PGC1α repression in IPF fibroblasts drives a pathologic metabolic, secretory and fibrogenic state

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Supplementary material

SUPPLEMENTARY MATERIAL

METHODS

Cell culture

Cells were maintained in EMEM (ATCC, Manassas, VA, USA) containing 10% FBS unless otherwise noted. All the experiments were performed with cells between passages 3 and 7. IMR-90 embryonic lung fibroblasts were purchased from ATCC. Primary human lung fibroblasts isolated by explant culture from the lungs of subjects diagnosed with IPF who underwent lung transplantation, or donors (HLF) whose organs were rejected for transplantation (non-IPF) were kindly provided by Peter Bitterman and Craig Henke at the University of Minnesota under a protocol approved by the University of Minnesota Institutional Review Board and by Carol Feghali-Bostwick at the Medical University of South Carolina under a protocol approved by the University of Pittsburg Institutional Review Board. Normal primary human lung fibroblasts (HLF) were also purchased by Lonza (Walkersville, MD, USA).

RNA interference

Transient RNA interference was performed with individual siGENOME siRNA (Dharmacon, Lafayette, CO, USA) specific for PPARGC1α (D-005111-01-0005; D-005111-03-0002; D-005111-04-0002) or, in control cells, with On-Target*plus* Non-targeting Pool (D-001810-10-05) by using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA). The day of transfection, the medium was switched to 0.5 % FBS and the cells were then cultured for 72 hours. For ECM

deposition assay, Traction Force Microscopy and OCR analysis, cells were transferred to the respective assay-adapted plates after 48 hours of transfection.

Plasmids and transfection

Transient transfection was performed with pcDNA4 Myc PGC1α (10974, Addgene, Cambridge, MA, USA) or empty vector control (EV) using Lipofectamine p3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA). Expression of the constructs in the transfections was determined by Western blot. The day of transfection, the medium was switched to 0.5 % FBS and the cells were then cultured for 72 hours. For iECM deposition assay, Traction Force Microscopy and OCR analysis, cells were transferred to the respectively assay adapted plates after 48 hours of transfection.

Immunofluorescence staining

Cells or slides were fixed in 3.7 % formalin (Sigma-Aldrich, St. Louis, MA, USA), permeabilized in 0.25 % Triton X-100 (Sigma-Aldrich, St. Louis, MA, USA), blocked with 1% BSA for 1 hour and incubated with α SMA (F3777, Sigma-Aldrich, St. Louis, MA, USA) or Collagen I (NB600-408, Novus Biologicals, Littleton, CO, USA) primary antibodies (diluted 1:200 in PBS with 1% BSA), followed by fluorescence-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) and DAPI to counterstain nuclei.

Traction force microscopy (TFM)

Fluorescent sulfate modified latex microspheres (0.2 μm, 505/515 ex/em) (FluoSpheres, Thermo Fisher Scientific, Waltham, MA, USA) were conjugated to the gel surfaces of polyacrylamide substrates with shear moduli of 6.4 kPa after treatment with 1 mg/ml of dopamine hydrochloride (Sigma-Aldrich, St. Louis, MA, USA) dissolved in 50 mM HEPES solution (pH 8.5). Cells were transferred on the gel plates overnight before traction force measurements. Images of gel surface-conjugated fluorescent beads were acquired for each cell before and after trypsinization using a Nikon ECLIPSE Ti microscope at x 10 magnification. Traction forces were estimated by measuring bead displacement fields and computing corresponding traction fields using TractionsForAll (http://www.mayo.edu/research/labs/tissue-repair-mechanobiology/software).

Real-time PCR

Total mRNA was isolated using RNeasy mini kit or RNeasy micro kit (Qiagen, Valencia, CA, USA) followed by Nanodrop concentration and purity analysis. cDNA was synthesized using SuperScript VILO (Thermo Fisher Scientific, Waltham, MA, USA); RT–PCR was performed using FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) and analyzed using a LightCycler 96 (Roche Diagnostics, Mannheim, Germany). RT-PCR primers used in this study (Integrated DNA Technologies, Coralville, IA, USA) are listed in Table 1.

Protein extraction and Western blotting analysis

Lysates were prepared with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher

Scientific, Waltham, MA, USA). Lysate Total protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Ten μg of protein was resolved by 7.5% SDS-page, transferred to polyvinylidene fluoride membranes and probed overnight at 4° C with primary antibodies: GAPDH (14C10, Cell Signaling Technology, Danvers, MA, USA), αSMA (ab5694, Abcam, Cambridge, MA, USA), PGC1α (sc-13067, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Myc-Tag (2272, Cell Signaling Technology, Danvers, MA, USA). Blots were then washed and incubated with appropriate IgG-HRP-conjugated secondary antibodies for 1 hour at room temperature. Bands were visualized by using Super Signal West Pico Plus (Thermo Fisher Scientific, Waltham, MA, USA) and ChemiDoc (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol.

Mouse model of bleomycin-induced lung injury

All animal experiments were carried out under protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and conforming to the ARRIVE guidelines. *Col1α1*-GFP transgenic mice (female, FVB strain) were generated as previously described (UC San Diego, La Jolla, CA) [1] and kindly provided by Dr. Derek Radisky. Bleomycin was delivered to the lungs as previously described [2]. Two month and 18 month old mice were anaesthetized and 1.2 U/kg bleomycin (APP Pharmaceutical, LCC Schaumburg, IL, USA) or PBS were intratracheally delivered using a MicroSprayer (Penn-Century, Philadelphia, PA, USA). Body weight was monitored daily.

FACS sorting

Col1α1-GFP mice were anaesthetized with ketamine/xylazine solution (100mg/kg and 10mg/kg, respectively) injected intraperitoneally and perfused via left ventricle with cold PBS 11, 21 or 30 days after bleomycin or PBS delivery. The lungs were immediately harvested and minced with a razor blade in a 100 mm petri dish in cold DMEM medium containing 0.2 mg/ml Liberase DL and 100 U/ml DNase I (Roche, Indianapolis, IN, USA). The mixture was transferred into 15 ml tubes and incubated at 37 °C for 35 min in a water bath to allow enzymatic digestion. Digestion was inactivated with DMEM medium containing 10% fetal bovine serum, the cell suspension was passed through a 40 µm cell strainer (Fisher, Waltham, MA, USA) to remove debris. Cells were then centrifuged (1,350 rpm, 10 min, 4 °C), and resuspended in 3ml red blood cell lysis buffer (Biolegend, San Diego, CA, USA) for 90 seconds to remove the remaining red blood cells and diluted in 9mL PBS after incubation. Cells were then centrifuged (1,350 rpm, 10 min, 4 °C) and resuspended in 0.2 ml of FACS buffer (1% BSA, 0.5 mM EDTA pH 7.4 in PBS). The single cell suspension was then incubated with anti-CD45:PerCp-Cy5.5 (1:200), anti-CD31:PE (1:200), anti-EpCAM:APC (1:200), and DAPI (1:1000) antibodies (Biolegend, San Diego, CA, USA) for 30 min on ice. After incubation, cells were washed with ice-cold FACS buffer and resuspended in 1 ml of FACS buffer. FACS sorting was conducted using a BD FACS Aria II (BD Biosciences, San Jose, CA, USA). To isolate CD45-, EpCAM-, CD31-, GFP positive population the following isolation strategy was used: debris exclusion (FSC-A by SSC-A), doublet exclusion (SSC-W by SSC-H and FSC-W by FSC-H), dead cell exclusion (DAPI by PE), CD45 positive cell exclusion (PerCP-Cy5.5 by GFP), EpCAM and CD31 positive cells exclusion (APC by

PE), and isolation of GFP positive cells (APC by GFP). FACS-sorted fibroblasts were subjected to mRNA isolation, cDNA synthesis and qPCR analysis.

Fibrosis evaluation

Hydroxyproline content was measured using a hydroxyproline assay kit (Biovision, Milpitas, CA, USA). Briefly, lung samples were transferred into glasses tubes and hydrolyzed with 200 µl 6N HCL at 110°C for 48 h. The hydrolyzed samples were evaporated to remove excess HCL, reconstituted with 400 µl H_2O and filtered in 1.5 ml centrifuge tubes equipped with a 0.45 µm semipermeable membrane filter. After samples were added to a 96 well micro-plate, Chloramine T solution was added and the plate was incubated at room temperature for 20 min. 100µl of Erlich's reagent was added to each well and the plate was incubated at 65°C for 18 min. This method gives an orange red color which is linear up to up to 6 µg of hydroxyproline. OD 550 nm was obtained, and compared to a hydroxyproline standard curve.

NAD+ assay

NAD+ biosynthesis was measured by using a colorimetric commercial kit (Cayman, Ann Arbor, Michigan, USA) following manufacturer's instructions. OD 450 nm was obtained, and compared to a NAD+ standard curve.

ECM deposition assay

Cells grown in clear-bottom 96-well plates were fixed in 3.7% formalin (Sigma-Aldrich), blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) for 60

min and incubated with primary Collagen I (NB600-408, Novus Biologicals, Littleton, CO, USA) and Fibronectin (sc-9068, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies (1:200 dilution in blocking buffer, overnight, 4°C). The day after, the cells were stained with secondary goat anti-mouse IgG IRDye™ 800 antibody and goat anti-rabbit IgG IRDye™ 680 antibody (1:750 dilution, 45 min, RT). The microplates were scanned with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and the integrated fluorescence intensities were acquired using the software provided with the imager station (Odyssey Software Version 3.0, LI-COR Biosciences).

Transmission electron microscopy

Cells were fixed in in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Number of mitochondria per cell was performed by using ImageJ software version 1.43 (NIH, Bethesda, MD, USA). At least 9 cells for each condition were used to count the number of mitochondria in human lung fibroblasts.

Mitochondrial mass

Cells were incubated with 20 nM MitoTracker Green FM (Molecular Probes, Eugene, OR, USA) and DAPI (1:1000) for 30 minutes at 37ºC, 5% CO_2. FACS analysis was performed with the following strategy: debris exclusion (FSC-A by SSC-A), doublet exclusion (SSC-W by SSC-H and FSC-W by FSC-H) and dead cell exclusion (DAPI by MitotrackerGreen).Data were analyzed with FlowJo version 10.6 software (Tree Star Inc., Ashland, OR, USA).

To quantify the mtDNA/gDNA ratio, qPCR analysis was used to amplify one gene of the

mitochondrial genome (*MT-ATP6*) and one gene of the nuclear genome (*GAPDH*). DNA was extracted using the QiaAMP DNA Mini- Kit (Qiagen, Valencia, CA, USA) followed by Nanodrop concentration and purity analysis.

Mitochondrial respiration measurements

Oxygen consumption rate (OCR) was measured using Seahorse XF^{e} 24 Extracellular Flux Analyxer (Seahorse Bioscience, North Billerica, MA, USA) in both basal condition and after the injection of mitochondrial respiration inhibitors in the system. The inhibitors used are: oligomycin (ATP uncoupler), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (accelerates electron transport chain), antimycin A (complex III inhibitor), rotenone (complex I inhibitor). The use of these inhibitors allows the determination of basal respiration, ATP production, maximal respiration and spare respiratory capacity of the cell. Real time OCR was recorded using Wave Desktop 2.3 without secondary data normalization, as described elsewere [3].

Cytokine Protein Array

Supernatants were used as the cytokine array samples. Cytokine array was performed using the Human XL Cytokine Array kit (Proteome Profiler Array, R&D Systems, Minneapolis, MN, USA) for the detection of 105 different cytokines, according to the manufacturer's protocol. Spots were detected by using ChemiDoc (Bio-Rad, Hercules, CA, USA).

Transforming growth factor-β1 quantification (ELISA)

TGF-β1 in CM was quantified by using a human Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's recommendation. The results are expressed as pg/ml.

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