Effects of mitochondrial biosynthesis regulation through the PGC-1α /ERRα signaling system on the pathogenesis of pulmonary fibrosis

Hyeon Woo Chung

Department of Medical Science

The Graduate School, Yonsei University

Effects of mitochondrial biosynthesis regulation through the PGC-1α /ERRα signaling system on the pathogenesis of pulmonary fibrosis

Directed by Professor Jae Myun Lee

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Hyeon Woo Chung

December 2022

This certifies that the Master's Thesis of
Hyeon Woo Chung is approved.

Thesis Supervisor : Jae Myun Lee

Thesis Committee Member#1 : Myung Hyun Sohn

Thesis Committee Member#2 : Ho-Geun Yoon

The Graduate School
Yonsei University

December 2022

ACKNOWLEDGEMENTS

First, I will give all my glory to the Lord for making me dream and guiding my way through life. Next, I would like to thank my advisor, Professor Jae-myun Lee, who gave me the correct guidance to begin my research, and encouraged me all the time. Next, I would like to thank Professor Myung-Hyeon Son and Professor Ho-Geun Yun for giving me kind advice and helping me to finish my thesis.

Furthermore, I would like to thank Ph.D Doo-hee Shim, Ph.D Hye-Ran Cha, Ph.D Sung-Gyu Jo, PhD Pil-gu Park and Ji-hee Seol for encouraging me all the time, and giving me an opportunity to participate in various studies. Also, I would like to thank my fellows SuJin, KyeoRe and Yuna, without colleges I wouldn't finish my race. Above all, I would like to thank to Sunghoon, who has given me good stimulation and comfort while walking this time with me.

Finally, I would like to say thank you and love you to my family. I was able to focus on my research under the best circumstances due to your love and sacrifices. Without the love of my family, I would not have been able to grow up like this.

The road ahead will likely be narrower and more difficult for me. In spite of this, I have learned patience, consideration, and love through this process, I will let go of the free grace I received. My sincere thanks.

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Effects of mitochondrial biosynthesis regulation through the PGC-1α /ERRα signaling system on the pathogenesis of pulmonary fibrosis

Hyeon Woo Chung

Department of Medical Science
The Graduate School Yonsei University

(Directed by Professor Jae Myun Lee)

Mitochondrial dysfunction has recently been linked to the onset and progression of pulmonary fibrosis. In patients with Idiopathic Pulmonary Fibrosis (IPF), fibroblasts exhibit mitochondrial dysfunction and decreased mitochondrial biogenesis.

Estrogen related receptor alpha (ERRα) can upregulate mitochondrial biogenesis by inducing transcriptional regulators for mitochondrial genes. Studies have revealed that ERRα requires the presence of a peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α.

The repression of PGC-1α has been demonstrated in the human IPF fibroblasts, leading to decreased mitochondrial biogenesis. However, research on ERRα and PGC-1α/ERRα-signaling systems in pulmonary fibrosis has been poorly reported. This study determined whether changes in ERRα affect mitochondrial status and fibroblast to myofibroblast transition (FMT) *in vitro*.

We treated the human fetus lung fibroblast cell line (MRC-5) with TGF-β1 to see the relevance between ERRα and FMT. MRC-5 cells were treated with ERRα inverse agonist (XCT790), and siRNA treatment was performed to determine whether the loss of ERRα results in mitochondrial dysfunction and FMT. To observe the resolution of FMT through enhancing ERRα, human IPF patients' lung fibroblast cell line (LL97A) were treated with PGC-1α agonists (thyroid hormone, rosiglitazone), and overexpression through pCMV6- ERRα vector were performed.

The PGC-1α /ERRα signaling axis and mitochondrial status were decreased in TGF-β1 treated human lung fibroblasts cell lines. The reduction of ERRα have induced FMT and led mitochondrial dysfunction. However, the elevation of ERRα via transfection and drug treatments has reduced FMT and ameliorated mitochondrial dysfunction. Furthermore, anti-fibrotic effects of ERRα was dependent on the coactivator PGC-1α.

In this study, we have demonstrated the relevance between the PGC-1α /ERRα signaling system and the pathogenesis of pulmonary fibrosis. FMT was induced by inhibition of ERRα and was enhanced by enhancing ERRα. These findings suggest that the downregulation of ERRα could contribute to pulmonary fibrosis and the resolution of mitochondrial biogenesis through the PGC-1α /ERRα signaling system can ameliorate pulmonary fibrosis through enhancing mitochondrial biogenesis.

Key Words : Pulmonary fibrosis, Mitochondrial Biogenesis, Estrogen related receptor alpha, peroxisome proliferator-activated receptor-gamma coactivator-1 alpha

Effects of mitochondrial biosynthesis regulation through the PGC-1α /ERRα signaling system on the pathogenesis of pulmonary fibrosis

Hyeon Woo Chung

Department of Medical Science
The Graduate School Yonsei University

(Directed by Professor Jae Myun Lee)

I. INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF), the most common type of idiopathic interstitial pneumonia, is a chronic, invariably progressive disease characterized by interstitial fibrosis and microscopic honeycombing.[1-4] As the disease progresses, gas exchange impairs, and lung function declines, eventually leading to death. The median age at diagnosis is 65 years suggesting IPF is an aging-related disease and has been reported 2 - 4 years of the median survival time from diagnosis.[2-4] IPF is diagnosed by histological or radiological criteria of a pattern of usual interstitial pneumonia.[5,6] Now, only two drugs are FDA-approved and treated for IPF patients: Nintedanib and Pirfenidone.[7-10] However, these cannot stop the progression of fibrosis but can only delay the progression of IPF and improve progression-free survival.[11]

The inciting events of IPF are now understood as repetitive alveolar epithelial injury-producing an abnormal wound-healing response.[12,13] The Increase of apoptotic type2 alveolar epithelial cells (AECs), type1 AECs, and increased myofibroblasts on the fibrotic lungs' epithelium, support this

hypothesis.[14,15] When the tissue gets injured, the wound-healing pathway is activated to repair itself from the stimulus. When wound healing begins in lung tissue, type2 AEC, which serves as stem cells in the lung, renewal type1 AEC, activated fibroblasts secrete extracellular matrix components such as collagens and fibronectins, which contribute to structural repair. [3] However, repetitive alveolar epithelial injury triggers abnormal wound-healing responses leading to fibroblast activation, myofibroblast differentiation, and abnormal extracellular matrix deposition, contributing to disease pathogenesis.[3,16]

IPF patients' damaged microenvironments show redox imbalance characterized as oxidative stresses through enhancing reactive oxygen species (ROS).[1718] Mitochondrial dysfunction plays a critical role in excessive ROS production, and it is a standard feature of IPF patients' lung epithelial cells, and fibroblasts.[19-24] The features of mitochondrial dysfunction are mitochondrial DNA damage, the reduction of the number of mitochondria, mitochondrial depolarization, morphological changes, decreased ETC activity, releasing apoptotic factors, and secreting Damage Associated Molecular Patterns (DAMPs) like mitochondrial DNA (mtDNA).[17,25,26]. Under normal conditions, mitochondrial ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide can be controlled by Glutathione (GSH), and superoxide dismutase (SOD). However, the expression levels of GSH and SOD are low in the IPF patients' lungs.[17] Additionally, NADPH oxidase-4 (NOX4), a critical ROS-producing enzyme, is overexpressed in IPF lung myofibroblast. NOX4 reduces mitochondrial biogenesis by directly inhibiting nuclear respiration factor (NRF)2.[18] Moreover, several transcriptional factors, and proteins that are involved in mitochondrial biogenesis and mitophagy, which are crucial for mitochondrial homeostasis, have been downregulated in IPF patients' lungs.[19-23]

Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α is the transcriptional coactivator of several nuclear receptors like peroxisome proliferator-activated receptors (PPAR) α, PPARβ, thyroid hormone (TH) receptor, and Estrogen-related receptors (ERRs). PGC-1α is an inducible regulator of the metabolic pathway, autophagic pathway, and mitochondrial biogenesis.[22,24-27] PGC-1α is also reported to increase NRF1/2 expression, which controls mtDNA replication and transcription via mitochondrial transcription factor A.[27-30] Fibroblast of IPF patients, down-regulation of PGC-1α have been reported leading to mitochondrial dysfunction. Treating Metformin, which activates AMPK, an upstream regulator of PGC-1α ameliorates fibrotic phenotype in the bleomycin-induced pulmonary fibrosis mouse model. [31] Transfecting PGC-1α plasmid vectors in the fibroblasts of IPF patients have also relieved fibrotic phenotype and knockdown of PGC-1α on fibroblast have worsened fibrotic phenotype.[22] Treating TH, which activates PGC-1α ameliorated fibrotic phenotype, but treating TH on PGC-1α knockout mouse have not improved fibrotic phenotypes.[23]

Among the targets of nuclear receptors of PGC-1α, ERR families comprise ERRα, ERRβ, and ERRγ. Unlike its naming, ERRs have no relationship between estrogen signaling pathways, and endogenous ligands have not been identified. ERRs are composed of three domains: DNA-binding domain that recognizes and binds a specific sequence TCAAGGTCA, known as estrogen-related response element (ERRE); a well-conserved C-terminal domain which is a site that coactivators and co-repressors bind and interact; and a less conserved N-terminal domain.[25,32-35] PGC-1α interacts with ERRα, and not only increases the activities of ERRα but also upregulates the transcription of ERRα via the ERRα gene itself bearing ERRE.[25] Increased ERRα can upregulate mitochondrial gene expression by inducing transcriptional regulators for mitochondrial genes such as NRF1, GABPα, a subunit of GABP/NRF2, and PPARα.[36,37]

The targets of ERRα include several genes related to mitochondrial activity: TCA cycle, fatty acid oxidation, and oxidative phosphorylation (OXPHOS). [32] It is evident that the ERRα binds to these mitochondrial genes because when ERRα is downregulated, the mitochondrial activity gets impaired, decreases ATP production and mitochondria lose their integrity.[37,38]ERRα not only increases mitochondrial biogenesis and function but also affects autophagic flux, which required for the maintenance of mitochondrial functions.[39-41] In the immune response of macrophages toward *Mycobacterium tuberculosis* (Mtb) infection, ERRα is required for transcriptional activation of autophagic genes and phagosomal maturation.[40] Also, ERRα is a critical factor for TFEB-induced activation of autophagy, which is crucial for maintaining mitochondrial functions through mitophagy in the intestinal epithelium.[40] Furthermore, several studies have revealed sirtuins such as SIRT3, and SIRT5 containing ERRE on its promoters through regulated by ERRα. Also, the activity of sirtuins is increased by ERRα activation due to increased NAD+ through activated OXPHOS. [39-41] However, since there is no research related to ERRα and IPF yet, the need for research between ERRα and IPF should be performed.

This study investigated the correlation between PGC-1α/ERRα signaling axis and pulmonary fibrosis in vitro. The activation of mitochondrial biogenesis through upregulating the PGC-1α/ERRα signaling axis could be an effective anti-fibrotic approach toward pulmonary fibrosis disease phenotypes.

II. MATERIALS AND METHODS

1. Transcriptosome analysis (Reanalysis of Public data)

RNA sequencing was performed by using RNA-seq data (GSE199949) from Gene Expression Omnibus (GEO). Briefly, samples were composed of IPF patients' biopsies and non-IPF donor lung biopsies that are not suitable for transplantation. Samples were separated into non-fibrotic central and fibrotic peripheral regions. RNA sequencing was performed using Illumina HiSeq 4000 and quality control was performed using FastQC and AfterQC. STAR version 2.5.3 was used for read alignments and count summaries. Reads count data were processed with R/Bioconductor "limma" package for TPM normalization'. Batch effects were adjusted using the "combat" function in R/Bioconductor "sva" package. To increase sensitivity and exclude noise, a TPM=1 cutoff was performed and row sum filters of 42 for the "all-samples" data sets were used as thresholds for low counting filters. Then, data were log2 transformed and P values cut off ($P \leq 0.05$) were performed to generate Differently Expressed Genes (DEGs). Gene Set Enrichment Analysis (GSEA) was performed with DEGs, and a list of GO (Gene Ontology) pathways was used to perform GO analysis.

2. Cell culture

MRC-5 was purchased in a Korean cell line bank and cultured in Minimal Essential Media (MEM; Hyclone, Logan, UT, USA), 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 1X Non-Essential Amino Acids (NEAA; Gibco, New York, NY, USA), 1mM Sodium Pyruvates (Gibco), and 1% penicillin/streptomycin (p/s; Hyclone). LL97A was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Kaighn's Modification of Ham's F-12 Medium (F-12K; ATCC) with 15% FBS (Hyclone),

and 1% p/s (Hyclone). Cells were cultured in complete media for 24 h and media were changed with 0.5% FBS medium for starvation. After 24 h of starvation cells were treated with recombinant human TGF-β1 protein (R&D Systems Inc, USA) in 0.5% FBS media and were cultured until 48 h. Cells were maintained at 37°C, 5% CO_2 incubator.

3. Cell transfection

Cells were seeded into plates to reach 80 ~ 90% confluence. For the knockdown analysis, cells were transfected with siRNA-negative control (siNC) and si-ESRRA using Lipofectamine RNAimax reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For the overexpression of ERRα, cells were transfected with pCMV6-ERRα plasmid vector (Origene, Rockville MD, USA) and pCMV6-EMTPY plasmid vector (Origene) using Lipofectamine 3000 reagent according to the manufacturer's protocol.

4. RNA isolation and quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) assay

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Briefly, cells were lysed with RLT buffer containing beta-mercaptoethanol and added 70% EtOH. Samples were transferred to the RNeasy mini spin column and after washing columns with RW1 buffer and RPE buffer, RNAs were diluted with DEPC-treated water and followed by Nanodrop concentration and purity analysis. cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA, USA), qRT-PCR was performed using KAPA STBR FAST (Roche Diagnostics, Mannheim, Germany), and analyzed with QuantStudio3 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). qRT-PCR primers used in this analysis are listed in Table 1.

5. DNA isolation and qRT-PCR assay

Total DNA was extracted using QIAamp Blood mini kit (Qiagen, Valencia, CA, USA). Briefly, cells were lysed with Protease K and added buffer AL and incubated at 56°C for 10 min. 100% EtOH was added to samples and samples were transferred to QIAamp mini spin column placed in a 2ml collection tube and centrifuged. After washing the columns with AW2 buffer, DNAs were diluted with DEPC-treated water and followed by Nanodrop concentration and purity analysis. qRT–PCR was performed using KAPA STBR FAST (Roche Diagnostics, Mannheim, Germany), and analyzed with QuantStudio3 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). qRT–PCR primers used in this analysis are listed in Table 1.

6. Western blot assay

Cell lysates were prepared RIPA buffer with protease and phosphatase inhibitors. Lysate sample concentration was calculated using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). 30 ug of protein was resolved by 4 ~ 12% SDS-PAGE and transferred to nitrocellulose (NC) membranes (Invitrogen). Blots were blocked with 5% skim milk for 1 h at room temperature, treated with primary antibodies, and incubated overnight at 4 °C. Blots were washed and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and washed. Bands were visualized using WesternBright ECL western blotting detection reagent (Advansta, Menlo Park, CA, USA) and ImageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Sweden).

7. Flow cytometry

Cells were scraped from the plate with a cell scraper and transferred to a 96-round-bottom plate then, washed with PBS. Cells were stained with MitoTracker Green FM (Invitrogen), and MitoTracker Red CMSROS (Invitrogen) and incubated for 30 min in a CO_2 chamber (37 °C, 5% CO_2). After staining, cells were washed twice with 100 μL PBS with 2% FBS buffer, resuspended, and analyzed using a flow cytometer (FACSCelesta, BD Biosciences, San Jose, CA, USA).

8. Luciferase promoter assay

3X ERRE Luciferase reporter construct was kindly provided by Rebecca Riggins (Addgene plasmid # 37851). pRL Renila Luciferase Control Reporter Vectors were used as a control for transfection efficiency. Transfections were performed with Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. After 24 h of transfection, cells were treated with growth factors and drugs for 48 h and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) following the manufacturer's protocol.

9. Statistical analysis

Results were visualized and analyzed with Prism software, version 9 (GraphPad Software, San Diego, CA, USA), Student's t-test (unpaired, two-tailed) was used to compare the two groups. One-way ANOVA was used to compare groups of more than three. Values of $P \leq 0.05$ were considered statistically significant. All P values less than 0.05 are summarized with asterisks (ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.001$).

Table 1. qRT-PCR Primers

qRT-PCR		
Gene Name	**Forward Primer(5' -> 3')**	**Reverse Primer(5' -> 3')**
ESRRA	CCACTATGGTGTGGCATCCTGT	GGTGATCTCACACTCGTTGGAG
PPARGC1A	CCAAAGGATGCGCTCTCGTTCA	CGGTGTCTGTAGTGGCTTGACT
TFAM	GGC AAG TTG TCC AAA GAA ACC	GCA TCT GGG TTC TGA GCT TTA
FN1	ACAACACCGAGGTGACTGAGAC	GGACACAACGATGCTTCCTGAG
ACTA2	GTGAAGAAGAGGACAGCACTG	CCCATTCCCACCATCACC
COL1A1	GATCCTGCCGATGTCGCTAT	TGTAGGCTAGCTGTTCTTGCA
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
mtDNA/gDNA Assay		
Gene Name	**Forward Primer(5' -> 3')**	**Reverse Primer(5' -> 3')**
tRNA-Leu(UUR)	CACCCAAGAACAGGGTTTGT	TGGCCATGGGTATGTTGTTA
B2-microglobulin	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT

III. RESULTS

1. PGC-1α /ERRα signaling axis and mitochondrial status were decreased in the lung of IPF patients

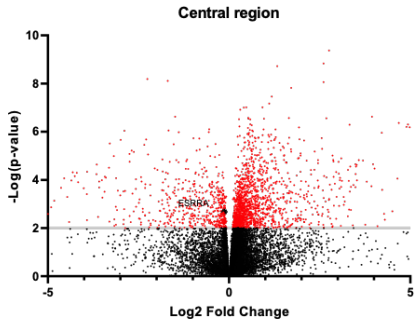
Several studies show that dysregulations on mitochondria are observed in the lung epithelium of pulmonary fibrosis patients. Above all, malfunction of mitochondrial biogenesis in alveolar epithelial cells and fibroblasts leads to critical alteration that affects the pathogenesis of lung fibrosis.

Reanalysis of Bulk RNA sequencing was performed using Gene Expression Omnibus (GEO) data from the National Center for Biotechnology Information (NCBI). GSE199949 database was composed of biopsies from visibly non-fibrotic central (IPF_C number) and fibrotic peripheral (IPF_P number) areas and non-IPF donor lung biopsies of central (Donor_C number) and peripheral (Donor_P number). Data were normalized using TPM normalization and ESRRA which codes ERRα was decreased in the central and peripheral region IPF patients' lung (fig.1A, B, G). However, other transcription factors known to regulate by PGC-1α were not reduced except for PPRAγ (fig.1G).

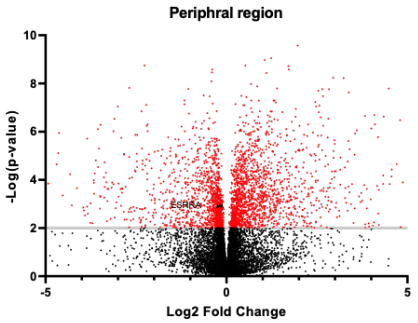
The GO analysis showed that the mitochondrial functions and biogenesis were significantly decreased in IPF patients' central region of lung biopsies compared to the donor's central region of lung biopsies (fig.1E). Meanwhile, the important metabolism pathways via mitochondria were decreased in the IPF patients' peripheral region of lung biopsies compared to donor's peripheral region of lung biopsies (fig.1F). The reduction in mitochondrial biosynthesis and dysfunction of cells in the non-fibrotic region, lead us to infer that the decrease in mitochondrial biosynthesis and disruption of function might be the pathogenesis of fibrosis.

Overall, the decrement in mitochondrial functions and the decrement in mitochondrial biogenesis were observed in the central and peripheral regions of IPF patients' lung samples.

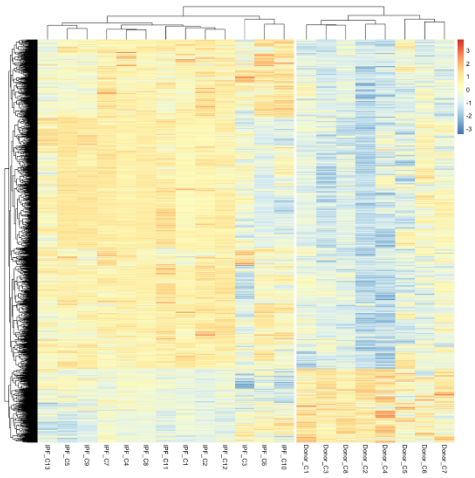
A



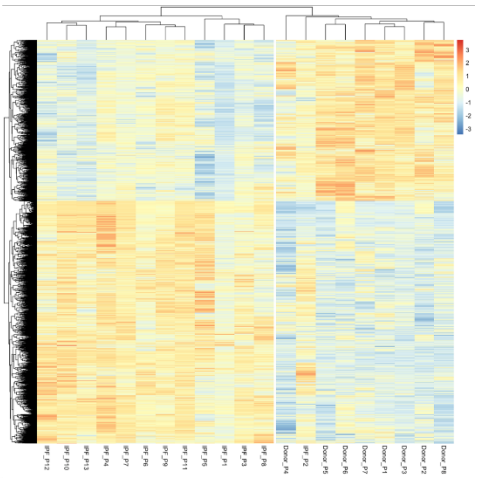
B



C



D



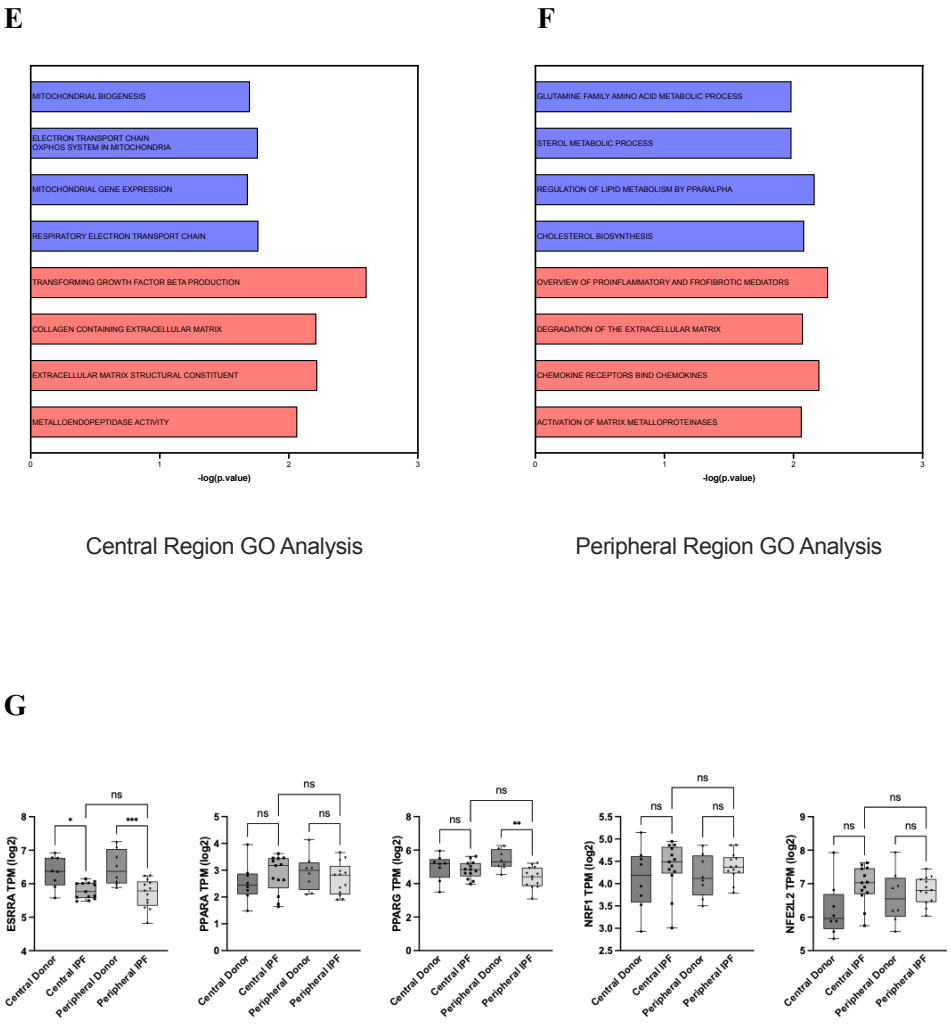


Figure 1. Downregulated expression of mitochondrial biogenesis and functions in IPF patients. (A) Differently expressed gene (DEG)s with a p-value less than 0.05 are shown in the volcano plots of the central region of IPF and donor biopsies. ESRRA is marked on the black stars symbol. (B) DEGs with a p-value less than 0.05 are shown in the volcano plots of the peripheral region of IPF and donor biopsies. ESRRA is marked on the black stars symbol. (C) Hierarchical clustering was performed on differentially expressed genes of the central region

of IPF and donor biopsies defined by ANOVA with FDR less than 0.05. The z-score scale bar represents relative expression ±3 SD from the mean. (D) Hierarchical clustering was performed on differentially expressed genes of the peripheral region of IPF and donor biopsies defined by ANOVA with FDR less than 0.05. The z-score scale bar represents relative expression ±3 SD from the mean. (E) Functional enrichment analysis with GO biological Processes was performed using Gene Set Enrichment Analysis with the p-value cut-off genes in the central region of IPF compared with donor lung biopsies. Gene sets that are downregulated are marked with blue color (respiratory electron transport chain, mitochondrial gene expression, electron transport chain oxphos system in mitochondria, mitochondrial biogenesis) and gene sets that are upregulated are marked with red color (metalloendopeptidase activity, extracellular matrix structural constituent, collagen-containing extracellular matrix, and transforming growth factor beta production). (F) Functional enrichment analysis with GO biological Processes was performed using Gene Set Enrichment Analysis with the p-value cut-off genes in the peripheral of IPF compared with donor lung biopsies. Gene sets that are downregulated are marked with blue color (cholesterol biosynthesis, regulation of lipid metabolism by PPARα, sterol metabolic process, glutamine family amino acid metabolic process), and gene sets that are upregulated are marked with red color (activation of matrix metalloproteinases, chemokine receptors bind chemokines, degradation of the extracellular matrix, an overview of proinflammatory and profibrotic mediators). (G) TPM (log2) expression value for the PPARA, PPARG, NRF1, NFE2L2, and ESRRA. These genes are regulated by PGC-1α and are known to regulate mitochondrial homeostasis.

2. PGC-1α /ERRα signaling axis and mitochondrial status were decreased in TGF-β1-induced myofibroblast *in vitro*

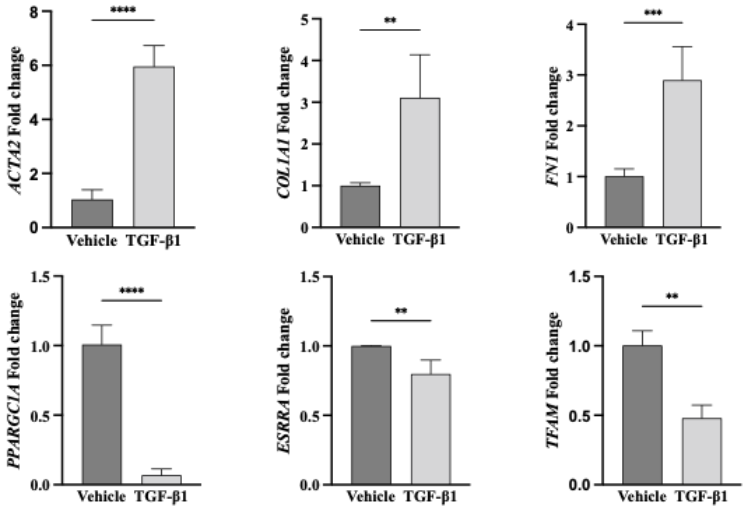
To Investigate changes in mitochondrial biogenesis-related molecules on pulmonary fibrosis *in vitro*, TGF-β1 was treated on a human fetus fibroblast cell line (MRC-5) for 48 h. The qRT-PCR results showed that the mRNA expression levels of the fibrotic markers such as FN1, ACTA2, and COL1A1 were increased after TGF-β1 treatment (fig.2A). The western blotting results also showed that the protein expression levels of the fibrotic markers such as fibronectin, alpha-smooth muscle actin (α-SMA), collagen-I were increased (fig.2B, C). Also, a cell contraction assay was performed to configure cell contraction, which is mediated by myofibroblast activation, and cell contraction was activated by treating TGF-β1 in a time-dependent manner (fig.2D, E). However, the decrement of mRNA expression and protein expression of ERRα were detected in TGF-β1-treated MRC-5 (fig.2A, B, C). In order to check the activity of ERRα, 3X ERRE promoter was transfected to MRC-5 and promoter assay was performed. As a result, it was confirmed that the activity of ERRα was decreased by TGF- β1 treatment (fig.2E).

Subsequently, several experiments were conducted to confirm the functional change of mitochondria in TGF-β1 treatment. Measuring mtDNA/gDNA, which is widely used as an indicator of mitochondrial mass and biogenesis was performed and mtDNA/gDNA ratios were decreased in TGF-β1 treated MRC-5 (fig.3A). Indirectly, decreased mitochondrial biogenesis can be confirmed through qRT-PCR that the mRNA level of TFAM, which is essential for the production of mitochondria, is reduced (fig.2A). To figure out decreased mitochondrial biogenesis-related molecules leading to aberrant mitochondrial function, Mitotracker Red CMXROS (Mitotracker Red) that stains the mitochondria-dependent on mitochondrial membrane potential and Mitotracker Green, which stains total mitochondrial mass were stained and analyzed with

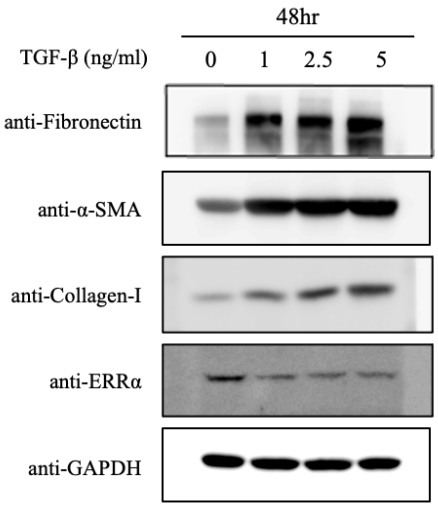
flow cytometry. Total mitochondrial masses and membrane potential were decreased in TGF-β1-treated MRC-5 (fig.3B, C). Furthermore, double staining Mitotracker Red CMX ROS and Mitotracker Green, which can determine mitochondrial functions through gating Mitotracker Green^{High} Mitotracker Red$^{High/Low}$ populations was performed. Results showed that treating TGF-β1 has increased dysfunctional mitochondria (Mitotracker Green^{High} Mitotracker Red^{Low}) but decreased functional mitochondria (Mitotracker Green^{High} Mitotracker Red^{High}) (fig.3D, E, F, G, H).

Overall, the decrement in mitochondrial functions and the decrement of mitochondrial biogenesis were observed on TGF-β1 treated lung fibroblasts.

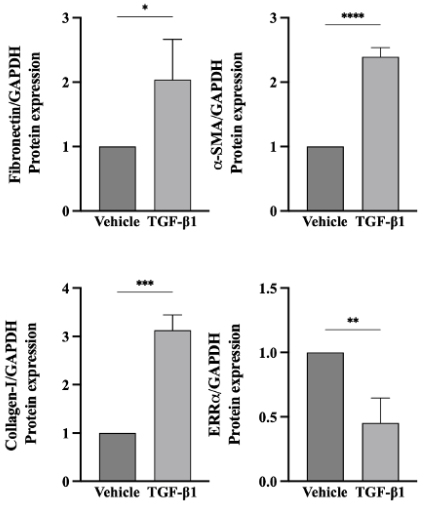
A



B



C



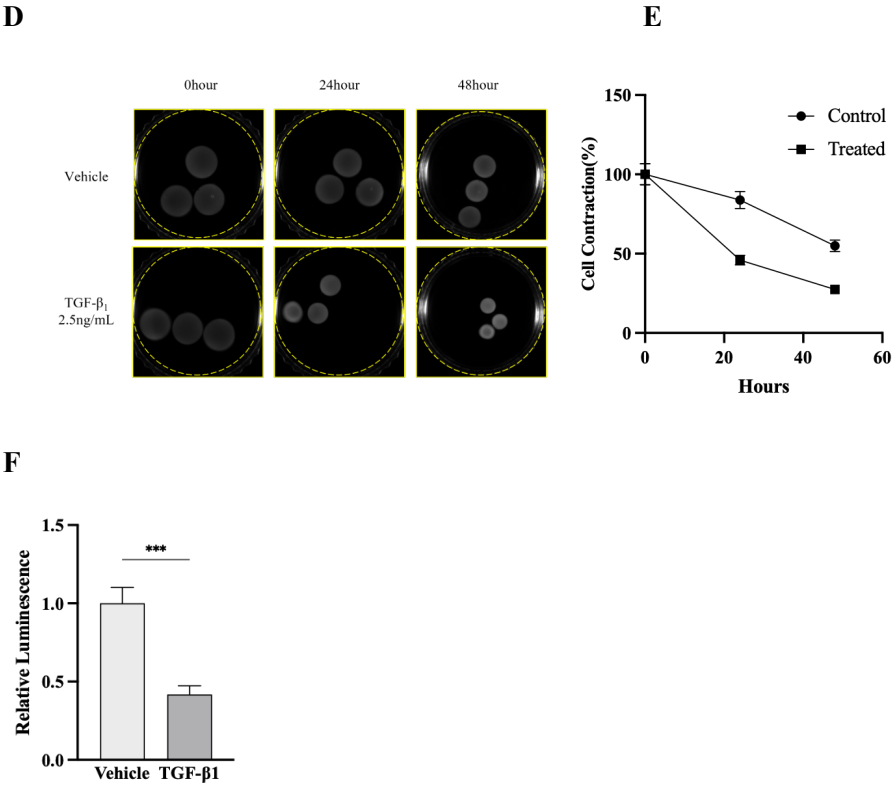


Figure 2. Downregulated expression of mitochondrial biogenesis-related genes in TGF-β1-treated human fetus lung fibroblasts (MRC-5). (A) qRT-PCR analysis of ACTA2, COL1A1, FN1, PPARGC1A, ESRRA, TFAM transcript expression in the vehicle-treated control and TGF-β1 5ng/mL treated MRC-5 (n=5). (B) Representative Western blot images of α-SMA, Collagen-I, Fibronectin, and ERRα in the vehicle-treated control and TGF-β1 (1, 2.5, 5ng/mL) treated MRC-5. (C) Relative density bar graphs of α-SMA, Collagen-I, Fibronectin, and ERRα in the vehicle-treated control and TGF-β1 (5ng/mL). GAPDH was used as a protein loading control (n=3 or more than 3). (D) Cell contraction assay of the vehicle-treated control and TGF-β1 2.5ng/mL treated MRC-5 (n=3). (E) Quantification of cell contraction assay (n=3). (F) Luciferase reporter analysis of *ESRRA* promoter activity in MRC-5 cells treated with TGF-β1 5.0ng/mL (n=3).

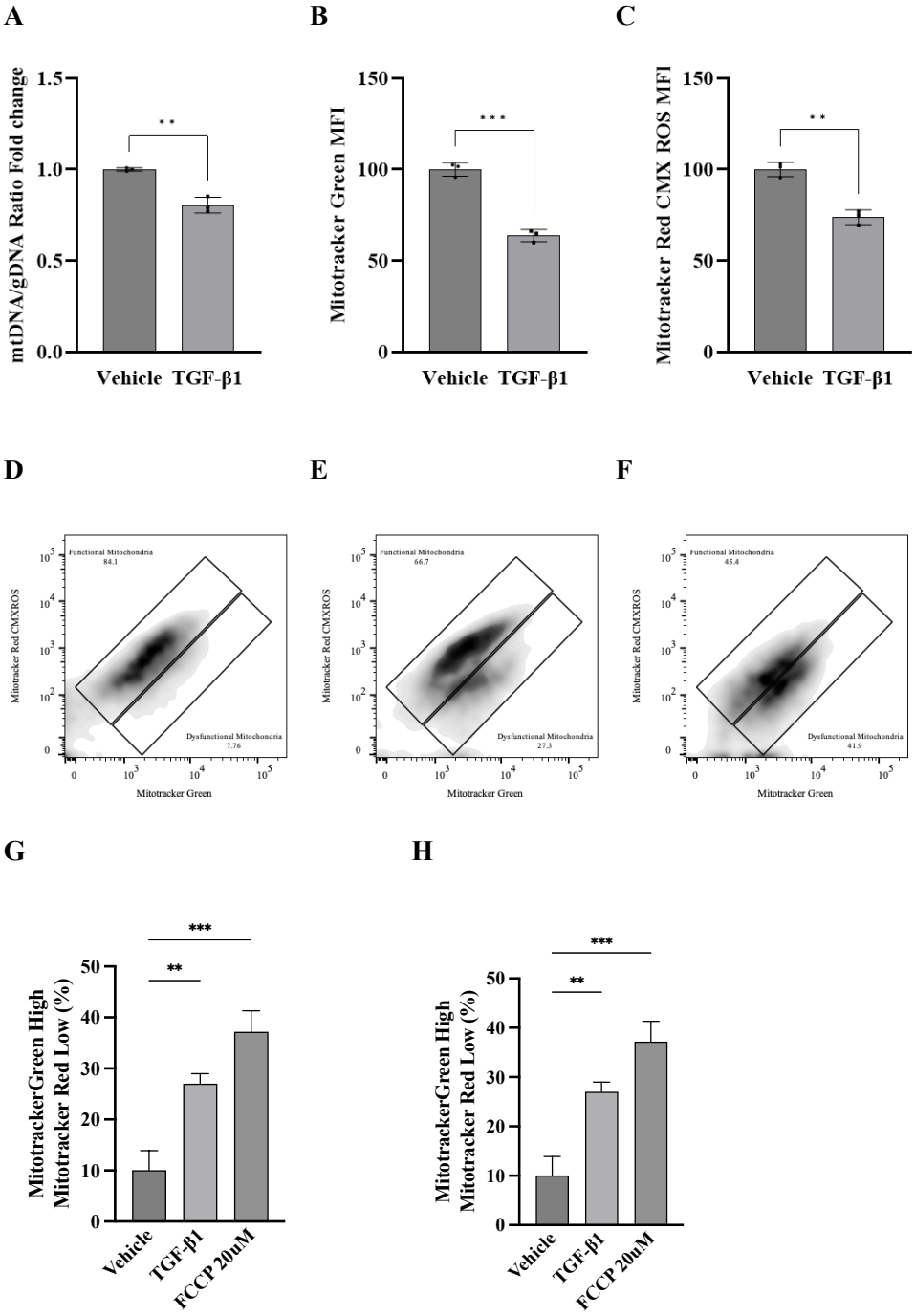


Figure 3. Mitochondrial dysfunction and reduction of mitochondrial biogenesis in TGF-β1-treated human fetus lung fibroblasts (MRC-5). (A) Quantification of mitochondrial mass and mitochondrial biogenesis by mt DNA/g DNA ratio in vehicle-treated control and TGF-β1 5ng/mL treated MRC-5 (n=3) (B) Quantification of Mito Tracker Green FM fluorescence intensity plotted against the number of cells and its quantification (geometric mean intensity) in vehicle-treated control and TGF-β1 5ng/mL treated MRC-5 (n=3). (C) Quantification of Mito Tracker Red CMXROS fluorescence intensity plotted against the number of cells and its quantification (geometric mean intensity) in vehicle-treated control and TGF-β1 5ng/mL treated MRC-5 (n=3). (D) Representative density plot of functional and dysfunctional mitochondria in vehicle-treated MRC-5. (E) Representative density plot of functional and dysfunctional mitochondria in TGF-β1 5ng/mL treated MRC-5. (F) Representative density plot of functional and dysfunctional mitochondria in FCCP 20uM treated MRC-5. Treating 20uM FCCP was used as a positive control for gating dysfunctional mitochondrial portions. (G) Quantification of the percentage of functional mitochondria in vehicle-treated control and TGF-β1 5.0ng/mL, and FCCP 20uM treated MRC-5 (n=3). (H) Quantification of the percentage of dysfunctional mitochondria in vehicle-treated control and TGF-β1 5.0ng/mL, and FCCP 20uM treated MRC-5 (n=3).

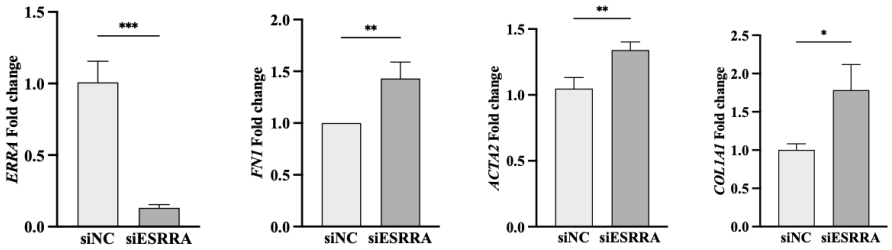
3. Loss of ERRα in human lung fibroblast promotes fibrogenic activation

To investigate the correlation between reduced ERRα and fibrogenesis, siRNA knockdown of ERRα in MRC-5 was performed. qRT-PCR analysis revealed that siRNA treatment for 72 h increased the expression of fibrotic markers (fig.4A). Also, western blotting results showed that except α-SMA, expression levels of Collagen-1 and fibronectin were increased (fig.4B, D). Similarly, XCT790, an inverse agonist of ERRα was treated on MRC-5 to determine if the inhibition of ERRα activation would induce myofibroblast transition. XCT790 inhibits the function of ERRα by blocking the binding site of PGC-1α and ERRα. Cells were treated with an optimal dose of XCT790 to inhibit the activity of ERRα for 48 h and the activity of ERRα was measured through promoter assay (fig.4E). Western blot results suggest that treating XCT790 on MRC-5 have induced myofibroblast transition (fig.4C).

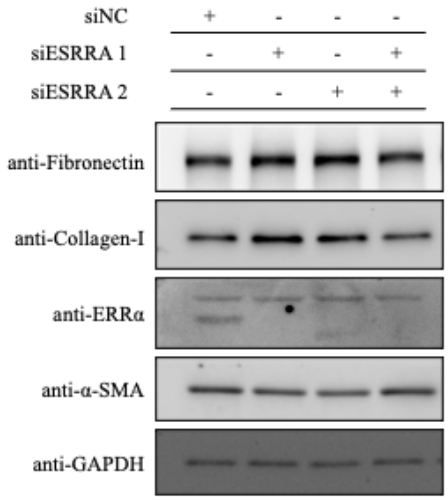
Next, it was confirmed whether ERRα knockdown and treating ERRα inverse agonist effects on mitochondrial function. To determine the mitochondrial mass, mtDNA/gDNA were measured through qRT-PCR and the result showed that the total mitochondrial masses were decreased in siESRRA transfected MRC-5 (fig.5A). Double staining Mitotracker Red and Mitotracker Green was performed to determine mitochondrial functions and it was confirmed that dysfunctional mitochondrial populations were increased and decreased functional mitochondrial populations in siESRRA transfected MRC-5 (fig.5B, C, D, E). Similarly, treating inverse agonist of ERRα have increased dysfunctional mitochondrial function and decreased functional mitochondria compared to DMSO-treated control (fig.5F, G, H, I).

Overall, decreasing ERRα and inhibiting function of ERRα led mitochondrial dysfunction and induced fibrogenesis *in vitro*.

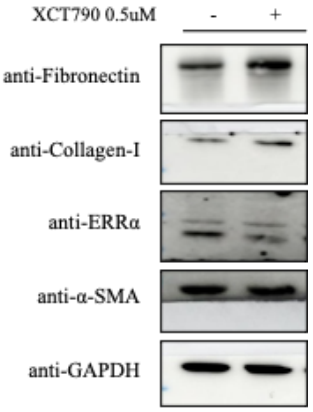
A



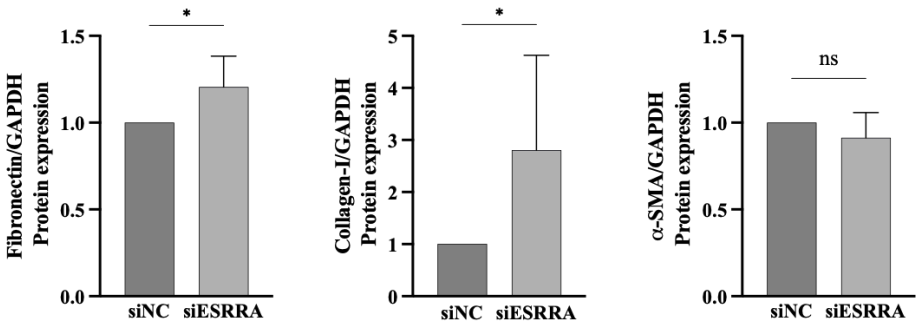
B



C



D



E

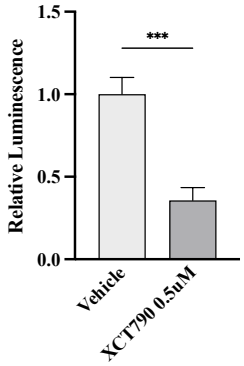
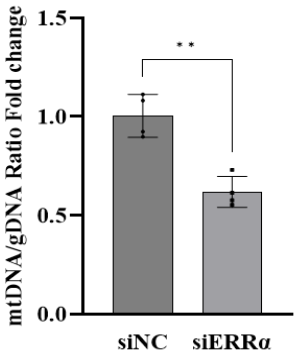
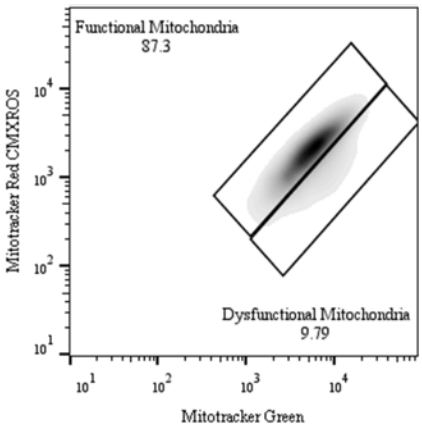


Figure 4. Loss of ERRα in human fetus lung fibroblasts (MRC-5) promotes their fibrogenic activation. (A) qRT-PCR analysis of ACTA2, COL1A1, FN1, ESRRA transcript expression in the negative control (siNC) and siESRRA-treated MRC-5 (n=3). (B) Representative Western blot images of α-SMA, Collagen-I, Fibronectin, and ERRα in the negative control SiNC and siESRRA-treated MRC-5. (C) Western blot analysis of Fibronectin, Collagen-I, ERRα, α-SMA protein expression in vehicle-treated control and TGF-β1 5.0ng/mL with/without XCT790 0.5uM-treated MRC-5. (D) Relative density bar graphs of α-SMA, Collagen-I, and Fibronectin in the negative control SiNC and siESRRA-treated MRC-5. GAPDH was used as a protein loading control (n=3). (E) Luciferase reporter analysis of *ESRRA* promoter activity in MRC-5 cells treated with 0.5uM XCT790 (n=3).

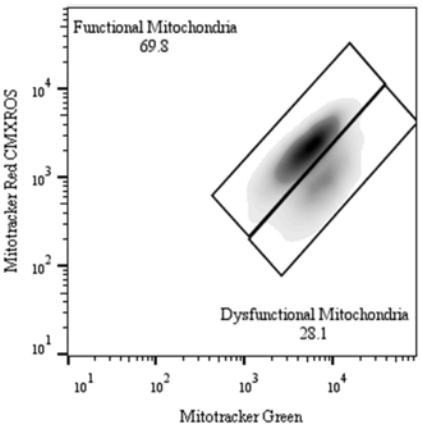
A



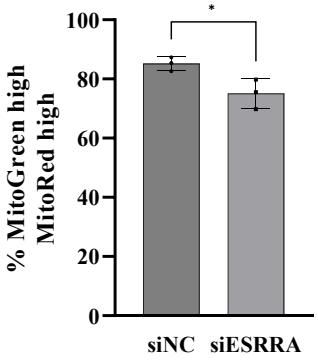
B



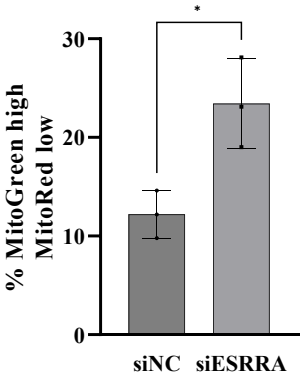
C



D



E



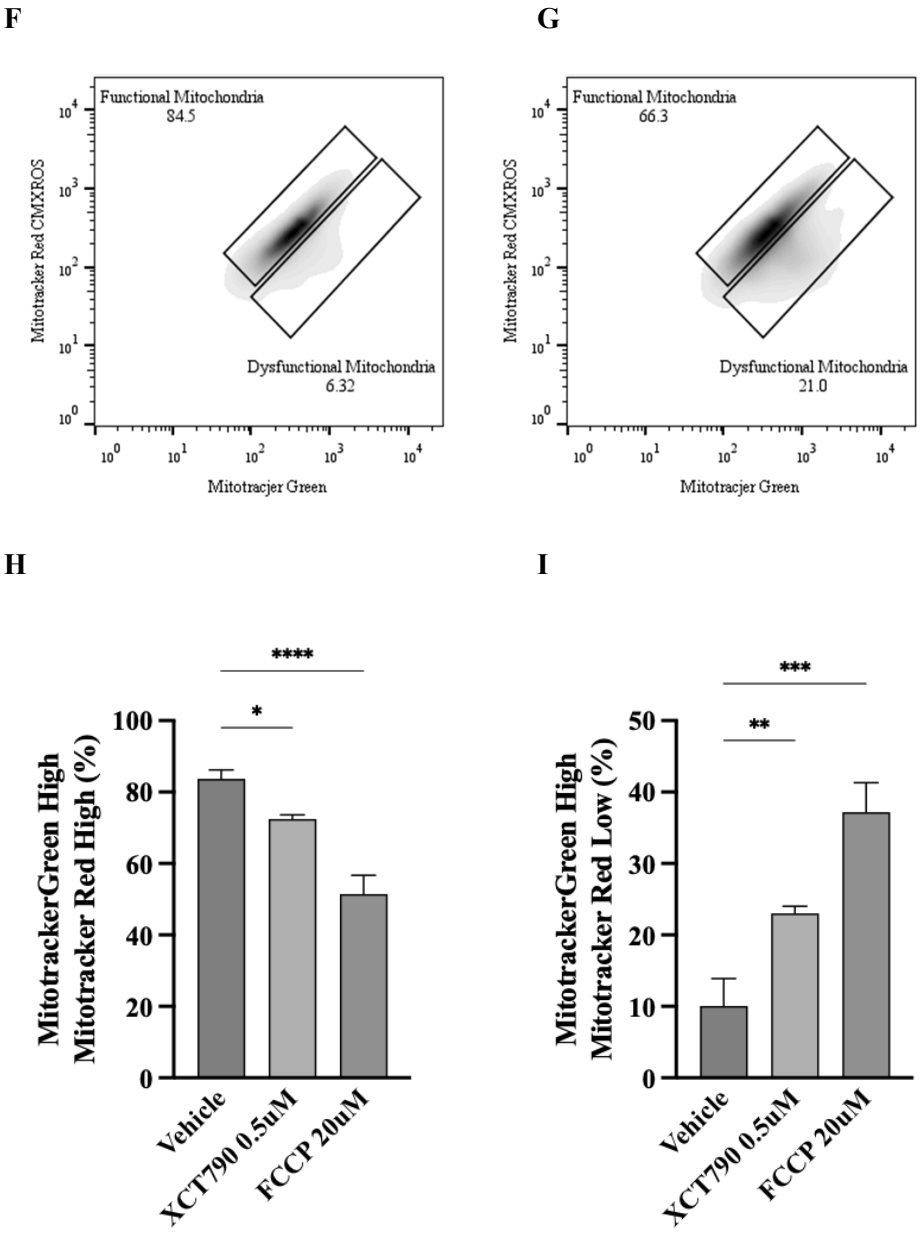


Figure 5. Mitochondrial dysfunction and reduction of mitochondrial biogenesis in ERRα inhibited human fetus lung fibroblasts (MRC-5). (A) Quantification of mitochondrial mass and mitochondrial biogenesis by mt DNA/g DNA ratio in siNC treated control and siESRRA treated MRC-5 (n=3). (B, C)

Representative density plots of functional and dysfunctional mitochondria in SiNC-treated control and siESRRA treated MRC-5 (n=3). (D) Quantification of the percentage of functional mitochondria in siNC-treated control and siESRRA treated MRC-5 (n=3). (E) Quantification of the percentage of dysfunctional mitochondria in siNC-treated control and siESRRA treated MRC-5 (n=3). (F) Representative density plot of functional and dysfunctional mitochondria in DMSO treated control MRC-5 (n=3). (G) Representative density plots of functional and dysfunctional mitochondria in XCT790(0.5uM) treated MRC-5 (n=3). (H) Quantification of the percentage of functional mitochondria in DMSO-treated control and XCT790(0.5uM) treated MRC-5 (n=3). FCCP was used as a negative control of functional mitochondria. (I) Quantification of the percentage of dysfunctional mitochondria in DMSO-treated control and XCT790(0.5uM) treated MRC-5 (n=3). FCCP was used as a positive control of dysfunctional mitochondria.

4. The elevation of ERRα ameliorated fibrogenic activation in PGC-1α dependent manner in human IPF lung fibroblast

Furthermore, effects of ERRα upregulation was verified through ERRα plasmid transfection using LL97A, which is IPF patients derived lung fibroblast. Interestingly, it was confirmed that fibrogenesis activated by TGF-β1 was effectively inhibited through ERRα transfection (fig.6A, C).

To confirm whether this was due to the restoration of mitochondrial function, the function of the mitochondria was checked through previous methods mtDNA/gDNA ratio was increased, suggesting that the increment of mitochondrial DNA, due to the increment of mitochondrial biogenesis (fig.7A). Mitochondrial membrane potentials were increased and mitochondrial function confirmed by gating mitotracker double positive populations were upregulated in ERRα transfected LL97A compared with mock vector transfected LL97A, both in the presence of TGF-β1 5.0ng/mL (fig.7B, C, E, F). Overall, it was confirmed that fibrogenesis was suppressed by increasing ERRα, via restoration of mitochondrial function.

Next, based on the results of previous studies that ERRα is dependent on PGC-1α, an experiment was conducted to overexpress ERRα and simultaneously inhibited the interaction between ERRα and PGC-1α via XCT790. As a result, it was confirmed through western blotting results that the anti-fibrotic effects of ERRα were ameliorated by XCT790 treatment after ERRα overexpression (fig.6B). Also, elevated mitochondrial biogenesis and functions via ERRα transfection was ameliorated in ERRα transfection and XCT790 co-treated group (fig.7B, C, D, E, F). Overall, it was confirmed that ERRα inhibited fibrogenesis dependent on the coactivator PGC-1α.

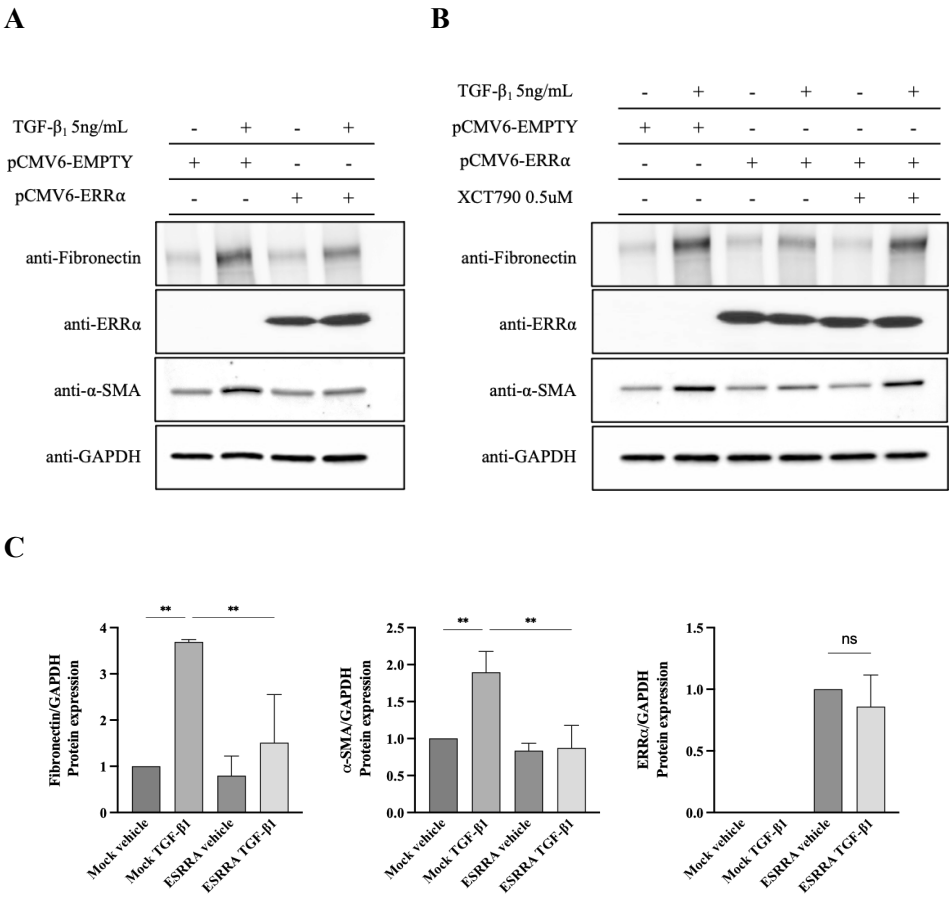


Figure 6. ERRα overexpression in TGF-β1-treated IPF patients' lung fibroblast (LL97A) ameliorates their fibrogenic activation in PGC-1α dependent manner. (A) Representative Western blot images of α-SMA, Collagen-I, Fibronectin, ERRα, and GAPDH in vehicle-treated control and TGF-β1 5.0ng/mL and transfecting pCMV6-ESRRA, p-CMV6-EMPTY plasmid vectors for 48 h in LL97A. (B) Western blot images of Fibronectin, ERRα, α-SMA, and GAPDH in vehicle-treated control and TGF-β1 5.0ng/mL with DMSO, XCT790 (0.5uM) and transfecting pCMV6-ESRRA, p-CMV6-EMPTY plasmid vectors for 48 h in LL97A. (C) Relative density bar graphs of α-SMA, and Fibronectin in vehicle-treated control and TGF-β1 5.0ng/mL and transfecting pCMV6-ESRRA, p-CMV6-EMPTY plasmid vectors for 48 h in LL97A (n=3).

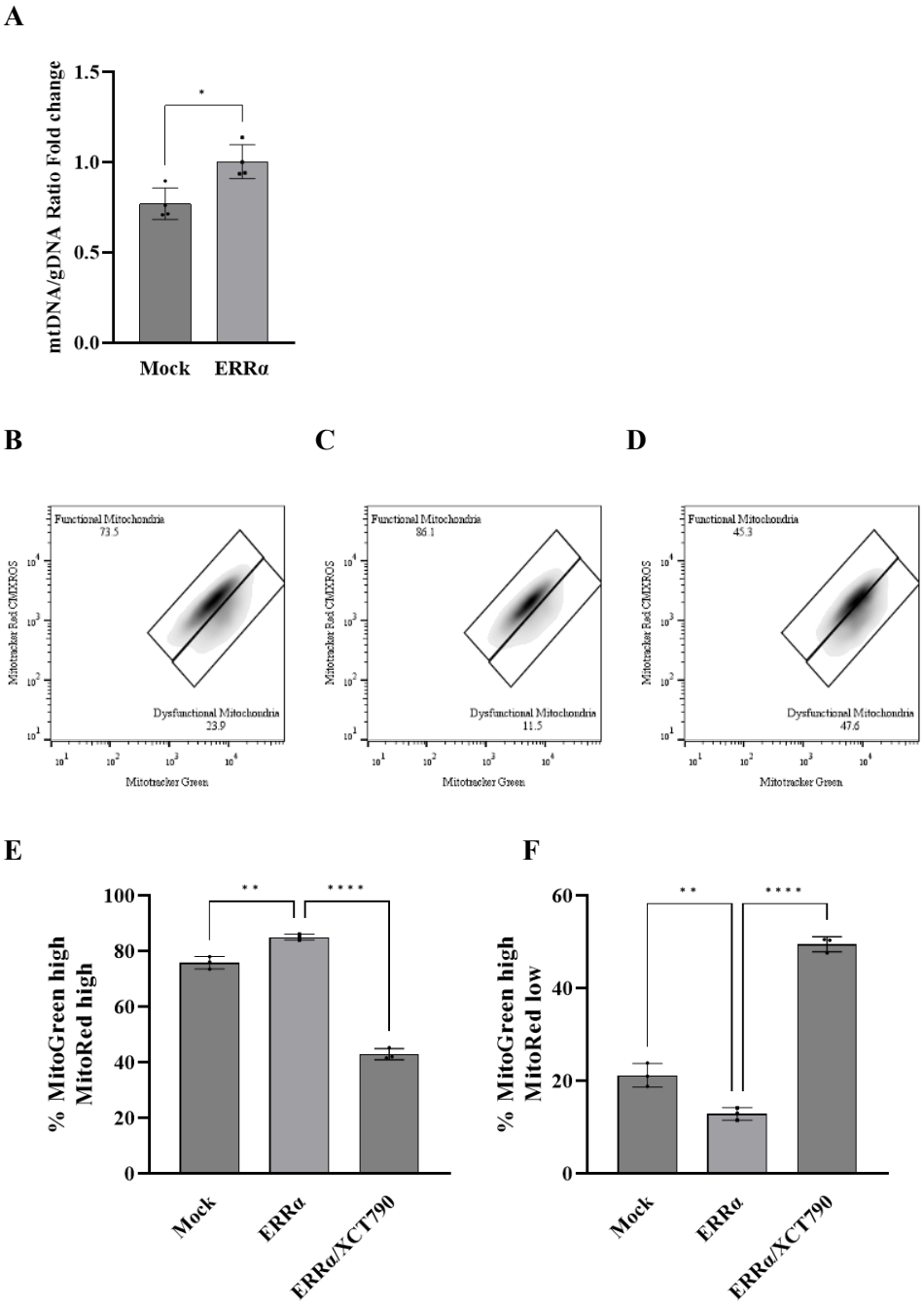


Figure 7. Restoration of Mitochondrial function and increments of mitochondrial biogenesis in pCMV6-ESRRA plasmid DNA transfected in

TGF-β1-treated IPF patients' lung fibroblast (LL97A) in PGC-1α dependent manner. (A) Quantification of mitochondrial mass by mt DNA/g DNA ratio in pCMV6-EMPTY and pCMV6-ESRRA plasmid DNA transfected LL97A in the presence of TGF-β1(n=3). (B) Representative density plot of functional and dysfunctional mitochondria in pCMV6-EMPTY plasmid DNA transfected LL97A in the presence of TGF-β1 (n=3). (C) Representative density plot of functional and dysfunctional mitochondria in pCMV6-ESRRA plasmid DNA transfected LL97A in the presence of TGF-β1 (n=3). (D) Representative density plots of functional and dysfunctional mitochondria in pCMV6-ESRRA plasmid DNA transfected with XCT790 (0.5uM) treated LL97A in the presence of TGF-β1 (n=3). (E) Quantification of the percentage of functional mitochondria in pCMV6-EMPTY and pCMV6-ESRRA plasmid DNA transfected with/without XCT790 treated LL97A in the presence of TGF-β1 (n=3). (F) Quantification of the percentage of dysfunctional mitochondria in pCMV6-EMPTY and pCMV6-ESRRA plasmid DNA transfected with/without XCT790 treated LL97A in the presence of TGF-β1 (n=3).

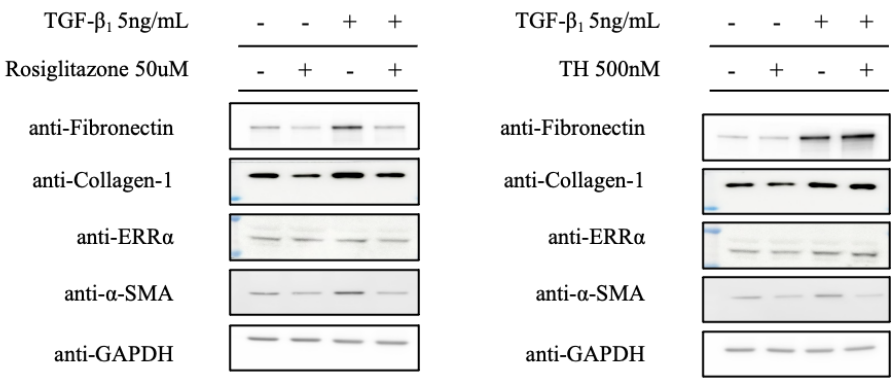
5. Pharmacological elevation of PGC-1α /ERRα attenuates lung fibrosis in the IPF lung fibroblast

Based on the previous experiment, experiments were conducted to verify whether the anti-fibrotic effect could be observed by increasing the activity of ERRα. However, since a drug that directly increases ERRα has not yet been developed, an experiment was conducted by treating Rosiglitazone and Thyroid hormone, which are known to activate PGC-1α referring to existing studies. Increment of protein levels in ERRα according to the increased PGC-1α activity was expected, but it was not shown in western blot, so to compensate for this, a promoter assay to confirm the change in ERRα activity when the drug was treated in the TGF-beta treatment situation has progressed. As a result, it was confirmed that the activity of ERRα was increased in both drugs (fig.8C).

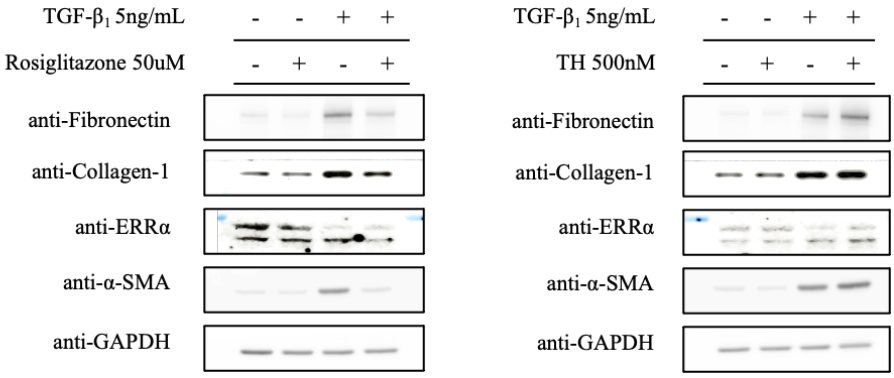
After confirming ERRα activity, western blotting was performed and data showed that Rosiglitazone has attenuated ECM deposition and cell contraction in MRC-5 and LL97A (fig.8A, B). However, treating thyroid hormone didn't have the anti-fibrotic effects as Rosiglitazone in MRC-5 and LL97A (fig.8A, B). Similarly, when a cell contraction assay was performed, it was confirmed that rosiglitazone effectively suppressed the contraction, especially in normal lung fibroblasts, but the such effect was almost absent when treated with thyroid hormone (fig.8D, F). Unexpected results were observed in the patient's lung fibroblasts, the protein expression level of myofibroblasts marker α-SMA was decreased, but it did not inhibit cell contraction (fig8E, G).

Finally, it was confirmed that the antifibrotic mechanism of rosiglitazone was reduced when the interaction between ERRα and PGC-1α was blocked by treatment with XCT790 (fig.8H).

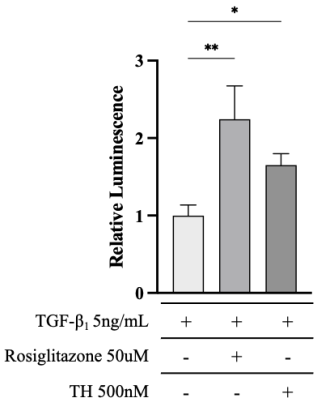
A



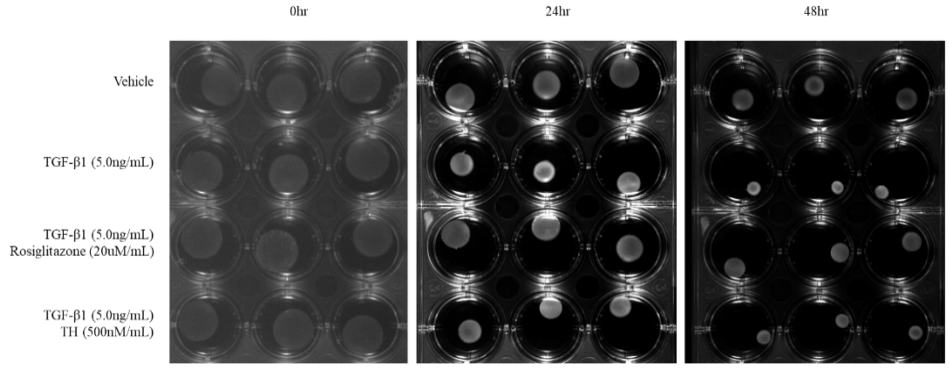
B



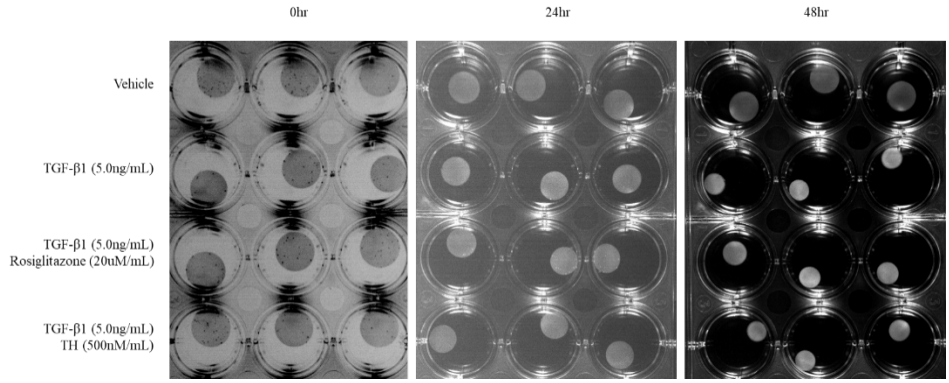
C



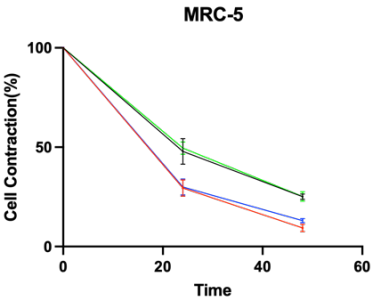
D



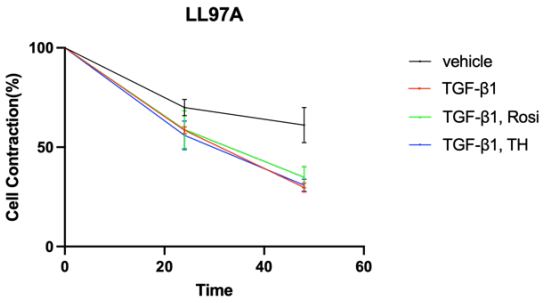
E



F



G



H

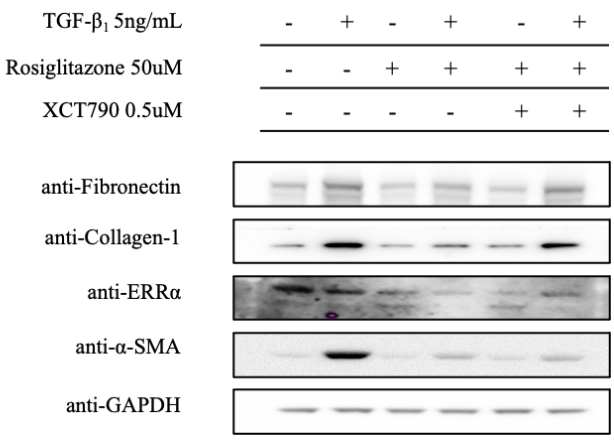


Figure 8. Pharmacological elevation of estrogen-related receptor alpha (ERRα) attenuates fibroblast activation in PGC-1α dependent manner.

(A) Western blotting of α-SMA, Fibronectin, collagen-1, and ERRα in MRC-5 treated with vehicle control and TGF-β1 5.0ng/mL with/without 500nM 3,3′,5-Triiodo-L-thyronine (T_3) and 50uM rosiglitazone for 48 h. (B) Western blotting of α-SMA, Fibronectin, collagen-1, and ERRα in LL97A treated with vehicle control and TGF-β1 5.0ng/mL with/without 500nM T_3 and 50uM rosiglitazone for 48 h. (C) Cell contraction assay of the vehicle-treated control and TGF-β1 5.0ng/mL treated MRC-5 with/without 500nM T_3 and 20uM rosiglitazone for 48 h (n=3). (D) Cell contraction assay of the vehicle-treated control and TGF-β1 5.0ng/mL treated LL97A with/without 500nM T_3 and 20uM rosiglitazone for 48 h (n=3). (E) Quantification of cell contraction assay (n=3) in MRC-5 treated with vehicle control and TGF-β1 5.0ng/mL with/without 500nM T_3 and 20uM rosiglitazone for 48 h. (F) Quantification of cell contraction assay (n=3) in LL97A treated with vehicle control and TGF-β1 5.0ng/mL with/without 500nM T_3 and 20uM rosiglitazone for 48 h. (H) Western blotting of α-SMA, Fibronectin, collagen-1, ERRα, PGC-1α in LL97A treated with vehicle control and TGF-β1 5.0ng/mL with/without 50uM rosiglitazone and XCT790 0.5uM for 48 h.

IV. DISCUSSION

It has been demonstrated that mitochondrial dysfunction contributes to the initiation and progression of lung fibrosis.[42] The mitochondrial function of epithelial cells, fibroblasts, and macrophages in the lungs of patients with IPF is dysregulated. Insufficient mitochondrial biogenesis and impaired mitophagy, resulting in mismatches in cellular energy metabolism, ROS production, senescence, and DNA damage, have been studied.[42-44] PGC-1α, one of the key regulators of mitochondrial biogenesis in human IPF fibroblasts has been repressed.[22] furthermore, it was confirmed that PGC-1α had an anti-fibrotic effect by promoting the expression of transcription factors such as PPARγ and inducing the differentiation of fibroblasts into lipofibroblasts.[45] Also, metformin, which is commercially available as a treatment for metabolic disorders, and rosiglitazone, which has not been commercialized due to the risk of heart failure, are being studied as a treatment for pulmonary fibrosis. [22,31,45,46]

After getting the idea that pulmonary fibrosis can be treated by controlling metabolism through the following studies, a reanalysis of public databases of RNA sequencing was performed to determine whether the configure mitochondrial dysfunction and downregulation were in correlation with ERRα. As a result, it was confirmed that ERRα was significantly decreased in the central region and peripheral region of IPF patients' lung biopsies. In the literature, it was confirmed that the fibrotic phenotype was reduced when ERRα was restored in kidney fibrosis, which is a representative fibrotic disease.[47] However, it has been confirmed that studies have not been established on ERRα, and thus the focus is on ERRα.

When experimenting focusing on the *in vitro* experiment, the experiment was carried out by treating MRC-5, a normal lung fibroblast cell line, or LL97A, an IPF-patient lung fibroblast, with TGF-β1 and inducing fibrogenesis. The

limitation of this experiment is that it was impossible to reenact the pulmonary fibrosis microenvironment, which is affected by a combination of various growth factors, cytokines, chemokines, and other cellular interactions between immune cells, epithelial cells, and fibroblasts.[48] However, TGF-β1 treatment is commonly used as an *in vitro* model design for epithelial-mesenchymal transition and fibroblast-myofibroblast transition and can observe dispositioning of extracellular matrix and cell contraction via TGF-β1 treatment.

To confirm a decrease in mitochondrial biogenesis and function according to fibrogenesis in various ways, comparing the mtDNA/gDNA ratios was performed. The widely used experimental design mtDNA/gDNA qRT–PCR data showed that TGF-β1 treatment leads to the decrement of mtDNA/gDNA ratio.[49] Then, flow cytometry assays were performed using Mitotracker, a fluorescent dye widely used as mitochondrial-specific dye on fluorescence microscopes. By staining the Mitotracker Green and Mitotracker Red CMXROS together, it was confirmed that the decrement of functional mitochondria was increased due to TGF-β1 treatment.[50] The experimental design could have been further supported by measuring mitostress by measuring oxygen consumption rates.[51]

After confirming the decrement of ERRα expression in mRNA and protein levels, determining whether this is the cause or result of fibrogenesis inhibiting ERRα was performed. siRNA treatment for 72 h and XCT790, an inverse agonist of ERRα, was used to inhibit ERRα. As a result, not only were mitochondrial function and biosynthesis decreased but also the progression of fibrosis was observed. Conversely, when ERRα was expressed through a plasmid vector, fibrogenic phenotypes were reduced and mitochondrial function was increased. However, it was confirmed that this improvement effect was reduced again when the interaction between PGC-1α and ERRα was inhibited through XCT790 treatments.[52]

Through previous experiments, drugs that increase ERRα were intended to be used to improve fibrosis. However, an important limitation of this concept is the fact that ERRα is an orphan nuclear receptor that requires a specific ligand to function and its activator has not yet been identified. Several papers have demonstrated that PGC-1α acts as its protein ligand; therefore, two candidate drugs that can increase PGC-1α were established, namely, rosiglitazone and thyroid hormone used as agonists of ERRα. [53,54] Rosiglitazone was revealed to activate PGC-1α by increasing PPARγ, and thyroid hormone, which translocates into the nucleus together with THRB and acts as a transcription factor, increases PGC-1α. Accordingly, it was thought that both drugs could effectively prevent fibrosis.

In the case of rosiglitazone, it was confirmed that it had an anti-fibrotic effect. However, rosiglitazone has been withdrawn from use as a drug by the FDA, as the cardiovascular risk is higher than its therapeutic effect. Another drug candidate, thyroid hormone, improved pulmonary fibrosis by increasing mitochondrial biosynthesis in a PGC-1α dependent manner in the bleomycin-induced pulmonary fibrosis animal model. However, thyroid hormone did not show the resolution of fibrosis *in vitro*. This is thought to be due to the absence of THRB, which delivers thyroid hormone into the nucleus. When comparing the public data, it was confirmed that the mRNA level of THRB was reduced.

Through this study, it is thought that there is a need for drug development that can directly target and increase ERRα. So far Additional studies should be conducted to determine the effects of the PGC-1α-ERRα axis on pulmonary fibrosis.

V. CONCLUSION

Through this study, we confirmed that mitochondrial dysfunction was observed in the lung tissue of IPF patients, and TGF-β1 treated lung fibroblasts. The correlation between lung fibrosis was investigated by reducing or enhancing ERRα, which regulates mitochondrial biogenesis. At this time, it was confirmed that ERRα, depends on PGC-1α, and the possibility of alleviating lung fibrosis by regulating the PGC-1α-ERRα axis was confirmed.

REFERENCE

1. King Jr TE, Pardo A, Selman M. Idiopathic pulmonary fibrosis. The Lancet 2011;378:1949-61.
2. Lederer DJ, Martinez FJ. Idiopathic pulmonary fibrosis. New England Journal of Medicine 2018;378:1811-23.
3. Martinez FJ, Collard HR, Pardo A, Raghu G, Richeldi L, Selman M, et al. Idiopathic pulmonary fibrosis. Nature Reviews Disease Primers 2017;3:1-19.
4. Richeldi L, Collard HR, Jones MG. Idiopathic pulmonary fibrosis. The Lancet 2017;389:1941-52.
5. Society AT. Idiopathic pulmonary fibrosis: diagnosis and treatment: international consensus statement. Am J Respir Crit Care Med 2000;161:646-64.
6. Raghu G, Remy-Jardin M, Myers JL, Richeldi L, Ryerson CJ, Lederer DJ, et al. Diagnosis of idiopathic pulmonary fibrosis. An official ATS/ERS/JRS/ALAT clinical practice guideline. American journal of respiratory and critical care medicine 2018;198:e44-e68.
7. Hilberg F, Roth GJ, Krssak M, Kautschitsch S, Sommergruber W, Tontsch-Grunt U, et al. BIBF 1120: triple angiokinase inhibitor with sustained receptor blockade and good antitumor efficacy. Cancer research 2008;68:4774-82.
8. Kurita Y, Araya J, Minagawa S, Hara H, Ichikawa A, Saito N, et al. Pirfenidone inhibits myofibroblast differentiation and lung fibrosis development during insufficient mitophagy. Respiratory research 2017;18:1-14.

9. Schaefer C, Ruhrmund D, Pan L, Seiwert S, Kossen K. Antifibrotic activities of pirfenidone in animal models. European Respiratory Review 2011;20:85-97.
10. Chaudhary N, Roth G, Hilberg F, Müller-Quernheim J, Prasse A, Zissel G, et al. Inhibition of PDGF, VEGF and FGF signalling attenuates fibrosis. European Respiratory Journal 2007;29:976-85.
11. Maher TM, Strek ME. Antifibrotic therapy for idiopathic pulmonary fibrosis: time to treat. Respiratory research 2019;20:1-9.
12. Katzenstein A-LA, Myers JL. Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. American journal of respiratory and critical care medicine 1998;157:1301-15.
13. Qunn L, Takemura T, Ikushima S, Ando T, Yanagawa T, Akiyama O, et al. Hyperplastic epithelial foci in honeycomb lesions in idiopathic pulmonary fibrosis. Virchows Archiv 2002;441:271-8.
14. Uhal BD, Joshi I, Hughes WF, Ramos C, Pardo A, Selman M. Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. American Journal of Physiology-Lung Cellular and Molecular Physiology 1998;275:L1192-L9.
15. Plataki M, Koutsopoulos AV, Darivianaki K, Delides G, Siafakas NM, Bouros D. Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis. Chest 2005;127:266-74.
16. Selman M, Pardo A. Revealing the pathogenic and aging-related mechanisms of the enigmatic idiopathic pulmonary fibrosis. an integral model. American journal of respiratory and critical care medicine 2014;189:1161-72.
17. Weidinger A, Kozlov AV. Biological activities of reactive oxygen and nitrogen species: oxidative stress versus signal transduction. Biomolecules 2015;5:472-84.

18. Jiang F, Liu G-S, Dusting GJ, Chan EC. NADPH oxidase-dependent redox signaling in TGF-β-mediated fibrotic responses. Redox biology 2014;2:267-72.
19. Patel AS, Song JW, Chu SG, Mizumura K, Osorio JC, Shi Y, et al. Epithelial cell mitochondrial dysfunction and PINK1 are induced by transforming growth factor-beta1 in pulmonary fibrosis. PloS one 2015;10:e0121246.
20. Springer W, Kahle PJ. Regulation of PINK1-Parkin-mediated mitophagy. Autophagy 2011;7:266-78.
21. Kobayashi K, Araya J, Minagawa S, Hara H, Saito N, Kadota T, et al. Involvement of PARK2-mediated mitophagy in idiopathic pulmonary fibrosis pathogenesis. The Journal of Immunology 2016;197:504-16.
22. Caporarello N, Meridew JA, Jones DL, Tan Q, Haak AJ, Choi KM, et al. PGC1α repression in IPF fibroblasts drives a pathologic metabolic, secretory and fibrogenic state. Thorax 2019;74:749-60.
23. Yu G, Tzouvelekis A, Wang R, Herazo-Maya JD, Ibarra GH, Srivastava A, et al. Thyroid hormone inhibits lung fibrosis in mice by improving epithelial mitochondrial function. Nature medicine 2018;24:39-49.
24. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. The Journal of clinical investigation 2006;116:615-22.
25. Schreiber SN, Emter R, Hock MB, Knutti D, Cardenas J, Podvinec M, et al. The estrogen-related receptor α (ERRα) functions in PPARγ coactivator 1α (PGC-1α)-induced mitochondrial biogenesis. Proceedings of the National Academy of Sciences 2004;101:6472-7.
26. Vernier M, Giguère V. Aging, senescence and mitochondria: the PGC-1/ERR axis. Journal of Molecular Endocrinology 2021;66:R1-R14.

27. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 1999;98:115-24.
28. Fisher RP, Lisowsky T, Parisi M, Clayton DA. DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. Journal of Biological Chemistry 1992;267:3358-67.
29. Scarpulla RC. Nuclear activators and coactivators in mammalian mitochondrial biogenesis. Biochimica et biophysica acta (BBA)-gene structure and expression 2002;1576:1-14.
30. Larsson N-G, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, et al. Mitochondrial transcription factor A is necessary for mtDNA maintance and embryogenesis in mice. Nature genetics 1998;18:231-6.
31. Rangarajan S, Bone NB, Zmijewska AA, Jiang S, Park DW, Bernard K, et al. Metformin reverses established lung fibrosis in a bleomycin model. Nature medicine 2018;24:1121-7.
32. Villena JA, Kralli A. ERRα: a metabolic function for the oldest orphan. Trends in Endocrinology & Metabolism 2008;19:269-76.
33. Audet-Walsh E, Giguére V. The multiple universes of estrogen-related receptor α and γ in metabolic control and related diseases. Acta Pharmacologica Sinica 2015;36:51-61.
34. Ranhotra HS. The estrogen-related receptor alpha: the oldest, yet an energetic orphan with robust biological functions. Journal of Receptors and Signal Transduction 2010;30:193-205.
35. Tang J, Liu T, Wen X, Zhou Z, Yan J, Gao J, et al. Estrogen-related receptors: novel potential regulators of osteoarthritis pathogenesis. Molecular Medicine 2021;27:1-12.
36. Mootha VK, Handschin C, Arlow D, Xie X, Pierre JS, Sihag S, et al. Errα and Gabpa/b specify PGC-1α-dependent oxidative

phosphorylation gene expression that is altered in diabetic muscle. Proceedings of the National Academy of Sciences 2004;101:6570-5.

37. Huss JM, Torra IP, Staels B, Giguere V, Kelly DP. Estrogen-related receptor α directs peroxisome proliferator-activated receptor α signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. Molecular and cellular biology 2004;24:9079-91.
38. Chaveroux C, Eichner LJ, Dufour CR, Shatnawi A, Khoutorsky A, Bourque G, et al. Molecular and genetic crosstalks between mTOR and ERRα are key determinants of rapamycin-induced nonalcoholic fatty liver. Cell metabolism 2013;17:586-98.
39. Kim S, Lee J-Y, Shin SG, Kim JK, Silwal P, Kim YJ, et al. ESRRA (estrogen related receptor alpha) is a critical regulator of intestinal homeostasis through activation of autophagic flux via gut microbiota. Autophagy 2020:1-20.
40. Kim SY, Yang C-S, Lee H-M, Kim JK, Kim Y-S, Kim Y-R, et al. ESRRA (estrogen-related receptor α) is a key coordinator of transcriptional and post-translational activation of autophagy to promote innate host defense. Autophagy 2018;14:152-68.
41. Yuk J-M, Kim TS, Kim SY, Lee H-M, Han J, Dufour CR, et al. Orphan nuclear receptor ERRα controls macrophage metabolic signaling and A20 expression to negatively regulate TLR-induced inflammation. Immunity 2015;43:80-91.
42. Li X, Zhang W, Cao Q, Wang Z, Zhao M, Xu L, et al. Mitochondrial dysfunction in fibrotic diseases. Cell death discovery 2020;6:1-14.
43. Zank DC, Bueno M, Mora AL, Rojas M. Idiopathic pulmonary fibrosis: aging, mitochondrial dysfunction, and cellular bioenergetics. Frontiers in medicine 2018;5:10.

44. Bueno M, Lai Y-C, Romero Y, Brands J, Croix CMS, Kamga C, et al. PINK1 deficiency impairs mitochondrial homeostasis and promotes lung fibrosis. The Journal of clinical investigation 2015;125:521-38.
45. Kheirollahi V, Wasnick RM, Biasin V, Vazquez-Armendariz AI, Chu X, Moiseenko A, et al. Metformin induces lipogenic differentiation in myofibroblasts to reverse lung fibrosis. Nature communications 2019;10:1-16.
46. Cheng D, Xu Q, Wang Y, Li G, Sun W, Ma D, et al. Metformin attenuates silica-induced pulmonary fibrosis via AMPK signaling. Journal of Translational Medicine 2021;19:1-18.
47. Dhillon P, Park J, Del Pozo CH, Li L, Doke T, Huang S, et al. The nuclear receptor ESRRA protects from kidney disease by coupling metabolism and differentiation. Cell metabolism 2021;33:379-94. e8.
48. Desai O, Winkler J, Minasyan M, Herzog EL. The role of immune and inflammatory cells in idiopathic pulmonary fibrosis. Frontiers in medicine 2018;5:43.
49. Rooney JP, Ryde IT, Sanders LH, Howlett E, Colton MD, Germ KE, et al. PCR based determination of mitochondrial DNA copy number in multiple species. Mitochondrial Regulation: Springer; 2015. p.23-38.
50. de Brito Monteiro L, Davanzo GG, de Aguiar CF, Moraes-Vieira PM. Using flow cytometry for mitochondrial assays. MethodsX 2020;7:100938.
51. Plitzko B, Loesgen S. Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in culture cells for assessment of the energy metabolism. Bio-protocol 2018;8:e2850-e.
52. Willy PJ, Murray IR, Qian J, Busch BB, Stevens Jr WC, Martin R, et al. Regulation of PPARγ coactivator 1α (PGC-1α) signaling by an estrogen-related receptor α (ERRα) ligand. Proceedings of the National Academy of Sciences 2004;101:8912-7.

53. Pardo R, Enguix N, Lasheras J, Feliu JE, Kralli A, Villena JA. Rosiglitazone-induced mitochondrial biogenesis in white adipose tissue is independent of peroxisome proliferator-activated receptor γ coactivator-1α. PloS one 2011;6:e26989.
54. Singh BK, Sinha RA, Tripathi M, Mendoza A, Ohba K, Sy JA, et al. Thyroid hormone receptor and ERRα coordinately regulate mitochondrial fission, mitophagy, biogenesis, and function. Science Signaling 2018;11:eaam5855.

ABSTRACT (IN KOREAN)

PGC-1α /ERRα 신호전달체계를 통한 미토콘드리아 생합성 조절이 폐섬유화 발병기전에 미치는 영향

<지도교수 이재면>

연세대학교 대학원 의과학과`

정현우

미토콘드리아 기능 장애와 특발성 폐섬유화증의 발병 및 진행이 관련됨이 규명된 연구 결과가 보고되었다. 특발성 폐섬유화증 환자들의 폐에 존재하는 세포들의 미토콘드리아의 생합성 감소와 기능 장애가 공통된 특징으로 나타난다. 일례로 인간 특발성 폐섬유화증 환자의 섬유아세포에서 PGC-1α 가 감소되고. 이로 인한 미토콘드리아의 생합성과 기능 감소가 규명된바 있다.

에스트로겐 관련 수용체 알파 (ERRα)는 미토콘드리아 유전자에 대한 전사 조절자를 유도함으로써 미토콘드리아 생합성을 상향조절할 수 있다. 연구에 따르면 ERRα 는 PGC-1α 의 의존적으로 작용함이 규명되었다. 하지만, 폐섬유증에서 ERRα와 PGC-1α /ERRα 신호전달 체계에 대한 연구는 보고된바 없어, 이에 대한 연구가 필요하다 생각되었다. 본 연구에서는 ERRα 의 변화가 미토콘드리아 상태 및 섬유아세포에서 근섬유아세포로의 전이에 어떠한 영향을 미치는지 확인했다.

폐 섬유아세포 세포주 (MRC-5)를 변형 성장 인자-베타 1 (TGF-β1)로 처리하여 ERRα 와 섬유아세포에서 근섬유아세포로의 전이 사이의 관련성을 확인했고, 그 결과 PGC-1α /ERRα 신호축과 미토콘드리아 상태는 TGF-β1 처리된 세포주에서 감소됨을 확인했다. 다음으로 ERRα 의 기능 소실과 미토콘드리아 기능 장애 및 섬유아세포에서 근섬유아세포로의 전이와의 관계를 규명하기 위해. 세포주에 ERRα 역작용제 (XCT790)를 처리하고, siRNA 처리하였다. 그 결과 ERRα 의 감소가 섬유아세포에서 근섬유아세포로의 전이를 유도시켰다. 이와 반대로, ERRα 강화를 통한 섬유아세포에서 근섬유아세포로의 전이의 개선을 확인하기 위해 MRC-5 세포에 pCMV6-ERRα 플라스미드 벡터를 이용한 유전자 과발현과 약물처리를 수행하였다. 그 결과 ERRα 를 유전적으로 혹은 약물을 이용하여 증가시킬 경우 섬유아세포에서 근섬유아세포로의 전이가 개선되는 것을 확인할 수 있었다. 그리고 이때, ERRα가 PGC-1α 에 의존적임을 규명했다.

본 연구를 통해 PGC-1α /ERRα 신호전달 시스템과 폐 섬유증의 발병기전 사이의 관련성을 입증했다. 이러한 발견은 감소된 PGC-1α /ERRα 신호 전달 시스템을 회복시킬 경우 폐 섬유증을 개선할 수 있음을 시사한다.

핵심되는 말 : 특발성 폐섬유화증, 미토콘드리아 생합성, 미토콘드리아 기능 장애, 에스트로겐 관련 수용체 알파 (ERRα), PGC-1α