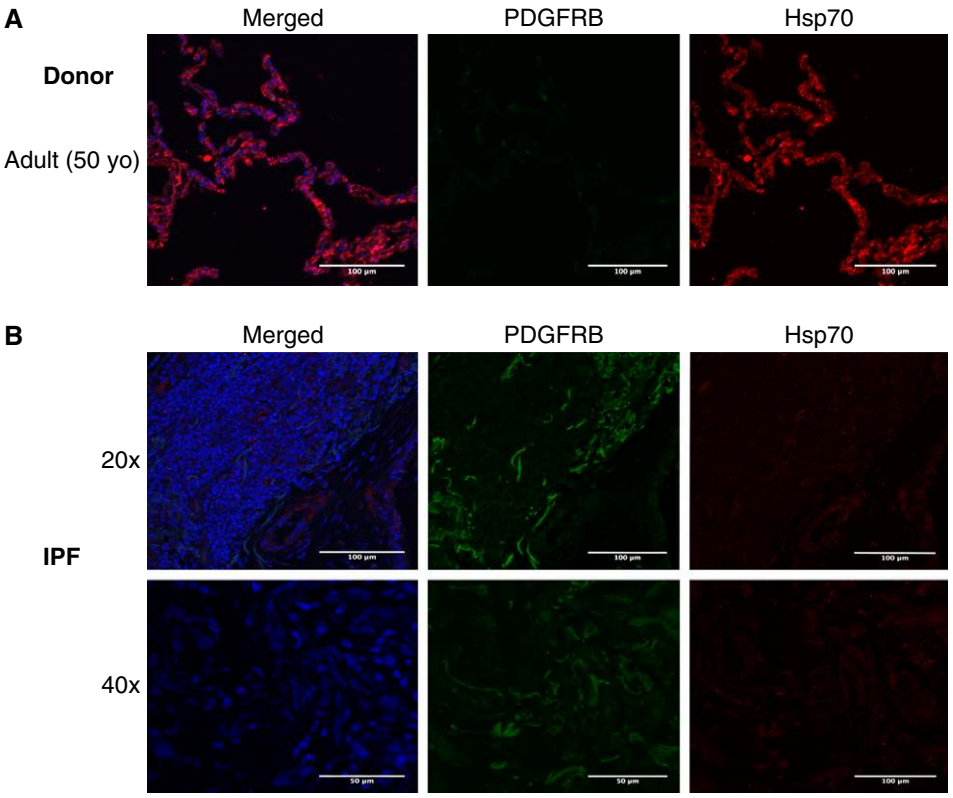

Figure 3. Total Hsp70 expression is higher in lungs from adult patients than in those with IPF. Representative immunofluorescence using anti-Hsp70 (red) and anti–PDGFR-β (PDGFRB, green) antibodies in lungs from (*A*) an adult patient without IPF (aged 50 yr) and (*B*) a patient with IPF (aged 74 yr). Hsp70 was highly expressed in lungs from adult patients but not in patients with IPF. In IPF, the absence of Hsp70 was observed in PDGFR-β–positive fibrotic areas. Corresponding negative controls (immunofluorescence with corresponding secondary antibody but without primary antibody) are included in Figure E2 in the data supplement. Scale bars: 100 μm and 50 μm. PDGFRB = platelet-derived growth factor-β.

We observed an overexpression of different relevant genes of TGF-β/SMAD pathway in Hsp70.1/3-knockout fibroblasts compared with wild-type control fibroblasts (Figure 4A). In addition, collagen and fibronectin protein (Figure 4B) were increased in cultured primary lung fibroblasts from Hsp70.1/3-knockout versus wild-type mice. These data suggest a mechanistic link between reduced inducible Hsp70 (Hsp72) and increased production of ECM components, a hallmark of fibrosis. This effect is likely associated with a loss of the inhibitory effect of Hsp70 in TGF-β/SMAD activation, as has previously been suggested (31).

Hsp72 Protein in Lung Fibroblasts Is Reduced by the Profibrotic Mediators IGFBP5 and TGF-β1

We next characterized Hsp70 inducible (Hsp72) and constitutive (Hsc70) protein concentrations in primary human normal lung fibroblasts in response to several growth factors and cytokines implicated in the regulation of the ECM (Figure 5). Two classical profibrotic mediators, IGFBP5 and TGF-β1, significantly decreased Hsp72 protein concentrations in cultured fibroblasts from adult normal donors after 48–72 hours, a time point at which expression of ECM proteins collagen and fibronectin are upregulated (18, 32). However, neither IGFBP5 nor TGF-β1 changed constitutive Hsc70 protein concentrations *in vitro*. Thus, two independent fibrogenic stimuli, IGFBP5 and TGF-β1, were each able partially to recapitulate the heat shock protein defect observed in cultured IPF fibroblasts. No effect on either Hsp72 or Hsc70 protein concentration was seen in response to PDGF, IL-4, or IL-13 (data not shown). Figure 5E demonstrates increased production of fibronectin (a major ECM structural protein) when Hsp72 protein expression was knocked down in normal lung fibroblasts transfected with *HspA1A* siRNA. This suggests that reduced expression of Hsp72 could increase fibronectin deposition in the lung.

Reduced Hsp72 Accelerates Bleomycin-induced Pulmonary Fibrosis in Young Mice

We hypothesized that reduced inducible Hsp70 (Hsp72) contributes to increased susceptibility to profibrotic stimuli *in vivo*. Hsp70.1/3 (Hsp72 ortholog)–knockout mice (23) and wild-type control animals were administered either 2 mg/kg bleomycin or sterile PBS vehicle control intranasally in a volume of 30 μl. Histological and biochemical analyses were performed at Postadministration Day 14. Blinded histopathological scoring by three independent observers using a modified Ashcroft scale (26) revealed more extensive fibrosis in the Hsp70.1/3-knockout mice than in the wild-type control animals at Day 14 (Figures 6A, 6B, and 6D). Hydroxyproline analysis confirmed a significant increase in collagen content in the bleomycin versus saline control group

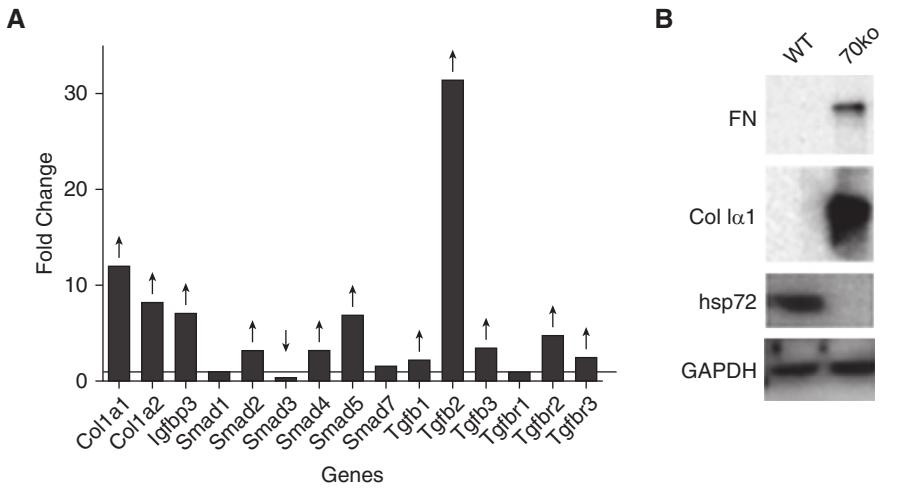

Figure 4. Reduced fibroblast inducible Hsp70 results in a fibrotic phenotype. Total RNA and whole-cell protein lysates were prepared from primary fibroblasts cultured from the lungs of Hsp70.1/3 (Hsp72 ortholog)–knockout (70ko) and wild-type (WT) mice and analyzed (*A*) for relative expression of relevant genes of the transforming growth factor-β (TGF-β)/SMAD pathway by reverse transcription–polymerase chain reaction and (*B*) for expression of fibronectin (FN) and collagen Iα1 (Col Ia1) proteins by Western blotting.

at Day 14 in the knockout mice but not in the wild-type control animals (Figure 6C). These data support the hypothesis that absence of Hsp72 results in accelerated development of lung fibrosis in response to bleomycin.

We next asked whether ECM accumulation would be affected by aging alone in the absence of inducible Hsp70, in the context of normal constitutive Hsp70 concentrations and without a second profibrotic insult such as bleomycin. We assessed 22–24-week-old Hsp70.1/3-knockout mice versus wild-type control animals and observed no spontaneous lung fibrosis by histology (Figure 6E) and no difference in hydroxyproline content (Figure 6F). This suggests that in the presence of normal constitutive Hsp70 expression, the absence of inducible Hsp70 alone is insufficient to promote abnormal ECM accumulation in advanced age.

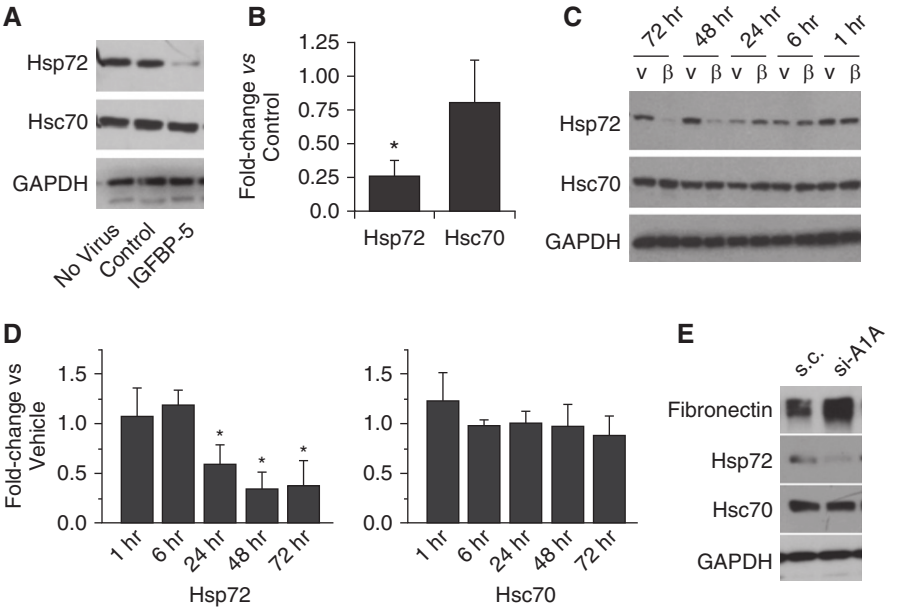

Figure 5. IGFBP5 (insulin-like growth factor–binding protein 5) and TGF-β1 reduce inducible Hsp70 expression in primary human fibroblasts. (*A* and *B*) Primary normal human lung fibroblasts were infected using an IGFBP5 expression vector or an empty control adenovirus, and cell lysates were prepared at 72 hours after infection for Western blot analysis of Hsp70 inducible (Hsp72) and constitutive (Hsc70) isoforms. (*C* and *D*) In separate experiments, primary normal human lung fibroblasts were stimulated for the indicated times with vehicle control (v) or recombinant human TGF-β1 (β) at a concentration of 10 ng/ml. Cell lysates were prepared, and Hsp72 and Hsc70 concentrations were analyzed by Western blotting. (*B* and *D*) Densitometry data from four or five independent experiments (*$P < 0.01$). (*E*) *HspA1A* siRNA suppressed Hsp72 expression in normal lung fibroblasts and consequently increased fibronectin production in cell lysates as analyzed by Western blotting. s.c. = scramble control siRNA; si-A1A = *HSPA1A* siRNA.

Discussion

Hsp70 is a vital cell-protective family of proteins involved in cell maintenance and repair. We have demonstrated that concentrations of two major Hsp70 isoforms, Hsp72 (inducible) and Hsc70 (constitutive), are significantly reduced *in vivo* in IPF lungs (Figures 2 and 3) and in primary fibroblasts cultured from IPF lung tissue (Figure 1), an environment in which it is well established that TGF-β1 and IGFBP5 concentrations are increased and promote fibrosis (33). Although reduced Hsp70 in IPF could merely be associated with the process of aging, a mechanistic link between deficient Hsp70 expression and fibrosis is supported by our observations that *1*) ECM production and secretion are increased in mouse lung fibroblasts lacking inducible Hsp70 (Figure 4) and in human lung fibroblasts with *HspA1A* siRNA–mediated reduction of Hsp72 expression (Figure 5E), and *2*) the profibrotic mediators TGF-β1 and IGFBP5 independently effect downregulation of inducible Hsp70 (Hsp72) expression *in vitro* in primary normal human lung fibroblasts (Figure 5). In addition, in knockout mice lacking inducible Hsp70, we demonstrated accelerated deposition of ECM in the lung in response to inhalational bleomycin injury (Figure 6), suggesting a deficiency of Hsp70 is relevant *in vivo*. Collectively, these observations support the hypothesis that regulation of the heat shock response is one pathway through which TGF-β and IGFBP5 affect ECM homeostasis and contribute to the development of fibrosis.

Hsp70 Has Been Proposed as a Potential Biomarker of Lifespan

Decreased serum Hsp70 concentrations have been observed with normal aging (34).

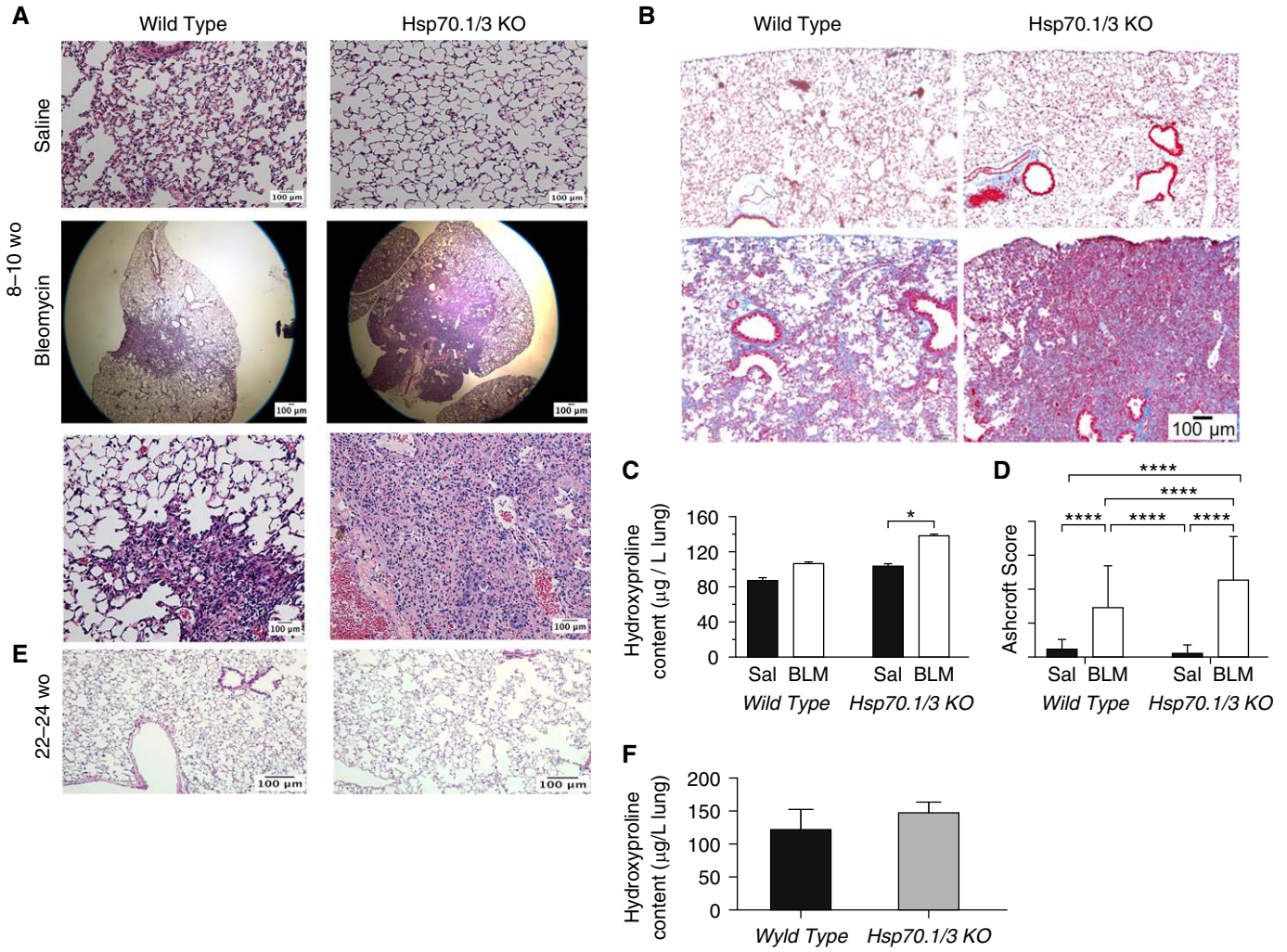

Figure 6. Absence of inducible Hsp70 accelerates bleomycin-induced pulmonary fibrosis in young mice. Hsp70.1/3 (Hsp72 ortholog)–knockout (KO) and wild-type control mice (8–10 wk old) were administered inhalational bleomycin (BLM) or sterile saline (Sal), and their lungs were harvested 14 days later for analysis. (*A* and *B*) Representative hematoxylin and eosin–stained sections and Masson's trichrome–stained sections. Scale bars: 100 μm. (*C*) Hydroxyproline analysis ($n = 4$–7 mice per group; $^*P < 0.05$). (*D*) Ashcroft scores of fibrosis measured at Day 14 ($^{****}P < 0.0001$). In addition, lungs from older Hsp70.1/3 (Hsp72 ortholog)–knockout and wild-type control mice (22–24 wk old) were harvested for analysis. (*E*) Representative hematoxylin and eosin–stained sections. Scale bars: 100 μm. (*F*) Hydroxyproline analysis ($n = 6$ mice per group).

In addition, there is a decrease of Hsp70 induction after an acute stress with aging (35, 36). The usual trigger of Hsp70 activation is the intracellular accumulation of incomplete or damaged proteins. If Hsp70 is decreased, its function as a chaperone will be impaired and may contribute to the progressive loss of proteostasis, one of the hallmarks of aging (8). IPF has been proposed to represent an "accelerated" form of aging of the lung (37). These observations, in conjunction with our data, suggest that Hsp70 deficiency associated with the aging lung may be one contributory mechanism to dysregulated injury repair and the development of fibrosis.

It has been described that Hsp70 has an inhibitory effect on the TGF-β/SMAD pathway (31). Tanaka and colleagues demonstrated that transgenic mice overexpressing Hsp70 were protected from lung inflammation and fibrotic lesions caused by bleomycin and developed lower TGF-β1 production and proinflammatory cytokine expression after bleomycin administration (12). Hsp70 appears to regulate several steps of the TGF-β/SMAD pathway, including Smad2 (38) and TGF-β receptor stability (28). This is consistent with our findings because fibroblasts from Hsp70.1/3-knockout mice overexpressed different genes from this pathway, including Col1, Smad2, TGF-β1 and TGF-β2, and TGF-β receptor 2 (Figure 4A). Concordantly, collagen and fibronectin protein expression and secretion (Figure 4B) were increased in cultured primary lung fibroblasts from Hsp70.1/3-knockout mice. We postulate that as the function of Hsp70 is reduced with aging, the TGF-β/SMAD pathway is activated and increases the risk of developing fibrosis in response to injury and stress. Furthermore, our data suggest a vicious profibrotic cycle in which TGF-β itself mediates additional downregulation of Hsp70 (Figure 5). Our data support a "two-hit" model of dysfunctional repair and accelerated lung fibrosis after injury in the context of Hsp70 deficiency (Figure 6), but suggest that Hsp70 deficiency alone is not sufficient to catalyze spontaneous fibrosis in the aging lung (Figures 6E and 6F).

In summary, to our knowledge, this is the first study to demonstrate reduced Hsp70 in IPF lung and primary cultured IPF fibroblasts. Furthermore, we provide evidence *in vitro* and *in vivo* supporting a mechanistic link between Hsp70 and ECM homeostasis that is mediated in part through TGF-β/SMAD activation. Small-molecule modulators of heat shock proteins have received much attention for the management of malignant disease (39) in which dysregulation of the ECM contributes to tumorigenesis and metastatic progression. This class of novel therapeutics may also hold promise for the treatment of fibrosing diseases that affect millions of individuals worldwide. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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