Transcriptomic Signature-Guided Depletion of Intermediate Alveolar Epithelial Cells Ameliorates Pulmonary Fibrosis in Mice

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Online Supplemental Materials

SUPPLEMENTAL METHODS

Antibodies and reagents

The following primary antibodies were used in this study: anti-Sprr1a (Thermo Fisher Scientific), anti-p16INK4a/CDKN2A (clone S04-8C9, Novus Biologicals), anti-p53 (Cell Signaling Technology), anti-p21 (Proteintech), anti-Ki67 (Abcam), anti-Collagen I (Novus Biologicals), anti-αSMA (American Research Products), anti-fibronectin (Proteintech), anti-GAPDH (Proteintech), anti-Krt8 (Thermo Fisher Scientific), anti-SFTPC (Abnova), anti-Krt5 (Proteintech), and anti-Krt17 (Proteintech). Secondary antibodies included Alexa Fluor 488-, 555-, and 647-conjugated goat anti-mouse or goat anti-rabbit IgG (Invitrogen). Additional reagents used were a 5'-FAM-labeled RNA fluorescence in situ hybridization probe targeting mSprr1a (NM_009264.2; Qiagen), X-gal substrate (Invitrogen), and diphtheria toxin (Sigma-Aldrich).

Plasmids

To construct mRNA expression vectors, cDNAs encoding mouse *Sftpc*, *Pdpn*, and *Sprr1a* were PCR-amplified from mouse lung cDNA and cloned downstream of and in-frame with *mCherry* in the lentiviral vector pCL245, generously provided by Dr. Chao Li (University of Alabama at Birmingham). In this construct, the *EF1α* promoter drives *mCherry* expression. Amplified fragments were inserted into BamHI-linearized pCL245 using the Gibson Assembly Cloning Kit (NEB, E5510S). Primer sequences used for amplification are listed in **Supplementary Table 2**. AAV vectors for sesRNA expression were derived from a previously reported AAV backbone (Addgene plasmid #184943). The vectors were linearized with the AgeI restriction enzyme, and synthesized sesRNA fragments were inserted using Gibson Assembly.

Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured at 37°C in a humidified incubator with 5% CO_2. For plasmid transfection, cells were seeded into 12-well plates at a density of 1×10^5 cells per well and allowed to adhere overnight, reaching approximately 70–80% confluency. Transfections was carried out using polyethyleneimine (PEI; Polysciences) following the manufacturer's instructions. Briefly, 1 µg of plasmid DNA was diluted in 100 µl of Opti-MEM (Gibco), and 3 µl of PEI was diluted in a separate 100 µl of Opti-MEM. The two solutions were combined, incubated at room temperature for 10–15 minutes to allow complex formation, and then added dropwise to the cells. After 6 hours of incubation, the transfection medium was replaced with fresh complete DMEM. Cells were harvested 24–72 hours post-transfection for downstream applications, including immunoblotting, fluorescence imaging, or RNA extraction.

Immunoblot and densitometry analysis

Cell lysates containing 10 - 20 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).

Fluorescence-activated cell sorting (FACS)

Whole-lung single-cell suspensions containing EGFP+ and/or tdTomato+ cells were prepared from enzymatically dissociated mouse lung tissue using established protocols. Following dissociation, cell suspensions were filtered sequentially through 100 μm and 40 μm strainers to remove debris. Cells were resuspended in cold FACS buffer consisting of PBS supplemented with 2% fetal bovine serum (FBS) and 2 mM EDTA, and maintained on ice throughout the procedure. Fluorescently labeled cells were isolated using a BD FACSymphony S6 cell sorter (BD Biosciences). Live single cells were identified and gated based on forward and side scatter profiles. EGFP and tdTomato fluorescence were detected in the FITC and PE-Texas Red channels, respectively. Sorted cells were collected directly into tubes containing FBS and kept on ice for immediate downstream applications.

Annexin V apoptosis assay

Apoptosis analysis of EGFP-labeled *Sprr1a*+ cells was performed using an Annexin V-APC/Propidium Iodide (PI) Apoptosis Detection Kit (Thermo Fisher, A35110), according to the manufacturer's instructions. After FACS sorting, EGFP+ cells were pelleted by gentle centrifugation (300 × g, 5 minutes), washed twice in cold PBS, and resuspended in 1× binding buffer at a concentration of approximately 1×10^6 cells/ml. To stain for apoptotic cells, 100 μl of

the cell suspension was transferred into a flow cytometry tube, followed by the addition of 5 µl of Annexin V-APC and 5 µl of propidium iodide solution. Samples were gently mixed and incubated for 15 minutes at room temperature in the dark. After incubation, 400 µl of 1× binding buffer was added to each tube, and samples were analyzed immediately by flow cytometry using a FACSymphony A3 instrument (BD). Data were analyzed using FlowJo software. Live cells were defined as Annexin V-/PI-, early apoptotic cells as Annexin V+/PI-, late apoptotic or necrotic cells as Annexin V+/PI+, and dead cells as Annexin V-/PI+. EGFP+ gating was used to restrict analysis specifically to the target cell population.

Cytospin preparation

Sorted EGFP+ cells were washed with PBS and resuspended at a concentration of 1×10^5 cells/mL. A 200 µl aliquot of the cell suspension was loaded into cytospin chambers and centrifuged at 500 rpm for 5 minutes using a Thermo Shandon Cytospin 4 cytocentrifuge. Cells were deposited onto Superfrost Plus microscope slides and immediately air-dried. Slides were then fixed with 4% paraformaldehyde (PFA) and subsequently processed for either β-galactosidase staining or immunofluorescence analysis.

β-Galactosidase (β-gal) staining

Senescence-associated β-galactosidase (SA-β-gal) activity in freshly isolated cells was assessed using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Sigma, 9630). Freshly isolated cells were cytospun onto slides using a Statspin Cytofuge, fixed, and immediately stained with X-gal. SA-β-gal-positive cells (blue staining) were counted under a Nikon Eclipse Ts2 Microscope and expressed as a percentage of total cells.

Fluorescence and confocal microscopy

Mouse lung tissue sections or cytospun slides were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature, followed by washing with PBS. Permeabilization was performed using 0.2% Triton X-100 in PBS for 10 minutes, after which slides were blocked with 5% normal goat serum in PBS for 1 hour at room temperature. Primary antibodies, diluted in blocking buffer, were applied and incubated overnight at 4 °C in a humidified chamber.

The following day, slides were washed three times with PBS and incubated with fluorophore-conjugated secondary antibodies for 1 hour at room temperature. After final washes, nuclei were counterstained with DAPI, and slides were mounted using antifade mounting medium. Fluorescence signals were detected using a confocal laser-scanning microscope Zeiss LSM710 confocal microscope equipped with a digital color camera (Oberkochen, Germany). All fluorescent images were acquired using sequential laser scanning, with only the corresponding single-wavelength laser line activated through acousto-optical tunable filters, to avoid cross-detection between fluorescence channels.

Lung histology, TUNEL assay, and immunofluorescent staining

Masson trichrome staining was performed to evaluate collagen deposition in mouse lung tissues according to the manufacturer's recommendation (Polysciences Inc, Warrington, PA, USA). Digital images of the stained sections were captured using a Nikon Eclipse TS2 microscope equipped with DS-Fi3 camera. For immunofluorescence staining, 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-Sftpc (1:200 dilutions), anti-Krt8 (1:200 dilutions), anti-p21

(1:200 dilutions), anti-p16 (1:200 dilutions), anti-p53 (1:200 dilutions), anti-Ki67 (1:100 dilutions), anti-Krt17 (1:200 dilutions), or anti-Sprr1a (1:200 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 (Invitrogen) were used according to the manufacturer's recommendation. TUNEL assay was performed with TdT-fluor kit (Trevigen). For co-staining, TUNEL was followed by IF using primary antibodies and fluorophore-conjugated secondaries. 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).

Hypoxyproline assay

Right lungs were homogenized in water, precipitated with TCA, hydrolyzed in 12 N HCl at 110°C for 14–18 h, and redissolved. Samples were incubated with chloramine T and Ehrlich's reagent, and OD measured at 550 nm.

Hydroxyproline assay

Lung collagen content was determined by an assay for hydroxyproline as previously described[1]. Briefly, Mouse right lungs were homogenized in 2.0 ml distilled water and incubated with 125 µl of 50% trichloroacetic acid on ice for 20 min. Samples were centrifuged and the pellets were mixed with 1 ml 12 N hydrochloric acid and baked at 110°C for 14-18 h. Dry samples were dissolved in 2 ml deionized water. 200 µl samples (or standards) were added to 500 µl 1.4% chloramine T (Fisher Scientific, Cat. AAA1204430) in 0.5 M sodium acetate/10%

isopropanol and incubated for 20 min at room temperature. 500 µl Ehrlich's solution (Sigma, Cat. 03891) was added, mixed, and incubated at 65°C for 15 min. Optical density of each sample and standard was measured at 550 nm and the concentration of lung hydroxyproline was calculated from a hydroxyproline standard curve.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: ***SPRR1A* is selectively expressed by KRT5-KRT17+ aberrant basaloid cells in human IPF**. Expression of *SPRR1A* across major lung cell populations in IPF and control subjects was analyzed from three independent scRNA-seq datasets available in the IPF Cell Atlas.

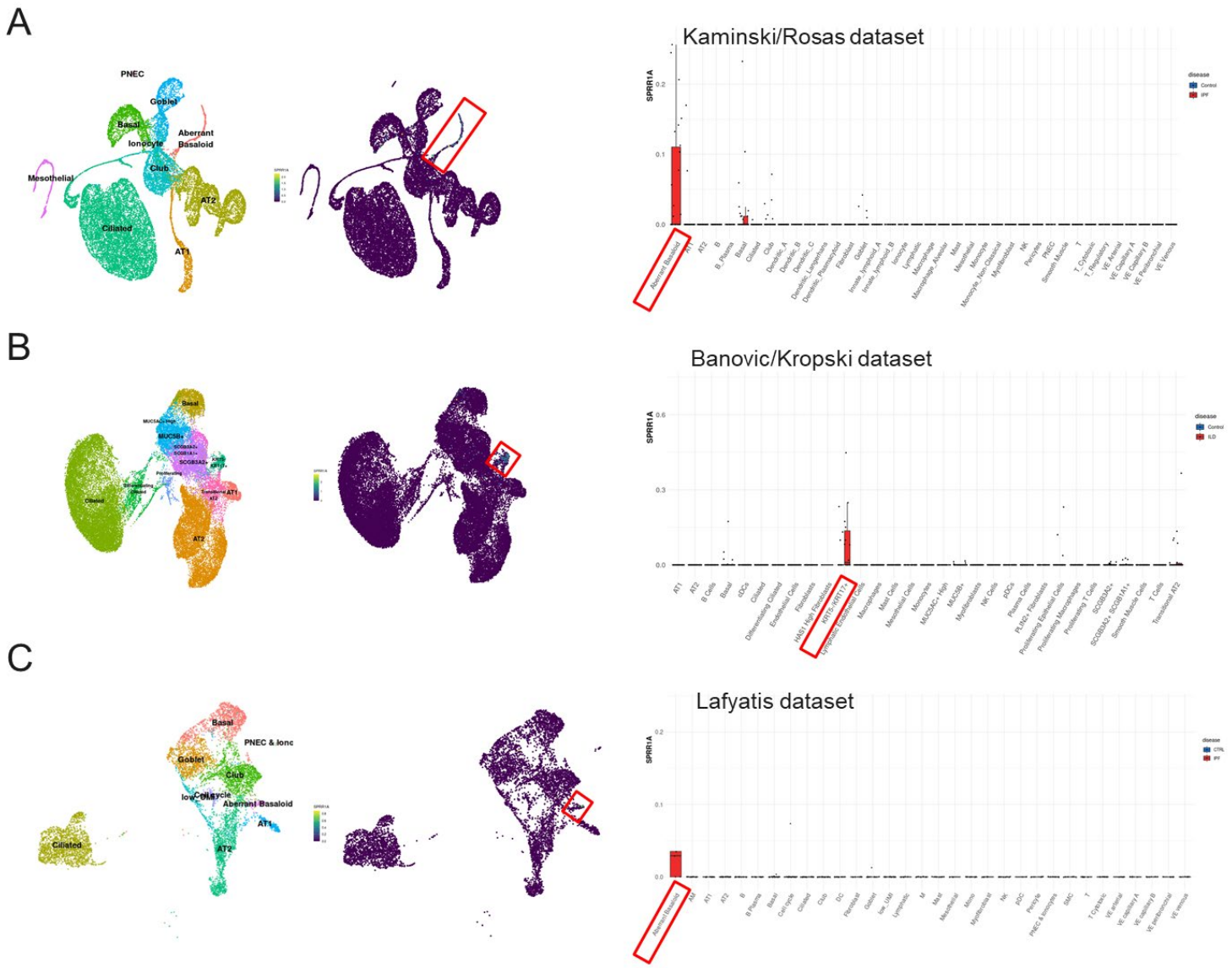
Supplemental Figure 2: ***Sprr1a*+ cells undergo spontaneous apoptosis during lung repair and regeneration following bleomycin injury in mice**. **A**, Flow cytometry analysis of ZsGreen1-labeled *Sprr1a*+ cells isolated from C57BL/6 mouse lungs at Days 12, 15, 17, 19, 24, and 29 after bleomycin administration; **B**, Quantification of apoptotic *Sprr1a*+ cells at each time point. Data are presented as mean ± SEM from 3 mice per group. *$p < 0.05$

Supplemental Table 1. SesRNA sequences used to target specific alveolar epithelial cell types

Sftpc SesRNA	5'-AGGAACAGGG TGGCAGGGAT CCTTGGTTTT GGGGGCACAA GGTTCCTGGG GTAGTGGCTG GCAAGGCTGC TTCACCTGCT GCTGGTGCAG CTGAGGAGGT AC-3'
Pdpn SesRNA	5'-TCTCGTTGGA GCCTCAGCAG CTACTGGGTT TTTTTGTGGG TTGTGAAAAG ATCAAAAACA AAACAAAACA AAACAAAAAA CTTCGAAGCC ACAGGCCCAG CCGGGAAGAG CTCGGGAGGA GGTTAGAAGG CGCTGAGCAA AAGTCTCAGC GCCAGAGAGT GCCCGGGCTT TCTGGACTCG CAGAGGCAGC AAGCACAGCA AACTTGGCAG CTGGCAACCT CTCGGCACAG TCGGCATTTA TGTCTCCGGT CCCGGGGCGG GAGCGGGTGG GCGTCAGGTG GTCCCGGAGA TCTAGGACCG-3'
Sprr1a SesRNA	5'-AGGAACAGGG TGGCAGGGAT CCTTGGTTTT GGGGGCACAA GGTTCCTGGG GTAGTGGCTG GCAAGGCTGC TTCACCTGCT GCTGGTGCAG CTGAGGAGGT AC-3'

Supplemental Table 2. Primer sequences used to amplify target mRNAs from mouse lungs

Sftpc	Forward: 5'-GTGGAGGAGA ATCCCGGCCC TGGATCCGGA CATGAGTAGC AAAGAGG-3'
	Reverse: 5'-ATACCGTCGA GATTAATTAA AGGATCCCAG GCTGCTTTAT TCTTTTGTG-3'
Pdpn	Forward: 5'-GTGGAGGAGA ATCCCGGCCC TGGATCCCGG TCCTAGATCT CCGGGA-3'
	Reverse: 5'-ATACCGTCGA GATTAATTAA AGGATCCAGG AAGAGGATGG GGAACAG-3'
Sprr1a	Forward: 5'-GTGGAGGAGA ATCCCGGCCC TGGATCCATG AGTTCCCACC AGCAGAA-3'
	Reverse: 5'-ATACCGTCGA GATTAATTAA AGGATCCCAT ACACTTGTCT CACTCCTC-3'



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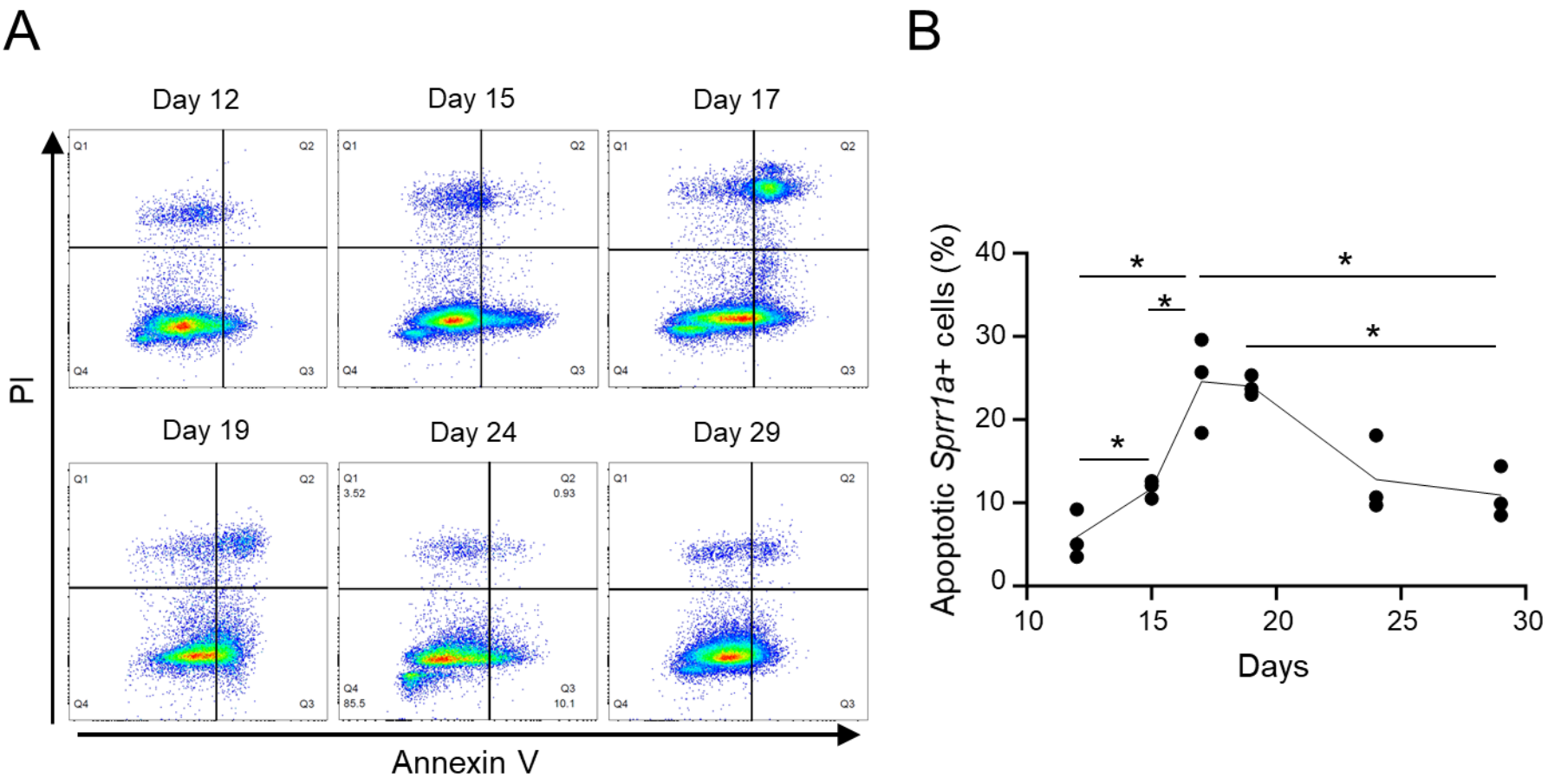

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