Received: 16 March 2022 | Revised: 13 July 2022 | Accepted: 19 July 2022

DOI: 10.1096/fj.202200406RR

THE FASEB JOURNAL

RESEARCH ARTICLE

Extracellular HSP90α promotes cellular senescence by modulating TGF-β signaling in pulmonary fibrosis

Wenshan Zhong[1] | **Weimou Chen**[1] | **Yuanyuan Liu**[1] | **Jinming Zhang**[1] | **Ye Lu**[1] | **Xuan Wan**[1] | **Yujie Qiao**[1] | **Haohua Huang**[1] | **Zhaojin Zeng**[1] | **Wei Li**[2] | **Xiaojing Meng**[3] | **Haijin Zhao**[1] | **Mengchen Zou**[4] | **Shaoxi Cai**[1] | **Hangming Dong**[1]

[1]Department of Respiratory and Critical Care Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China

[2]Department of Dermatology, The USC-Norris Comprehensive Cancer Center, University of Southern California Keck Medical Center, Los Angeles, California, USA

[3]Guangdong Provincial Key Laboratory of Tropical Disease Research, Department of Occupational Health and Occupational Medicine, School of Public Health, Southern Medical University, Guangzhou, China

[4]Department of Endocrinology and Metabolism, Nanfang Hospital, Southern Medical University, Guangzhou, China

Correspondence
Shaoxi Cai and Hangming Dong, Department of Respiratory and Critical Care Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China.
Email: hxkc@smu.edu.cn and dhm@smu.edu.cn

Funding information
NSFC | National Natural Science Foundation of China, Grant/Award Number: 81870058 and 82170032

Abstract

Recent findings suggest that extracellular heat shock protein 90α (eHSP90α) promotes pulmonary fibrosis, but the underlying mechanisms are not well understood. Aging, especially cellular senescence, is a critical risk factor for idiopathic pulmonary fibrosis (IPF). Here, we aim to investigate the role of eHSP90α on cellular senescence in IPF. Our results found that eHSP90α was upregulated in bleomycin (BLM)-induced mice, which correlated with the expression of senescence markers. This increase in eHSP90α mediated fibroblast senescence and facilitated mitochondrial dysfunction. eHSP90α activated TGF-β signaling through the phosphorylation of the SMAD complex. The SMAD complex binding to p53 and p21 promoters triggered their transcription. In vivo, the blockade of eHSP90α with 1G6-D7, a specific eHSP90α antibody, in old mice attenuated the BLM-induced lung fibrosis. Our findings elucidate a crucial mechanism underlying eHSP90α-induced cellular senescence, providing a framework for aging-related fibrosis interventions.

KEYWORDS

cellular senescence, HSP90 heat-shock proteins, mitochondria, pulmonary fibrosis, reactive oxygen species, transforming growth factor beta

Abbreviations: ATP, adenosine triphosphate; BALF, bronchoalveolar lavage fluid; BLM, bleomycin; eHSP90α, extracellular heat shock protein 90α; IL6, interleukin 6; IPF, idiopathic pulmonary fibrosis; MMP, mitochondrial membrane potential; mt-ROS, mitochondrial-derived reactive oxygen species; PAI1, plasminogen activator inhibitor-1; rHSP90α, recombinant human HSP90α; ROS, reactive oxygen species; SA-βgal, senescence-associated beta-galactosidase; SASP, senescence-associated secretory phenotype; TGF-β, transforming growth factor β.

Wenshan Zhong, Weimou Chen, and Yuanyuan Liu contributed equally to this work.

1 | INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and age-related lung disease with a median age at diagnosis of 66 years and estimated survival of 3–4 years.[1] IPF is characterized by repetitive injury to the lung epithelium, activation, and proliferation of (myo)fibroblasts, and extracellular matrix deposition, which destroy lung architecture and function.[2] However, current IPF treatment regimens have limited efficacy. A better understanding of the pathological mechanisms of fibrosis is essential to investigate effective therapies.

Heat shock protein 90 (HSP90) is a ubiquitously expressed chaperone involved in the posttranslational folding and stability of proteins. In addition, HSP90, which has an extracellular function, is found in two isotypes: inducible alpha and beta, which is constitutive.[3] Recently, extracellular HSP90α (eHSP90α) was shown to be upregulated in pulmonary fibrosis and interact with adjacent cells' surfaces, leading to increased myofibroblast activation.[4] Our previous experiments also showed that eHSP90α blockade was beneficial for pulmonary fibrosis.[5] Overall, eHSP90α is an essential factor for the development of pulmonary fibrosis, but the associated mechanisms are unclear.

Emerging studies have implicated aging as a significant risk factor for IPF, and elderly individuals are also more sensitive to lung injuries.[6] Notably, HSP90 inhibitors have been reported as senolytic agents, indicating a new role in aging.[7] However, whether eHSP90α involve in the process of aging remains unknown. Cellular senescence, a hallmark of aging, has been thought to be involved in the pathogenesis of lung fibrosis.[8–10] A type of cell fate, cellular senescence, is characterized by permanent growth arrest and other phenotypic alterations, including the development of a pro-inflammatory secretome.[11] Moreover, elevated expression of p16, p21, and p53 are observed in senescent cells and used as miscellaneous senescence biomarkers.[12] Upregulated expression of p16 and p21, Senescence-associated beta-galactosidase (SA-βgal) activity, and a senescence-associated secretory phenotype (SASP) were observed in human samples and mice models of lung fibrosis.[10,13] Recent studies have demonstrated that the accumulation of senescent fibroblasts drives pulmonary fibrosis.[10] It is well established that mitochondria that accumulate in senescence often show a decreased membrane potential, increased mitochondrial mass, and simultaneously produce increased reactive oxygen species (ROS).[14,15] Mitochondrial dysfunction refers to the damage of mitochondrial structure, respiratory chain defects, biogenic dysfunction, gene damage, and changes in oxidative protein activity in cells and tissues.[16] However, the mechanisms of fibroblast senescence are poorly understood.

Transforming growth factor β (TGF-β) is one of the most potent profibrotic factors in the development of pulmonary fibrosis.[17–19] Active TGF-β binds to its receptors leading to activation of canonical SMAD signaling.[20] The SMAD complex translocates to the nucleus to regulate the transcription of target genes.[21] Although SMADs contribute to p21 transcription,[22–24] their role in fibroblast senescence is unknown.

In this study, we assessed the pathophysiological role of eHSP90α in cellular senescence and the impact of eHSP90α blockade in the bleomycin (BLM)-induced injury model for understanding how TGF-β signaling activation in senescence controls the expression of p21 and p53. We further demonstrate that the function of eHSP90α may offer new opportunities for targeting eHSP90α as a therapeutic option for age-related fibrotic disease.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

IMR90 human fibroblasts (ATCC, CCL-186) were used between 25 and 40 population doublings. The cells were cultured in EMEM (ATCC, 30-2003) with 10% FBS (GIBCO, 10100147) at 37°C in a humidified atmosphere containing 5% CO_2. The laboratory of Dr. Wei Li donated the F-5 fragment and anti-HSP90α monoclonal antibody (1G6-D7). For experiments employing extracellular HSP90α-induced senescence, IMR90 human lung fibroblasts were treated 2 h with 1G6-D7(20 μg/ml) before F-5 (10 μg/ml) for 48 h. 1G6-D7 was developed against the dual lysine region of human Hsp90α and raised in mice. The isotype is IgG1κ. Species Reactivity was tested in humans and mice.[5,25] For experiments utilizing etoposide-induced senescence, IMR90 human lung fibroblasts were treated for 48 h with 20 mM etoposide (Sigma–Aldrich, E1383). For experiments abrogating mtROS, cells were treated for 48 h with F-5 after Mito-Tempo (10 μM, MedChemExpress, HY-112879) treatment. For experiments inhibiting, cells were treated for 48 h with F-5 after RepSox (7.5 μM, Selleck, S7223) treatment.

2.2 | Animals

All the animal experiments were approved by the Committee on the Ethics of Animal Experiments of Southern Medical University in Guangzhou, China and performed under standard guidelines for the Care and Use of Laboratory Animals. Male mice (C57BL/6) were kept on a 12 h light–dark cycle with free access to food and water. Mice were randomly distributed to indicated groups.

2.2.1 | Mouse model for bleomycin-induced lung fibrosis

For bleomycin administration, C57BL/6 mice, 6 to 8 weeks of age were anesthetized with 2, 2, 2-tribromoethanol (Sigma-Aldrich) followed by intratracheal instillation of bleomycin (5 U/kg, i. t.) in 50 μl PBS, as previously described.[26] Mice received the first dose of PBS (control group) or 1G6-D7 8 days after bleomycin, as described in the main text. PBS or 1G6-D7 was then administered every day for an additional 13 days.

2.2.2 | Mouse model for age-related lung fibrosis

For bleomycin administration, 2-month-old and 18-month-old C57BL/6 mice were anesthetized with 2, 2, 2-tribromoethanol (Sigma-Aldrich) followed by intratracheal instillation of bleomycin (5 U/kg, i. t.) in 50 μl PBS. 18-month-old mice received the first dose of PBS (control group) or 1G6-D7 8 days after bleomycin treatment. PBS or 1G6-D7 was then administered every day for an additional 13 days.

2.3 | Hydroxyproline assay

Hydroxyproline content of the whole lung was measured by a hydroxyproline (HYP) kit (Nanjing Jian Cheng Institute, Nanjing, China) in accordance with the manufacturer's recommendations.

2.4 | Lung histology and Immunohistochemistry

Harvested lungs were formaldehyde fixed, paraffin-embedded, and stained with H&E or Masson's trichrome stain as previously described.[27]

Sections (5-μm-thick) were deparaffinized and rehydrated, endogenous peroxidases were inactivated with methanol containing 0.3% hydrogen peroxide for 30 min. Antigen retrieval was performed by steaming in citrate buffer (pH 8.0) for 20 min. After incubation in blocking solution for 10 min at room temperature, the slides were incubated with an anti-fibronectin antibody (Abcam, ab2413), anti-p16 antibody (Abcam, ab51243), or anti-α-SMA antibody (Abcam, ab5694) overnight at 4°C. After three washes, the sections were incubated with biotinylated anti-IgG at 37°C for 60 min, followed by Streptavidin-peroxidase conjugate (Zhongshan Golden Bridge Biotechnology). Immunoreactivity was detected using 3, 3′ diaminobenzidine, and the sections were counterstained with hematoxylin for observation by microscopy.

2.5 | Pulmonary function test

Pulmonary function was characterized at the endpoint using a Buxco FinePointe RC system as previously described.[28,29] Mice were euthanized by an intraperitoneal injection of 2, 2, 2-tribromoethanol and connected to the Buxco system after tracheotomy. Dynamic lung compliance and resistance values were obtained from the respiratory system.

2.6 | ELISA

Bronchoalveolar lavage fluid (BALF) and serum samples from mice and cell culture medium from cells were collected as described previously.[30] The HSP90aA1 (CloudClone, Buckingham, UK) and HSP90α (Enzo) ELISA kits were used following the manufacturer's recommendations.

2.7 | Western blot

Western blot analyses were performed as described previously.[5] Briefly, cell lysates or lung homogenates were prepared using a protein extraction kit (KeyGEN BioTECH). After SDS–PAGE, PVDF membranes were probed with specific antibodies described in the figure legends followed by a corresponding secondary antibody (LI-COR). Bands were quantified with ImageJ software. Antibodies are listed as below: anti-fibronectin antibody (Abcam, ab2413); anti-p16 antibody (Abcam, ab51243); anti-α-SMA antibody (Abcam, ab5694); anti-p21 antibody (Santa Cruz, sc-6246); anti-β-actin antibody (Proteintech, 66009-1-Ig); anti-phospho-SMAD3 antibody (Ser423/425, Cell Signaling Technology, 9520T); anti-phospho-SMAD2 antibody (Ser465/Ser467, Cell Signaling Technology,18 338 T); anti-SMAD3 antibody (Proteintech, 25 494-1-AP); anti-SMAD2 antibody (Proteintech, 12 570-1-AP); anti-CollagenI antibody (Affinity, AF7001); anti-SMAD4 antibody (Cell Signaling Technology, 46 535). anti-GAPDH antibody (Proteintech,10 494-1-AP).

2.8 | SA-β-gal

The SA-β-gal activity was detected as described[31] using a commercial Senescence Cells Histochemical Staining Kit (Sigma–Aldrich).

2.9 | RNA extraction and RT-qPCR

Mouse lung tissue RNAs were extracted using TRIzol reagent (Takara). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). The iTaq Universal SYBR Green Supermix qPCR Kit (Bio-Rad) was used to perform RT-qPCR. Primers used are listed as follows:

IL6 (Forward: TAGTCCTTCCTACCCCAATTTCC, Reverse:TTGGTCCTTAGCCACTCCTTC); TGF-β (Forward: CCACCTGCAAGACCATCGAC, Reverse: CTG GCGAGCCTTAGTTTGGAC); PAI1 (Forward: TCTGGGA AAGGGTTCACTTTACC, Reverse: GACACGCCATAGGG AGAGAAG).

2.10 | Transmission electron microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer with 0.1 M NaCl, pH 7.5 for 20 min at room temperature, as previously described.[32]

2.11 | Mitochondrial mass

The mitochondrial mass was assessed using MitoTracker (Invitrogen, M22425) in accordance with the manufacturer's recommendations. Briefly, cells were incubated with 20 nM Mito Tracker for 20 min at 37°C. Images were obtained using Olympus FV1000 Confocal Laser Scanning Microscopy (Tokyo, Japan).

2.12 | Mitochondrial ROS assessment

The levels of mitochondrial ROS were detected using the fluorescent probes MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen, M36008), and fluorescent intensity was obtained by Olympus FV1000 Confocal Laser Scanning Microscopy (Tokyo, Japan).

2.13 | Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were conducted using the SimpleChIP Enzymatic Chromatin IP Kit (CST, 9003) in accordance with the manufacturer's recommendations. Chromatin was immunoprecipitated with the immunoglobulin G (CST, #2729; as a negative control) or SMAD2/3 (Abcam, ab202445). In all, 10% of total DNA was used for input evaluation. DNA enrichment in the ChIP samples was determined by reverse transcription and semi-quantitative PCR (RT-PCR) with PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, China) following the manufacturer's protocol. PCR products of immunoprecipitated and input samples were analyzed on a 2% agarose gel. Primers used are listed as follow: p16 promoter P1 (Forward: GCTACGTAAGAGTGATCGCTAAA, Reverse: CTCGGTACAAACCCAAGACAA); p16 promoter P2 (Forward: CCTTTCCTTGCCCTGCTTT, Reverse: CTTCCA CATCACCGATCCTTTC); p21 promoter P1 (Forward: CAT GGGAGGAGCTACAACTATATG, Reverse: CTTCCTGTTC CTGGCTCTAAC); p21 promoter P2 (Forward: CACCTGAA TACCTGGGACTACA, Reverse: GCCTGGCCAATATGGT GAAA); TP53 promoter P1 (Forward: GACCAGGAACCAC TGAGAAATC, Reverse: CTTGGCGACCCAGGTTTATT); TP53 promoter P2 (Forward: GAGTCCCGCGGTAATTCT TAAA, Reverse: CTGAAGCCTGGAGAATGAGATG).

2.14 | Statistical analysis

Prism 8.0 software (GraphPad) was used to analyze data. The experiment was set up to use 3 to 7 samples/repeats for each experiment/group/condition. Representative images for IF staining, IHC staining, and immunoblot are shown. Student's *t* test was used to analyze the differences between 2 groups, one-way ANOVA analysis was used to compare the differences between more than 2 groups. *p*-values of less than .05 were regarded as statistically significant.

3 | RESULTS

3.1 | Extracellular HSP90α is upregulated in pulmonary fibrosis and associated with cellular senescence

A recent study demonstrated that HSP90α was increased in serum from patients with IPF compared with healthy controls.[4] To further characterize HSP90α and cellular senescence in IPF, we generated a BLM-induced mouse model (Supporting Information Figure S1). Bodyweight monitoring, an essential indicator of pathological severity following the BLM challenge, revealed that mice that received bleomycin lost substantial body mass relative to mice that received PBS (Figure 1A). HSP90α levels were increased in the bronchoalveolar lavage fluid (BALF) of the BLM-treated mice from day14 to day21 (Figure 1B). Western blot analysis revealed that senescence marker (p16 and p21) levels were upregulated in lung tissue from the BLM-treated mice (Figure 1C,D). Interestingly, the level of senescence markers p21 gradually increased from day 7 (the inflammatory phase) up to day 21 (established fibrosis), accompanied by the upregulation of

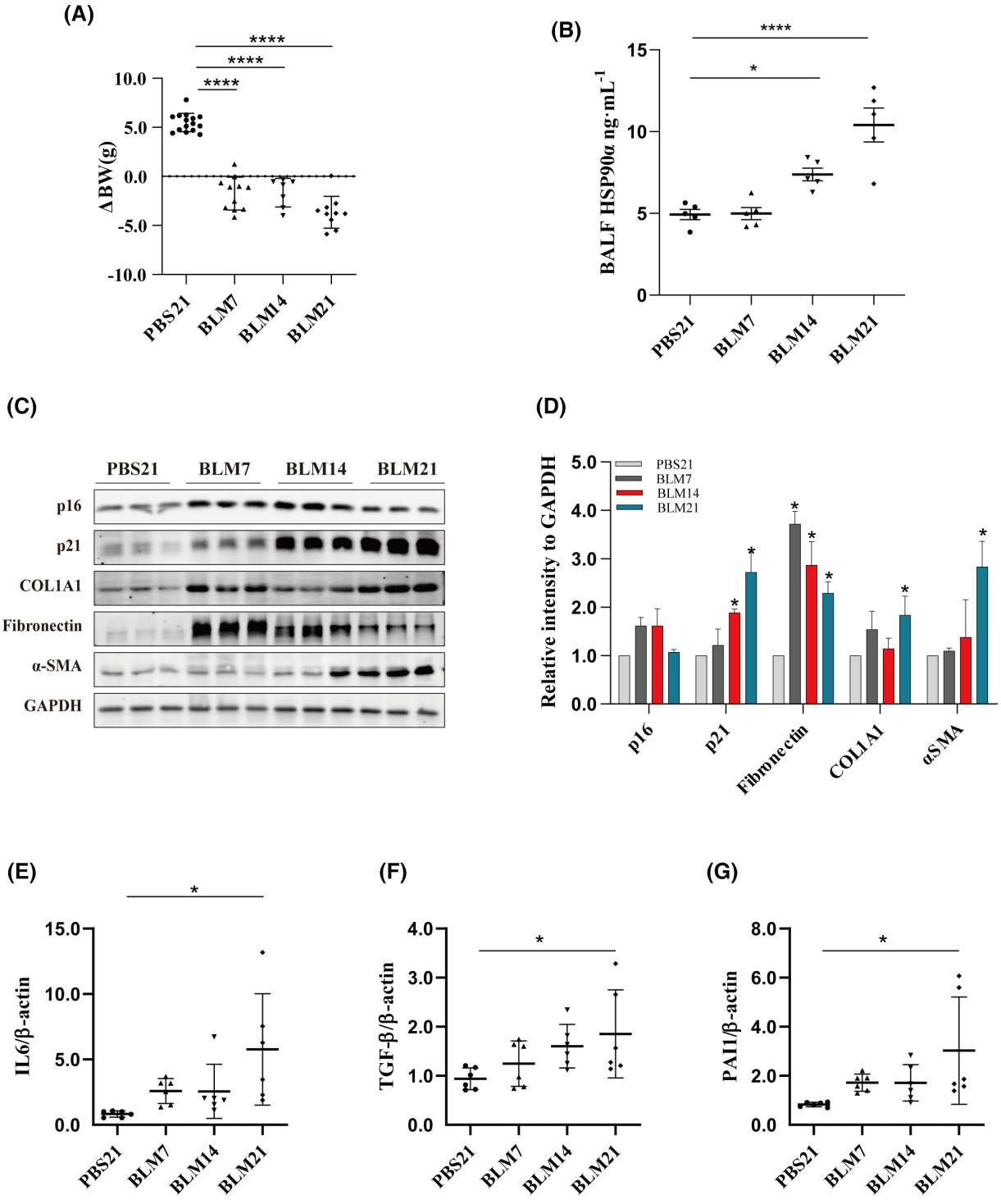

FIGURE 1 The level of extracellular HSP90α was increased and in the same trend as senescence markers in the BLM-induced model. C57/BL6 mice were treated with BLM or PBS as control. (A) Body weight (BW) is depicted as a change in grams relative to baseline. Data presented as mean ± SD; PBS: $n = 8$; BLM day 7: $n = 8$; BLM day 14: $n = 7$; BLM day 21: $n = 8$. (B) Bronchoalveolar lavage fluid (BALF) levels of HSP90α were measured by ELISA. Data presented as mean ± SD; PBS: $n = 5$; BLM day 7: $n = 5$; BLM day 14: $n = 5$; BLM day 21: $n = 5$. (C, D) Western blot analysis of p16, p21, COL1A1, Fibronectin, and α-SMA expression release by mice lung slices. Data presented as mean ± SD. IL6 (E), TGF-β (F), and PAI1 (G) expression by qPCR, normalized to β-actin expression. Data presented as mean ± SD. ****$p < .0001$, ***$p < .001$, **$p < .01$, and *$p < .05$ between indicated groups.

fibronectin, type collagen I, and αSMA (Figure 1C,D), suggesting that cellular senescence is associated with disease severity.

To further confirm the relationship between HSP90α and cellular senescence in BLM-treated mice, we next detected several SASP factors via qPCR. Results showed that

expression of Interleukin 6 (IL6), transforming growth factor β(TGF-β), and plasminogen activator inhibitor-1 (PAI1) were upregulated (Figure 1E–G), which has the same trend as the level of HSP90α. Taken together, these results demonstrate that eHSP90α is upregulated in pulmonary fibrosis in correlation with cellular senescence.

3.2 | Extracellular HSP90α promotes fibroblast senescence

In order to explore the role of eHSP90α in cellular senescence, we established an etoposide-induced senescent model.[10] Exposure of human IMR90 fibroblasts to etoposide treatment-induced senescence observable after 48 h, which was confirmed by staining for SA-β-gal (Figure 2A,B) and expression of senescence effectors, p21 (Figure 2C,D). The secretion of HSP90α was next assessed in vitro in etoposide-induced fibroblasts. Etoposide-induced senescent cells exhibited a 2-fold increase in HSP90α compared to control (Figure 2E). This result was also confirmed by western blot analysis (Supporting Information Figure S2A). However, no change in the total level of the HSP90α protein was observed (Figure 2C,D), suggesting intracellular HSP90α might not contribute to cellular senescence. Therefore, we hypothesise that eHSP90α might mediate cellular senescence.

To identify whether eHSP90α regulates cellular senescence, we treated IMR90 fibroblasts with recombinant human HSP90α (rHSP90α). rHSP90α-treated fibroblasts exhibited increased SA-β-gal activity and p16 and p21 expression relative to those of control fibroblasts (Supporting Information Figure S2B–D). These results suggest that eHSP90α regulates cellular senescence. To further confirm this phenotype, we used a monoclonal antibody, 1G6-D7, which binds to the F-5 region of HSP90α and inhibits the extracellular HSP90α function.[25,33] The previous study has demonstrated that F-5, a 115-amino acid fragment within the linker region and middle domain of Hsp90α, retains the extracellular function of HSP90α.[34]

Similarly, in our study F-5 treatment increased SA-β-gal activity, while 1G6-D7 reduced F-5-induced SA-β-gal activity (Figure 2F,G). F-5 treatment increased p16 and p21 protein expression, but this effect was blocked by 1G6-D7 (Figure 2H,I). Taken together, our results demonstrate that eHSP90α stimulates a senescence phenotype in healthy human fibroblasts.

3.3 | Extracellular HSP90α-induced fibroblast senescence is associated with mitochondrial dysfunction

Previous studies have demonstrated that mitochondrial function is responsible for cellular senescence in IPF.[35–37] To clarify whether eHSP90α affects mitochondrial function, we used MitoTracker Red (MitoRed) to assess mitochondrial mass. Confocal microscopy revealed an increase in mitochondrial mass and the formation of highly elongated mitochondria after F-5 treatment (Figure 3A,B), while 1G6-D7 reduced the effect of eHSP90α. Fibroblasts treated with F-5 exhibited an increased number of abnormal mitochondria compared to cells treated with F-5 combined 1G6-D7 (Figure 3C,D). Significantly, the F-5 treatment enhanced mitochondrial ROS production shown by MitoSOX Red staining (Figure 3E,F). These results demonstrate that eHSP90α leads to mitochondrial dysfunction and damaged mitochondria in senescent cells.

3.4 | Extracellular HSP90α activates TGF-β signaling to promote fibroblast senescence

TGF-β signaling is the master regulator of pulmonary fibrosis[18,19] and has reportedly been linked to the phenotypes of cellular senescence.[38] We next investigated whether eHSP90α activates TGF-β signaling to promote fibroblast senescence. We found that F-5 treatment induced more activation of TGF-β signaling, including phosphorylation of SMAD2 and SMAD3 and expression of SMAD4 (Figure 4A–D). Notably, the induction of TGF-β by F-5 was entirely abolished by the blockade of extracellular HSP90α with 1G6-D7 (Figure 4A–D), suggesting eHSP90α improves TGF-β signaling activation. To further validate that eHSP90α promotes cellular senescence by TGF-β signaling, we stimulated IMR90 with RepSox, an inhibitor of TGF-β signaling, in the presence or absence of F-5. We found that expression of p16 and p21 (Figure 4E,F) and activity of SA-β-gal (Figure 4G,H) were abrogated by RepSox. Similarly, SMAD4 knockdown also attenuated senescence induced by eHSP90α (data not shown). Taken together, eHSP90α promotes cellular senescence through TGF-β signaling.

3.5 | mtROS is involved in TGF-β activation

As we observed mtROS upregulation was accompanied by eHSP90α-induced senescence and ROS has been reported to be involved in TGF-β activation.[39] To elucidate whether mtROS is involved in eHSP90α-TGF-β signaling activation, we cultured IMR90 in media containing Mito-Tempo, an inhibitor of mtROS, together with F-5. Results showed that Mito-Tempo suppressed the activation of TGF-β signaling induced by F-5 (Figure 5A–D), which suggests mtROS partly help with TGF-β signaling activation.

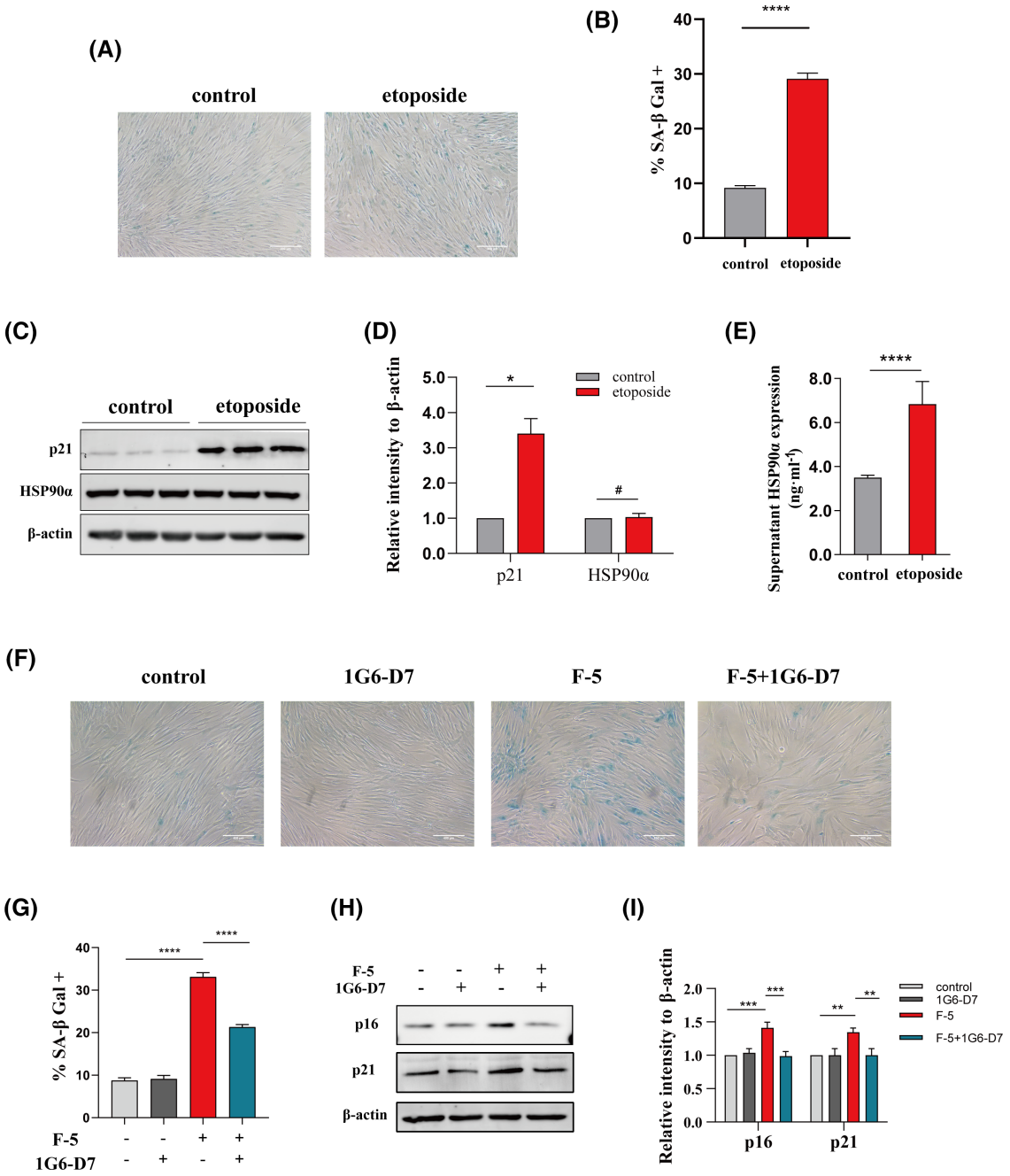

FIGURE 2 Extracellular HSP90α induces senescence in fibroblasts. (A, B) Cytochemical evaluation of the senescence marker SA-β-gal activity of IMR90 lung fibroblasts exposed to etoposide. (C, D) Representative immunoblots of p21, HSP90α, and β-actin protein levels in IMR90 lung fibroblasts exposed to etoposide. Data presented as mean ± SD. (E) supernatant levels of HSP90α were measured by ELISA. Data presented as mean ± SD. SA-β-gal activity (F, G) and (H, I) representative immunoblots of p16, p21, and β-actin protein levels in IMR90 lung fibroblasts exposed to F-5 for 48 h after pre-treat with 1G6-D7. Data presented as mean ± SD. ****$p < .0001$, ***$p < .001$, **$p < .01$, and *$p < .05$ between indicated groups.

3.6 | The SMAD2/3 complex regulates p53 and p21 transcription

The SMAD complex involved in TGF- β signaling has been reported as a vital transcript factor to regulate gene expression.[18] Some researchers found that p21 transcription can be mediated by SMADs.[22,24] These results inspired us to investigate whether the expression of senescence markers in fibroblasts exposed to eHSP90α is also under regulation of the SMAD complex. To validate the interaction between SMADs and senescence markers, chromatin immunoprecipitation analysis was performed utilizing IMR90 cells treated with F-5. First, using the NCBI website and JASPAR program, we analyzed the p16, p21,

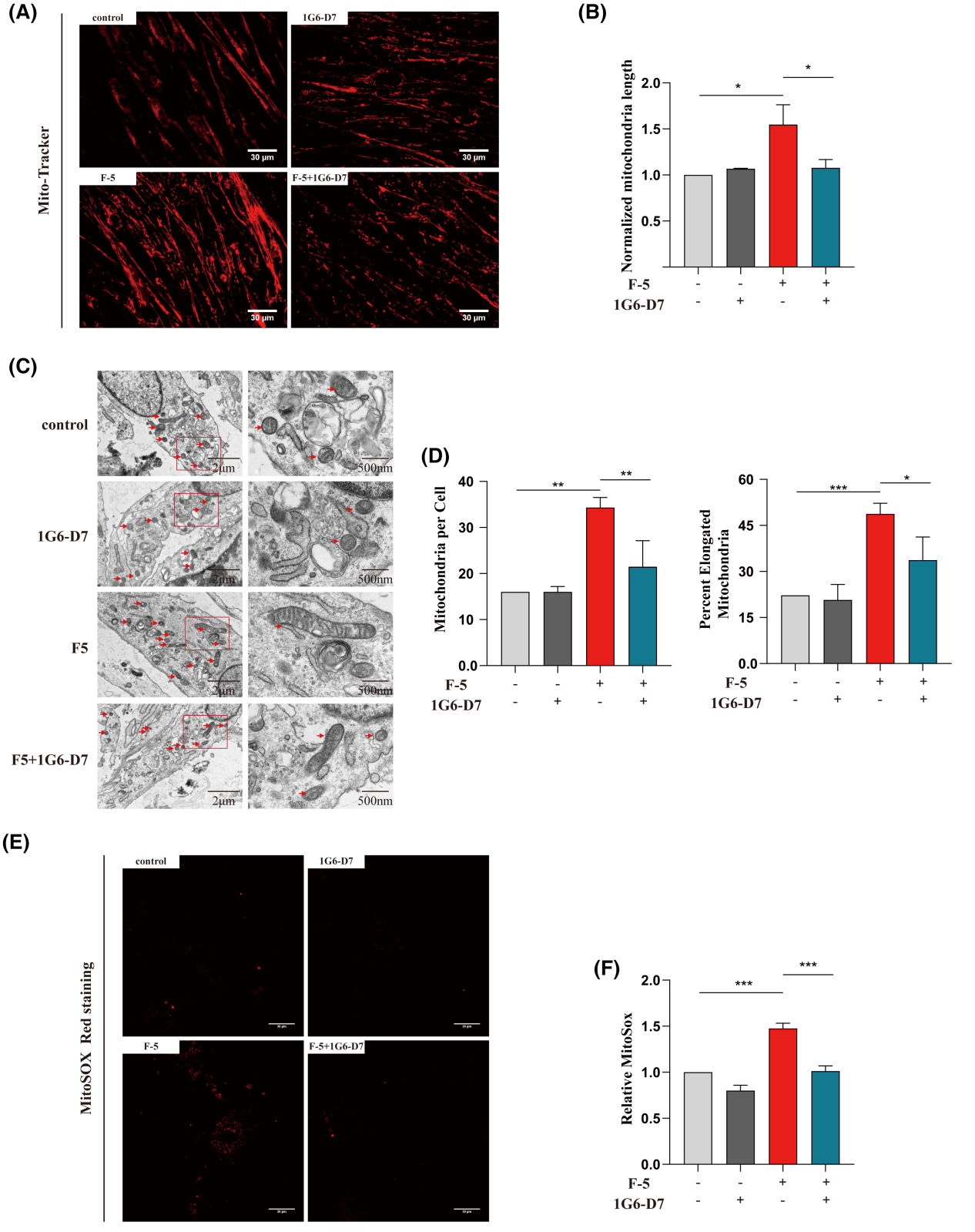

FIGURE 3 Extracellular HSP90α contributes to abnormal mitochondrial structure and mitochondrial ROS accumulation. (A) Immunofluorescence and (B) quantitative analysis for Mito-Tracker in IMR90 after 1G6-D7 or PBS exposure and treatment with or without F-5. Ten fields were randomly selected and acquired from each group. Data presented as mean ± SD ($n = 3$). (C, D) Representative transmission electron microscopy (TEM) images. Red arrows indicate damaged and swollen mitochondria. Ten fields were randomly selected and acquired from each group. Data presented as mean ± SD ($n = 3$). (E) MitoSOX Red staining and (F) quantitative analysis for mitochondrial ROS production. Fluorescence microscopy detection was performed. Ten fields were randomly selected and acquired from each group. Data presented as mean ± SD ($n = 3$). ****$p < .0001$, ***$p < .001$, **$p < .01$, and *$p < .05$ between indicated groups.

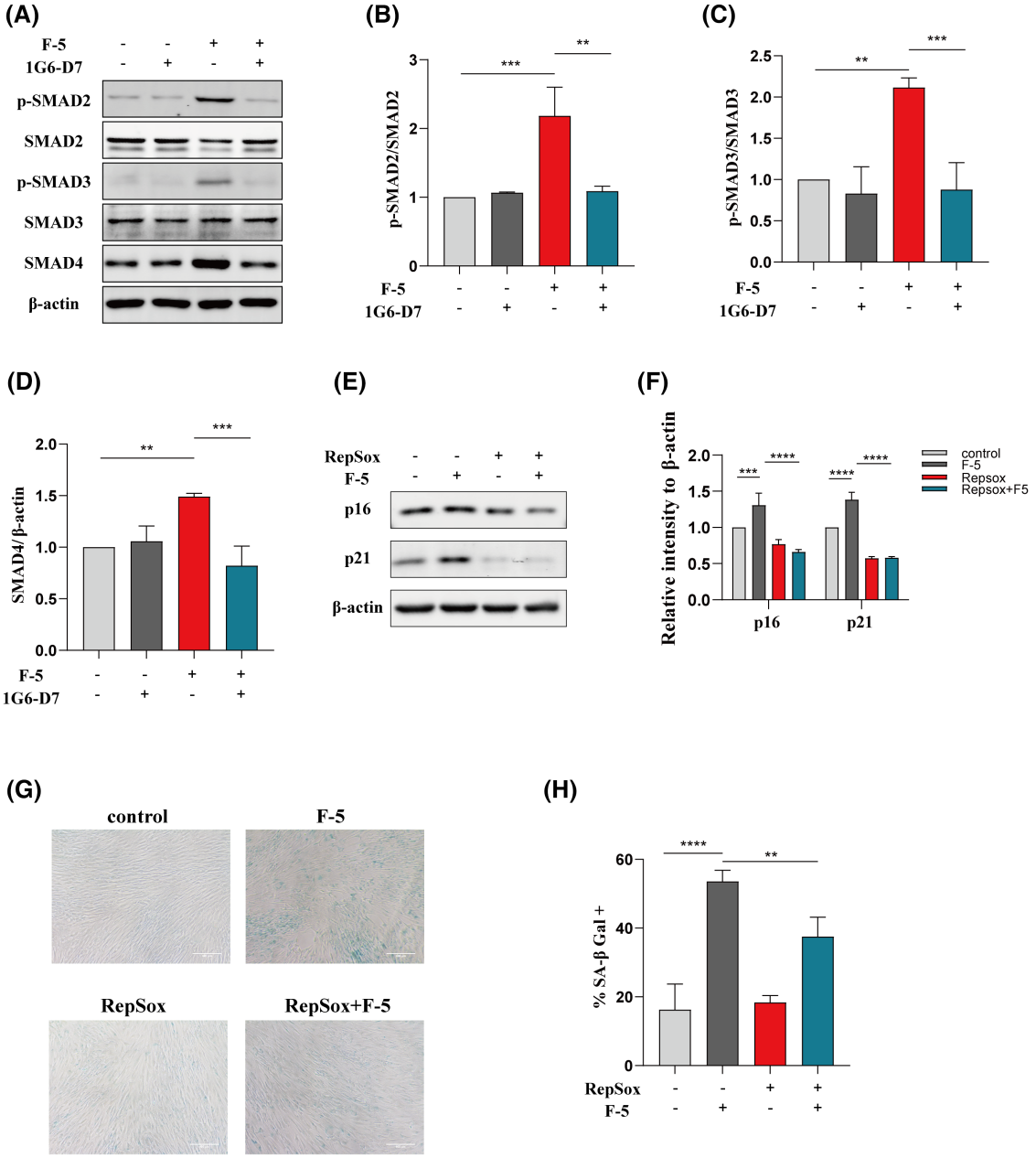

FIGURE 4 Extracellular HSP90α promotes cellular senescence through mtROS-dependent TGF-β signaling. Representative western blots (A) and quantitative analysis (B–D) show the amounts of p-SMAD2, p-SMAD3, SMAD2, SMAD3, SMAD4, and β-actin in IMR90 fibroblasts stimulated with F-5 in the presence or absence of 1G6-D7. Data presented as mean ± SD. Representative western blots (E) and quantitative analysis (F) show the amounts of p16, p21, and β-actin in IMR90 fibroblasts stimulated with F-5 in the presence or absence of RepSox. Data presented as mean ± SD. (G, H) SA-β-gal activity in IMR90 lung fibroblasts exposed to F-5 for 48 h after pre-treat with RepSox. ****$p < .0001$, ***$p < .001$, **$p < .01$, and *$p < .05$ between indicated groups.

and p53 promoter sequences to identify predicted transcription factor-binding sites (Figure 6A). As shown in Figure 6B–D, P1 and P2 regions from senescence markers could be amplified. Our data showed that F-5 increased the enrichment of SMADs at the binding sites in p21 (Figure 6F) and p53 (Figure 6G), but no increase at the p16 promoter (Figure 6E). They suggested that the SMAD complex regulates p21 and p53 transcription to improve cellular senescence.

3.7 | Targeting eHSP90α with 1G6-D7 prevented pulmonary senescence and fibrosis

Our results strongly support the hypothesis that eHSP90α promotes cellular senescence in vitro. We further quantify whether eHSP90α regulates senescence in a BLM-induced lung fibrosis model. Indicated dose of 1G6-D7 was administered from day 8 after BLM injury (Figure 7A). H&E

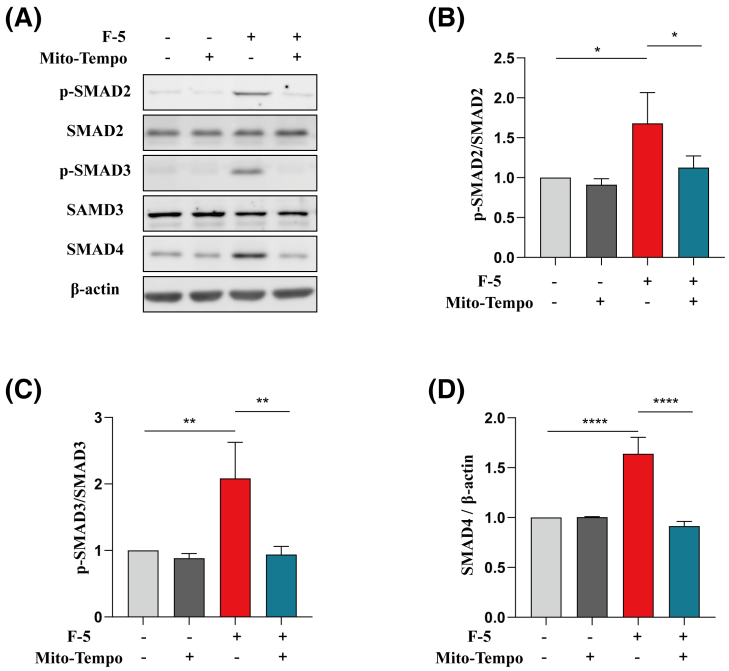

FIGURE 5 mtROS is involved in TGF-β signaling activation. Representative western blots (A) and quantitative analysis(B–D) show the amounts of p-SMAD2, p-SMAD3, SMAD2, SMAD3, SMAD4, and β-actin in IMR90 fibroblasts stimulated with F-5 in the presence or absence of Mito-Tempo. Data presented as mean ± SD.

staining showed lungs from BLM-treated mice exhibited more severe pulmonary fibrosis compared with those from BLM plus 1G6-D7-treated mice (Figure 7B).

1G6-D7 treatment attenuated BLM-induced body weight loss and lung function (Figure 7C–E). Furthermore, mice that received 1G6-D7 demonstrated significant reductions in several pro-fibrotic markers, including total lung hydroxyproline (Figure 7F)and the expression of α-SMA and fibronectin (Figure 7G–K). Similar results were shown in immunohistochemical analysis (Figure 7O).

In order to illustrate the role of eHSP90α in TGFβ activation in vivo, we performed western blot to test the phosphorylation of SMAD2 and SMAD3. We observed that 1G6-D7 inhibits the effect of bleomycin in the phosphorylation of SMAD2 and SMAD3 (Figure 7L–N). These data suggest that blockade of extracellular HSP90α by 1G6-D7 attenuates BLM-induced lung fibrosis even though fibrosis was established.

Importantly, immunohistochemical and western blot analyses revealed that 1G6-D7 treatment reduced the amounts of p16 and p21 (Figure 7C–H). Moreover, as shown in Figure 7P, the expression of γH2AX was decreased after 1G6-D7 administration. Taken together, these findings suggest that 1G6-D7 attenuated lung fibrosis and cellular senescence in vivo.

3.8 | 1G6-D7 reduced lung fibrosis in older mice

Aging is a well-known risk factor for IPF and cellular senescence has been reported as a crucial hallmark of aging.[1,40] Thus, we next investigate whether blocking eHSP90α by 1G6-D7 attenuates age-related lung fibrosis in vivo. The levels of circulating HSP90α were measured in the sera of old (18 months) and young (2 months) mice. HSP90α levels were upregulated in aged mice compared to young mice (Figure 8A), suggesting that eHSP90α might contribute to aging-induced fibrosis. However, histological analysis showed that the lungs from both old and young mice appeared without inflammation or fibrosis (data not shown).

In order to examine the effect of 1G6-D7 in older mice, 18-month-old and 2-month-old mice were administered low-dose BLM and received 1G6-D7 from day8. H&E and Masson trichrome staining (Figure 8B,C) further confirmed increased fibrosis in the lungs of the 18-month-old mice, while blockade by 1G6-D7 reduced lung fibrosis. Similarly, we observed increased expression of α-SMA in 18-month-old mice through immunohistochemistry (Figure 8D) and western blot analysis (Figure 8E–G) compared to 2-month-old mice. These results suggested that the older mice were more sensitive to BLM, and aging is involved in fibrosis development. In contrast, 1G6-D7 abrogated the fibrotic effects of BLM in the older mice (Figure 8B,C). Immunohistochemistry (Figure 8D) and western blot (Figure 8E–G) show diminished amounts of α-SMA and fibronectin. These data suggest that 1G6-D7 intervention prevents severe lung fibrosis in older mice.

4 | DISCUSSION

In this study, we demonstrate that eHSP90α contributes to fibroblast senescence, accompanied by abnormal

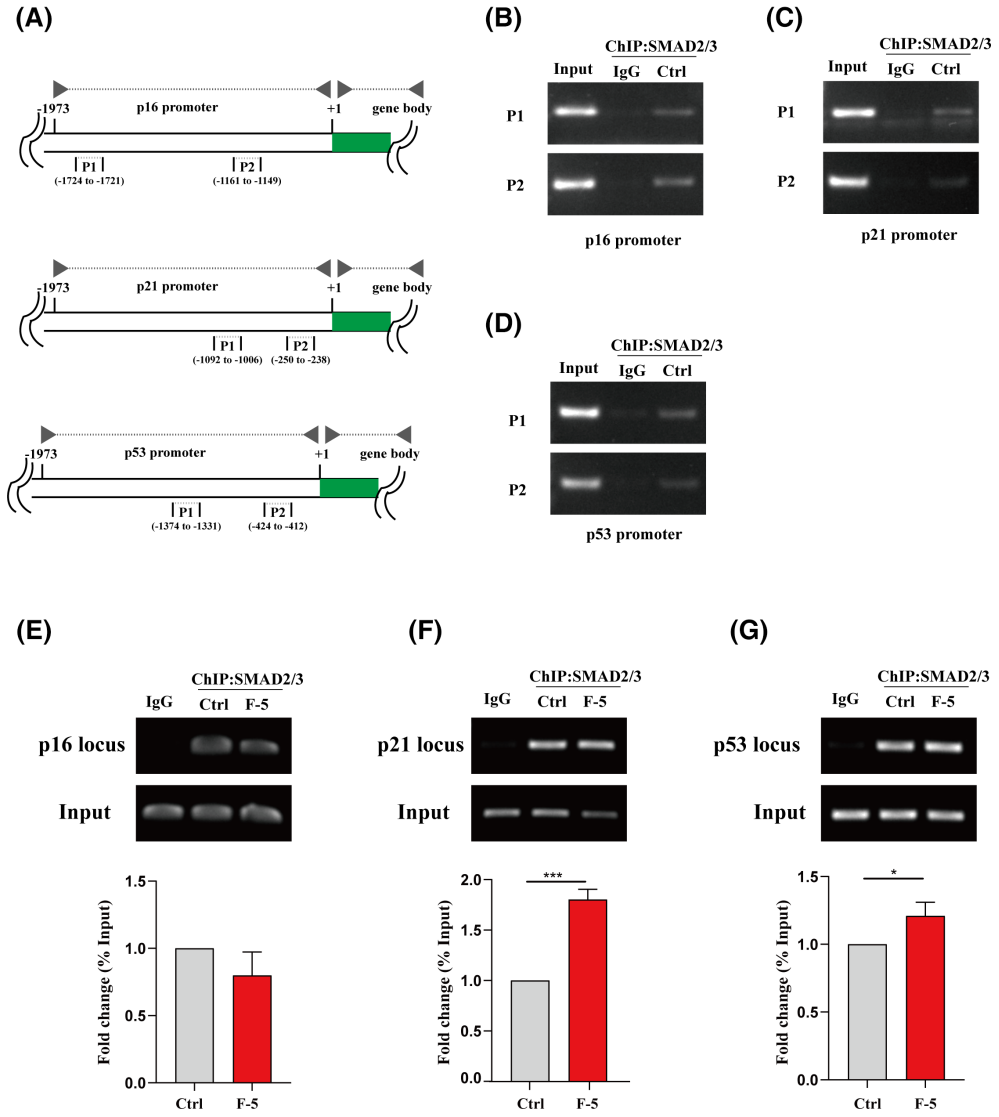

FIGURE 6 The SMAD2/3 complex binds to p21 and TP53 promoters. (A) The schematic representation of p16, p21, and TP53 promoter with the indicated regions. ChIP analysis of p16 promoter (B), p21 promoter (C) and p53 promoter (D) of control cells. Immunoprecipitation was performed using anti-SMAD2/3 and IgG antibodies followed by PCR analysis. ChIP analysis of p16 promoter (E), p21 promoter (F), and p53 promoter (G) of cells exposed to F-5 or PBS. Immunoprecipitation was performed using anti-SMAD2/3 and IgG antibodies followed by PCR analysis. Data presented as mean ± SD. ****$p < .0001$, ***$p < .001$, **$p < .01$, and *$p < .05$ between indicated groups.

mitochondrial structure and mtROS accumulation. eHSP90α activates TGF-β signaling to enhance the transcription of senescence genes (Figure 9). We found that eHSP90α blockade by 1G6-D7 decreased age-related lung fibrosis.

Our study showed that the level of serum HSP90α was upregulated in BLM-injured mice and associated with disease severity, which is consistent with published data.[4] Administration of rHSP90a had a pro-fibrotic effect, as shown in our previous study[5] and Martin's study,[4] which highlights the importance of eHSP90α in pulmonary fibrosis. Previous data demonstrated that eHSP90α might activate ERK signaling. However, whether eHSP90α mediates the senescence phenotype in pulmonary fibrosis is unknown. Interestingly, the level of HSP90α was correlated with the levels of SASP factors, such as IL-6, TGFβ1, and PAI1. Furthermore, the expression of p16 and p21 was upregulated from day 7, suggesting that senescent cells contribute to the inflammatory and fibrotic phases.

HSP90 has been shown to affect cellular senescence. Destabilizing the CDK6 interaction with HSP90 promotes premature cellular senescence.[41] A recent study showed that HSP90β interacts with MDM2 to suppress p53-dependent senescence.[42] Moreover, HSP90 inhibitors have been identified as novel senolytics.[7,43] In contrast, our in vitro experiments showed that eHSP90α, but not intracellular HSP90α, was upregulated in the secretome of etoposide-exposed fibroblasts. We also found that

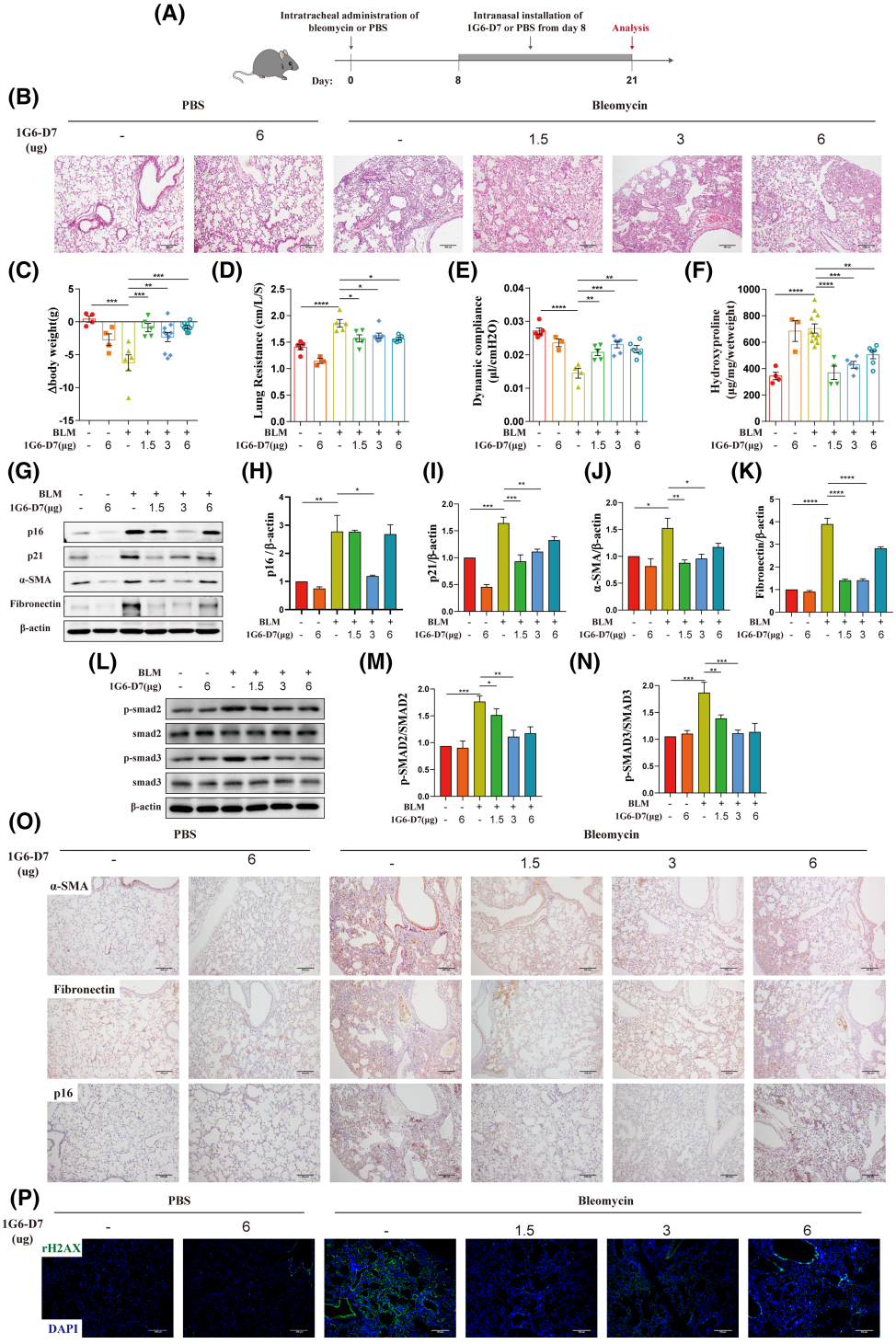

FIGURE 7 Blockade of eHSP90α by 1G6-D7 attenuates cellular senescence and lung fibrosis in vivo. (A) Outline the design of therapeutic dosing of 1G6-D7 in mice with established fibrosis following bleomycin-induced lung injury. (B) Representative images show hematoxylin and eosin (H&E) staining of lung sections from the indicated groups of mice. (C) Twenty-one-day body weight was compared with baseline body weight. Lung resistance (D) and compliance (E) were measured by a pulmonary function test. (F) Hydroxyproline (HYP) contents in lung tissues from mice. Representative western blots (G) and quantitative analysis (H–K) show the amounts of p16, p21, α-SMA, fibronectin and β-actin in lung homogenates from mice treated as shown in (A). Data presented as mean ± SD. ****$p < .0001$, ***$p < .001$, **$p < .01$, and *$p < .05$ between indicated groups. Representative western blots (L) and quantitative analysis (M, N) show the amounts of Psmad2, Psmad3 in lung homogenates. (O) Representative images show α-SMA, fibronectin, and p16 staining of lung sections from the indicated groups of mice. (P) Immunofluorescence show γH2AX staining.

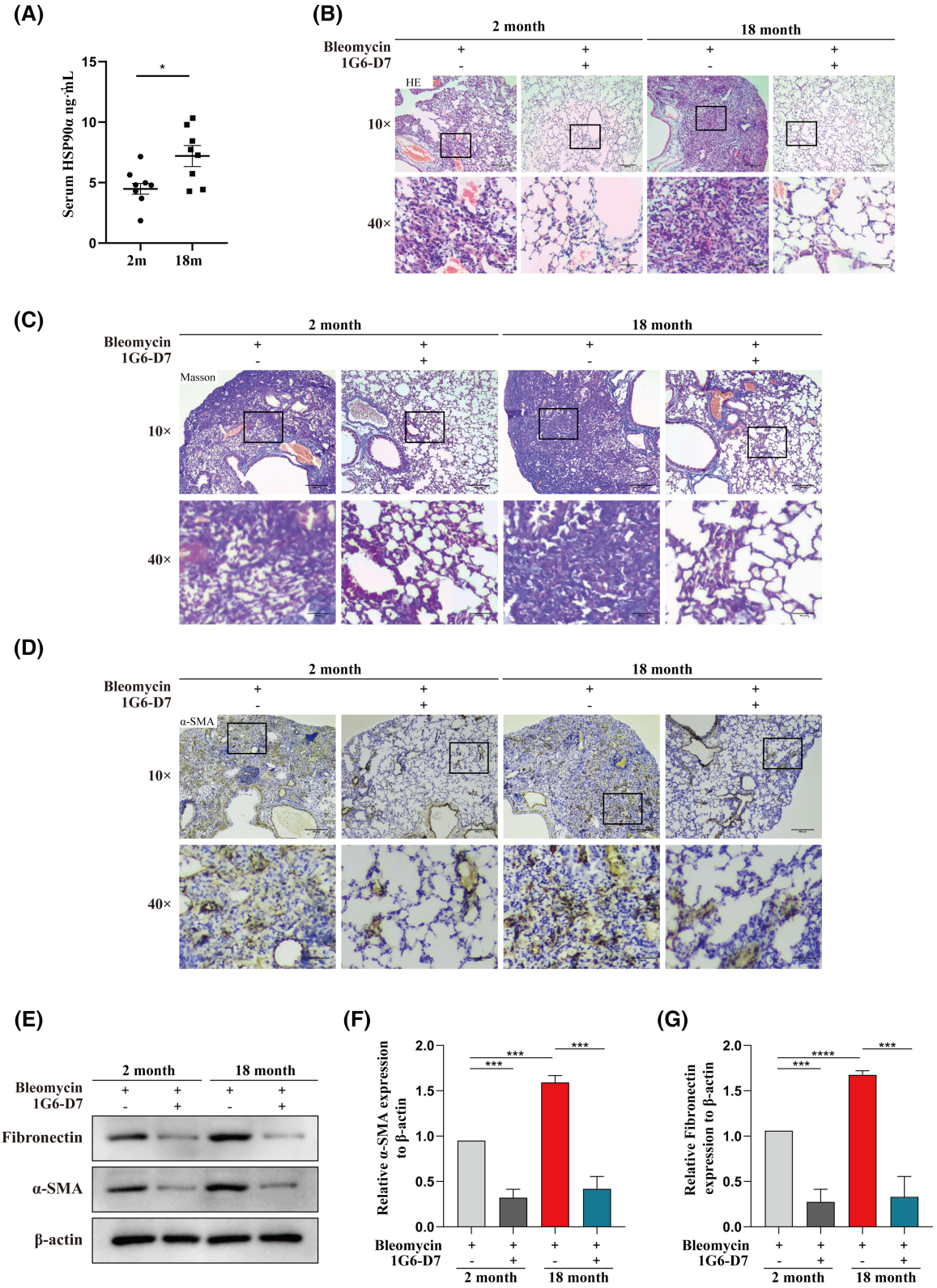

FIGURE 8 Blockade of eHSP90α by 1G6-D7 accelerates resolution of bleomycin-induced lung fibrosis in aged mice. (A) Serum level of HSP90α was measured by ELISA on 2-month-old and 18-month-old mice. Representative images show H&E (B), Masson's trichrome (C), and α-SMA(D) staining of lung sections from the indicated groups of mice. (E–G) Representative immunoblots of α-SMA, fibronectin, and β-actin in whole-lung homogenates. Data presented as mean ± SD. ****$p < .0001$ and *$p < .05$ between indicated groups.

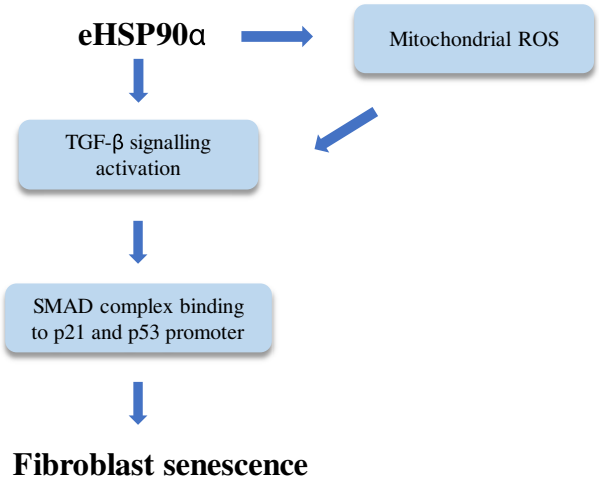

FIGURE 9 Schematic illustration of mechanisms of eHSP90α in cellular senescence.

eHSP90α stimulated a fibroblast senescence phenotype that included, for example, increased p16 and p21 expression and SA-β-gal activity. Using a model of BLM-induced lung fibrosis, we demonstrated that treatment with the anti-HSP90α antibody 1G6D7 significantly decreased cellular senescence. Thus, HSP90α functional diversity may depend on cell type and disease. Several studies have confirmed that the effect of eHSP90α is mediated by low-density lipoprotein receptor-related protein 1 (LRP1).[44–46] Intracellular HSP90β may stabilize the TGF-βRII receptor to promote smad2/3 translocation into the nucleus.[47] However, the receptor to which eHSP90α binds to activate downstream pathways remains unknown. The SASP is an important pathological feature of senescence released through nuclear factor κB (NF-κB) signaling activation.[48] In this study, eHSP90α also augmented SASP factor expression, which promotes senescence. Overall, our findings highlight the importance of eHSP90α in regulating fibroblast senescence.

Several studies have demonstrated the importance of TGFβ signaling in pulmonary fibrosis.[17,49] We showed that eHSP90α activated TGFβ signaling in a manner dependent on mitochondrion-derived ROS accumulation. TGFβ signaling activation triggers translocation of the SMAD complex into the nucleus to promote target gene transcription.

SMADs have been implicated in mediating p21 transcription.[22–24] Our data showed that SMAD2/3, but not SMAD4, binds a specific sequence in the promoters of senescence markers. Furthermore, eHSP90α promoted p53 and p21 transcription by enhancing the binding of SMAD2/3 to their promoters. As p53 also acts as a transcription factor to control p21 transcription, we demonstrated that eHSP90α induces p21 transcription in direct and indirect ways. The accumulation of cytosolic p53, in particular, promotes mitochondrial dysfunction.[50]

Our previous experiments evaluated the preventive effect of the anti-HSP90a antibody 1G6-D7 in a BLM-induced injury model.[5] To accurately assess anti-fibrotic efficacy, the intervention should inhibit fibrosis without impacting early inflammation.[51] In this study, our therapeutic model employed 1G6-D7 administration from day 8 to day 20, which led to improved lung function and decreased collagen deposition. While aging is a major risk factor for IPF and cellular senescence has been proposed as a typical feature,[52] we observed a normal lung structure with the absence of inflammation or fibrosis in 18-month-old mice. In contrast, some studies found significant alveolar changes with age.[53,54] Previous research[55] demonstrated that old mice (above 18-month-old) are more sensitive to bleomycin injury which is consistent with our results. A recent study performed an analysis on human lungs from donors age between 16 and 76 years. They found damage of alveolar epithelial cell and an increasing proportion of fibroblasts with age.[56] Relationship between fibroblast proliferation and cellular senescence should be further explored. Interestingly, 18-month-old mice presented high levels of circulating HSP90α than 2-month-old mice, suggesting eHSP90α might be involved in injury response. Additionally, eHSP90α blockade significantly reduced lung fibrosis in the old model mice. The effect of 1G6-D7 on BLM-injured old mice highlights its capacity to reduce age-related fibrosis and cellular procession.

In conclusion, the present study has demonstrated that eHSP90α causes mitochondrial dysfunction, activating mtROS-dependent TGF-beta signaling to induce fibroblast senescence. eHSP90α blockade reduced lung fibrosis in both young and old mode mice. This study adds to the literature and identifies eHSP90α as an essential contributor to fibroblast senescence in pulmonary fibrosis, suggesting an anti-hsp90a antibody as a potential treatment for age-related fibrotic disease.

AUTHOR CONTRIBUTIONS

Wenshan Zhong, Weimou Chen, and Yuanyuan Liu designed and performed experiments, analyzed data, interpreted the results, and wrote the manuscript. Jinming Zhang and Ye Lu performed experiments and analyzed data. Xuan Wan, Yujie Qiao, Haohua Huang, and Zhaojin Zeng performed experiments. Xiaojing Meng, Wei Li, Haijin Zhao, and Mengchen Zou interpreted the results and edited the manuscript. Hangming Dong and Shaoxi Cai designed and supervised the study, interpreted the results, wrote, and edited the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China [grant numbers 81870058, 82170032].

DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or Supporting Information of this article.

ORCID

Shaoxi Cai https://orcid.org/0000-0003-2816-8262
Hangming Dong https://orcid.org/0000-0002-4476-9829

REFERENCES

1. Martinez FJ, Collard HR, Pardo A, et al. Idiopathic pulmonary fibrosis. *New Engl J Med.* 2001;345(7):517-525.
2. Spagnolo P, Kropski JA, Jones MG, et al. Idiopathic pulmonary fibrosis: disease mechanisms and drug development. *Pharmacol Ther*. 2021;222:107798.
3. Barginear MF, Van Poznak C, Fau-Rosen N, et al. The heat shock protein 90 chaperone complex: an evolving therapeutic target. *Curr Cancer Drug Targets*. 2008;8(6):522-535.
4. Bellaye PS, Shimbori C, Yanagihara T, et al. Synergistic role of HSP90α and HSP90β to promote myofibroblast persistence in lung fibrosis. *Eur Respir J*. 2018;51(2):1700386. doi:10.1183/13993003.00386-2017
5. Dong H, Luo L, Zou M, et al. Blockade of extracellular heat shock protein 90α by 1G6-D7 attenuates pulmonary fibrosis through inhibiting ERK signaling. *Am J Physiol-Lung Cell Mol Physiol*. 2017;313(6):L1006-L1015.
6. Schuliga M, Read J, Knight DA. Ageing mechanisms that contribute to tissue remodeling in lung disease. *Ageing Res Rev*. 2021;70:101405.
7. Dutta Gupta S, Pan CH. Recent update on discovery and development of Hsp90 inhibitors as senolytic agents. *Int J Biol Macromol.* 2020;161:1086-1098.
8. Lehmann M, Korfei M, Mutze K, et al. Senolytic drugs target alveolar epithelial cell function and attenuate experimental lung fibrosis ex vivo. *Eur Respir J*. 2017;50(2):1602367. doi:10.1183/13993003.02367-2016
9. Hernandez-Gonzalez F, Faner R, Rojas M, Agustí A, Serrano MA-O, Sellarés JA-O. Cellular senescence in lung fibrosis. *Int J Mol Sci*. 2021;22(13):7012. doi:10.3390/ijms22137012
10. Schafer MJ, White TA, Iijima K, et al. Cellular senescence mediates fibrotic pulmonary disease. *Nature Commun*. 2017;8(1):1.
11. McHugh D, Gil JA-O. Senescence and aging: causes, consequences, and therapeutic avenues. *J Cell Biol*. 2018;217(1):65-77.
12. Calcinotto A, Kohli J, Zagato E, Pellegrini L, Demaria M, Alimonti A. Cellular senescence: aging, cancer, and injury. *Physiol Rev.* 2019;99(2):1047-1078.
13. Yanai H, Shteinberg A, Porat Z, et al. Cellular senescence-like features of lung fibroblasts derived from idiopathic pulmonary fibrosis patients. *Aging (Albany NY)*. 2015;7(9):664.
14. Korolchuk VI, Miwa S, Carroll B, von Zglinicki T. Mitochondria in cell senescence: is mitophagy the weakest link? *BioMedicine*. 2017;21:7-13.
15. Lee HC, Yin P, Fau CC-W, Fau CC, Wei Y-H, Wei YH. Increase in mitochondrial mass in human fibroblasts under oxidative stress and during replicative cell senescence. *J Biomed Sci*. 2002;9(6):517-526.
16. Li X, Zhang W, Cao Q, et al. Mitochondrial dysfunction in fibrotic diseases. *Cell Death Discov.* 2020;6(1):1-4.
17. Tatler AL, Jenkins G. TGF-β activation and lung fibrosis. *Proc Am Thor Soc.* 2012;9(3):130-136.
18. Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-β: the master regulator of fibrosis. *Nature Rev Nephrol.* 2016;12(6):325-338.
19. Chanda D, Otoupalova E, Smith SR, Volckaert T, De Langhe SP, Thannickal VJ. Developmental pathways in the pathogenesis of lung fibrosis. *Mol Aspects Med.* 2019;65:56-69.
20. Kim KK, Sheppard D, Chapman HA. TGF-β1 signaling and tissue fibrosis. *Cold Spring Harb Perspect Biol.* 2018;10(4):a022293. doi:10.1101/cshperspect.a022293
21. Hill CS. Nucleocytoplasmic shuttling of Smad proteins. *Cell Res.* 2009;19(1):36-46.
22. Pouliot F, Labrie C. Role of Smad1 and Smad4 proteins in the induction of p21WAF1,Cip1 during bone morphogenetic protein-induced growth arrest in human breast cancer cells. *J Endocrinol.* 2002;172(1):187-198.
23. Pardali K, Kowanetz M, Fau HC-H, Fau HC, Moustakas A, Moustakas A. Smad pathway-specific transcriptional regulation of the cell cycle inhibitor p21(WAF1/Cip1). *J Cell Physiol.* 2005;204(1):260-272.
24. Pardali K, Kurisaki AF, Morén A, et al. Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J Biol Chem*. 2000;275(38):29244-29256.
25. Zou M, Bhatia A, Dong H, et al. Evolutionarily conserved dual lysine motif determines the non-chaperone function of secreted Hsp90alpha in tumour progression. *Oncogene.* 2017;36(15):2160-2171.
26. Chen W, Zhang J, Zhong W, et al. Anlotinib inhibits PFKFB3-driven glycolysis in myofibroblasts to reverse pulmonary fibrosis. *Front Pharmacol*. 2021;12:744826. doi:10.3389/fphar.2021.744826
27. Hecker L, Fau VR, Jones T, et al. NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. *Nat Med*. 2009;15(9):1077-1081.
28. Clougherty JE, Fau RC, Lawrence J, et al. Chronic social stress and susceptibility to concentrated ambient fine particles in rats. *Environ Health Perspect*. 2010;118(6):769-775.
29. Mishra AA-O, Majid D, Kandikattu HK, Yadavalli CA-O, Upparahalli VS. Role of IL-18-transformed CD274-expressing eosinophils in promoting airway obstruction in experimental asthma. *Allergy.* 2022;77(4):1165-1179. doi:10.1111/all.15180
30. Yao L, Zhao H, Tang H, et al. The receptor for advanced glycation end products is required for β-catenin stabilization in a chemical-induced asthma model. *Br J Pharmacol.* 2016;173(17):2600-2613.
31. Dimri GP, Lee X, Fau BG, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Nat Acad Sci.* 1995;92(20):9363-9367.
32. Graham L, Orenstein JM. Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. *Nat Prot.* 2007;2(10):2439-2450.
33. Dong H, Zou M, Bhatia A, et al. Breast cancer MDA-MB-231 cells use secreted heat shock protein-90alpha (Hsp90α) to survive a hostile hypoxic environment. *Sci Rep.* 2016;6(1):1-9.

34. Fuentealba RA, Liu Q, Kanekiyo T, Zhang J, Bu G. Low density lipoprotein receptor-related protein 1 promotes anti-apoptotic signaling in neurons by activating Akt survival pathway. *J Biol Chem*. 2009;284(49):34045-34053.
35. Kurundkar A, Thannickal VJ. Redox mechanisms in age-related lung fibrosis. *Redox Biol*. 2016;9:67-76.
36. Tsubouchi K, Araya J, Kuwano K. PINK1-PARK2-mediated mitophagy in COPD and IPF pathogeneses. *Inflamm Regen*. 2018;38(1):1-9.
37. Al Fau M, Bueno M, Fau BM, Rojas M, Rojas M. Mitochondria in the spotlight of aging and idiopathic pulmonary fibrosis. *J Clin Invest*. 2017;127(2):405-414.
38. Tominaga K, Suzuki HA-O. TGF-β signaling in cellular senescence and aging-related pathology. *Int J Mol Sci*. 2019;20(20):5002. doi:10.3390/ijms20205002
39. Zhang D, Jin W, Wu R, et al. High glucose intake exacerbates autoimmunity through reactive-oxygen-species-mediated TGF-β cytokine activation. *Immunity*. 2019;51(4):671-681.
40. Meiners S, Eickelberg O, Königshoff M. Hallmarks of the ageing lung. *Eur Respir J*. 2015;45(3):807-827.
41. Kanugovi AV, Joseph C, Siripini S, Paithankar K, Amere SA-OX. Compromising the constitutive p16(INK4a) expression sensitizes human neuroblastoma cells to Hsp90 inhibition and promotes premature senescence. *J Cell Biochem*. 2020;121(4):2770-2781.
42. He MY, Xu SB, Qu ZH, et al. Hsp90β interacts with MDM2 to suppress p53-dependent senescence during skeletal muscle regeneration. *Aging Cell*. 2019;18(5):e13003.
43. Fuhrmann-Stroissnigg H, Ling YY, Zhao J, et al. Identification of HSP90 inhibitors as a novel class of senolytics. *Nat Commun*. 2017;8(1):1-4.
44. Seclì L, Avalle L, Poggio P, et al. Targeting the extracellular HSP90 co-chaperone Morgana inhibits cancer cell migration and promotes anti-cancer immunity. *Cancer Res*. 2021;81(18):4794-4807. doi:10.1158/0008-5472.CAN-20-3150
45. Tian Y, Wang C, Chen S, Liu J, Fu Y, Luo YA-O. Extracellular Hsp90α and clusterin synergistically promote breast cancer epithelial-to-mesenchymal transition and metastasis via LRP1. *J Cell Sci*. 2019;132(15):jcs228213. doi:10.1242/jcs.228213
46. Tsen F, Fau BA, O'Brien K, et al. Extracellular heat shock protein 90 signals through subdomain II and the NPVY motif of LRP-1 receptor to Akt1 and Akt2: a circuit essential for promoting skin cell migration in vitro and wound healing in vivo. *Mol Cell Biol*. 2013;33(24):4947-4959.
47. Sibinska Z, Tian X, Korfei M, et al. Amplified canonical transforming growth factor-β signalling via heat shock protein 90 in pulmonary fibrosis. *Eur Respir J*. 2017;49(2):1501941. doi:10.1183/13993003.01941-2015
48. Faget DA-O, Ren Q, Stewart SA-O. Unmasking senescence: context-dependent effects of SASP in cancer. *Nat Rev Cancer*. 2019;19(8):439-453.
49. Sime PJ, Xing ZF, Graham FL, et al. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest*. 1997;100(4):768-776.
50. Hoshino A, Mita Y, Okawa Y, et al. Cytosolic p53 inhibits Parkin-mediated mitophagy and promotes mitochondrial dysfunction in the mouse heart. *Nat Commun*. 2013;4(1):1-2.
51. Kolb PA-O, Upagupta C, Vierhout M, et al. The importance of interventional timing in the bleomycin model of pulmonary fibrosis. *Eur Respir J*. 2020;55(6):1901105. doi:10.1183/13993003.01105-2019
52. Selman M, Pardo A. Revealing the pathogenic and aging-related mechanisms of the enigmatic idiopathic pulmonary fibrosis. an integral model. *Am J Respir Crit Care Med*. 2014;189(10):1161-1172.
53. Schulte H, Mühlfeld C, Brandenberger C. Age-Related Structural and Functional Changes in the Mouse Lung. Frontiers in physiology. 2019 Dec 4;10:1466.
54. Shin HJ, Kim SH, Park HJ, Shin MS, Kang I, Kang MA-OX. Nucleotide-binding domain and leucine-rich-repeat-containing protein X1 deficiency induces nicotinamide adenine dinucleotide decline, mechanistic target of rapamycin activation, and cellular senescence and accelerates aging lung-like changes. Aging cell. 2021 Jul;20(7):e13410
55. Stout-Delgado HW, Cho SJ, Chu SG, et al. Age-dependent susceptibility to pulmonary fibrosis is associated with NLRP3 inflammasome activation. *Am J Respir Cell Mol Biol*. 2016;55(2):252-263.
56. Lee SA-O, Islam MA-O, Boostanpour K, et al. Molecular programs of fibrotic change in aging human lung. *Nat Commun*. 2021;12(1):1-10.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Zhong W, Chen W, Liu Y, et al. Extracellular HSP90α promotes cellular senescence by modulating TGF-β signaling in pulmonary fibrosis. *The FASEB Journal*. 2022;36:e22475. doi:10.1096/fj.202200406RR