Stiff Matrix Instigates Type I Collagen Biogenesis by Mammalian Cleavage Factor I Complex-Mediated Alternative Polyadenylation

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Online Supplemental Materials

SUPPLEMENTAL METHODS

Antibodies and reagents

Anti-CFIm68 (Cat# A301-356A-T) and anti-CFIm59 (Cat# A301-359A) antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Anti-CFIm25 (Cat# 10322-1-AP) antibodies were purchased from Proteintech (Rosemont, IL). Anti-collagen I alpha I (Cat# NBP1-30054), anti-pro-collagen I alpha I (Cat# ABT257MI) and anti-Pdgfrα (Cat# 50-112-2095) antibodies, and actinomycin D were from Fisher Scientific (Hampton, NH). Anti-GFP antibodies (Cat# ab13970) were from Abcam (Cambridge, MA). Anti-fibronectin (Cat# sc-9068) and anti-GAPDH (Cat# sc-25778) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-αSma (Cat# 03-61001) antibody was from American Research Products (Waltham, MA). Validated CFIm68, CFIm59 and CFIm25 siRNAs were from Origene (Rockville, MD). LIVE/DEAD cell viability assay kit (Cat# L3224) was from ThermoFisher Scientific (Waltham, MA).

Isolation of primary lung fibroblasts

Lungs were minced in sterile phosphate-buffered saline and tissue pieces were placed in 100-mm tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/glutamine, nonessential amino acids, and sodium pyruvate (supplemented DMEM). Medium was replenished every 3 d. After 14 d, cells growing out of the explants were trypsinized and plated in supplemented DMEM. Lung fibroblasts were used between passages 6 and 10.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (ThermoFisher Scientific, Waltham, MA). 1 µg total RNA was reversely transcribed into cDNA with a cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA). Quantitative PCR reactions were carried out in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). Each sample was run in triplicate. Relative quantification was calculated using the comparative C_T method[1]. Delta CT values of target gene were normalized to GAPDH and subjected to statistical analysis. The ratio of distal transcripts/common transcripts was calculated as $\Delta CT = (CT_{distal} - CT_{GAPDH}) - (CT_{common} - CT_{GAPDH}) = CT_{distal} - CT_{common}$.

Construction of CFIm subunit-expressing lentiviruses and cell infection

The full-length cDNAs of human CFIm68, CFIm59, and CFIm25 corresponding to NCBI Reference sequence NM_007007.3, NM_001136040.3, and NM_007006.3 were amplified by PCR with primer pairs as follows: CFIm68, forward 5'-AAGCT AGCCA CCATG GCGGA CGGCG TGGAC CACAT AGAC-3', reverse 5'-GGACC GGTCG ATGAC GATAT TCGCG CTCTC GGTCA-3'; CFIm59, forward 5'-AAGCT AGCCA CCATG TCAGA AGGAG TGGAC TTGAT TGAT-3', reverse 5'-GGACC GGTTG GTGCC GGTCC CGTTC TCTA-3'; CFIm25, forward 5'-AAGCT AGCCA CCATG TCTGT GGTAC CGCCC AATCG CT-3', reverse 5'-GGACC GGTTT GTAAA TAAAA TTGAA CCTGC TCAAC-3'. The full-length cDNAs were subsequently cloned into a lentiviral expression vector, pLJM1-EGFP (Addgene, Cambridge, MA) and were confirmed by DNA sequencing. Viruses were packaged in 293T cells by co-transfection with lentiviral helper plasmids psPAX2 and pMD2.G (both from Addgene). Virus-containing conditioned medium was harvested 72 hrs after transfection, filtered, and used

to infect recipient lung fibroblasts in the presence of 8 µg/mL polybrene (Sigma, Louis, MO). Infected cells were selected with 2 µg/mL puromycin (Sigma, St. Louis, MO).

siRNA-mediated gene knockdown

100 pmol CFIm siRNAs or scrambled siRNAs were transfected into 1×10^6 lung fibroblasts by a Nucleofector device (Lonza, Basel, Switzerland) according to the manufacturer's protocol. Transfected cells were cultured in DMEM supplemented with 10% FBS for 72 h before harvesting.

Immunoblot and densitometry analysis

Cell lysates containing 10 - 40 µg total proteins were loaded onto SDS-polyacrylamide gels under reducing conditions. After electrophoresis, proteins were electrophoretically transferred from the gels to nitrocellulose at 100 V for 1.5 hr at 4°C. Membranes were blocked in casein solution (1% casein, 25 mM Na_2HPO_4, pH 7.1) for 1 hr at room temperature. Primary antibodies were diluted in TBS-T and casein solution (1:1) at a working concentration recommended by manufactures. Membranes were incubated with primary antibodies at room temperature for 1 hr. After extensive washing, membranes were incubated with peroxidase-conjugated secondary antibodies (0.1 µg/ml) diluted in TBS-T for 1 hr at room temperature. Immunodetection was carried out by chemiluminescence. Blot images were scanned. Bands were quantified by ImageJ (NIH, Bethesda).

AFM measurements of lung tissue elasticity

AFM probes were composed of silicon nitride coating with Au (TR400PB, spring constant ranging 10–50pN/nm) (Oxford Instruments Asylum Research Inc, Goleta, CA). The AFM system was calibrated according to the manufacturer's instruction before each indentation measurement, and the cantilever spring constant was confirmed by the thermal fluctuation method. Force-indentation profiles were acquired at an indentation rate of 20 μm/s separated by 4 μm spatially in a 16 × 16 sample grid covering a 20 × 20–μm area. The elasticity (Young's modulus) at each point on the grid was calculated from fitting force-indentation data using a Hertz-based model: $F = 2E\delta^2\tan(\alpha)/\pi(1-v^2)$, where indentation force (F) was calculated by using Hooke's law ($F = \kappa\Delta x$), where κ and Δx denote the AFM probe's spring constant and the probe's measured deflection, respectively. The indentation depth (δ) is calculated from the difference in the z-movement of the AFM piezo and the deflection of the probe. E is the elastic modulus of lung tissues being studied, and v denotes the Poisson ratio (0.4). α represents the shape of the probes that were considered to be conical with an approximated half-angle of 35 degrees in this report. A contour map was plotted to visualize spatial patterns of elasticity collected in Force-Map mode using the Matlab R2019a (MathWorks, Inc., Natick, MA).

Lung histology and immunofluorescent staining

Picrosirius red staining was performed to identify collagen content in mouse lung tissue samples according to the manufacturer's recommendation (Abcam, Cambridge, UK). Digital images of the stained sections were captured using an Olympus light microscopy.. Images were obtained with a Nikon Eclipse TE 300 microscope equipped with Spot Insight CCD camera and MetaMorph software version 6.2 r4 (Universal Imaging Corp., Downington, PA).

30 μm cryostat sections were rehydrated in PBS for 10 minutes. Tissue sections were blocked with 5% normal goat serum and co-stained with anti-αSma (1:200 dilutions) and anti-Pdgfrα (1:300 dilutions) antibodies diluted in PBS containing 1% goat serum, 0.3% Triton X-100, and 0.01% sodium azide according to manufacturer's instructions. Fluorochrome-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL) were used according to the manufacturer's recommendation. Nuclei were stained with DAPI (Thermo Fisher Scientific, Waltham, MA). Fluorescent signals were detected using a confocal laser-scanning microscope Zeiss LSM710 confocal microscope equipped with a digital color camera (Oberkochen, Germany).

SUPPLEMENTAL REFERENCES

1.   Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif* **25**, 402-408 (2001).