The role and mechanism of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis

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The role and mechanism of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis

Directed by Professor Soo Han Bae

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With sincere and warm regards,

Chanho Lee

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ABSTRACT

The role and mechanism of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a fatal disease with a prognosis comparable to major malignant tumors, yet its pathogenic mechanism remains unclear. Although it is widely accepted that autophagy is impaired in alveolar epithelial type 2 cells (AEC2) and fibroblasts in the lungs of IPF patients, the molecular mechanism through which impaired autophagy contributes to IPF remains elusive. SQSTM1/p62 (Sequestosome 1) is a key protein that links cellular senescence and redox homeostasis to autophagy through selective autophagic degradation of GATA4 and KEAP1. However, the role of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis has not yet been explored. This study aims to elucidate the role and working mechanism of SQSTM1/p62 in pulmonary fibrosis.

Lung samples from IPF patient registry and public transcriptomic and single-cell RNA sequencing datasets were analyzed to determine the expression of SQSTM1/p62. Using bleomycin-induced lung fibrosis as the main in vivo model for pulmonary fibrosis, SQSTM1/p62 conventional knockout mice and AEC2-specific knockout mice were employed to validate the role of SQSTM1/p62 in vivo. Lung-specific deletion rescue using

adeno-associated virus 9 vectors was also employed.

SQSTM1/p62 was transcriptionally downregulated in patients with IPF, and its protein was rather accumulated due to impaired autophagic degradation. Moreover, mRNA expression of SQSTM1/p62 was positively correlated to better lung function in patients with IPF. SQSTM1/p62 conventional knockout mice exhibited increased pulmonary fibrosis compared to wild-type mice, whereas deletion-rescued mice were protected from pulmonary fibrosis compared to empty-vector-transduced mice. AEC2-specific knockout mice were more susceptible to bleomycin-induced injury and lethality. Furthermore, SQSTM1/p62 knockout led to impaired autophagy and KEAP1 degradation, suggesting a potential mechanistic link.

In conclusion, this study demonstrates that SQSTM1/p62 plays a protective role in the pathogenesis of pulmonary fibrosis, shedding light on a potential therapeutic target for IPF.

Key words : IPF, Autophagy, SQSTM1/p62, pulmonary fibrosis, RNA sequencing, Alveolar epithelial type 2 cell, senescence, redox homeostasis.

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I. INTRODUCTION

Idiopathic pulmonary fibrosis(IPF) is a fatal disease with a prognosis comparable to major malignant tumors.[1] This chronic progressive interstitial lung disease is more common in the elderly and is imposing a growing burden on humanity.[2] Despite the clinical advances in the diagnosis and treatment of IPF[3], little is known about its molecular pathogenic mechanism.

It has not been very long since the traditional idea of inflammation causing IPF was rejected. Clinical investigations have shown that anti-inflammatory therapy, including local and systemic corticosteroids, had little efficacy on IPF patients, and inflammation is not prominent in lungs of IPF patients.[4] It is now widely accepted that repetitive micro-injury to the lungs leads to impairment of self-renewal and over-activation of interstitial fibrosis.[2]

Alveolar epithelial type 2 cells, responsible for self-renewing of alveolar epithelial cells inside the human adult lung, have gained attention lately in IPF research.[5] Recent discoveries revealed that senescent alveolar epithelial type 2 cells impair recovery mechanism in the alveoli of IPF lungs.[6] Also, dysfunctional autophagy in alveolar epithelial

type 2 cell may lead to cellular senescence and dysfunctional mitochondria.[2, 5] However, the molecular mechanism through which impaired autophagy contributes to IPF remains unclear.

The KEAP1 (Kelch like ECH associated protein 1) - NFE2L2/NRF2 (nuclear factor, erythroid 2 like 2) pathway is one of the most well-known pathway that controls intracellular redox homeostasis.[7-10] NFE2L2 is a major controller of the cellular antioxidant mechanism; it binds to the antioxidant response element(ARE) to promote transcription of antioxidant enzymes. KEAP1, in conjunction with CUL3 (Cullin 3), induces proteasomal degradation of NFE2L2.[7-9]

SQSTM1/p62 (Sequestosome 1) is a key protein that links cellular senescence and autophagy. SQSTM1/p62 binds to KEAP1 and competitively protects NFE2L2 from degradation.[7, 9, 10] SQSTM1/p62 binding to KEAP1 can cause selective autophagic degradation of KEAP1, which can up-regulate NFE2L2 and promote antioxidants in stressful conditions.[7, 9] SQSTM1/p62 is also known to promote autophagic degradation of GATA4 and inhibit cellular senescence.[11] There are findings that SQSTM1/p62 can actually promote autophagic flux itself.[10]

Collectively, SQSTM1/p62 is a key protein which can control autophagy, redox homeostasis, and cellular senescence. Nevertheless, the role of SQSTM1/p62 in the pathogenesis of IPF is still unknown (Figure 1).

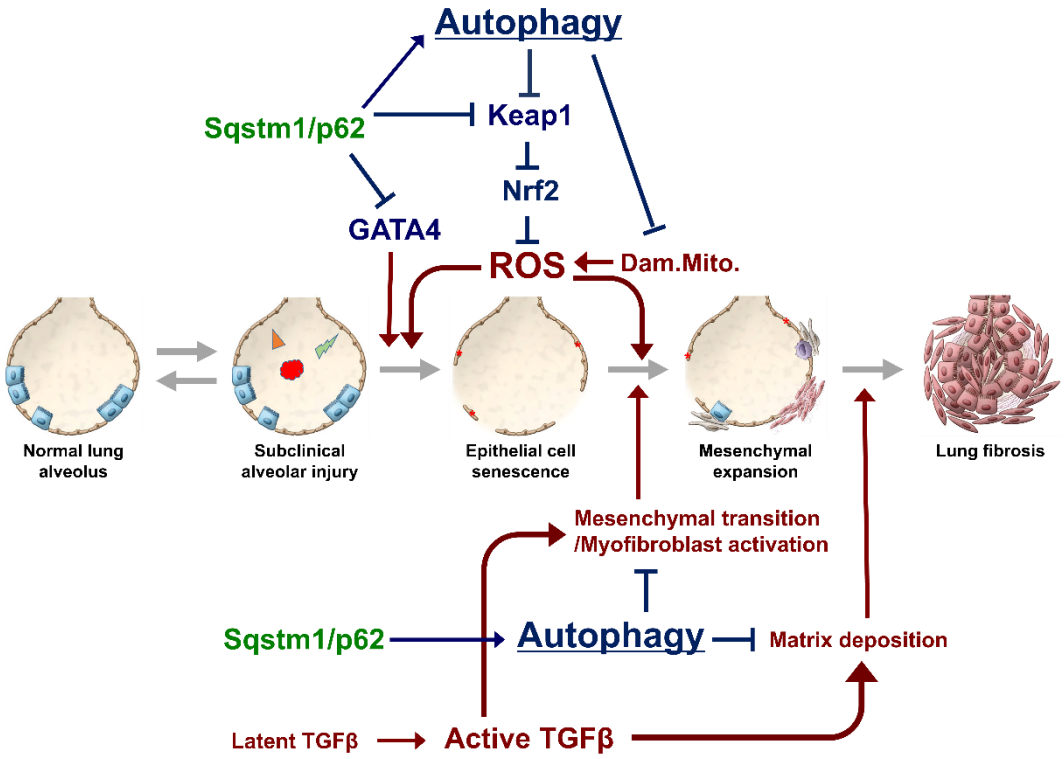

Figure 1 Illustration of the working hypothesis of the role of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis.

Abbreviations. Dam. Mito., damaged mitochondria; GATA4, GATA Binding Protein 4; Keap1, Kelch like ECH associated protein 1; Nrf2, nuclear factor, erythroid 2 like 2; ROS, Reactive oxygen species; Sqstm1/p62, Sequestosome 1; TGF, Transforming growth factor

Therefore, this study investigated the role of SQSTM1/p62 in the pathogenic process of IPF. Expression levels of the SQSTM1/p62 gene in both mRNA and protein levels in human lung were compared between IPF patients and healthy controls. Using conventional and conditional in vivo genetic deletion of SQSTM1/p62, I investigated cell-specific roles of SQSTM1/p62 in mouse models of pulmonary fibrosis. To further validate the causal relationship between genetic deletion of SQSTM1 and pulmonary fibrosis, I employed SQSTM1/p62 gene rescue using an adeno-associated virus 9 (AAV9) vector in the conventional knockout mouse model. Based on the robust role of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis, I investigated molecular mechanisms such as autophagy, the KEAP1-NFE2L2/NRF2 pathway, cellular senescence, and possibly novel interacting protein pathways. In addition, I discussed the clinical value of SQSTM1/p62 expression as a therapeutic target.

II. MATERIALS AND METHODS

1. Human subjects and ethical considerations

Patients with IPF were enrolled in registries previously established at Severance Hospital. These patient registries collected baseline characteristics, including age, sex, comorbidities, and diagnostic evaluation results at the time of enrollment. Lung sections obtained from surgical lung biopsy or lung transplantation, which were surplus after obtaining sufficient tissues for diagnosis, were stored in a liquid nitrogen tank until use. Patients included in this registry underwent the same medical procedures as those not part of the registry, with no additional medical procedures performed to obtain the tissue.

For the control group, human lung specimens were obtained from patients undergoing lobectomy or pneumonectomy for clinical necessities. Only remnant non-neoplastic sections were used.

All patients provided written informed consent. All study procedures were reviewed and approved by the Institutional Review Board at Severance Hospital (IRB numbers: 4-2012-0685, 4-2013-0770, 4-2019-0447).

The publicly available transcriptomic datasets with accession numbers GSE47460, GSE53845, and GSE150910 were retrieved from online sources and subsequently analyzed.[12-14] Also, single-cell RNA sequencing datasets with web-based analysis tools were analyzed online.[15, 16] It is noteworthy that all of these datasets were subjected to anonymization procedures to ensure the protection of individual privacy. Thereby, the need for consent was waived for these analyses.

2. Animal subjects and ethics

SQSTM1/p62 knockout(KO) mice in a C57BL/6 background were provided by Dr. J. Shin (Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine,

Suwon, Republic of Korea) and Dr. J.W. Ryu (Department of Biomedical Sciences, Yonsei University College of Medicine, Seoul, Republic of Korea).[17] Wild type and knockout littermates from same ancestry were used for the experiments. Surfactant protein C(Sftpc)-cre mice which express Cre-ERT2 recombinase from the Sftpc gene locus were kindly provided by Dr. B.C. Cho (Division of Medical Oncology, Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Republic of Korea) and Dr. Harold Chapman (University of California, San Francisco, CA, USA).[18] SQSTM1/p62 floxed mice of a C57BL/6 background were provided by Dr. Yanagawa (Department of Oral and Maxillofacial Surgery, Faculty of Medicine, Tsukuba University, Tsukuba-shi, Japan) and Dr. M.S. Lee (Department of Biomedical Sciences, Yonsei University College of Medicine, Seoul, Republic of Korea).[19, 20]

The breeding environment of all mice was in agreement with the guideline from National Research Council (Washington, DC, USA).[21] Mice were maintained in individually ventilated cages in a specific-pathogen-free barrier facility at Yonsei Biomedical Research Institute of Yonsei University College of Medicine. The facility has been fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) since 2003. The mice were housed in cages that accommodated no more than 5 animals per cage. All materials for breeding were sterilized. They had free access to water and food in rooms maintained at 23 ± 2 ℃ and 50–70 % humidity with a 12 h light/dark cycle.

All experimental procedures in this study were reviewed thoroughly and approved by Yonsei University Health System Institutional Animal Care and Use Committee (IACUC number: 2020-0099, 2023-0112).

3. Determination of genotypes

All littermates were genotyped and allocated to separate cages before the age of 6 weeks old. Ear tissue obtained from ear-tagging was hydrolyzed in 75μL of 25mM NaOH and

0.2mM EDTA aqueous solution at 98℃ for 1 hour. After cooling to room temperature, 75μL of 40mM Tris HCL (pH 5.5) was added to neutralize. After centrifugation at 4,000 rpm for 3 minutes, an aliquot of resulting supernatant containing the genomic DNA was subjected to polymerase chain reaction (PCR) using Platinum™ II Hot-Start PCR Master Mix (14000012; Invitrogen™, Thermo Fisher Scientific, Inc.). The sequences of the primers used for genotyping were as follows: Sftpc-cre primer 1 ,5'-TCGCCTTCTATCGCCTTCTTG-3'; Sftpc-cre primer 2, 5'-CCTTTTGCTCTGTTCCCCATTA-3'; Sftpc-cre primer 3, 5'-TGGTTCCGAGTCCGATTCTTC-3'; SQSTM1/p62 flox primer 1, 5'-GGCAATGGCTGGTCTACTTT-3'; SQSTM1/p62 flox primer 2, 5'-GGACTGAGCCTCTGAGCAAC-3'; SQSTM1/p62 KO primer 1, 5'-CCCGTACCTTTCAAGGGTCC-3'; SQSTM1/p62 KO primer 2, 5'-CCGCTGGATGTTAGATGTAACCTG-3'; SQSTM1/p62 KO primer 3, 5'-AGCAGCCTCTGTTCCACATACACTT-3'. The amplified PCR products were analyzed by agarose gel electrophoresis, and the genotype was decided as per the known sequence length of each genotype (Figure 2).

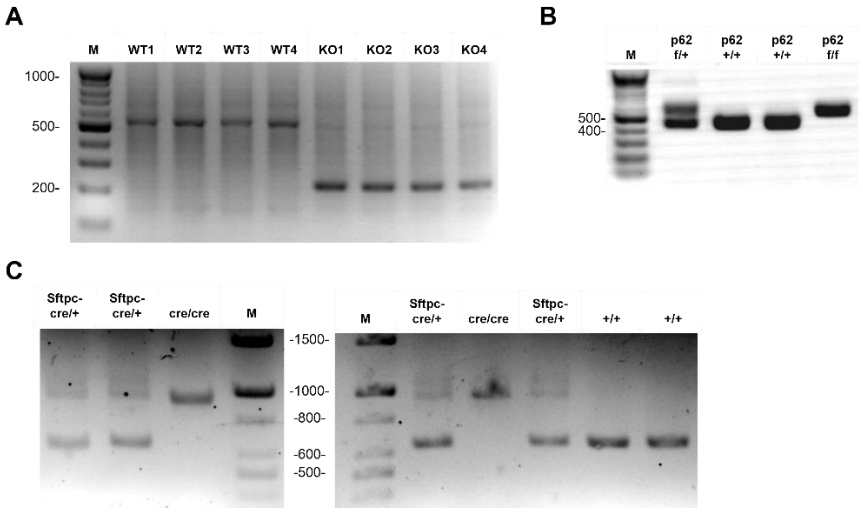

Figure 2 Genotyping of mice used in this study.

(A) Genotypes of SQSTM1/p62 conventional knockout mice were confirmed by PCR. The wild type (WT) allele shows a 510-base pairs (bp) band, whereas the knockout (KO) allele shows a 220-bp band.

(B) Genotypes of SQSTM1/p62 floxed mice were confirmed by PCR. The expected size of SQSTM1/p62 flox allele is 547 bp and the wild type allele is 449 bp.

(C) Genotypes of Sftpc-creERT2 mice were confirmed by PCR. The Cre allele is a 1000-bp band, and the wild type allele is a 700-bp band.

Abbreviations. PCR, polymerase chain reaction; Sftpc, surfactant protein C.

4. Bleomycin induced mouse lung fibrosis model

Male mice aged 7 to 9 weeks were utilized for the experiments. Briefly, each mouse received an oropharyngeal aspiration of either 2U/kg of bleomycin (B5507; Sigma-Aldrich, Inc., St. Louis, MO, USA) dissolved in 50uL of injection-quality normal saline or a corresponding vehicle under 4% isoflurane-induced anesthesia. The detailed method of inducing aspiration in mice were described elsewhere.[22] Starting from the day of bleomycin administration, body weights were recorded three times weekly before sacrifice. Fourteen days post-instillation, the animals were anesthetized using intraperitoneal injection of 80 mg/kg alfaxalone with 10 mg/kg xylazine, and humanely euthanized to collect tissue samples. The lungs were subjected to perfusion with 6 mL of ice-cold sterile phosphate-buffered saline (PBS) through right ventricle and subsequently processed as described below.

5. Induction of conditional knockout and deletion rescue

Cre-ERT2 recombinase of Sftpc-cre mice was activated by oral injection of 100mg/kg tamoxifen 6 times over 2 weeks. Male mice having one allele of Sftpc-cre were selected for the experiments in both the SQSTM1/p62 floxed group and the control group to exclude the effect of the known toxicity of Cre recombinase expression in alveolar epithelial type 2 cells.[23]

For deletion rescue of SQSTM1/p62, SQSTM1/p62 knockout mice were orally aspirated with $1*10^{10}$ genome copies (GC) of AAV9 vectors dissolved in 50μL of PBS. The control mice were transfected with an empty AAV9 vector expressing a GFP reporter (7007; Vector Biolabs, Malvern, PA, USA), and the deletion-rescue mice were administered an AAV9 vector stably expressing mouse SQSTM1/p62 with a GFP reporter (AAV9-273112; Vector Biolabs).

6. Immunoblot analysis

The tissue and cell samples were lysed in an ice-cold lysis buffer composed of the following components: 20 mM HEPES-KOH (pH 7.9), 120 mM NaCl, 0.5% Nonidet P-40, 0.3% Triton X-100, 10% glycerol, 5 mM NaF, 1 mM Na3VO4, 1 mM ethylenediaminetetraacetic acid, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 2 mM phenylmethylsulfonyl fluoride. The right lobe of each mouse lung was homogenized using a TissueLyser II (QIAGEN GmbH, Hilden, Germany). The resulting lysate was subjected to centrifugation for 15 minutes at 17,177 g, and the supernatant was analyzed for sample concentration using a Bradford assay. Each protein sample underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with the separated proteins subsequently transferred to a polyvinylidene fluoride membrane (IPVH00010; Millipore™, Sigma-Aldrich, Inc.).

After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with specific primary antibodies overnight at 4 °C. This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. Protein visualization was achieved using an enhanced chemiluminescence solution (34580; Thermo Scientific™, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Semi-quantitative densitometry analysis was performed using Image J (version 1.53; National Institutes of Health, Bethesda, MD, USA).

The following primary antibodies were used: anti-COL1A1 (91144S, 72026S; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-ACTB (A5316; Sigma-Aldrich, Inc.), anti-LC3B (2775S; Cell Signaling Technology), anti-GFP (sc-9996; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Keap1 (10503-2-AP; Proteintech Group, Inc., Rosement, IL, USA), anti-Fibronectin (ab2413; Abcam plc, Cambridge, UK), anti-ACTA2 (A5228; Sigma-Aldrich, Inc.), anti-SQSTM1 for human samples (39749; Cell Signaling

Technology), and anti-SQSTM1 for murine samples (H00008878-MO1; Abnova Corporation, Taipei City, Taiwan).

7. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Total RNA was prepared using TRIzol™ Reagent (15596018; Invitrogen™, Thermo Fisher Scientific, Inc.) for cultured cells or RNeasy Plus Mini kits (74134; QIAGEN) for lung tissues according to the manufacturer's protocol. After quantitation with NanoDrop 2000 (Thermo Scientific™, Thermo Fisher Scientific, Inc.), cDNA was synthesized using 1 μg RNA and PrimeScript™ RT Master Mix (RR036A; Takara Bio Inc., Shiga, Japan) and was analyzed using SYBR™ Green PCR Master Mix (Applied Biosystems™, 4301955) and specific paired primers for each genes. The primers were synthesized by Bioneer Corporation (Daejeon, Republic of Korea). The comparative Ct method was employed to normalize the gene expression in each sample. The 18S ribosomal RNA (*RN18S*) gene was used as an internal control for both human and mouse samples.

8. Biochemical measurements of lung fibrosis

The amount of soluble collagen in the right lung of each mouse was quantified using the Sircol Soluble Collagen Assay (S1000; Biocolor Ltd, Carrickfergus, N. Ireland) as per the manufacturer's protocol. Hydroxyproline content in the lung samples was measured using Picosens™ Hydroxyproline Assay Kit (BM-HYP-100; Biomax, Gyeonggi-do, Republic of Korea) according to the manufacturers' protocol.

9. Histological analysis

Mouse left lungs were intra-tracheally infused with low-melting agarose, immersed in 10% formalin for 24hr, and embedded in paraffin. The fixed lung tissue samples were cut into 5-μm sections, slide-mounted, stained, and analyzed. Masson's trichrome staining was

used to visualize collagen accumulation indicative of fibrosis. All slides were scanned using an Aperio AT2 (Leica Biosystems Nussloch GmbH, Nussloch, Germany)., and the scanned images were processed and analyzed using Aperio ImageScope (Leica Biosystems).

10. Mouse lung mechanics measurements

Lung mechanics of each mouse was measured using flexiVent (SCIREQ Scientific, Montreal, Canada) according to the manufacturer's protocol. Briefly, fully anesthetized mice were injected with 1mg/kg vecuronium to inhibit spontaneous breathing. After performing tracheostomy using an 18G catheter, inspiratory capacity and static compliance were sequentially measured four times utilizing the pre-built measurement sets of the flexiVent.

11. Transcriptomic analysis

For GEO datasets without any normalization, data normalization and differential expression analysis were performed using DESeq2 in R (version 4.2.1; R Foundation for Statistical Computing, Vienna, Austria).[24] Gene Set Enrichment Analysis v.4.3.2 (Broad Institute, Inc., Cambridge, MA, USA) with MSigDB gene sets (release 7.5.1, Broad Institute, Inc.) was utilized to clarify the functions of differentially expressed genes and to understand the implications at the transcriptomic level.

12. Statistical analysis

A T-test or Mann-Whitney test was employed to compare variables between the two groups. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons adjustment was performed to compare data between three or more groups. Results from

repeated measurements of body weights were analyzed by two-way ANOVA. A p-value of <0.05 was considered significant. In scatter dot plotted graphs, the bar graph with error bar represents mean ± standard error of the mean unless otherwise mentioned in the figure legend. Each dot represents one subject (mouse or human). Linear regression between SQTSM1 mRNA expression and the percent predicted forced vital capacity (FVC) or the percent predicted diffusion capacity of the lung for carbon monoxide (DLCO) was performed to address the role of SQSTM1/p62 in the clinical setting. All statistical analyses were performed and visualized using the built-in analysis package in Prism 10 (GraphPad Software, San Diego, CA, USA).

III. RESULTS

1. The transcription of SQSTM1/p62 gene is downregulated in lung epithelial cells of patients with IPF.

To compare the expression levels of SQSTM1/p62 in the lungs of patients with IPF, transcriptomic analyses data from public database were analyzed. In GSE47460, RNA samples from lungs of 582 subjects with different respiratory diseases were analyzed by microarray. Patients with IPF showed 7% lower expression of SQSTM1/p62 mRNA levels compared to other three groups (Figure 3A). This difference was bigger in GSE53845, which compared 40 patients with IPF to 8 controls and analyzed RNA by microarray, showing 14% lower mRNA expression (Figure 3B). In the most recent study, GSE150910, 103 patients with IPF were compared to same number of controls using RNA-sequencing. The result was in the same trend that patients with IPF had about 14% lower expression of SQSTM1/p62 mRNA (Figure 3C). Therefore, SQSTM1/p62 transcription is downregulated in the lungs of patients with IPF.

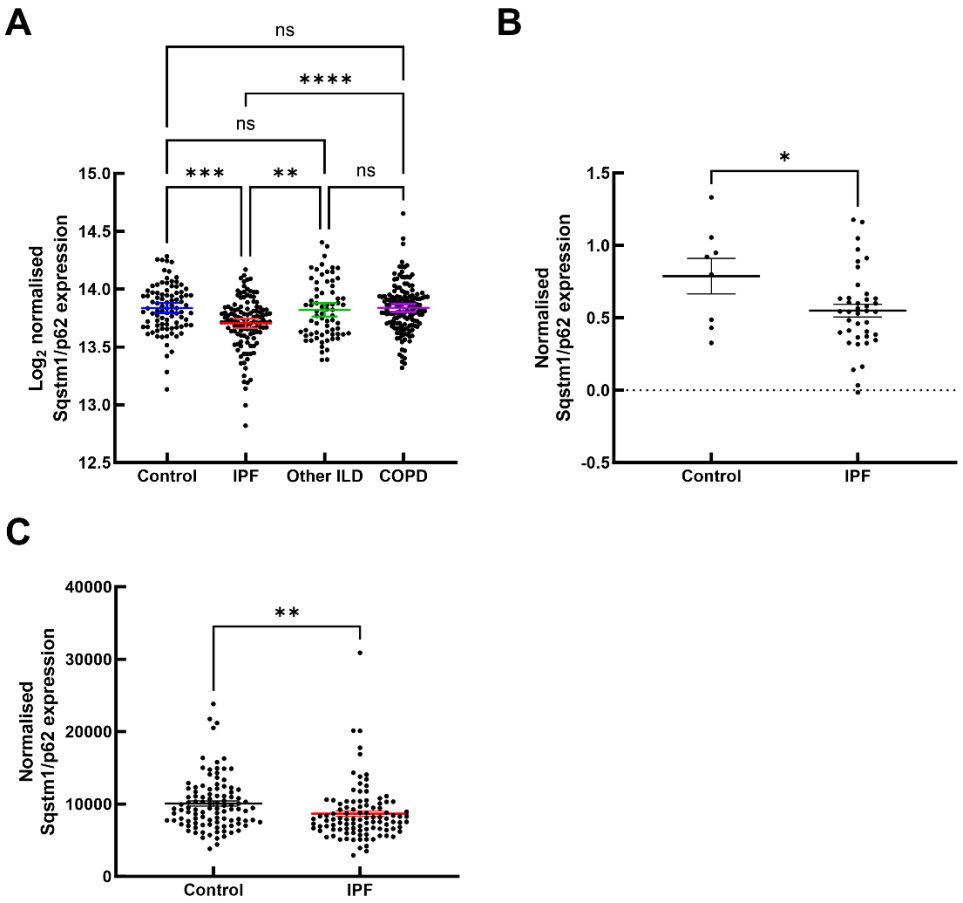

Figure 3 Transcriptomic analyses reveals downregulation of SQSTM1/p62 mRNA in patients with IPF

Public transcriptomic datasets available via Gene Expression Omnibus were analyzed.

(A) SQSTM1/p62 expression data from GSE47460 are plotted. The subjects were diagnosed with IPF (n=122), chronic obstructive pulmonary disease (n=220), other interstitial lung diseases (n=132), or healthy controls (n=108).

(B) SQSTM1/p62 expression data from GSE53845. Control (n=8), IPF (n=40).

(C) SQSTM1/p62 expression data from GSE150910 are plotted. The numbers of subjects were Control (n=103) and IPF (n=103).

COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Since the nature of bulk RNA sequencing or microarray is limited to estimating the average amount of mRNA over whole lung, I took a further step by analyzing single-cell RNA sequencing datasets, which are also publicly available. In the data from Kaminski and Rosas[15], the mRNA expression levels of SQSTM1/p62 in different cell types was analyzed (Figure 4A). There was a clear trend that SQSTM1 was expressed mainly in the epithelial cells, not stromal or immune cells.

Considering this epithelial cell preference, I found that the dataset from Königshoff[16] was the most suitable option since this dataset analyzed EPCAM-positive, a one of the best well-known epithelial cell marker, cells for single-cell RNA sequencing. This data revealed that the difference between control and IPF subjects was the biggest in alveolar epithelial type 2 cells (Figure 4B). Basal cells and deuterosomal cells did also showed some decrease in the SQSTM1 expression. However, deuterosomal cells had low expression of SQSTM1 in the average which may lead to bigger noise in the result. Basal cells, on the other hand, were not supported by other datasets. In summary, SQSTM1 expression is downregulated in alveolar epithelial type 2 cells of patients with IPF.

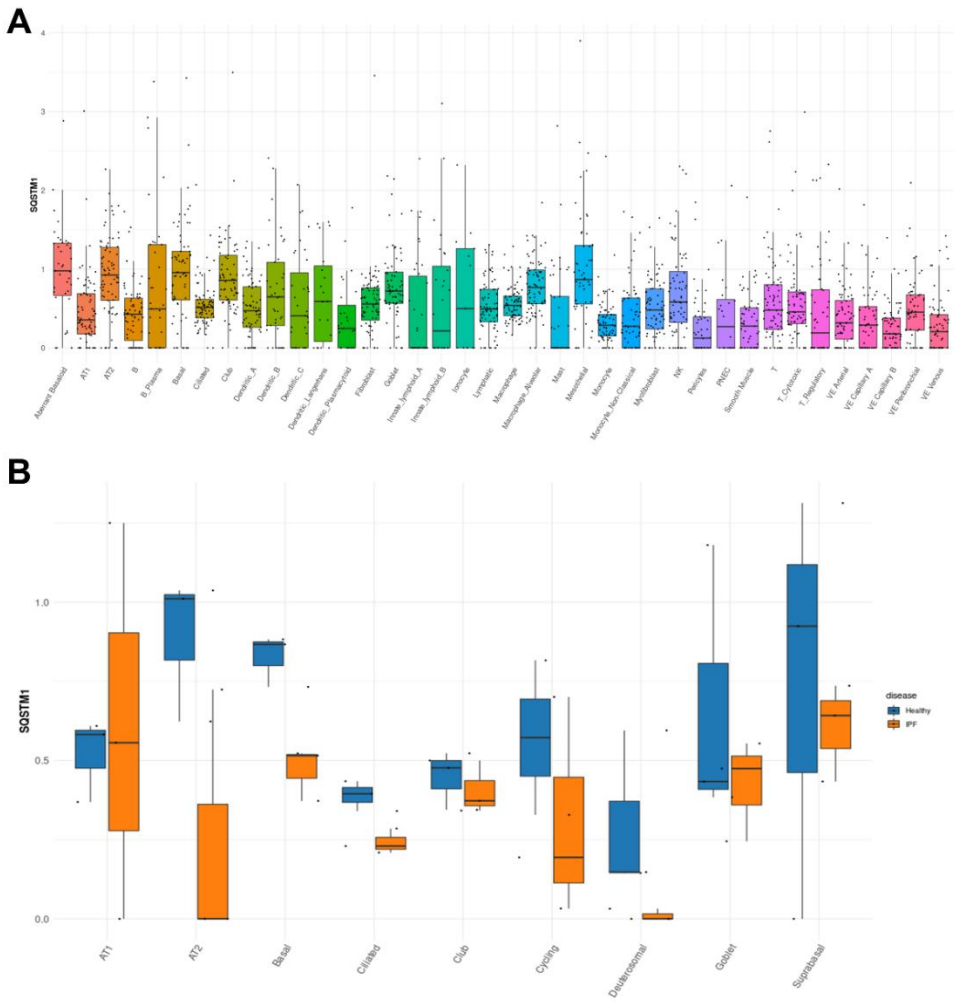

Figure 4 Analysis of published single-cell RNA sequencing datasets reveals alveolar epithelial type 2 cells as the main cell type for the downregulation of SQSTM1 transcription in patients with IPF.

(A) Single-cell RNA sequencing data from Kaminski and Rosas are visualized as box plots. Each dot represents one cell.

(B) Single-cell RNA sequencing data published from Königshoff are visualized as box plots. Each dot represents one person.

2. The discrepancy in the SQSTM1/p62 mRNA level between in vivo model and patients with IPF indicates the potential role of SQSTM1/p62 in the pathogenesis of IPF.

To investigate the sequential effects of decreased SQSTM1/p62 mRNA levels in alveolar epithelial cells of patients with IPF, lung tissue samples were first analyzed to confirm whether the protein expression is downregulated. Surprisingly, the lung samples from the IPF patient registry revealed an accumulation of SQSTM1/p62 protein in the patients' lungs (Figure 5A, B). The fibrosis markers, Collagen 1a1 (COL1A1), Fibronectin (FN), and alpha-smooth muscle actin 2 (ACTA2), were increased in the IPF group, which reflects the validity and representativeness of the patient samples. This 4.5-fold increase in SQSTM1 protein level indicates a decrease in the degradation rather than an upregulation, considering the reduction in mRNA levels.

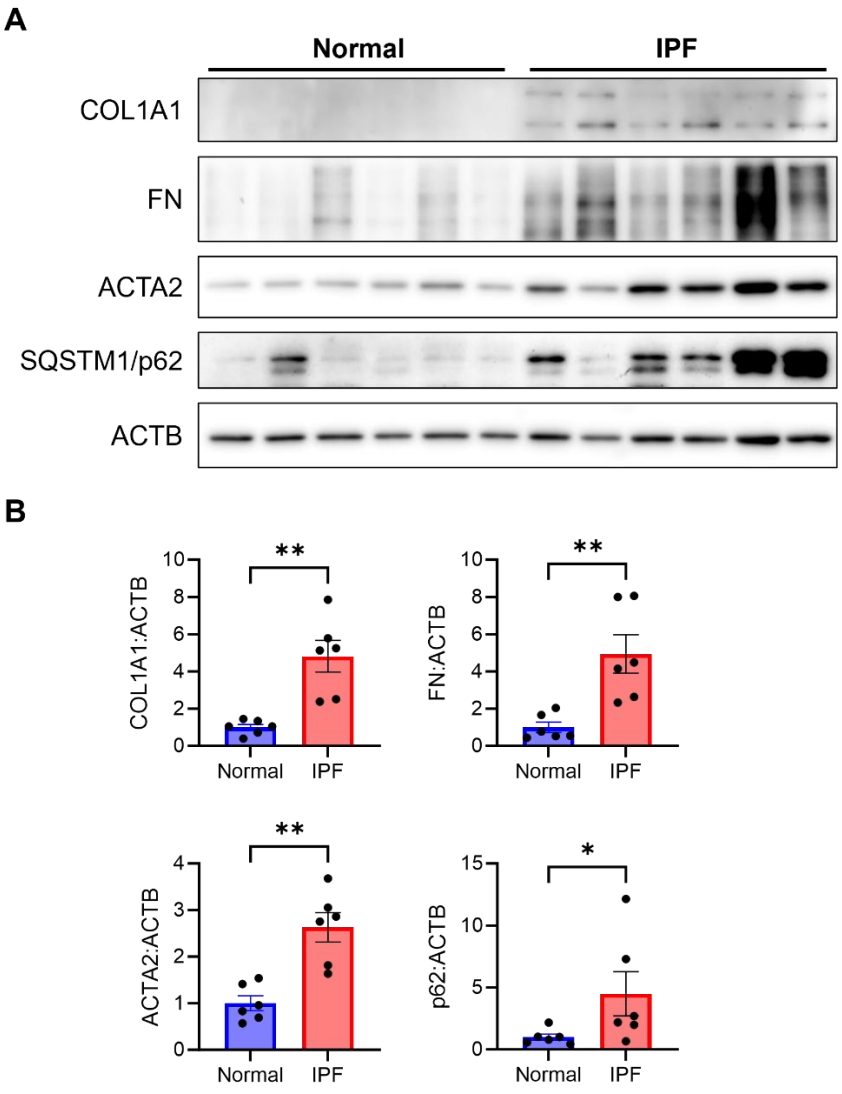

Figure 5 SQSTM1/p62 protein is accumulated in lungs of patients with IPF

Lung tissues from 6 patients with IPF and 6 controls were subjected to immunoblot analysis.

(A) Representative blots are shown.

(B) Densitometry analysis of immunoblot data in (A) is presented as mean ± standard error of the mean with individual data points.

To verify whether the increase in SQSTM1/p62 protein expression is a common process during lung fibrosis, we examined the bleomycin-induced mouse pulmonary fibrosis model throughout its chronological progression. Using bleomycin-injured mice sacrificed at various time points, we observed an increase in SQSTM1/p62 protein levels from day 0 to 56 after bleomycin injury (Figure 6A). Notably, the SQSTM1/p62 mRNA levels increased approximately two-fold at 14 days after bleomycin and remained at a similar level at 21 days after bleomycin (Figure 6B).

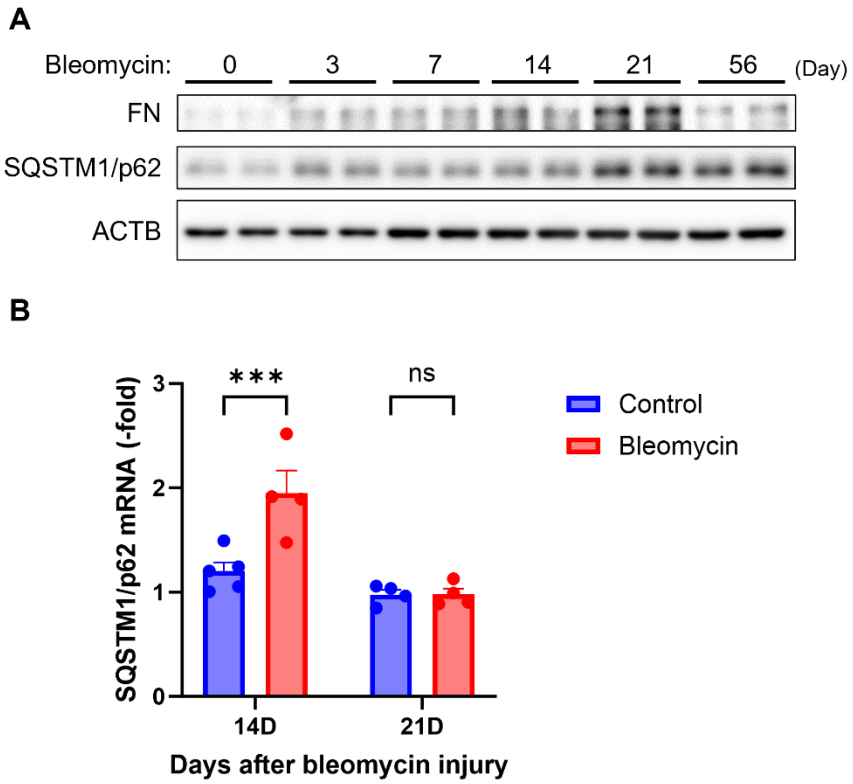

Figure 6 SQSTM1/p62 is upregulated in both mRNA and protein level in bleomycin-induced pulmonary fibrosis mouse model.

Lung tissues from mice sacrificed at different time points after bleomycin injury were analyzed by western blot and RT-qPCR.

(A) SQSTM1/p62 protein level was gradually increased after the initial bleomycin injury. Representative blots are shown.

(B) RT-qPCR analysis revealed that SQSTM1 mRNA level was increased at day 14 after bleomycin, while there is no difference at day 21.

Consequently, a discrepancy emerged in the SQSTM1/p62 mRNA levels between the mouse model and IPF patients. This incongruity gave rise to the hypothesis that the transcription of the SQSTM1/p62 gene may be impaired in patients with IPF, potentially contributing to the pathological outcomes. With this idea in mind, I aimed to uncover the protective role of SQSTM1/p62 in pulmonary fibrosis.

3. Genetic deletion of SQSTM1/p62 in mice reveals its protective role in bleomycin-induced pulmonary fibrosis.

To investigate the potential protective role of SQSTM1/p62 during pulmonary fibrosis, the bleomycin in vivo model with conventional knockout mice was employed. Especially, considering that the SQSTM1/p62 mRNA was upregulated at day 14 after bleomycin, I used this model throughout the whole study. Overall schedule of the animal experiment in this section is illustrated (Figure 7A). Body weight trends indicated that SQSTM1/p62 knockout mice exhibited slower recovery after bleomycin injection (Figure 7B).

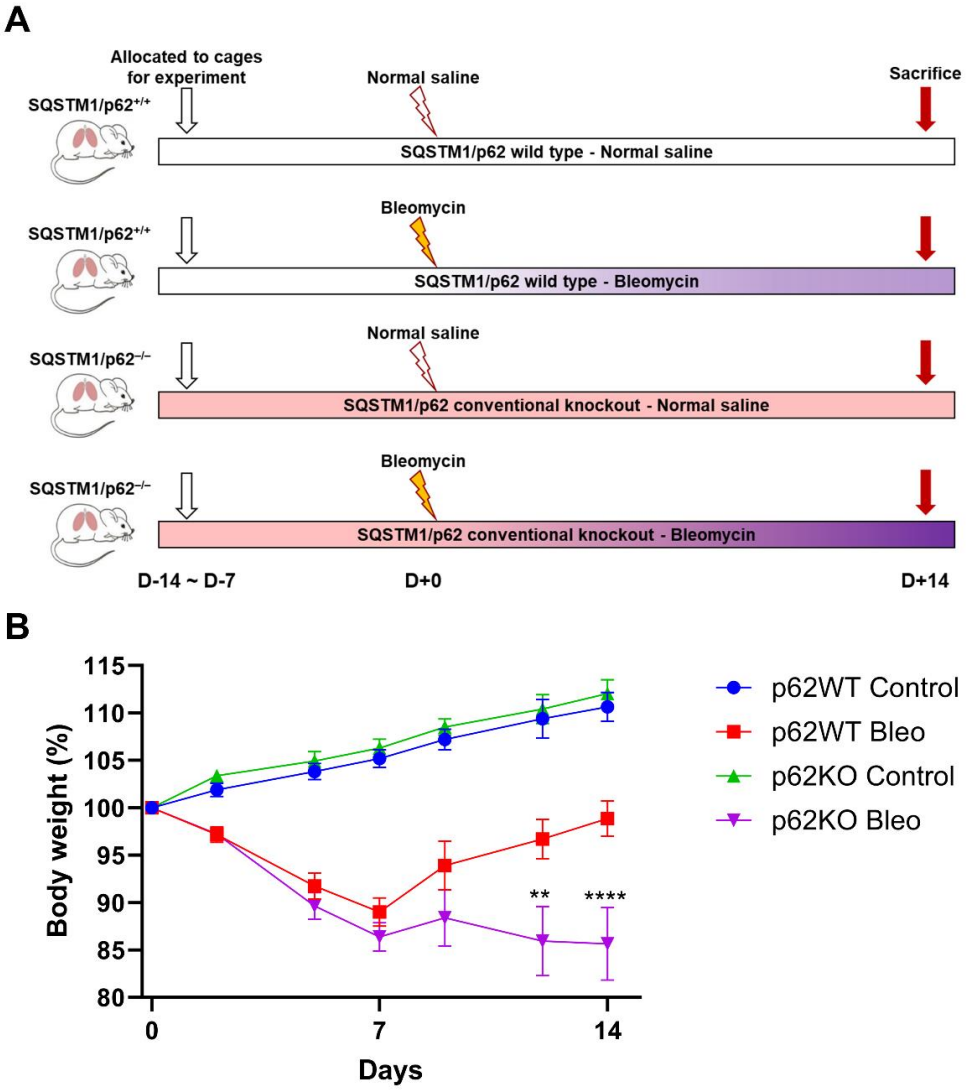

Figure 7 SQSTM1/p62 knockout mice recovers slower from bleomycin injury.

(A) The timeline and experimental design are illustrated.

(B) Measured body weights were normalized to each mouse's initial body weight. n=16–21. Only statistically significant differences between the 'WT Bleo' group and the 'KO Bleo' group are indicated with asterisks.

Bleo, bleomycin; KO, knockout; WT, wild type; **, p<0.01; ****, p<0.0001.

To probe the extent of pulmonary fibrosis after bleomycin aspiration, the amount of collagen accumulated in the mouse lungs were measured by two different methods. The amount of soluble collagen, as measured by the Sircol assay, showed that knockout mice had 14% more pulmonary fibrosis compared to wild-type mice (Figure 8A). Histological evaluation reached the same conclusion, with SQSTM1/p62-deleted mice displaying more collagen-positive areas in Masson's trichrome-stained slides compared to wild-type mice (Figure 8B).

To validate this biochemical and histological evaluation of pulmonary fibrosis, protein and mRNA levels in lung lysates were measured by immunoblots and RT-qPCR. Immunoblots confirmed that COL1A1 expression was further increased in the knockout-bleomycin group compared to the wild-type-bleomycin group (Figure 9A). The deletion of SQSTM1/p62 expression was also verified. This pattern was similar in the RT-qPCR results, where bleomycin-injected knockout mice exhibited a 20% increase in COL1A1 mRNA level compared to bleomycin-aspirated wild-type mice (Figure 9B). Absolute deletion of SQSTM1/p62 mRNA in the knockout mouse was also affirmed.

To sum up, conventional knockout of SQSTM1/p62 aggravated bleomycin-induced pulmonary fibrosis.

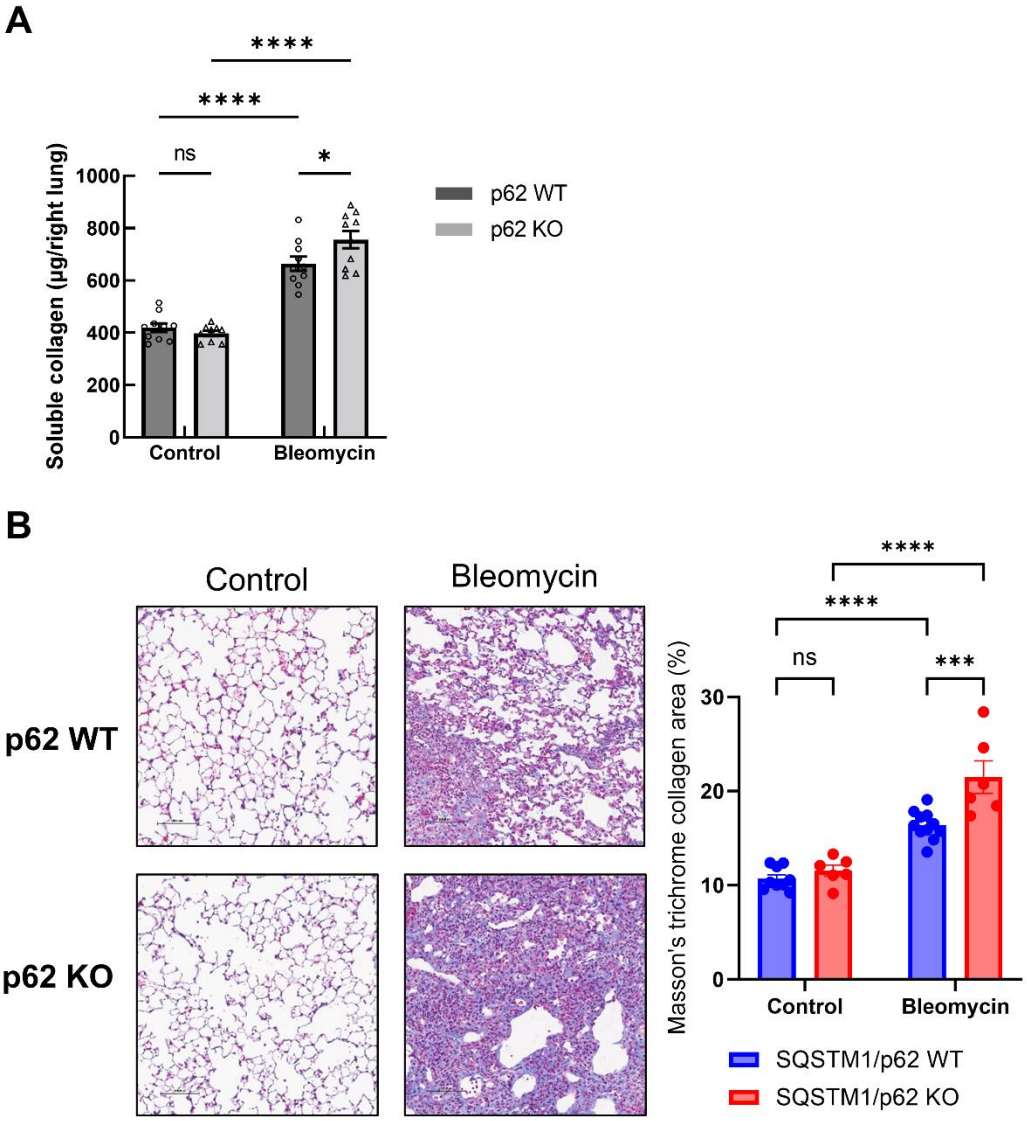

Figure 8 SQSTM1/p62 conventional knockout mice develops more pulmonary fibrosis after bleomycin injury.

The amount of collagen was measured by two assays to compare the extent of pulmonary fibrosis.

(A) Sircol assay revealed that soluble collagen was more accumulated in knockout

mice after bleomycin injury. n=9–10.

(B) Masson's trichrome stain revealed that collagen-stained area was larger in the bleomycin-aspirated knockout mice than their counterpart. Representative scanned images of Masson's trichrome stain from paraffin-embedded slide sections of the left lungs are presented. n=6–8.

ns, not significant; *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$.

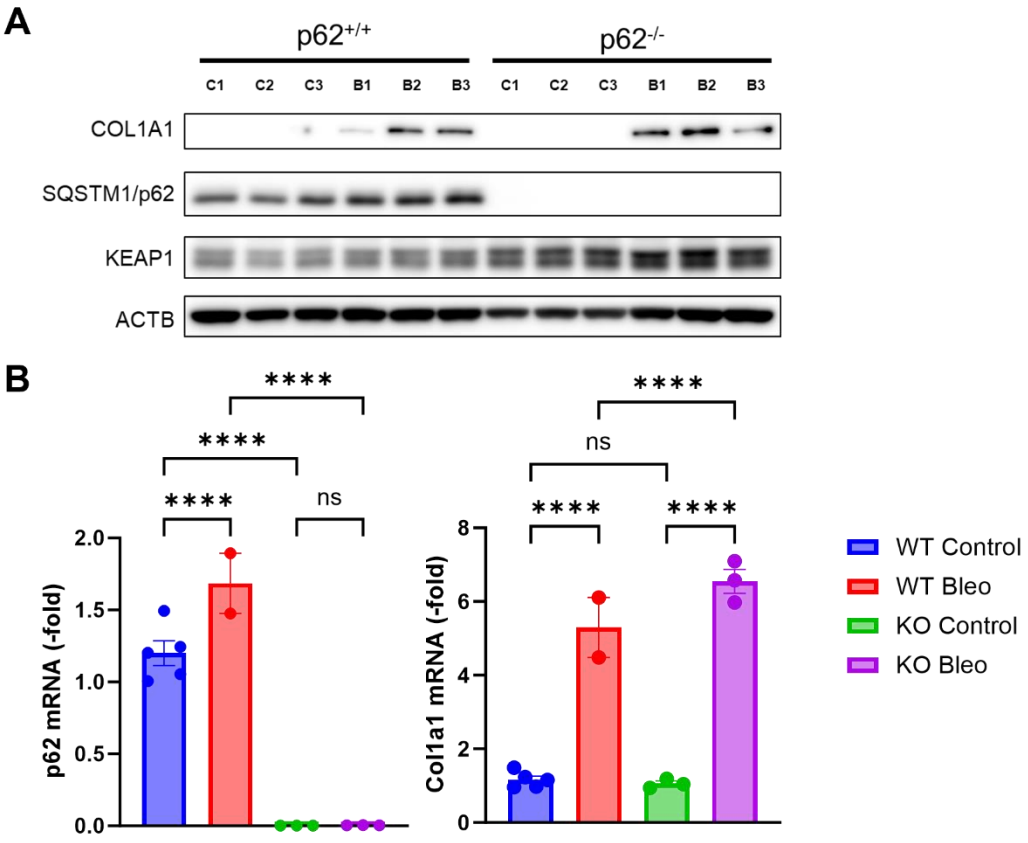

Figure 9 Increased COL1A1 protein and mRNA level in the bleomycin-induced pulmonary fibrosis model of SQSTM1/p62 knockout mice.

Lung tissues from 2 to 5 mice from each group were analyzed by immunoblots and RT-qPCR.

(A) Representative blots from immunoblot analysis are shown.

(B) RT-qPCR reveals increased Col1a1 mRNA in the KO Bleo group compared to the WT Bleo group. Sqstm1 deletion was also validated in this result.

Bleo, bleomycin; KO, knockout; WT, wild type; ns, not significant; ****, p<0.0001.

4. Lung-specific deletion rescue of SQSTM1/p62 in conventional knockout mice confirms its protective role in pulmonary fibrosis.

To determine whether the role of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis is lung specific, AAV9 vectors expressing mouse SQSTM1/p62 were transfected directly to the lung by oropharyngeal aspiration. AAV9 serotype is known for its high transduction rate and low immunogenicity in the lungs, therefore the effect of inflammation caused by AAV infection itself may not impact the extent of pulmonary fibrosis.[25, 26] It has been reported that AAV9 transduced lungs stably express the delivered gene at least for 6 weeks.[27] Therefore, SQSTM1/p62 knockout mice were transduced with mouse SQSTM1-expressing AAV9 (mSQSTM1-AAV9) or Green fluorescent protein(GFP)-expressing AAV9 (GFP-AAV9) 2 weeks before bleomycin injury. The mice were sacrificed 2 weeks after the bleomycin aspiration (Figure 10A).

Body weight trends exhibited less decrease in the deletion rescued mice compared to the empty-vector transduced knockout mice (Figure 10B). This indicated that the potential protective role of SQSTM1/p62 may relate to the early phase of bleomycin, which is basically the injury and inflammation caused by epithelial cell senescence and cell death.[28]

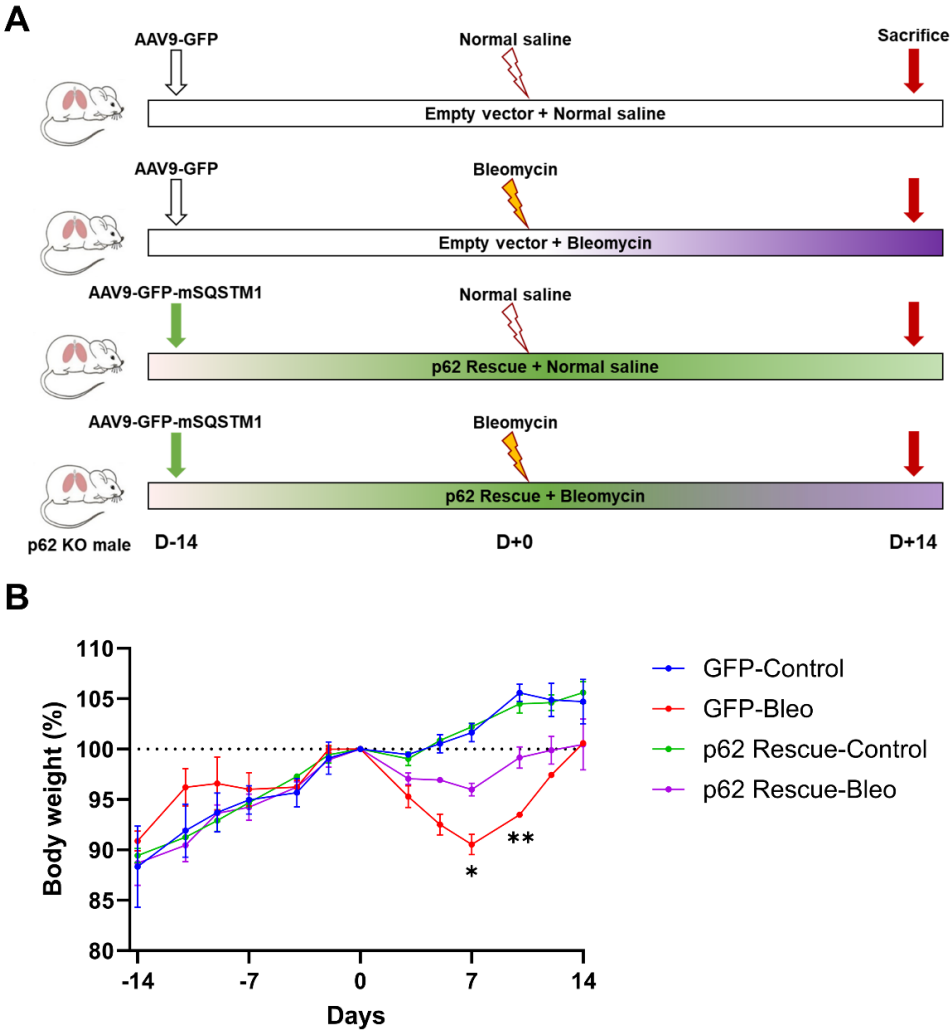

Figure 10 SQSTM1/p62 rescued knockout mice suffers less from body weight loss after bleomycin injury.

(A) The timeline and experimental design are illustrated.

(B) Measured body weights were normalized to each mouse's initial body weight. n=3 per group. Only statistically significant differences between the 'GFP-Bleo' group and the 'p62 Rescue-Bleo' group are indicated with asterisks.

Bleo, bleomycin; *, $p<0.05$; **, $p<0.01$.

To probe the lung-specific protective role of SQSTM1/p62 against pulmonary fibrosis, the amount of collagen was measured by 3 different assays; 1) Masson's trichrome stain with quantification by automated algorithms pre-built in the program Image Scope, 2) Sircol assay, and 3) Hydroxyproline assay, which is known as the gold standard method that best reflects pulmonary fibrosis.[29] The results were all in similar trends. Masson's trichrome stain revealed that the deletion-rescue protected mice from fibrosis (Figure 11A, B). The amount of soluble collagen and hydroxyproline was lower in the 'Rescue-bleomycin' group than the 'control-bleomycin' group (Figure 11C, D). These findings indicate that SQSTM1/p62 deletion rescue successfully prevented pulmonary fibrosis in the bleomycin mouse model.

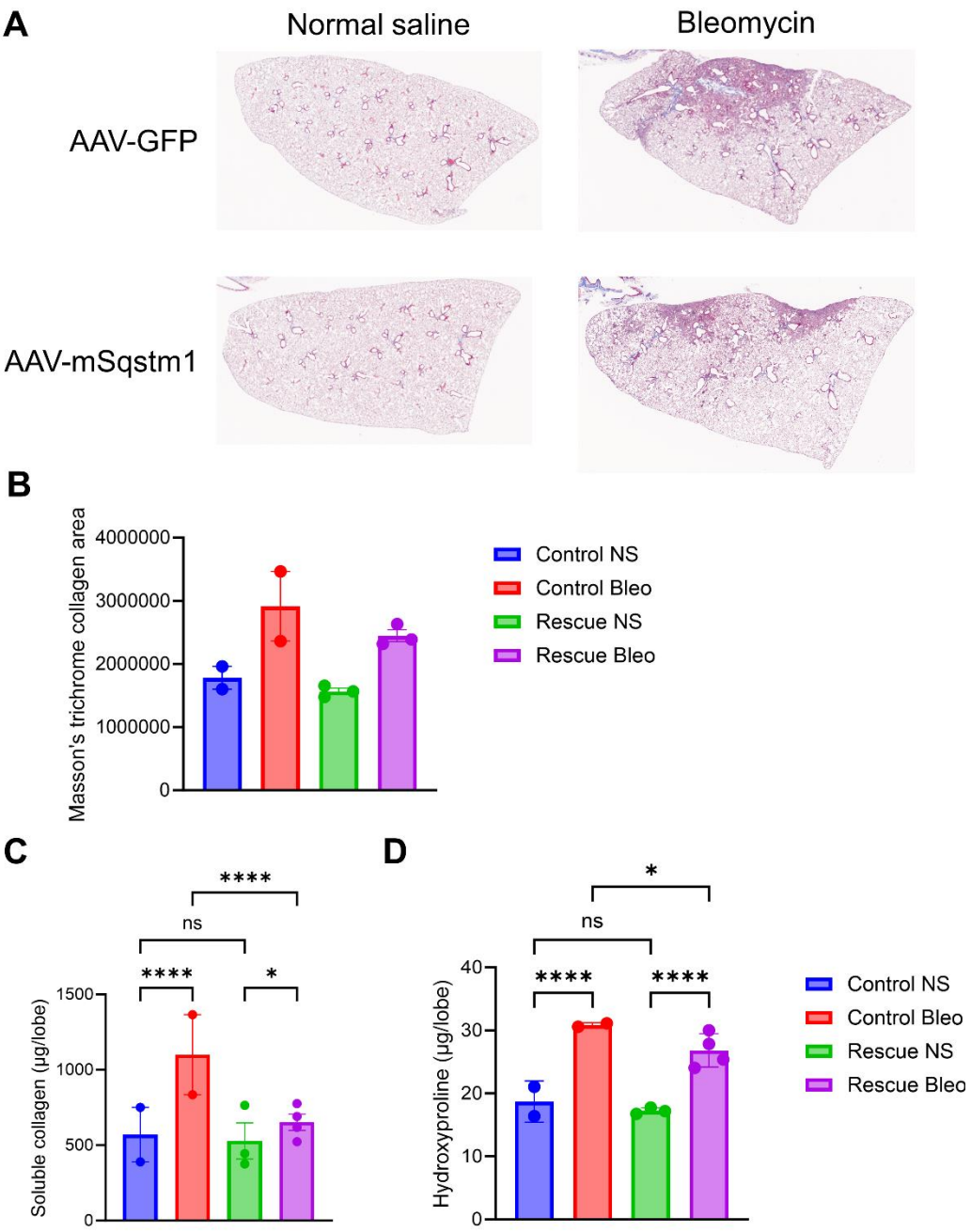

Figure 11 Lung-specific SQSTM1/p62 deletion rescue protects mice from bleomycin-induced pulmonary fibrosis.

The amount of collagen in the lungs of mice was analyzed by 3 different methods.
(A) Representative scanned images of Masson's trichrome stain slides of left lungs are shown.
(B) Collagen-stained area from the scanned image was quantified by automated built-in algorithm of ImageScope program. Statistical analysis was omitted due to very small number of subjects.
(C) The amount of soluble collagen in the right upper lobe of each mouse was measured by Sircol assay.
(D) hydroxyproline assay revealed less fibrosis in the 'Rescue Bleo' group compared to the control counterpart.
Bleo, bleomycin; NS, normal saline; ns, not significant; *, $p<0.05$; ****, $p<0.0001$.

5. Alveolar epithelial type 2 cell-specific knockout of SQSTM1/p62 results in more lethality and toxicity of bleomycin injury, but no more fibrosis.

To investigate the cell-specific role of SQSTM1/p62 in pulmonary fibrosis, alveolar epithelial type 2 cell (AEC2)-specific genetic deletion of SQSTM1/p62 was introduced using Sftpc-cre mice and SQSTM1/p62 floxed mice. After 6 consequent injections of tamoxifen to activate the Cre recombinase, bleomycin was aspirated to each mouse (Figure 12A). The body weight trends showed a substantial delay in the recovery process (Figure 12B). Also, 2 AEC2-specific SQSTM1/p62 knockout mice were dead before sacrifice on day 14, which led to considerable differences in the survival rates (Figure 12C). All the other mice survived throughout the experiment process.

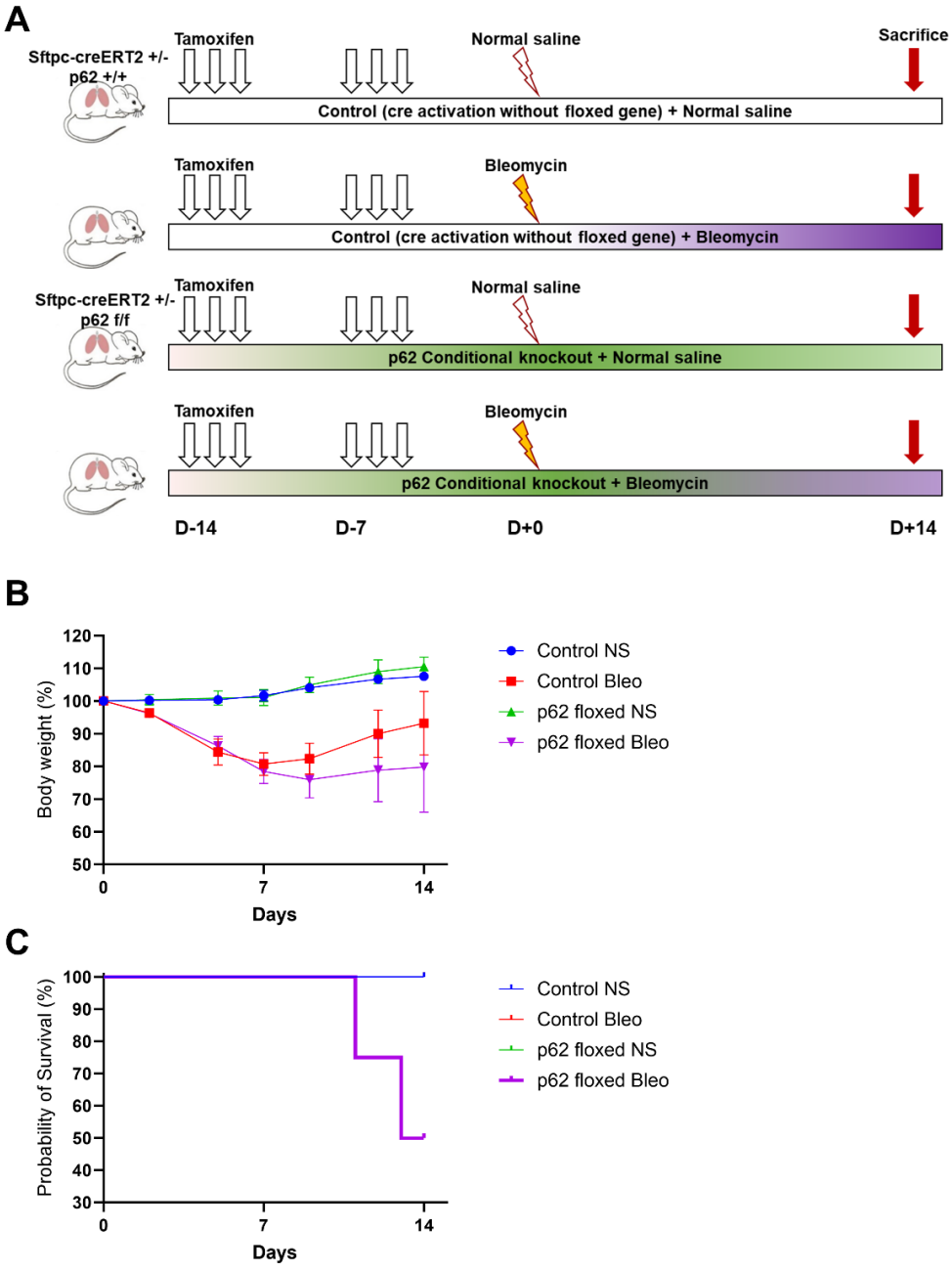

Figure 12 Alveolar epithelial type 2 cell-specific deletion of SQSTM1/p62 results in more lethality and delayed recovery.

(A) The timeline and experimental design are illustrated.
(B) Measured body weights were normalized to each mouse's initial body weight. n=2–3 per group.
(C) Survival curves are shown, with only lethality in the AEC2-specific SQSTM1/p62 knockout bleomycin group.
Statistical analysis was omitted due to very small number of subjects. Bleo, bleomycin; NS, normal saline;

Surprisingly, the amount of fibrosis did not differ between the conditional SQSTM1/p62 knockout mice and the Cre mice after bleomycin injury. Although it seemed that conditional knockout mice exhibit wider area to be injured by bleomycin (Figure 13A), the quantified results did not differ significantly (Figure 13B). This was the same in the hydroxyproline assay, that the two bleomycin groups were not distinguishable.

However, when the conditional knockout mice were evaluated with lung function, which is one of the most clinical-setting relevant diagnostic tools, bleomycin-aspirated SQSTM1/p62 AEC2-specific knockout mice showed worse lung function in both static compliance and inspiratory capacity (Figure 14A, B).

In summary, AEC2-specific SQSTM1/p62 knockout mice were more susceptible to bleomycin injury, but not fibrosis.

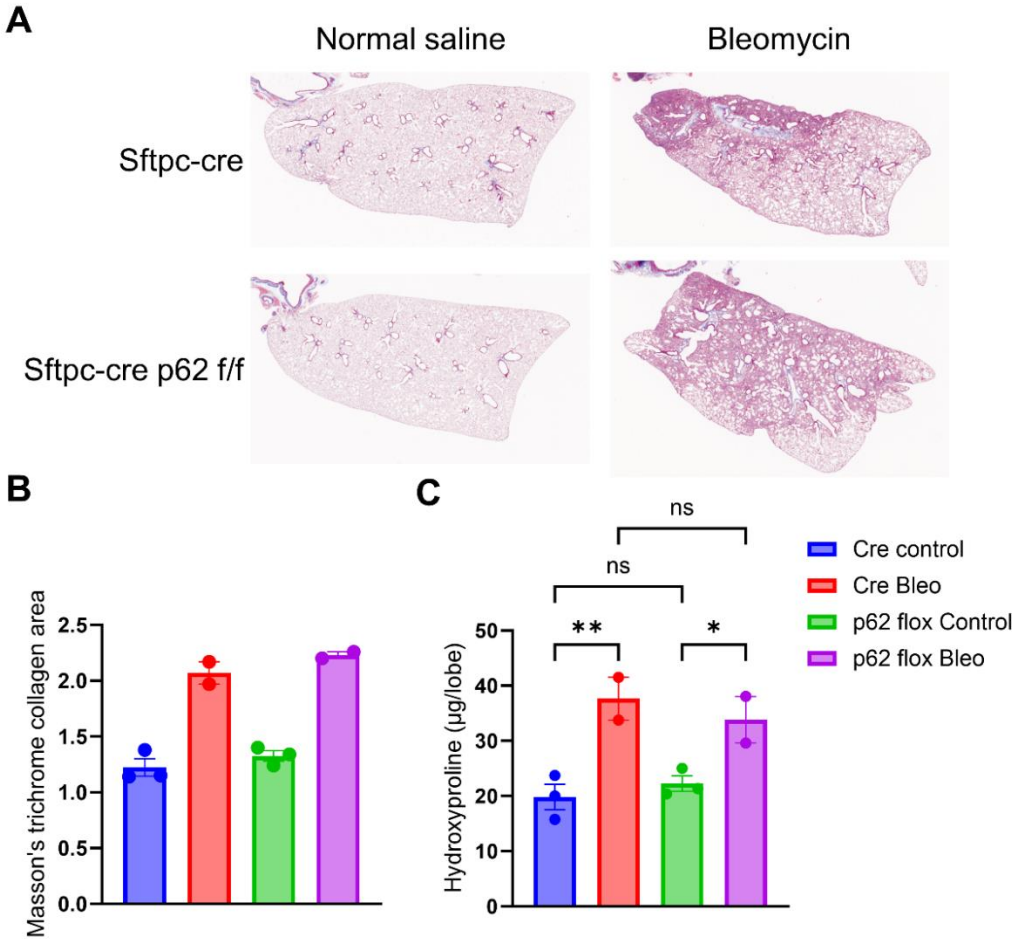

Figure 13 AEC2-specific SQSTM1/p62 knockout does not aggravate bleomycin-induced pulmonary fibrosis

The amount of collagen in the lungs of mice was analyzed by 2 different methods.

(A) Representative scanned images of Masson's trichrome stain slides of left lungs are shown.

(B) Collagen-stained area from the scanned image was quantified by automated built-in algorithm of ImageScope program. Statistical analysis was omitted due to very small number of subjects.

(C) Hydroxyproline assay did not show any difference between conditional knockout

mice and control mice.

Bleo, bleomycin; NS, normal saline; ns, not significant; *, $p<0.05$; **, $p<0.01$.

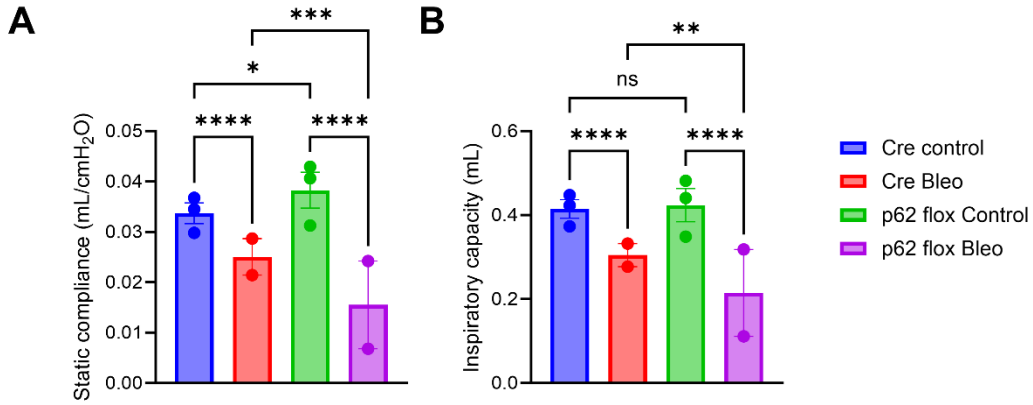

Figure 14 AEC2-specific SQSTM1/p62 knockout mice exhibits lower lung function measurements after bleomycin injury compared to the Cre mice.

Lung function of each mouse was measured by FlexiVent. The average results from 4 consecutive measurements were analyzed. (A) Static compliance and (B) Inspiratory capacity reveals that SQSTM1/p62 floxed mice shows lower lung function after bleomycin injury. n=2–3.

Bleo, bleomycin. ns, not significant. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

6. Increased SQSTM1/p62 mRNA levels are correlated to better lung function and less mRNA expression of fibrosis genes in patients with IPF.

To validate the role of SQSTM1/p62 in terms of its protective effect against lung function decline during lung injury, as demonstrated by in vivo models, a public transcriptomic dataset with sufficient clinical information, GSE47460, was employed for linear regression analysis. Using the data from the control and IPF groups, SQSTM1/p62 mRNA expression levels were found to be positively correlated with higher percent predicted FVC and DLCO (Figure 15 A, B). Moreover, when fibrosis marker genes were analyzed together in a correlation matrix, SQSTM1/p62 mRNA, as well as the lung function markers, exhibited a negative correlation with the mRNA expression of the fibrosis marker genes, including COL1A2, ACTA2, and COL3A1 (Figure 15C). Therefore, this analysis confirms the protective role of SQSTM1/p62 in the pathogenesis of pulmonary fibrosis with clinical relevance.

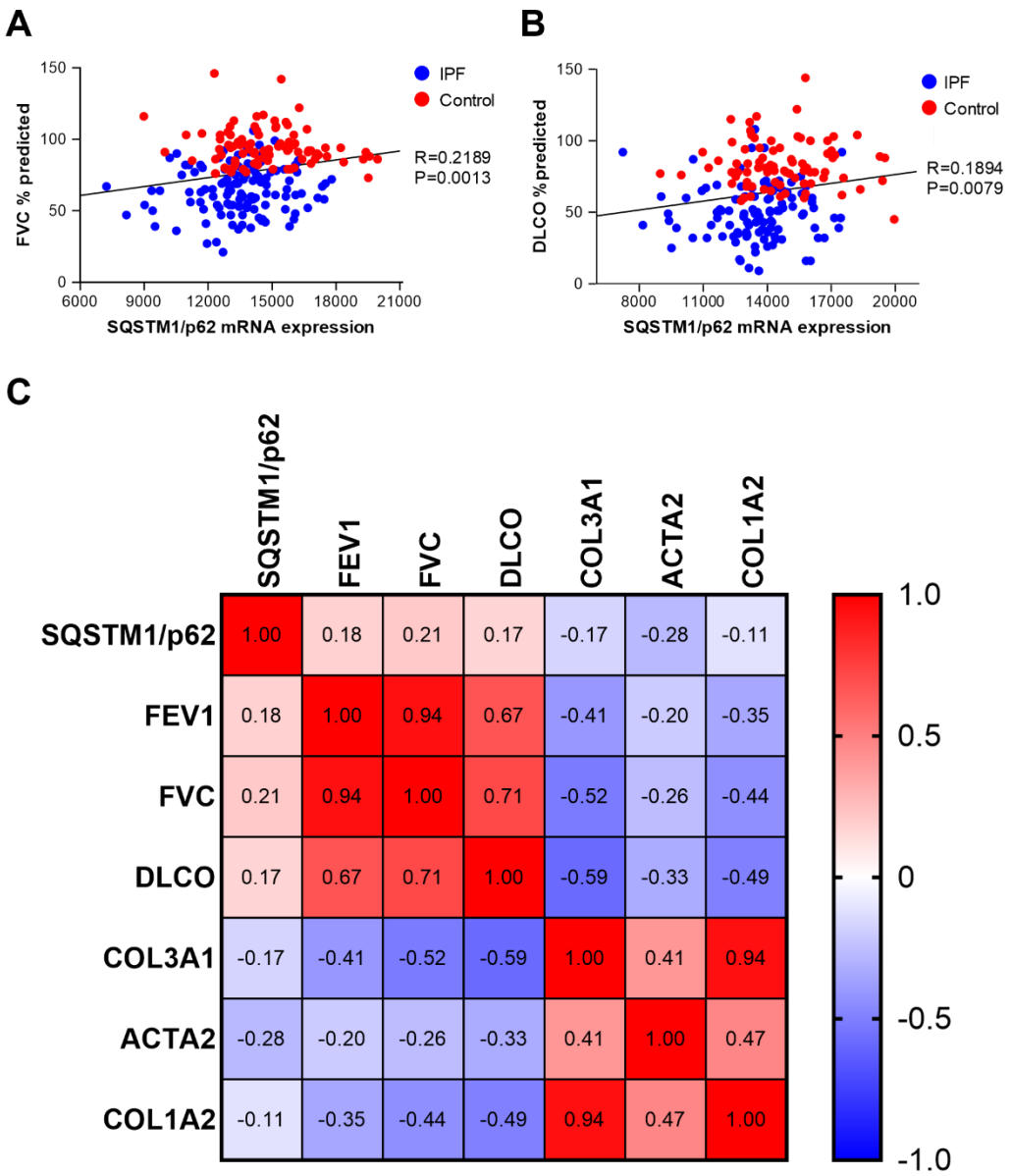

Figure 15 Correlation of SQSTM1/p62 mRNA expression with lung function markers and mRNA levels of fibrosis genes.

Patient characteristics in addition to the transcriptomic data from GSE47460 were analyzed.

(A) The percent predicted FVC and (B) the percent predicted DLCO show a positive linear relationship with SQSTM1/p62 mRNA expression.

(C) A correlation matrix was constructed to analyze the relationship between SQSTM1/p62 mRNA expression levels, lung function markers, and the mRNA expression of COL1A2, COL3A1, and ACTA2. The correlation coefficients are presented within the cells.

DLCO, diffusion capacity of the lung for carbon monoxide; FEV1, forced expiratory volume in one second; FVC, forced vital capacity;

7. Impaired autophagy in patients with IPF and the in vivo model explains the regulation of SQSTM1/p62 during pulmonary fibrosis

To explore the mechanisms related to the protective role of SQSTM1/p62 in pulmonary fibrosis, gene set enrichment analysis (GSEA) was conducted on the dataset GSE150910 to compare transcriptomic regulation between controls and patients with IPF. When gene sets from the Gene Ontology were employed for GSEA, the cellular components of autophagosomes were observed to be prominently downregulated in patients with IPF (Figure 16).

To further investigate autophagic activity, lung samples from IPF patient registry were analyzed via western blot to validate dysfunctional autophagy. The levels of KEAP1, LC3B-II, and total LC3B were substantially increased compared to the controls (Figure 17A, B). Notably, the prominent accumulation of total LC3B and LC3B-II suggests chronic dysfunction of autophagy.[30] Moreover, the ratio of SQSTM1/p62 to LC3B-II was lower in patients with IPF (Figure 17C). Since autophagy is the major degradation process for SQSTM1/p62, this could indicate upstream downregulation of SQSTM1/p62 gene translation or transcription, or selective degradation of SQSTM1/p62.

To further explore this hypothesis, the bleomycin model was analyzed for its chronological patterns. Immunoblots revealed accumulation in KEAP1 and LC3B in murine lungs post-bleomycin injury, which were consistent with those in patients with IPF (Figure 18A). These observations mirror trends noted in patients with IPF where autophagy appears compromised, leading to an accumulation of KEAP1 due to decreased degradation Furthermore, a decline in the ratio of SQSTM1/p62 to LC3B-II was evident in mice on days 7 and 14 post-bleomycin injury (Figure 18B). Notably, no such decrease in the SQSTM1/p62:LC3B-II ratio was observed in mice on days 21 and 56.

In conclusion, our results highlight dysfunctional autophagy and propose a potential compromise in the upstream regulation mechanisms of SQSTM1/p62 in IPF patients.

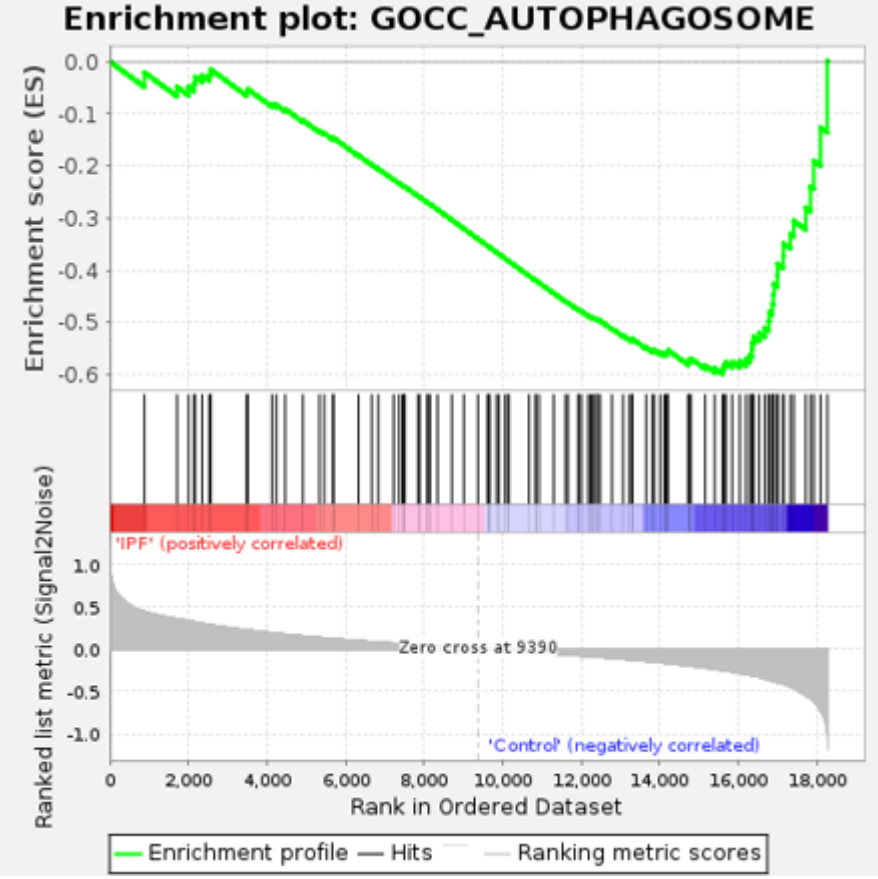

Figure 16 Gene set enrichment analysis on the data from GSE150910 reveals autophagy inhibition in patients with IPF in the transcriptomic level.

Gene set enrichment analysis was performed using Gene Ontology gene sets from MsigDB with the transcriptomic data from GSE150910.

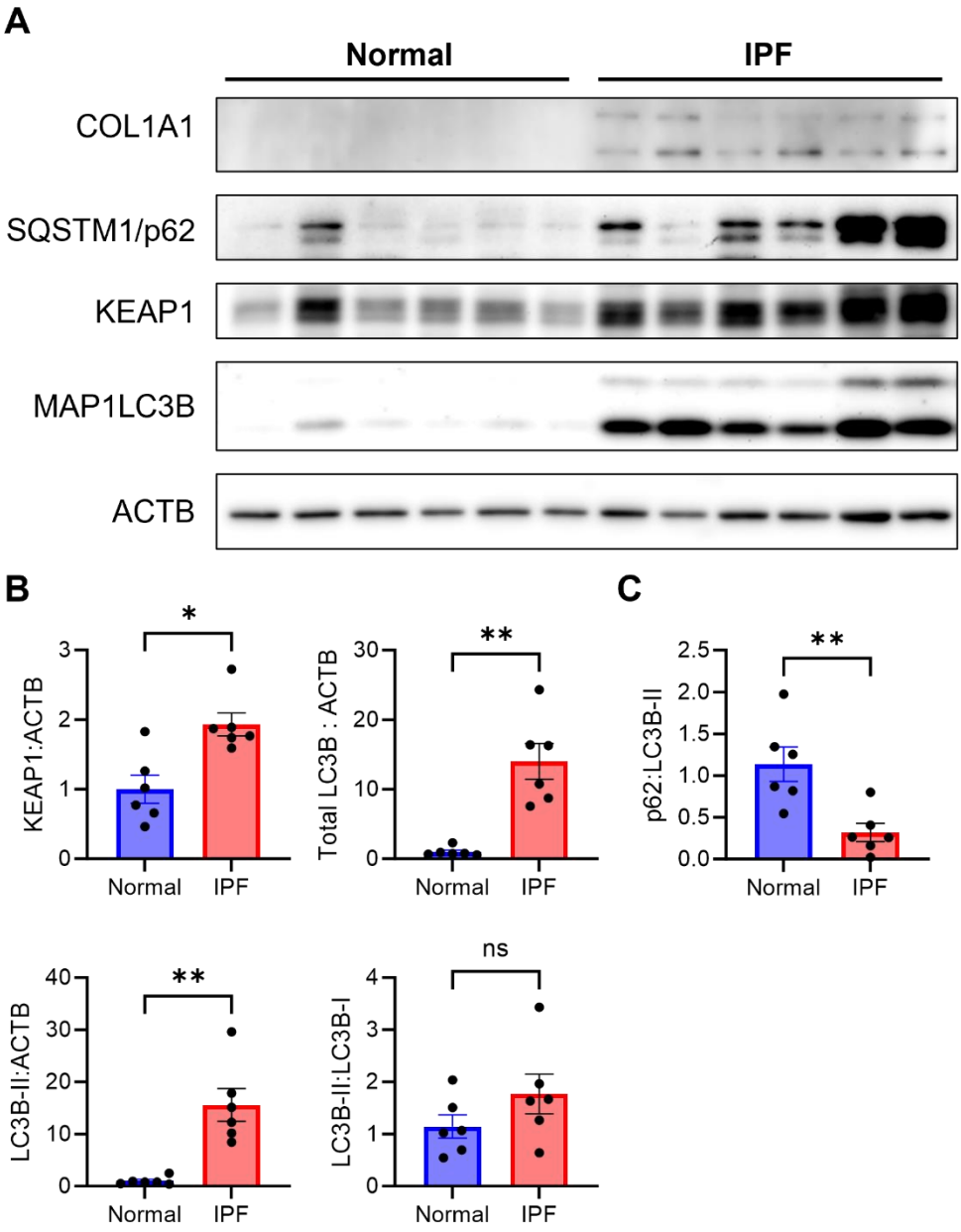

Figure 17 Dysfunctional autophagy with Keap1 accumulation is prominent in lung samples from patients with IPF.

Lung tissues from 6 patients with IPF and 6 controls were subjected to immunoblot

analysis.

(A) Representative blots are shown.

(B) Densitometry analysis of immunoblot data in (A) is presented as mean ± standard error of the mean with individual data points.

(C) The ratio of SQSTM1/p62 and total LC3B is presented.

ns, not significant; *, $p<0.05$; **, $p<0.01$.

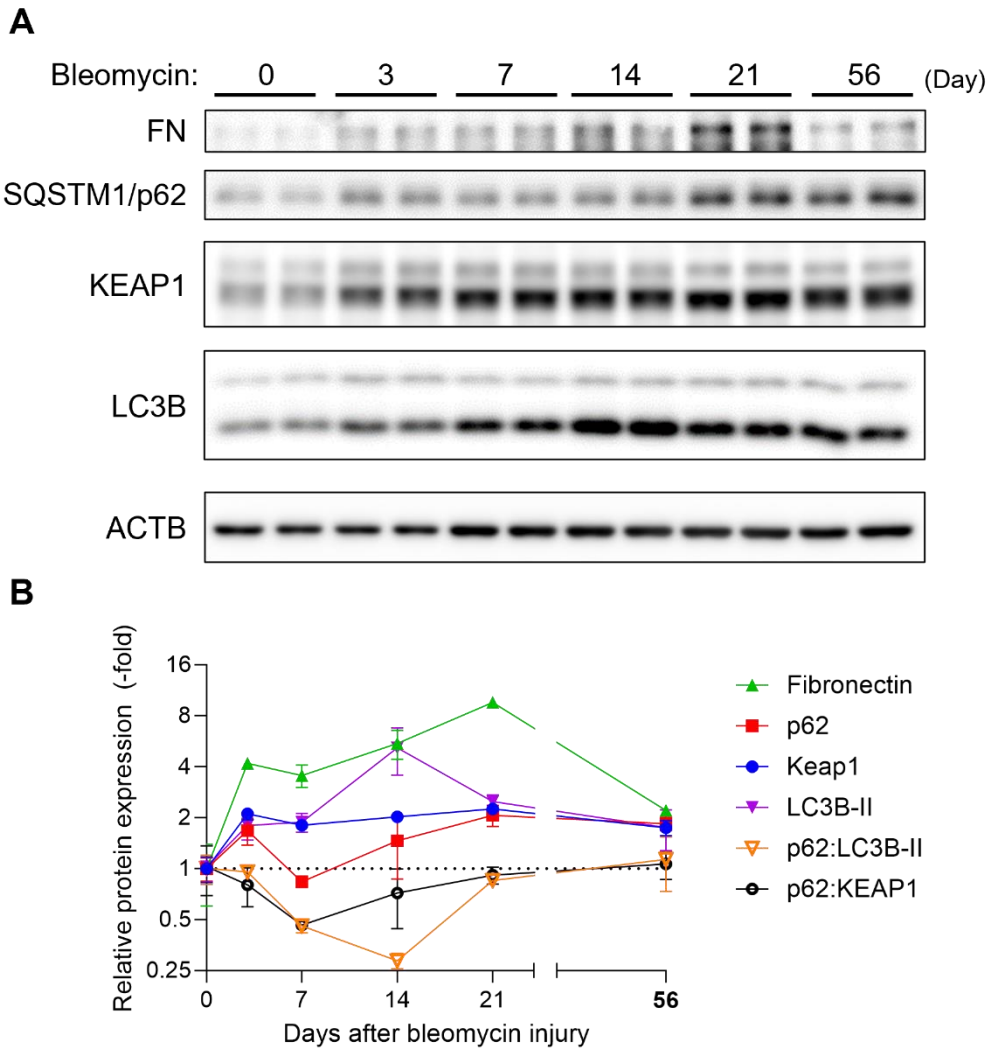

Figure 18 Chronological changes in protein expression of murine lungs after bleomycin injury show SQSTM1/p62-specific degradation, its spontaneous recovery, and indications of autophagy impairment.

Lung tissues from mice sacrificed at different time points after bleomycin injury were subject to immunoblots.

(A) Representative blots are shown.

(B) Densitometry analysis of immunoblot data in (A) is presented as mean ± standard error of the mean with individual data points and connecting lines

(C) The ratio of SQSTM1/p62 and total LC3B is presented.

ns, not significant; *, $p<0.05$; **, $p<0.01$.

IV. DISCUSSION

To the best of available knowledge, this study represents the first comprehensive assessment of SQSTM1/p62's role in the pathogenesis of pulmonary fibrosis and its underlying mechanisms. Through a comprehensive analysis of previously published transcriptomic datasets, the transcriptional downregulation of SQSTM1/p62 was confirmed in patients with IPF. Utilizing pre-established patient tissue registries and in vivo models, this pathologic alteration was observed predominantly at the transcriptional level rather than at the protein level, supported by the observed disparity between in vivo models and patient samples. Genetic deletion of SQSTM1/p62 in mice, both in a conventional manner and in a cell-type specific manner to alveolar epithelial type 2 cells (AEC2), coupled with meticulous verification through lung-specific deletion rescue using adeno-associated virus serotype 9 (AAV9), consistently substantiated the notion that SQSTM1/p62 plays a protective role in the pathogenesis of pulmonary fibrosis. This protective role was further corroborated by a transcriptomic dataset that underwent clinical validation, revealing a positive correlation between SQSTM1/p62 expression and improved lung function. The presence of impaired autophagy in patients with IPF was reaffirmed. The alterations in protein expression levels suggested the potential involvement of other upstream mechanisms in the downregulation of SQSTM1/p62 in patients with IPF.

When comparing results from IPF patients with the chronological progress of the in vivo model, SQSTM1/p62 protein appeared to accumulate less than the autophagosome-membrane protein, LC3B. Given that LC3B accumulation might suggest an overload of autophagosomes and autolysosomes, a decreased SQSTM1/p62 to LC3B-II ratio could indicate either the active selective degradation or down-regulation of SQSTM1/p62 protein. Within the in vivo model, SQSTM1/p62 mRNA showed up-regulation on day 14 post-bleomycin, coinciding with the protein's lowest level. Moreover, these changes in SQSTM1/p62 expression are normalized at day 21 post-bleomycin. This suggests that the SQSTM1/p62 protein undergoes active degradation in the model, with increased

transcription possibly compensating for this deficiency. In contrast, IPF patients demonstrated a decreased SQSTM1/p62 to LC3B-II ratio and a decline in SQSTM1/p62 mRNA. This observation leans more towards interpreting it as down-regulation rather than active degradation. Such insights might shed light on the protective role SQSTM1/p62 plays during pulmonary fibrosis.

Previous studies examining the relationship between SQSTM1/p62 and IPF have primarily focused on assessing the expression levels of SQSTM1/p62 protein as a marker of autophagic activity. One of these investigations reported an increased expression of SQSTM1/p62 in the epithelial cells within fibrotic areas of IPF patients.[31] Additionally, they also noted the prominent accumulation of LC3B, a key autophagic marker, primarily in alveolar epithelial type 2 cells (AEC2s), as determined through morphological analyses in immunohistochemistry staining. Another group of researchers also observed enhanced expression of SQSTM1/p62 protein within IPF fibroblastic foci and epithelial cells in fibrotic alveolar septae as demonstrated through immunohistochemistry assays.[32] My observations were consistent with those recent findings, indicating that AEC2s are the major cell-type exhibiting autophagic impairment and SQSTM1/p62 accumulation in IPF.

The significant discrepancy between the mRNA and protein levels of SQSTM1/p62 has prompted a hypothesis suggesting the presence of a transcriptomic disturbance related to autophagy-associated genes, including SQSTM1/p62, in patients with IPF. One possibility is the chronic deficiency of NFE2L2/NRF2, which acts as the downstream protein in the SQSTM1/p62-KEAP1-NRF2 pathway, potentially leading to the downregulation of SQSTM1/p62 mRNA. This hypothesis finds support in numerous prior studies that have established NRF2 as one of the transcription factors responsible for upregulating the transcription of the SQSTM1/p62 gene.[10, 33] The observed accumulation of KEAP1, a critical component in the degradation machinery for NRF2, lends credence to this notion. Nevertheless, further research is required to investigate the specific target genes regulated by NRF2.

My recent findings, which have been presented elsewhere, involve the repositioning of the anti-cholesterol drug ezetimibe to an anti-fibrotic treatment for IPF. These observations demonstrated that ezetimibe activates autophagy in both in vitro and in vivo pulmonary fibrosis models, resulting in beneficial effects in survival rates of patients with IPF.(abstracts and/or the paper will be cited after publication Given that SQSTM1/p62 is a known target of ezetimibe, the expression of SQSTM1/p62 could serve as a potential therapeutic target for this newly suggested IPF treatment.[33]

While this study has been supported by genetic deletion, lung-specific deletion rescue, and inducible conditional knockout models, the detailed mechanism of SQSTM1/p62 remains to be fully determined. Further research is needed to elucidate cell-specific expression levels of SQSTM1/p62 and its related proteins, which will help validate the proposed role and mechanism of SQSTM1/p62 in this paper. Additionally, to confirm the molecular mechanism of this protein, in vitro models using knockout cells and experiments on protein-protein interactions should be explored.

Using previously obtained tissue samples from in vivo models and patients with IPF, transcriptomic screening of novel mehcanisms as well as immunoprecipitation experiments are planned to be performed. Also, establishment of knockout cell-line using CRISPR/Cas9 is currently under progress. These forthcoming experiments are expcted to provide additional support for various aspects of current study’s results.

V. CONCLUSION

In conclusion, SQSTM1/p62 plays a protective role in the pathogenesis of pulmonary fibrosis. It may be used as a therapeutic target for the treatment of IPF.

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ABSTRACT(IN KOREAN)

폐 섬유화의 발병 기전에서 SQSTM1/p62의 역할 및 기전 연구

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특발폐섬유증은 주요 악성 종양과 유사한 수준의 예후를 가진 치명적인 질병이나, 그 병인 메커니즘이 명확히 알려진 바가 없다. 특히, 특발폐섬유증 환자의 폐에서 2형 폐포상피세포(AEC2)와 섬유모세포의 자가포식 기능이 손상되어 있다는 것은 알려져 있으나, 이러한 손상된 자가포식이 병인 과정에 기여하는 분자 메커니즘은 불분명하다.

SQSTM1/p62 (Sequestosome 1)은 GATA4와 KEAP1의 선택적 자가포식 분해를 통해 세포 노화와 산화환원 균형에 관여하는 주요 단백질이다. 그러나 SQSTM1/p62가 폐 섬유화의 발병 메커니즘에서 어떠한 역할을 하는지는 아직 밝혀지지 않았다. 따라서, 본 연구는 폐 섬유화에서 SQSTM1/p62의 역할과 작용 메커니즘을 규명하고자 하였다.

특발폐섬유증 환자 레지스트리에서 폐 조직 샘플과 공개적으로 이용 가능한 전사체 및 단일세포 RNA sequencing 데이터셋을 분석하여 SQSTM1/p62의 발현을 비교하였다. 폐 섬유화의 잘 알려진 in vivo 모델인 블레오마이신 유발 폐 섬유증 마우스 모델을 사용하였고, SQSTM1/p62의 conventional KO 마우스와 AEC2-특이적인 유전자 결핍 마우스를 사용하여 SQSTM1/p62의 in vivo에서의 역할을 확인하였다. 또한, Adeno-associated virus 9 (AAV9) 벡터를 사용한 폐 특이적 KO rescue 실험도 진행하였다.

SQSTM1/p62는 특발폐섬유증 환자에서 mRNA 전사가 감소되어 있었다. 한편, 단백질은 오히려 축적되어 있었는데, 이는 손상된 자가포식의 분해 기전으로 인한 것으로 확인되었다. SQSTM1/p62의 mRNA 발현은 특발폐섬유증 환자에서 폐 기능과 양의 상관관계를 보였다. SQSTM1/p62의 conventional KO

마우스에서 야생형 마우스에 비해 폐 섬유증이 증가되었고, Rescue 마우스에서는 empty vector만 전달된 마우스에 비해 폐 섬유화로부터 보호되었다. AEC2-특이적 유전자 결핍 마우스는 블레오마이신에 의핸 폐 손상과 사망에 더 취약한 양상을 보였다. SQSTM1/p62는 자가포식 및 KEAP1 분해에 장애를 초래하여, 본 연구를 통해 잠재적인 메커니즘을 제안할 수 있었다.

결론적으로, 본 연구는 SQSTM1/p62가 폐 섬유화의 발병에서 보호하는 역할을 한다는 것을 입증하였으며, 이는 향후 폐섬유증의 치료 과정에 중요한 타겟으로 고려될 수 있다.

핵심되는 말 : 특발폐섬유증, 자가포식, SQSTM1/p62, 폐 섬유화, RNA sequencing, 2형 폐포 상피세포 (AEC2), 세포 노화, 산화환원 균형

PUBLICATION LIST

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