

Supplemental information

The CCL20–integrin $\alpha 5\beta 1$ interaction enhances TGF- β /Smad signaling to promote fibroblast activation in pulmonary fibrosis

Authors

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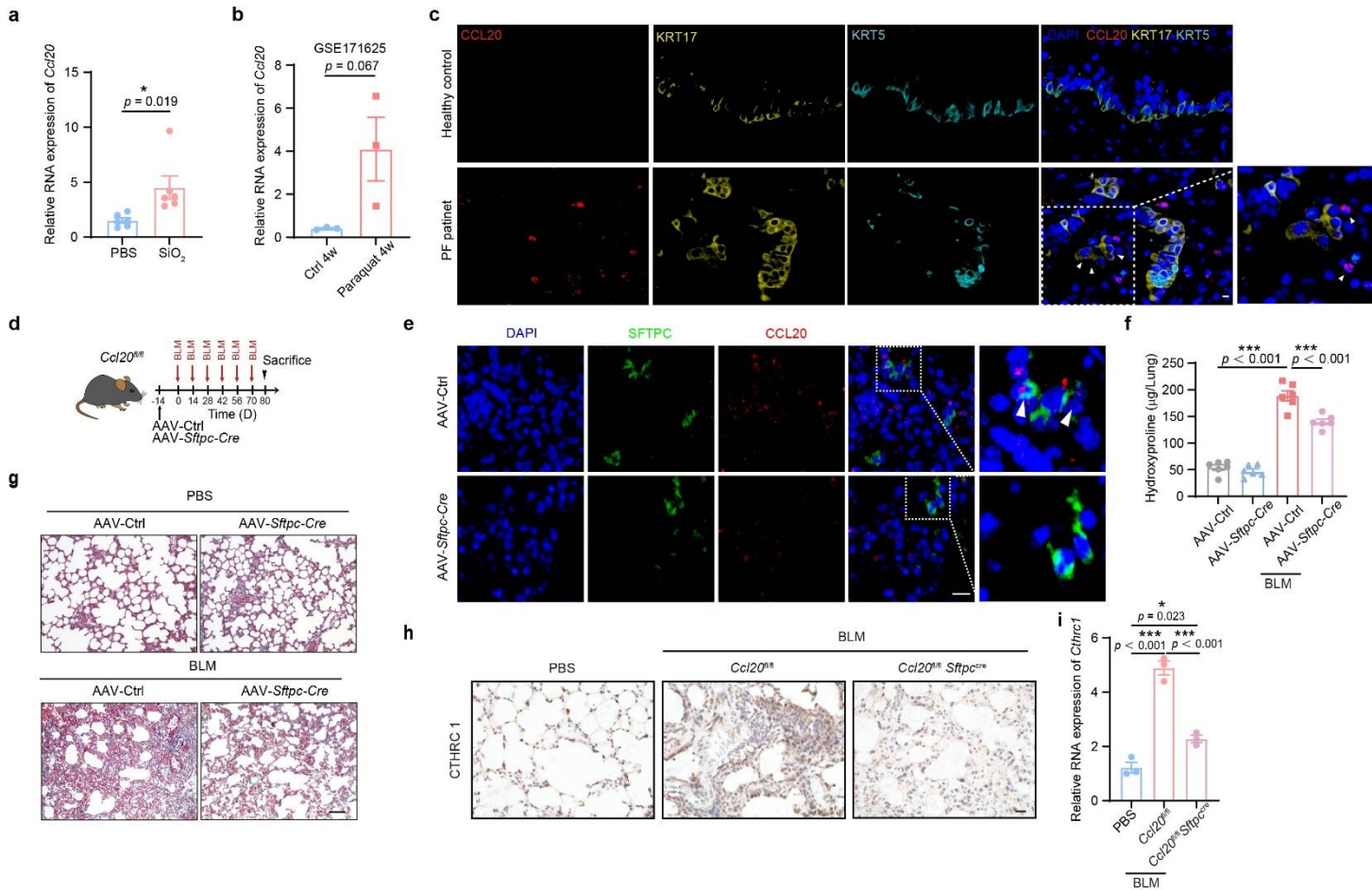
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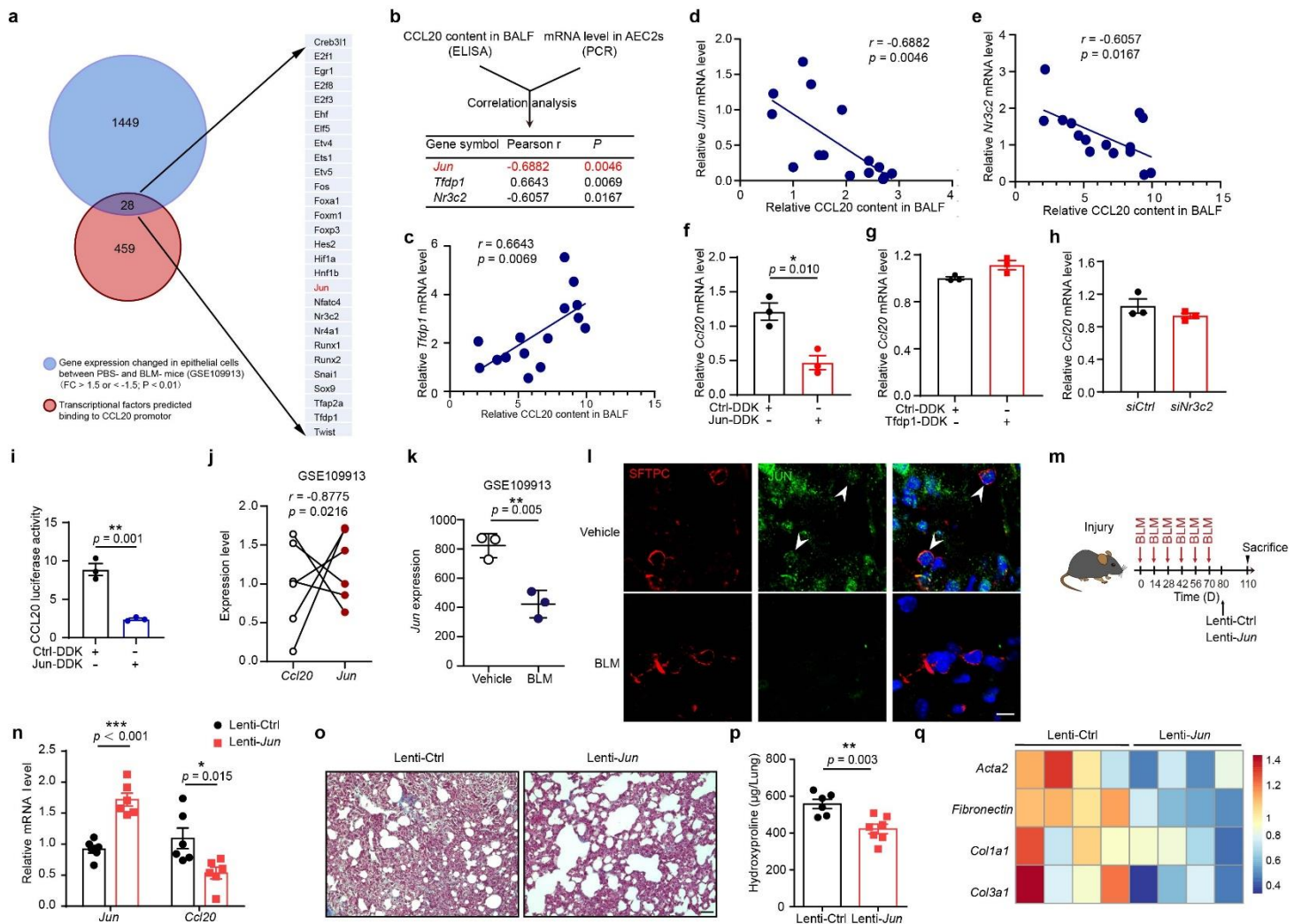


Supplementary Figure 1. CCL20 is secreted by AEC2s in PF. **a** mRNA expression levels of *Ccl20* in lung tissues from control and SiO₂-exposed mice (n = 6 samples per group). **b** *Ccl20* expression in lung tissues from control or paraquat-challenged mice. Data were obtained from an open dataset (GSE171625) (n = 3 samples per group). **c** IF of CCL20, KRT17 and KRT5 in lung tissues from patients with PF and age-matched controls. Scale bars, 20 μm. **d** Schematic overview of the experimental design for (e–g). **e** IF of SFTPC and CCL20 in lung tissues from AEC2-specific *Ccl20* knockout (KO) mice and controls. Scale bars, 10 μm. **f** Hydroxyproline content in lung tissues from the indicated mice (n = 6 samples per group). **g** Masson staining of lung tissues from the indicated mice. Scale bars, 50 μm. **h** IHC staining of CTHRC1 from the lung tissue of the indicated mice. Scale bars, 20 μm. **i** RT–PCR analysis of *Cthrc1* expression in primary lung fibroblasts from the indicated mice (n = 3 samples per group). The data are presented as

35 means $\pm$ SEM. The data in (a) and (b) were analyzed by two-tailed Student's t test. The data in (f) and (i)
36 were analyzed by one-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001. Source data are provided as a
37 Source Data file.

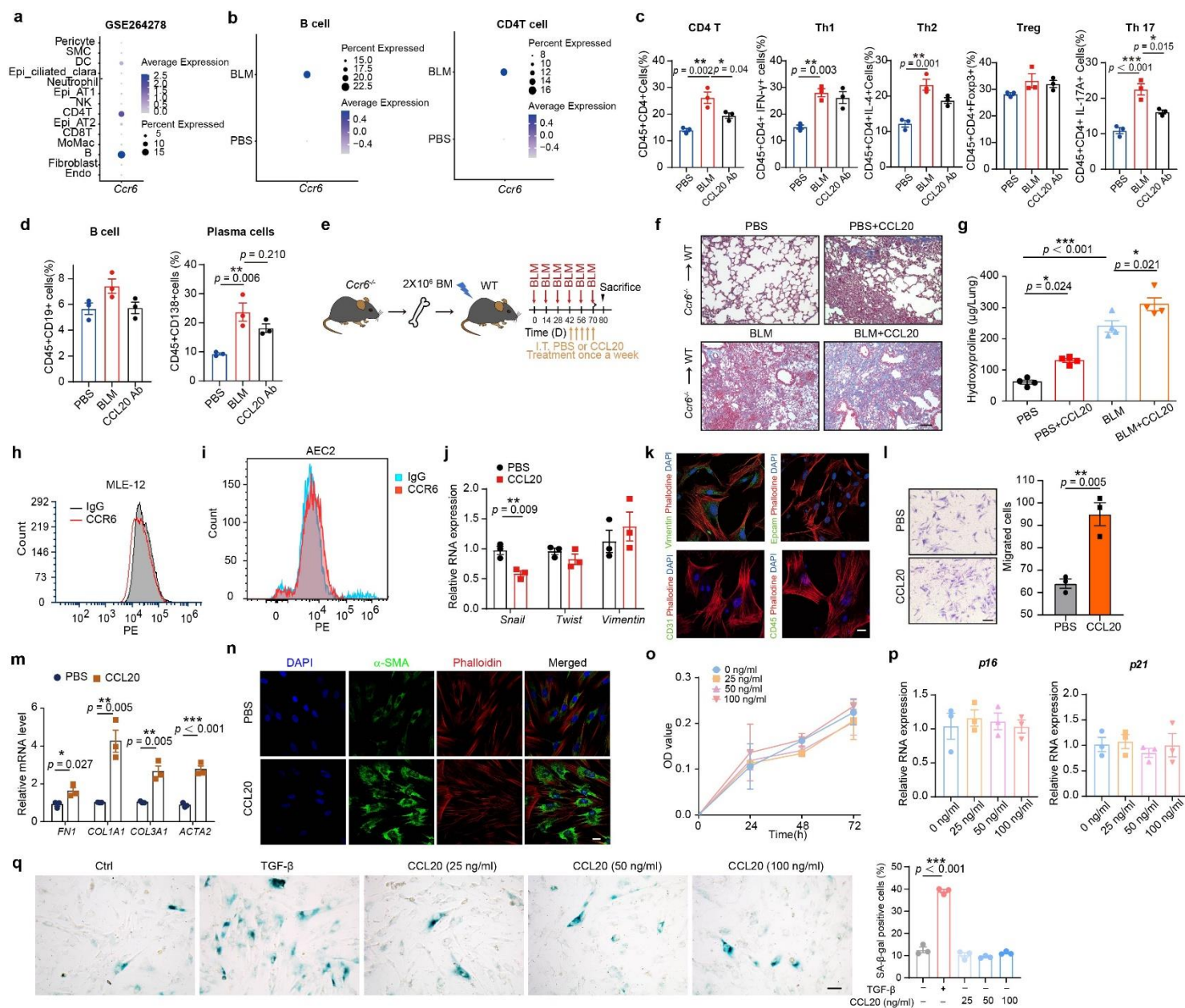
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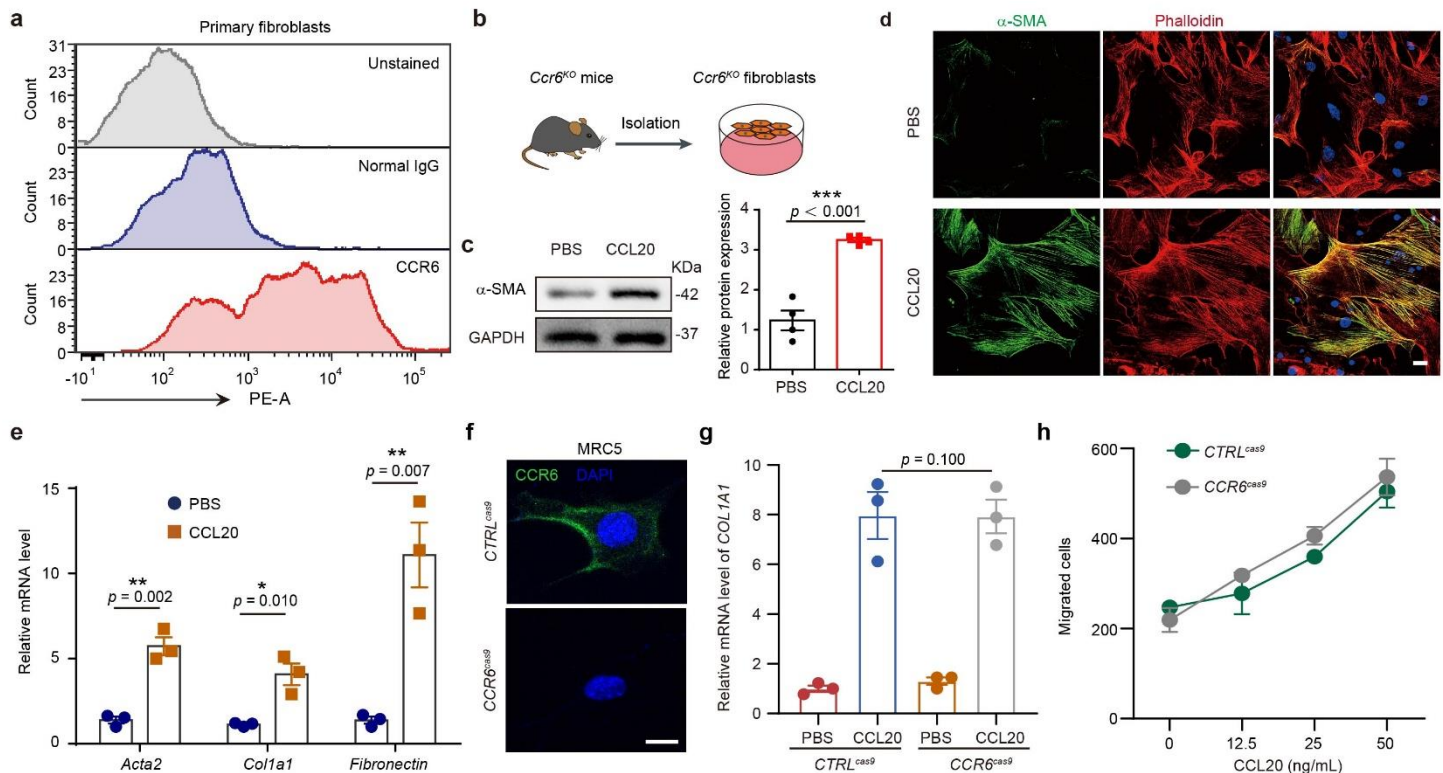
Supplementary Figure 2. JUN negatively regulates CCL20 expression in AEC2s. **a** Venn diagram of genes whose expression changed in lung epithelial cells from PBS- and BLM-treated mice and transcription factors predicted to bind to the CCL20 promoter. **b** Correlation analysis between the CCL20 content in the BALF and the expression of transcription factors in AEC2s. **c** Correlation analysis between CCL20 content in BALF and relative *Jun* expression in AEC2s (n = 15 samples per group). **d** Correlation analysis between the CCL20 content in BALF and the relative *Nr3c2* mRNA level in AEC2s (n = 15 samples per group). **e** Correlation analysis between the CCL20 content in the BALF and the relative *Tfdp1* mRNA level in AEC2s (n = 15 samples per group). **f** Relative *Ccl20* expression in MLE-12 cells with or without *Jun* overexpression (n = 3 technical replicates per group). **g** Relative *Ccl20* expression in *Tfdp1*-

50 overexpressing MLE-12 cells (n = 3 technical replicates per group). **h** Relative *Ccl20* expression in *Nr3c2*-
51 silenced MLE-12 cells. **i** CCL20 luciferase activity in MLE-12 cells with or without *Jun* overexpression (n
52 = 3 technical replicates per group). **j** Correlations between *Ccl20* and *Jun* expression levels in alveolar
53 epithelial cells from PBS- and BLM-challenged mice. Data were obtained from an open dataset
54 (GSE109913) (n = 6 samples per group). **k** *Jun* expression in alveolar epithelial cells from PBS- and BLM-
55 challenged mice. Data were obtained from an open dataset (GSE109913) (n = 3 samples per group). **l** IF
56 staining of SFTPC and JUN in lung tissues from PBS- and BLM-challenged mice. Scale bar, 20 μ m. **m**
57 Schematic overview of the experimental design for (**n–q**). **n** *Jun* and *Ccl20* expression in lung tissue from
58 PBS- and BLM-challenged mice intratracheally injected with Lenti-control or Lenti-*Jun* virus (n = 6 samples
59 per group). **o** Masson staining of lung tissues from the indicated mice. Scale bar, 100 μ m. **p** Hydroxyproline
60 content in lung tissues from the indicated mice (Lenti-*Ctrl*, n = 6; Lenti-*Jun*, n = 7). **q** Expression levels of
61 *Col1a1*, *Col3a1*, *fibronectin* and *Acta2* in lung tissues from the indicated mice (n = 4 samples per group).
62 The data are presented as means $\pm$ SEM. Data were analyzed by two-tailed Student's t tests in (**f–i**), (**k**),
63 (**n**) and (**p**) and Two-tailed Pearson correlation in (**b–e**) and (**j**). *p < 0.05; **p < 0.01; ***p < 0.001. Source
64 data are provided as a Source Data file.

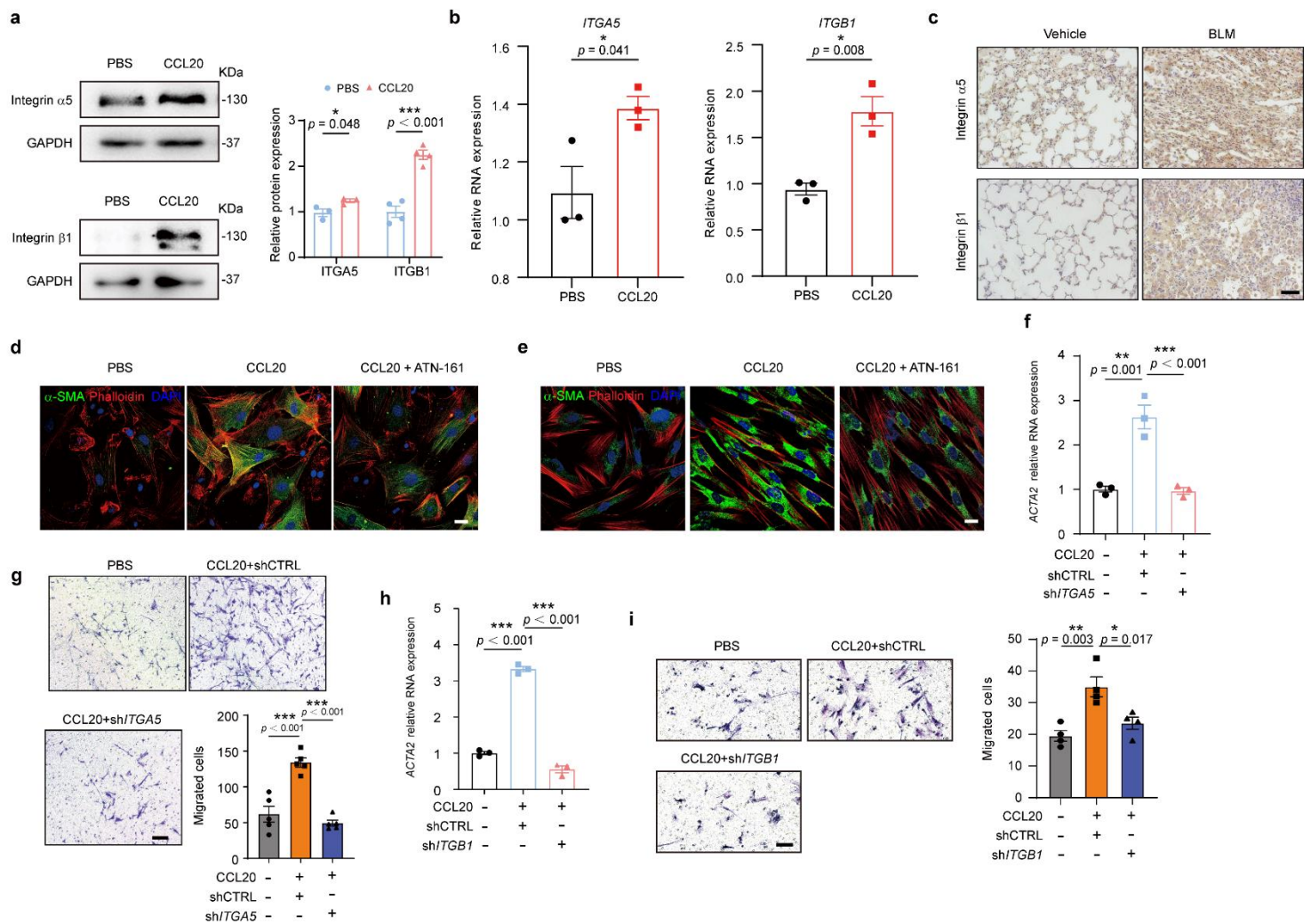


Supplementary Figure 3. CCL20 promotes PF by activating lung fibroblasts. **a** Dot plot visualization of *Ccr6* in different cell types. **b** Dot plot visualization of *Ccr6* in B cells and CD4⁺ T cells between BLM-induced PF and control lungs. Data were obtained from an open dataset (GSE264278). **c** Percentages of CD4⁺ T cells and Th1, Th2, Treg and Th17 subsets in the lungs of the indicated groups (n = 3 samples per group). **d** Percentages of B cells and plasma cells in lungs from the indicated groups. **e** Scheme of BM chimeric mouse construction and experimental design (n = 3 samples per group). **f**, **g** Masson staining (**f**)

and hydroxyproline content (**g**) in the lungs of the indicated mice. Scale bars, 200 μ m. **h** Flow cytometry analysis of CCR6 expression in MLE-12 cells. **i** Flow cytometry analysis of CCR6 expression in AEC2s. **j** Quantitative analyses of the mRNA levels of the indicated EMT-associated genes by RT-PCR in MLE-12 cells treated with or without CCL20 (n = 3 technical replicates per group). **k** IF staining of vimentin, EpCAM, CD45, CD31 or phalloidin in isolated lung fibroblasts. Scale bars, 20 μ m. **l** Migration analysis of MRC-5 cells treated with or without CCL20 (n = 3 technical replicates per group). Scale bars, 100 μ m. **m** PCR analysis of the mRNA levels of genes associated with fibrosis in MRC-5 cells treated with or without CCL20 (n = 3 technical replicates per group). **n** IF of α -SMA in MRC-5 cells treated with or without CCL20. Scale bars, 20 μ m. **o** CCK-8 analysis of MRC-5 cells after CCL20 stimulation (n = 3 technical replicates per group). **p** Relative expression of *p16* and *p21* in MRC-5 cells after CCL20 stimulation (n = 3 technical replicates per group). **q** β -Gal staining of MRC-5 cells after CCL20 stimulation (n = 3 technical replicates per group); scale bars, 50 μ m. The data are presented as the means $\pm$ SEM. The data were analyzed by two-tailed Student's t tests in (**j**), (**i**), and (**m**). The data in (**b**, **c**, **g**, **j**, **p**, **q**) were analyzed by one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001. Source data are provided as a Source Data file.

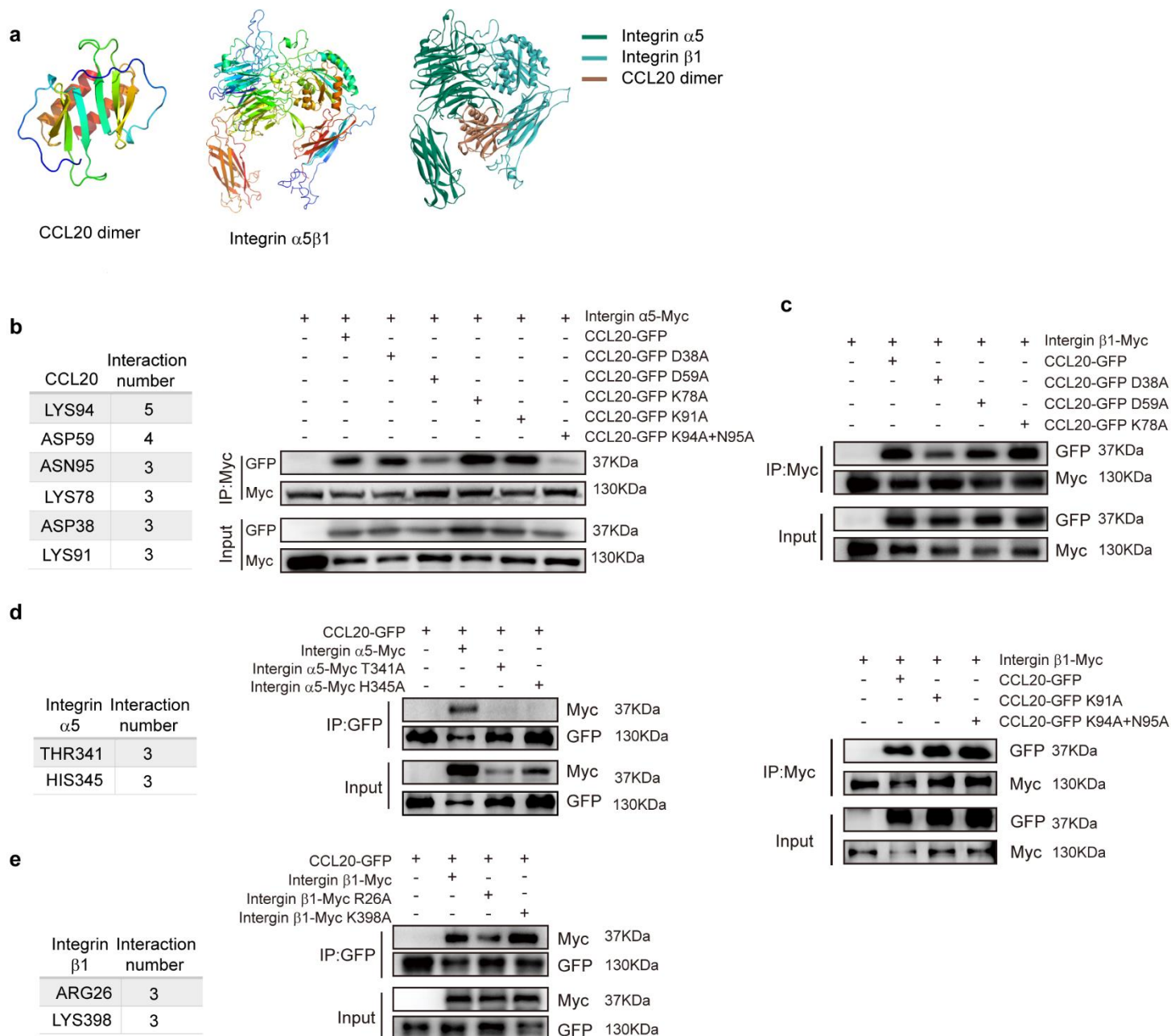


Supplementary Figure 4. CCL20 activates lung fibroblasts independent of CCR6. **a** Flow cytometry analysis of CCR6 expression in primary mouse lung fibroblasts. **b** Schematic diagram of the isolation of primary lung fibroblasts from *Ccr6*^{KO} mice. **c** Immunoblotting of α -SMA in primary lung fibroblasts isolated from *Ccr6*^{KO} mice (n = 3 samples per group). **d** IF of α -SMA in primary lung fibroblasts isolated from *Ccr6*^{KO} mice. Scale bars, 20 μ m. **e** Expression of *Acta2*, *Col1a1* and *fibronectin* in primary fibroblasts treated with or without CCL20 after CCR6 knockout (n = 3 technical replicates per group). **f** IF of CCR6 in control or CCR6 knockout MRC-5 cells. Scale bars, 10 μ m. **g**, **h** Expression of COL1A1 (**g**) and migration ability analysis (**h**) of control or CCR6 knockout MRC-5 cells in the presence of CCL20 (n = 3 technical replicates per group). The data are presented as the means $\pm$ SEM. The data were analyzed by two-tailed Student's t test in (**e**). The data in (**g**) were analyzed by one-way ANOVA. n.s., not significant; ** $p < 0.01$; *** $p < 0.001$. Source data are provided as a Source Data file.



Supplementary Figure 5. CCL20 binds to integrin $\alpha 5\beta 1$ to activate lung fibroblasts. **a** Immunoblotting of integrin $\alpha 5$ and integrin $\beta 1$ in MRC-5 cells in response to CCL20 stimulation (n = 3 technical replicates per group). **b** Expression of *ITGA5* and *ITGB1* in MRC-5 cells in response to CCL20 stimulation (n = 3 technical replicates per group). **c** IHC staining of integrin $\alpha 5$ and integrin $\beta 1$ in lung tissues from PBS- or BLM-challenged mice. Scale bar, 100 μ m. **d** IF of α -SMA in primary lung fibroblasts treated with or without ATN-161 in the presence of CCL20. Scale bars, 20 μ m. **e** IF of α -SMA in MRC-5 cells treated with or without ATN-161 in the presence of CCL20. Scale bars, 20 μ m. **f** Quantitative analysis of *ACTA2* mRNA expression in CCL20-treated MRC-5 cells transfected with Lv-shCTRL or Lv-sh/*TGA5* virus (n = 3

12 technical replicates per group). **g** Migration analysis of CCL20-treated MRC-5 cells transfected with Lv-
13 shCTRL or Lv-sh/*TGA5* virus (n = 5 technical replicates per group). Scale bars, 20 μ m. **h** Quantitative
14 analysis of *ACTA2* in CCL20-treated MRC-5 cells transfected with Lv-shCTRL or Lv-sh/*TGB1* virus (n = 3
15 technical replicates per group). **i** Migration analysis of CCL20-treated MRC-5 cells transfected with Lv-
16 shCTRL or Lv-sh/*TGB1* virus (n = 4 technical replicates per group). Scale bars, 50 μ m. The data are
17 presented as the means $\pm$ SEM. The data were analyzed by two-tailed Student's t tests (**a–b**) and one-
18 way ANOVA (**f–i**). *p < 0.05; **p < 0.01; ***p < 0.001. Source data are provided as a Source Data file.



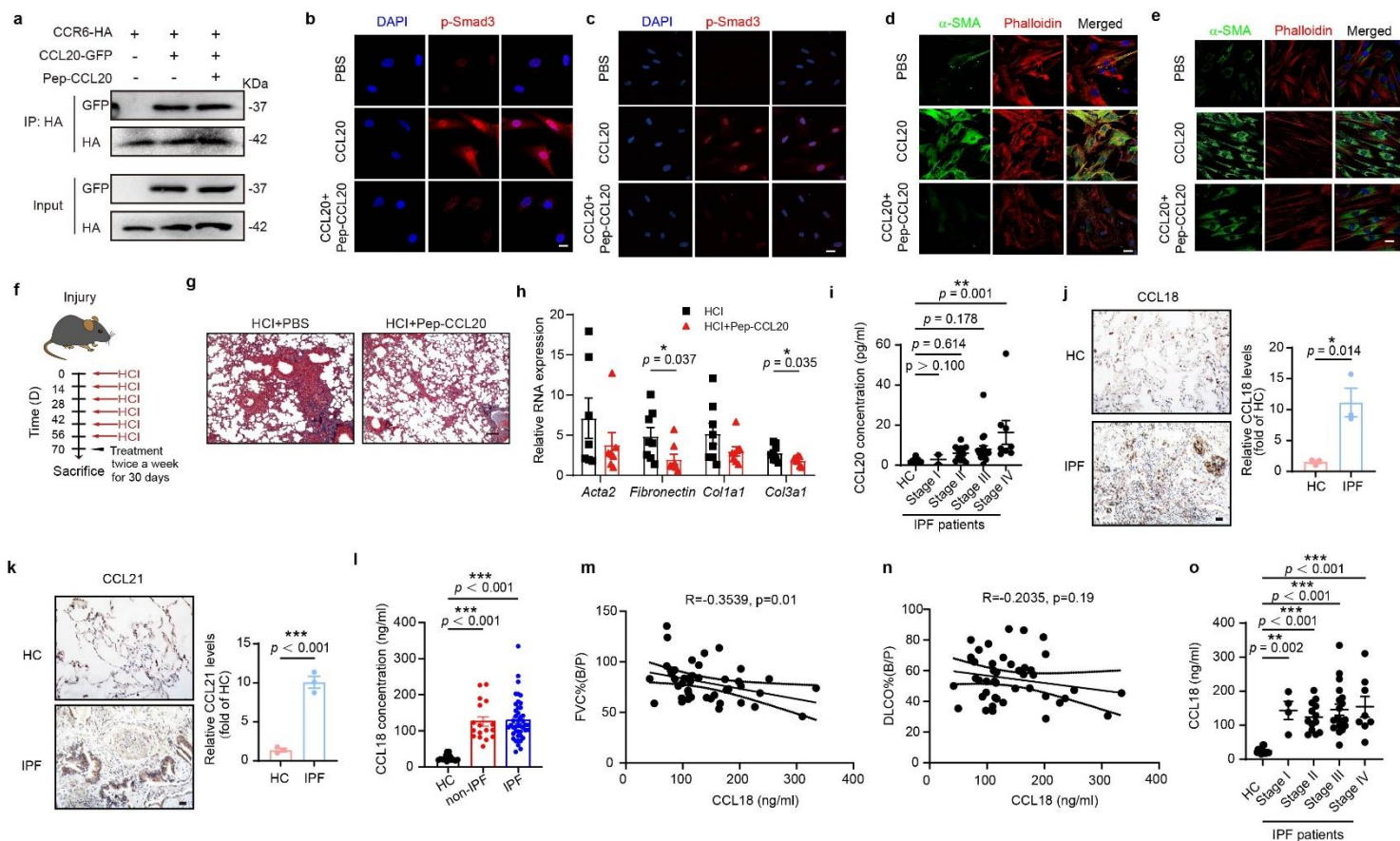
Supplementary Figure 6. Identification of the binding sites of CCL20 with integrin α5 or integrin β1.

a Model interaction between CCL20 and integrin α5β1 using the crystal structures of CCL20 (PDB: 2HCI) and integrin α5β1 (PDB: 3VI3) as templates. **b** Mapping of the CCL20 amino acid site involved in integrin α5β1 binding. Sample blot showing the interaction of each mutant with integrin α5. **c** Sample blot showing the interaction of the CCL20 mutant with integrin β1. **d** Mapping of integrin α5 amino acid sites involved in

27 CCL20 binding; sample blot showing the interaction of each mutant with CCL20. **e** Mapping of integrin β 1
28 amino acid sites involved in CCL20 binding; sample blot showing the interaction of each mutant with
29 CCL20. Source data are provided as a Source Data file.

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Supplementary Figure 7. Disruption of the CCL20 and integrin $\alpha 5 \beta 1$ interaction reduces PF. **a** IP assay analyzing the interaction between CCL20 and CCR6 in the presence of Pep-CCL20. **b, c** IF of phosphorylated Smad3 in primary lung fibroblasts (**b**) and MRC-5 cells (**c**) treated with or without Pep-CCL20 in response to CCL20 stimulation. Scale bars, 20 μ m. **d, e** IF of α -SMA in primary lung fibroblasts (**d**) and MRC-5 cells (**e**) treated with or without Pep-CCL20 in response to CCL20 stimulation. Scale bars, 20 μ m. **f** Schematic diagram for evaluating the therapeutic effects of Pep-CCL20 in the HCL-induced PF model. **g, h** Masson staining (**g**) and mRNA expression of genes associated with fibrosis (**h**) ($n = 7$ samples per group for *Acta2*; $n = 8$ samples per group for *Fibronectin*, *Col1a1*, *Col3a1*) in the lungs of PBS- or Pep-CCL20-treated PF mice. Scale bars, 200 μ m (**g**). **i** Graph showing the concentrations of CCL20 in the serum of healthy subjects and IPF patients at different stages based on DLCO% predicted ($n = 13$ samples for HC; $n = 2$ samples for stage1; $n = 13$ samples for stage2; $n = 19$ samples for stage3; $n = 8$ samples

for stage4). **j, k** IHC staining of CCL18 (**j**) and CCL21 (**k**) in lung tissues from patients with PF and age-matched controls (n = 3 samples per group). Scale bars, 50 μ m. **l** ELISA analysis of the CCL18 content in the serum of patients with IPF (n = 44 samples), non-IPF ILD patients (n = 17 samples), and healthy controls (n = 13 samples). **m, n** Analysis of the correlation between CCL18 levels and lung function in IPF patients (n = 44 samples), as measured by the FVC% (**m**) and DLCO% (**n**). **o** Graph showing the concentrations of CCL18 in the serum of healthy subjects and IPF patients at different stages based on DLCO% Predicted (n = 13 samples for HC; n = 4 samples for stage1; n = 13 samples for stage2; n = 19 samples for stage3; n = 8 samples for stage4). The data are presented as the means $\pm$ SEM. The data were analyzed by two-tailed Student's t test in (**h, j, k**). The data in (**i, l, o**) were analyzed by one-way ANOVA and Two-tailed Pearson correlation in (**m, n**) *p < 0.05; **p < 0.01. Source data are provided as a Source Data file.

Supplementary Table 1. Characteristics of the serum donors.

Characteristics	HCs	IPF patients	non-IPF ILD patients
	(n = 13)	(n = 44)	(n = 17)
Age, years	64.15 ± 9.75	63.73 ± 7.15	58 ± 9.77
Male/female (n)	10/3	39/5	8/9
ILD diagnosis			
IPF	/	44	0
CTD-ILD	/	0	11
NSIP	/	0	2
IPAF	/	0	4
Pulmonary function			
FVC, % predicted	/	79.49 ± 18.49	81.91 ± 21.97
DLCO, % predicted	/	54.86 ± 15.25	54.74 ± 22.39
HRCT patterns			
UIP	/	39	4
NSIP	/	5	13
Comorbidities			
Chronic bronchitis	0	3	0
Diabetes	0	8	1
Hypertension	4	13	3
Coronary heart disease	0	2	0
Gouty nephropathy	0	1	0
Osteoarthritis	0	1	0

58 The data are presented as the means $\pm$ SEM or medians (interquartile ranges).
59 Abbreviations: HCs, healthy controls; IPF, idiopathic pulmonary fibrosis; ILD, interstitial lung disease; FVC,
60 forced vital capacity; DLCO, diffusion capacity of the lung for carbon monoxide; UIP, usual interstitial
61 pneumonia; NSIP, non-specific interstitial pneumonia; CTD-ILD, connective tissue disease-related
62 interstitial lung disease; IPAF: interstitial pneumonia with autoimmune features.

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Supplementary Table 2. Characteristics of the donors for lung tissues.

Characteristics	HC-1	HC-2	HC-3	PF-1	PF-2	PF-3	PF-4
Age, y	63	63	68	45	65	51	73
FVC, % predicted	/	/	/	88.5	90.3	90.7	85.8
DLCO, % predicted	/	/	/	47.3	46.4	46.9	46.1
GAP score	/	/	/	4	4	4	4
Diagnosis	/	/	/	IPF	IPF	SSc-ILD	SSc-ILD

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Abbreviations: SSc-ILD, systemic sclerosis-interstitial lung disease.

Supplementary Table 3. Primers used for RT-PCR.

Gene	Forward	Reverse
Human <i>GAPDH</i>	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
Human <i>ITGA5</i>	GCCGATTACATCGCTCTCAAC	GTCTTCTCCACAGTCCAGCAAG
Human <i>ITGB1</i>	GGATTCTCCAGAAGGTGGTTTCG	TGCCACCAAGTTTCCCATCTCC
Human <i>ACTA2</i>	TGCTGACAGAGGCACCACTGAA	CAGTTGTACGTCCAGAGGCATAG
Human <i>COL1A1</i>	GATTCCCTGGACCTAAAGGTGC	AGCCTCTCCATCTTTGCCAGCA
Human <i>COL3A1</i>	TGGTCTGCAAGGAATGCCTGGA	TCTTTCCCTGGGACACCATCAG
Human <i>FN1</i>	ACAACACCGAGGTGACTGAGAC	GGACACAACGATGCTTCCTGAG
Human <i>P16</i>	CTCGTGCTGATGCTACTGAGGA	GGTCGGCGCAGTTGGGCTCC
Human <i>P21</i>	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG
Mouse <i>Tfdp1</i>	TTGAAGCCAACGGAGAACTAAAG	TGGAAGTGTCCGAAGGTTTTTG
Mouse <i>Nr3c2</i>	AACCACACGGTGACCTGTCATC	AGTGACACCCAGAAGCCTCATC
Mouse <i>Fn1</i>	CCCTATCTCTGATACCGTTGTCC	TGCCGCAACTACTGTGATTCCG
Mouse <i>Col1a1</i>	CCTCAGGGTATTGCTGGACAAC	CAGAAGGACCTTGTTTGCCAGG
Mouse <i>Ccl20</i>	GTGGGTTTCAACAAGACAGATGGC	CCAGTTCTGCTTTGGATCAGCG
Mouse <i>Jun</i>	CAGTCCAGCAATGGGCACATCA	GGAAGCGTGTTCTGGCTATGCA
Mouse <i>Snail</i>	ATGGCCATTTCTGTGGAGGG	CAAAAACCCACGCAGACAGG
Mouse <i>Twist</i>	ATTCAGACCCTCAAGCTGGC	AGCTTGCCATCTTGGAGTCC
Mouse <i>Vimentin</i>	AACTTAGGGGCGCTCTTGTC	ATTCAAGTCTCAGCGGGCTC
Mouse <i>Acta2</i>	TGCTGACAGAGGCACCACTGAA	CAGTTGTACGTCCAGAGGCATAG
Mouse <i>Col3a1</i>	GACCAAAAGGTGATGCTGGACAG	CAAGACCTCGTGCTCCAGTTAG
Mouse <i>Gapdh</i>	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

Mouse <i>p16</i>	TGTTGAGGCTAGAGAGGATCTTG	CGAATCTGCACCGTAGTTGAGC
Mouse <i>Cthrc1</i>	TG TTCAGGACCTCTTCCCATCG	GCCACATCTACCAATCCAGCAC