Supplementary Methods

Prostaglandin $F_{2\alpha}$-FP signaling facilitates bleomycin-induced pulmonary fibrosis independently of TGF-β

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Preparation and FACS analysis of cells from the lung. Analysis of cells from the whole lung was performed according to the method as reported previously[1]. On Day 21, lungs of WT and *Ptgfr*$^{-/-}$ mice were removed, minced and then incubated with collagenase A in high–glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ℃ for 2 h. The collagenase digests were filtered, and single–cell suspensions were obtained. After hemolysis, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Pharmingen). Cells were stained with rabbit anti–collagen I (Col I) antibody (Cedarlane, Ontario, Canada) or rat PE–conjugated anti–mouse CD11b antibody, or both. Col I^{+} cells were visualized with Alexa Flour 488–labeled anti–rabbit IgG, and were subjected to flow cytometry.

Western blot analysis. The lung was removed from anesthetized mice, and homogenized in 1 ml of ice–cold RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5% sodium deoxycholate) containing a protease inhibitor cocktail (Nacalai tesque) and a phosphatase inhibitor cocktail (Roche). After centrifugation at 10,000 × g for 10 min at 4 ˚C, the supernatant were boiled in Laemmli buffer for 5 min. A 6 l-aliquot of each sample was loaded and subjected to electrophoresis in an 8% poly-acrylamide gel. After electrophoresis, separated proteins were electrophoretically transferred to a nitrocellulose membrane (Protran, Whatman Gmbh). The membrane were blocked in 5% BSA-0.1% Tween20 in TBS for 1 h at room temperature and incubated with anti–Smad2/3 antibody (#3102, Cell Signaling) diluted 1:1000 in blocking buffers or blocked in 5% Skim Milk (Difco) and incubated with anti–phospho-Smad2 antibody (#3101, Cell Signaling) diluted 1:200, at 4 ˚C overnight. The membrane was washed three times for 5 min with 0.1% Tween20 TBS and treated with a 1:1000 dilution of anti–rabbit

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IgG HRP–linked whole antibody (#NA934, Amersham Biosciences). After three washes with 0.1% Tween20 TBS, the membrane was incubated with ECL plus reagents (GE lifesciences) for 5 min at room temperature. The blot was exposed to Hyperfilm ECL (GE lifesciences).

DNA microarray analysis. RNA was extracted from the lung of WT and *Ptgfr*$^{-/-}$ mice on Days 0, 7 and 14 (n = 4–5 for each group at each point) after bleomycin instillation, and converted to biotinylated cRNA using commercially available reagent kits (Affymetrix, Inc, Santa Clara, CA) and were hybridized to Affymetrix mouse 430 2.0 microarrays (45,101 probes). Filtering was performed on the hybridization signals after normalization according to GeneChip Operating Software 1.4 to eliminate signals that were absent in any of the 4 or 5 samples or were smaller than the normalized intensity of 111. This filtering procedure left 25,503 probe sets for analysis. We then calculated their mean signal intensities in each group, and selected genes that differed in intensity both by more than 2–fold (fold change) and by difference of more than 111 in comparison of any one pair chosen from the six groups of samples. This procedure defines 2,217 genes as fluctuating genes, which were subjected to the hierarchical cluster analysis based on Pearson correlation (Spotfire Decision Site (http://spotfire.tibco.com/index.cfm)).

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. Staining β-galactosidase activity was performed according to the method as reported previously[2]. Mice were fixed with perfusion with 4% paraformaldehyde through intracardiac catheter and lungs were isolated, and cut 8 m thick. Sections were incubated with 0.5 mg ml^{-1} X-gal for 96 h at 37 ℃ in PBS containing 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide and 2 mM $MgCl_2$. After washing in PBS, sections were incubated with 3% hydrogen peroxide in methanol for 10 min. For immuno-staining, sections after X-gal staining were incubated overnight with anti-α-smooth muscle actin (SMA)

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antibody (Sigma). Immune complexes were detected by Histofine Mouse Stain Kit (Nichirei, Tokyo, Japan), followed by exposure to 0.01% diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-HCl, pH 7.5. The sections were further stained with nuclear fast red and examined without xylene extraction.

Preparation and culture of primary lung fibroblasts. Lungs were isolated from mice without any treatment, perfused with PBS, and minced with scalpels. Minces were digested with 1 mg ml^{-1} collagenase A (Roche Diagnostics) in high–glucose DMEM supplemented with 10% FBS at 37 ℃ for 2 h. The digests were passed through nylon mesh. Dissociated cells were washed with DMEM containing 10% FBS, and plated in a 10–cm plastic dish. After overnight culture, non–adherent cells were removed. Cells were then cultured in high–glucose DMEM with 10% FBS, 100 g ml^{-1} streptomycin and 100 units ml^{-1} penicillin at 37 ℃ under 5% CO_2 in a humidified incubator, and passed at least two to three times before use.

Human cell culture. IMR90 and WI38 cells were maintained in high–glucose DMEM with 10% FBS, 100 g ml^{-1} streptomycin and 100 units ml^{-1} penicillin at 37 ℃ under 5% CO_2 in a humidified incubator. LL29 cells were maintained in Ham's F12 with 45 mg l^{-1} ascorbic acid, 18 mg l^{-1} inositol and 15% FBS.

Collagen production assay. Cells were grown to confluence in 60–mm dishes and the medium was replaced with DMEM or Ham's F12 containing 0.5% FBS. The cells were incubated with either TGF-β1 (R&D Systems, Minneapolis, MN) or $PGF_{2\alpha}$ (Cayman Chemical, Ann Arbor, MI) or both for 48 h at 37 ℃. In some experiments, SB-505124 (Sigma, St. Louis, MO), AL8810 (Cayman Chemical, Ann Arbor, MI), Y-27632 (Calbiochem), SIS3 (Calbiochem) and other inhibitors (**Supplementary Fig. 5g**) was added 30 min or 1 h before stimulation, respectively. The amount of total soluble collagen

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in culture supernatants was measured by the Sircol dye binding assay kit (BioColor Ltd., Northern Ireland, UK) according to the manufacturer's direction[3].

Quantitative real–time RT–PCR. For analysis of *PTGFR* expression, the cells were treated with or without human recombinant TGF-β1 for 24 h at 37 ℃ before harvest. For analysis of *COL1A2* expression, the cells were treated with $PGF_{2\alpha}$ or vehicle for 6 h at 37 ℃ after 24 h of incubation in DMEM with 0.5% FBS, and harvested. In another experiment, the cells were first cultured in DMEM with 0.1% FBS for 24 h. The culture medium was then aspirated, and the cells were incubated in 1 ml PBS with or without the lung homogenates from bleomycin–challenged mice for 6 h at 37 ℃. AL8810 was added 1 h before addition of the homogenate, and supplemented also with the homogenate in the AL8810–treated group. Complementary DNA was synthesized using Superscript II (Invitrogen, Carlsbad, CA). The quantification of *Ptgfr*, *PTGFR*, and *COL1A2* expression is performed on a LightCycler™2.0 (Roche Molecular Diagnostics, Mannheim, Germany), and was normalized by expression of the house–keeping gene *Gapdh* and *GAPDH*, respectively. The *Ptgfr* and *Gapdh* expression was detected by the LightCycler FastStart DNA Master SYBR Green I kit (Roche), and the *PTGFR*, *COL1A2* and *GAPDH* expression was detected by the LightCycler TaqMan Master kit (Roche). Primers used for amplifications were as follows: *Ptgfr* forward, 5'-CTG GCT TGT GCC CAC T-3'; *Ptgfr* reverse, 5'-GAC GGC ATT GCA CGA GA-3'. *Gapdh* forward, 5'-TGA ACG GGA AGC TCA CTG G-3'; *Gapdh* reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. *PTGFR* forward, 5'-GGC AGA TCT CAT CAT TTG GAA-3'; *PTGFR* reverse, 5'-TCC AAT GTT GGC CAT TGT AA-3'. *COL1A2* forward, 5'-TCT GGA GAG GCT GGT ACT GC-3'; *COL1A2* reverse, 5'-GAG CAC CAA GAA GAC CCT GA-3'. *GAPDH* forward, 5'-AGC CAC ATC GCT CAG ACA C-3'; *GAPDH* reverse, 5'-GCC

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CAA TAC GAC CAA ATC C-3'. The Probe sets of #17, #79 and #60 of the Universal Probe Library Set, Human (Roche), were used for *PTGFR*, *COL1A2* and *GAPDH* respectively.

Proliferation assay. To examine effects of $PGF_{2\alpha}$ on proliferation of mouse lung fibroblasts, WT and *Ptgfr*$^{-/-}$ fibroblasts were cultured on a 96–well plate at a density of 8×10^3 per well. After cells were starved in RPMI-1640 with 0.5% FBS for 48 h, either $PGF_{2\alpha}$ or PDGF-BB or both were added and incubated for 48 h. [^{3}H]thymidine, 0.5 Ci per well, was added to the cells at 24 h, and the culture was continued for another 24 h. The acid–insoluble radioactivity was determined by liquid scintillation counting. IMR90 cells were cultured on a 24–well plate at a density of 2×10^4 per well. After cells were starved in DMEM with 0.5% FBS for 24 h, either $PGF_{2\alpha}$ or PDGF-BB, or both were added. After 48 h and 96 h, the cells were harvested and subjected to flow cytometry for cell counting.

Promoter assay. IMR90 cells were transfected with the plasmid construct containing 3.5kb *COL1A2* gene promoter linked to the CAT reporter gene (the –3500*COL1A2/CAT* construct)[4,5]. To correct variations in trasfection efficacy, pSV-β-galactosidase vector (Promega, Madison, USA) was also transfected. Briefly, IMR90 cells were grown and seeded at 2×10^5 cells per well in 2 ml DMEM with 0.5% FBS on the day of transfection in 6–well plates. Before transfection, the plasmid DNA, each 2.5 g, was mixed with 2.5 l Plus reagent (Invitrogen) in 500 l Opti-MEM I Reduced Serum Medium (Invitrogen) for 5 min. Five l Lipofectamine LTX (Invitrogen) was then added, mixed, and incubated for an additional 25 min before being added to cells in each well for transfection. After transfection, IMR90 cells were cultured at 37 ℃ for 18 h, and then incubated with $PGF_{2\alpha}$, TGF-β1, or both. After 24 h of incubation, media were removed and the cells were washed 3 times with PBS and lysed in 2 ml lysis buffer of the CAT or

β-galactosidase ELISA kit (Roche Applied Science, Germany). CAT and β-galactosidase protein levels in cell lysates were measured by ELISA according to the manufacturer's protocol, and the CAT/β-galactosidase ratio was used as the *COL1A2* promoter activity.

Supplementary Reference

1. Hashimoto, N. *et al*. Bone marrow–derived progenitor cells in pulmonary fibrosis. *J. Clin. Invest.* **113**, 243–252 (2004).
2. Bell, P. *et al*. An optimized protocol for detection of E. coli β-galactosidase in lung tissue following gene transfer. *Histochem. Cell Biol.* **124**, 77–85 (2005).
3. Yurovsky, V.V. Tumor necrosis factor-related apoptosis-inducing ligand enhances collagen production by human lung fibroblasts. *Am. J. Respir. Cell Mol. Biol.* **28** 225–231 (2003).
4. Jinnin, M. *et al*. Potential regulatory elements of the constitutive up–regulated α2(I) collagen gene in scleroderma dermal fibroblasts. *Biochem. Biophys. Res. Commun.* **343**, 904–909 (2006).
5. Ihn, H. *et al*. Transcriptional regulation of the human α2(I) collagen gene. Combined action of upstream stimulatory and inhibitory cis-acting elements. *J. Biol. Chem.* **271**, 26717–26723 (1996).

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Supplementary Table 1 Flow cytometry analysis of cells of the whole lung of WT and *Ptgfr*$^{-/-}$ mice on Day 21.

	WT (n = 11)	*Ptgfr*$^{-/-}$ (n = 12)
Total number of cells ($\times 10^5$)	29.7 ± 5.7	14.9 ± 2.5*
Col I$^+$ cells ($\times 10^5$)	14.8 ± 2.3	8.1 ± 1.2*
CD11b$^+$ cells ($\times 10^5$)	8.7 ± 1.7	5.0 ± 0.7

* $P < 0.05$ versus WT mice.

Supplementary Table 2 Flow cytometry analysis of lymphocyte subsets in BALF of WT and *Ptgfr*$^{-/-}$ mice on Day 7.

	WT (n = 4)	*Ptgfr*$^{-/-}$ (n = 5)
Total number of cells ($\times 10^5$)	41.4 ± 8.8	32.8 ± 7.2
T cell ($\times 10^4$)	52.5 ± 11.9	50.9 ± 18.4
B cell ($\times 10^4$)	4.2 ± 0.9	8.8 ± 5.4
$CD4^+$ cells ($\times 10^4$)	17.8 ± 3.9	18.7 ± 8.2
$CD8^+$ cells ($\times 10^4$)	18.2 ± 4.1	24.1 ± 8.6

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Supplementary Table 3 Expression of representative genes in Clusters 13, 14 and 15 during bleomycin-induced pulmonary fibrosis.

Cluster	Gene encoding	WT			*Ptgfr*$^{-/-}$		
		Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
13	elastin	901±113	8235±1255	12510±763	1030±212	3642±680	4773±1612*
	collagen, type I, α1	1078±59	5225±194	8374±1282	1491±81	3043±326	3620±685*
	collagen, type IV, α1	3781±111	7113±603	9876±353	4277±296	5696±318	6235±727*
	collagen, type VI, α2	3187±211	6535±179	6700±176	3465±183	5341±171	5130±391*
	syndecan 1	795±148	1153±134	1863±164	813±81	1066±43	1371±77
	matrix metalloproteinase 2	1222±134	2868±249	5047±335	1448±76	2345±102	3655±592
	matrix metalloproteinase 19	186±31	510±34	853±70	180±26	417±33	404±53*
14	chemokine ligand 2	59±8	700±118	202±21	68±15	606±70	101±15*
	chemokine ligand 17	357±44	2161±572	657±109	364±41	1807±444	475±64
	chemokine ligand 22	126±30	420±57	291±34	175±19	385±58	238±18
	chemokine (C-X-C motif) ligand 10	110±13	971±153	443±11	152±25	1025±38	205±34
	S100 calcium binding protein A4	1493±57	6173±543	3020±294	1760±101	5152±493	1791±360
	tissue inhibitor of metalloproteinase 1	298±7	5197±1004	2156±220	394±24	3908±577	754±132*
15	collagen, type I, α2	5612±284	13651±681	15507±939	6287±478	11020±552	10256±960*
	collagen, type V, α1	716±55	2398±217	2283±165	819±82	1860±209	1213±178*
	collagen, type V, α2	1978±97	9312±1058	8118±433	2323±235	7008±815	4257±713*
	fibronectin 1	6418±205	19823±2227	19219±746	7286±608	15276±1351	12584±1605*
	tenascin C	217±25	3651±470	2857±230	265±36	2261±383	829±199*
	fibrillin 1	2123±74	6787±526	6520±325	2403±229	5327±490	3798±607*
	thrombospondin 1	1437±119	6185±860	4783±484	1529±180	4195±193	3278±311*
	insulin-like growth factor 1	328±90	1307±425	1400±251	285±67	1011±346	686±320*
	matrix metalloproteinase 12	148±12	2004±329	2694±226	253±58	1333±214	1463±494
	chemokine (C-X-C motif) ligand 12	1444±38	4423±209	3901±198	1440±110	3817±234	2141±240*
	plasminogen activator inhibitor 1	600±48	1689±164	1096±122	575±18	1070±46*	753±70
	cyclin-dependent kinase inhibitor 1A	1138±258	4635±634	4605±471	916±147	3649±401	2602±515*
	connective tissue growth factor	6493±610	13188±1525	10537±706	8170±581	9396±173*	10622±477

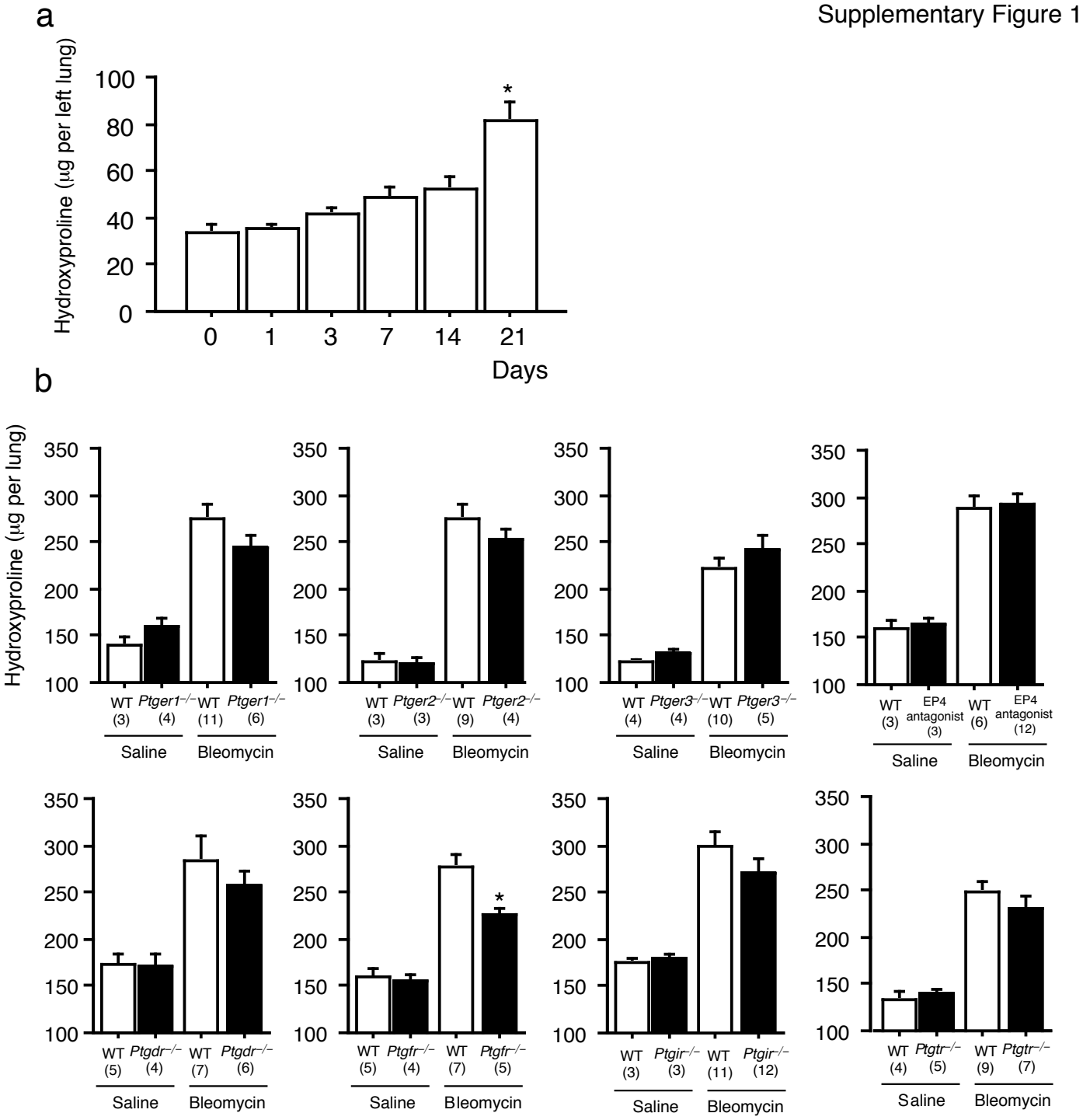
Numbers indicate signal intensities in 4-5 mice. * $P < 0.05$ versus WT.

Supplementary Table 4 Arachidonic acid metabolites in the lung during bleomycin-induced pulmonary fibrosis.

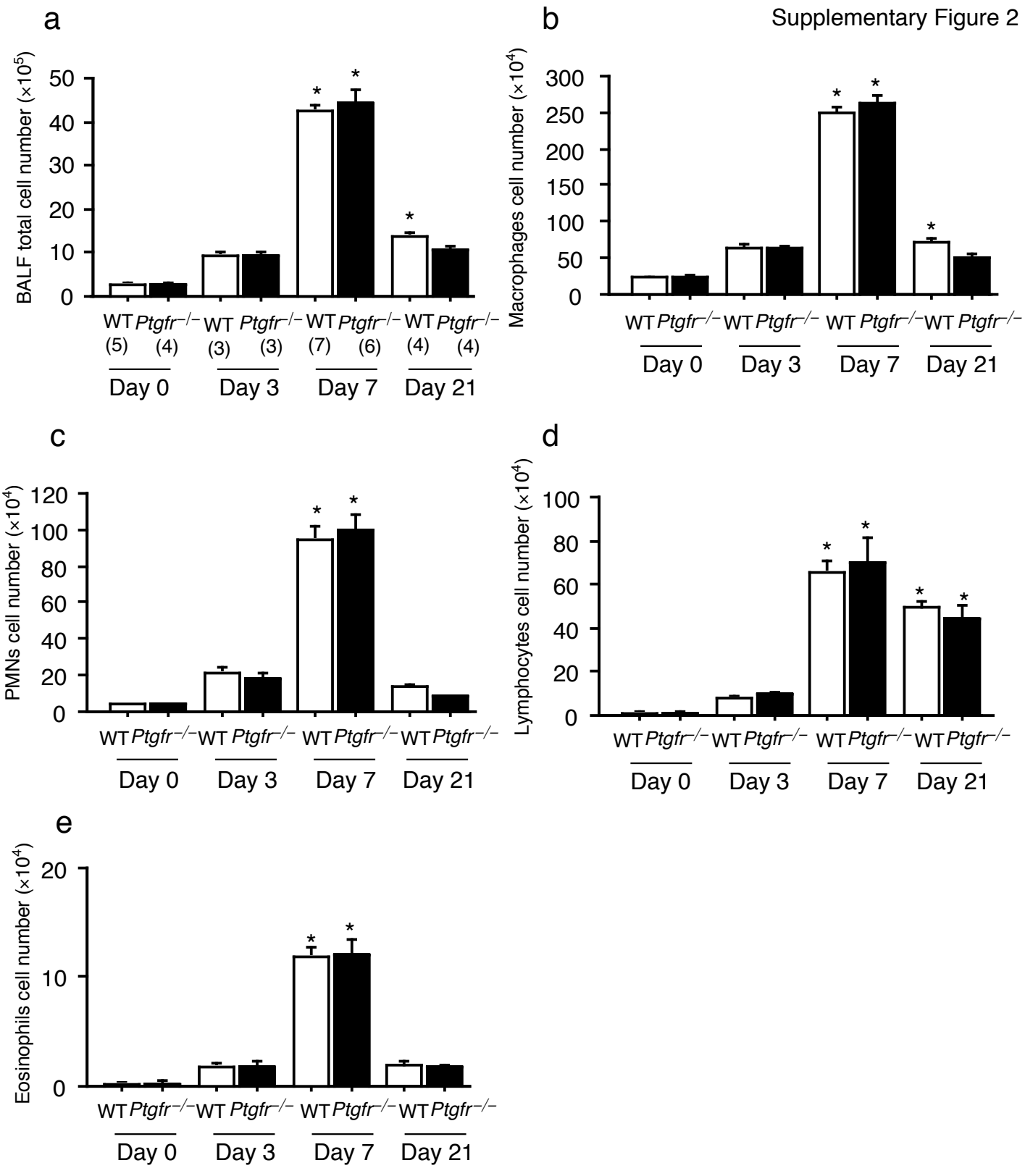
	Day 0	Day 7	Day 14	Day 21
PGD_2	83±35	96±26	132±29	85±25
PGE_2	76±27	141±55	421±184	68±6
$PGF_{2\alpha}$	25±8	27±9	46±11	25±5
6-keto-$PGF_{1\alpha}$	1078±358	1233±521	2416±1037	544±176
TXB_2	38±38	108±14	151±64	66±24
LTB_4	9±8	14±5	9±5	4±4
LTC_4	55±2	35±12	83±26	35±14
LTD_4	0±0	0±0	8±5	2±2
5-HETE	3±2	33±14	43±12	15±7
8-HETE	8±8	5±3	12±5	9±2
11-HETE	144±52	142±17	311±72	87±13
12-HETE	218±67	280±64	276±56	141±37
15-HETE	96±29	100±22	188±70	50±14
13,14-dihydro-15keto-PGD_2	23±23	10±10	79±31	79±30
13,14-dihydro-15keto-PGE_2	832±832	2204±737	4891±1069	982±378
13,14-dihydro-15keto-$PGF_{2\alpha}$	43±43	69±40	0±0	64±64
PGJ_2 + 12-deoxy-PGJ_2	6±6	6±3	9±5	5±2
15-deoxy-PGJ_2	54±26	91±47	103±20	114±38
12-HHT	68±68	106±80	559±106	33±33

Results are presented as mean±s.e.m. (n = 3-4). (pg per left lung)

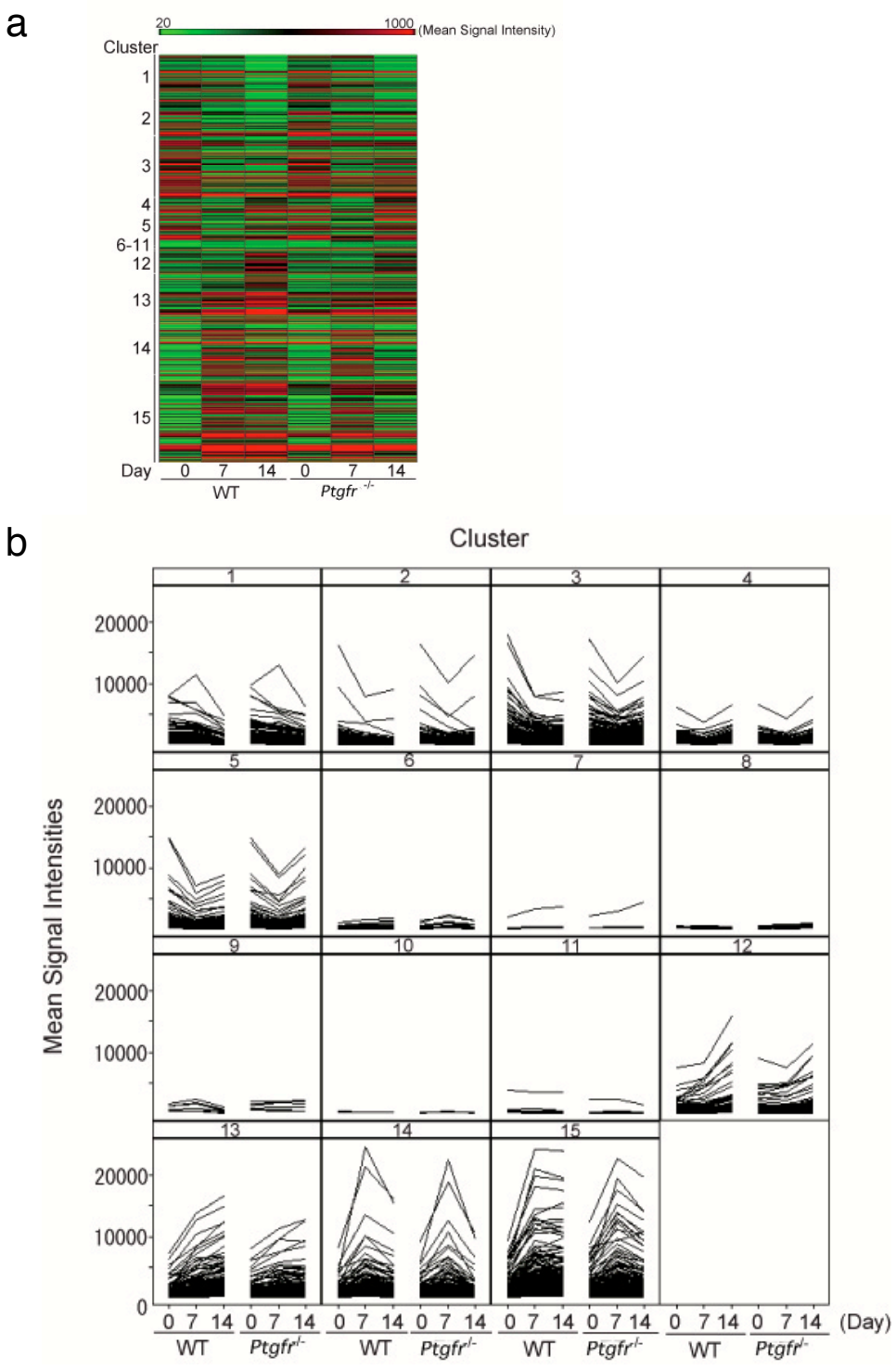
HETE, hydroxyeicosatetraenoic acid. HHT, 12-hydroxyheptadecatrienoic acid.



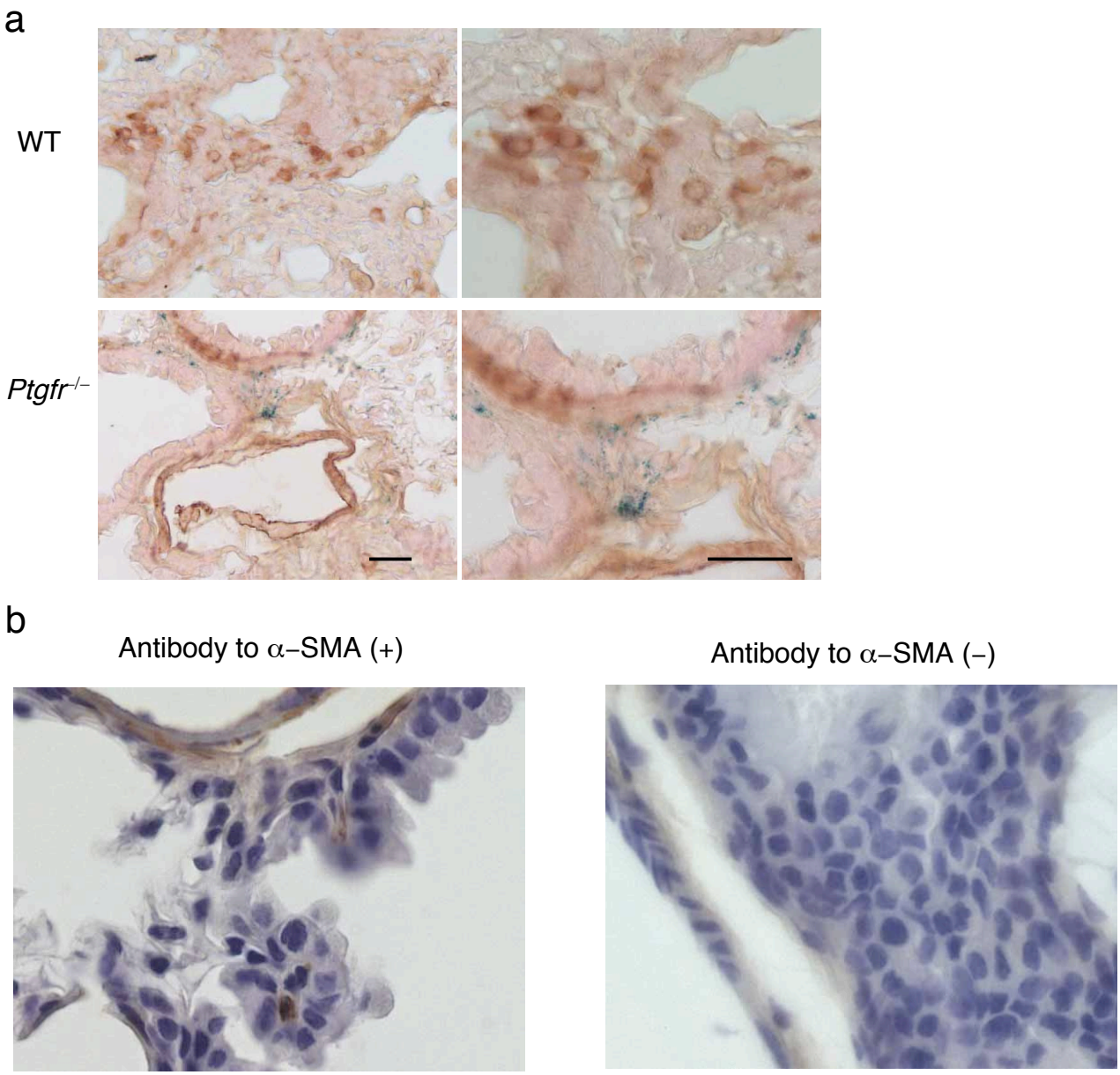
Supplementary Figure 1 Pulmonary fibrosis in mice deficient in the prostanoid receptors. (**a**) Time course of the hydroxyproline contents in the lung of WT mice. WT mice were instilled intratracheally with bleomycin on Day 0, and killed on Day 0, 1, 3, 7, 14 and 21 (n = 3-4 for each day). The left lung was dissected and subjected to the measurement of collagen by quantifying hydroxyproline contents. $^*P < 0.01$ versus Day 0, 1, 3, 7 and 14. (**b**) Effects of the prostanoid receptor deficiency and antagonism on the lung hydroxyproline contents. *Ptger1*$^{-/-}$, *Ptger2*$^{-/-}$, *Ptger3*$^{-/-}$, *Ptgdr*$^{-/-}$, *Ptgfr*$^{-/-}$, *Ptgir*$^{-/-}$ and *Ptgtr*$^{-/-}$ mice, and WT mice with or without treatment of ONO-AE3-208 at 10 mg kg^{-1} per day were instilled intratracheally with saline or bleomycin on Day 0, and killed on Day 21. Both lungs were dissected and subjected to the hydroxyproline measurement. Numbers in parentheses indicate the number of mice of each group. $^*P < 0.05$ versus the WT mice.



Supplementary Figure 2 Pulmonary inflammation in *Ptgfr*$^{-/-}$ mice. BAL was performed on Day 0, 3, 7 and 21 after the bleomycin instillation in WT and *Ptgfr*$^{-/-}$ mice. (**a**) The total number of cells and (**b–e**) the number of different types of cells in BALF. Numbers in parentheses indicate the number of each group. *$P < 0.05$ versus each mice group on Day 0.

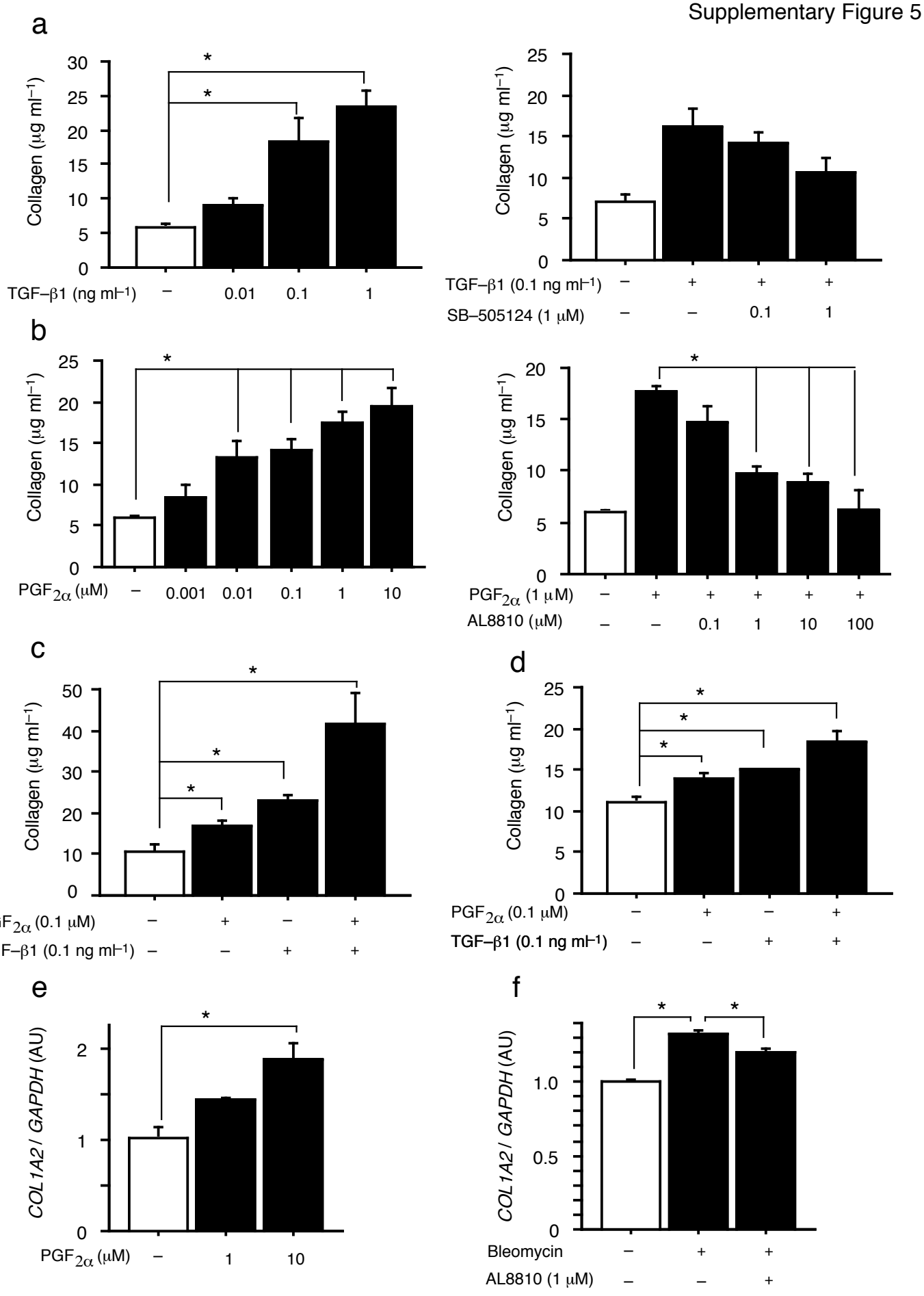


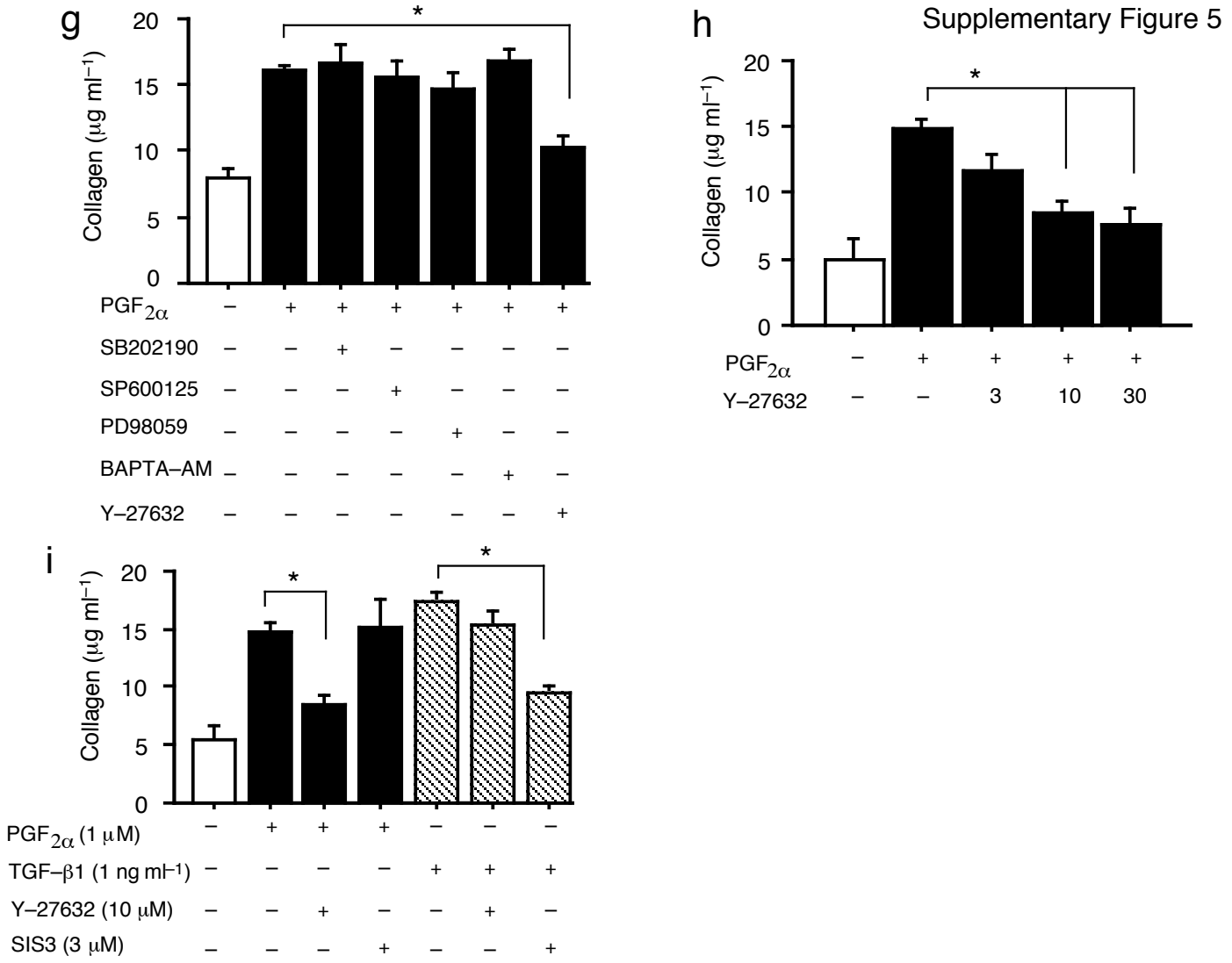
Supplementary Figure 3 Microarray analysis. (**a**) Cluster analysis. RNA was prepared from the lung of WT and *Ptgfr*$^{-/-}$ mice on Day 0, 7 and 14 (n = 4-5 for each group at each point), and used for hybridization with Affymetrix mouse 430 2.0 microarray. After normalization and filtering, 2,217 genes showing more than 2-fold changes in intensity or the difference of more than 111 in signal intensities in at least one pair-wise comparison were identified and subjected to hierarchical cluster analysis based on Pearson correlation, and divided into the 15 clusters. Expression of genes belonging to each cluster is shown with graded color from green to red. (**b**) Time-dependent changes in expression of genes belonging to each of the 15 clusters in *Ptgfr*$^{-/-}$ mice group are compared with those in WT mice.



Supplementary Figure 4 Staining of *Ptgfr*$^{-/-}$ lungs. (**a**) X-gal staining. The lung was isolated from WT and *Ptgfr*$^{-/-}$ mice on Day 21 after bleomycin treatment, and stained for the β−galactosidase activity (blue) and α−SMA (brown). Nuclear fast red staining (pink) was also carried out. Due to the β−galactosidase staining, the image was examined without xylene extraction. Left and right panels indicate lower and higher power images, respectively. Bar, 25 μm. (**b**) α−SMA staining. *Ptgfr*$^{-/-}$ lung was dissected and stained with (left) or without (right) antibody to α−SMA. The preparation was counterstained with hematoxylin and cleared with xylene. The X-gal staining was found in the alveolar thickening areas of *Ptgfr*$^{-/-}$ lung, where α−SMA staining was also found.

Supplementary Figure 5





Supplementary Figure 5 FP-dependent collagen production in human lung fibroblast cell lines. (**a**) Effects of TGF−β1 on collagen production in IMR90 cells. Cells were treated with indicated concentrations of TGF −β1 (left, n = 3-4) or with 0.1 ng ml^{-1} TGF−β1 in the presence of indicated concentrations of SB-505124, a TGF receptor type I kinase antagonist (right, n = 3) for 48 h, and secreted soluble collagen was measured. SB-505124 was added 30 min before stimulation with TGF−β1. (**b**) Effects of $PGF_{2\alpha}$ on collagen production in IMR90 cells. Cells were treated with indicated concentrations of $PGF_{2\alpha}$ (left, n = 3-4), or with 1 μM $PGF_{2\alpha}$ in the presence of indicated concentrations of AL8810, an FP antagonist (right, n = 3). AL8810 was added 1 h before stimulation of $PGF_{2\alpha}$. (**c,d**) Additive effects of $PGF_{2\alpha}$ and TGF−β1 on collagen production in LL29 (**c**) and WI38 cells (**d**). Cells were incubated either with $PGF_{2\alpha}$ or TGF−β1, or both, and secreted soluble collagen was measured (n = 3). (**e,f**) Real-time PCR for *COL1A2* expression. IMR90 cells were treated with indicated concentrations of $PGF_{2\alpha}$ (n = 2-4) (**e**) or with the lung homogenates of mouse instilled with or without bleomycin (n = 2-3) (**f**) for 6 h, and *COL1A2* expression levels were quantified by RT-PCR and normalized to that of *GAPDH*. AL8810 was added 1 h before addition of the homogenates. (**g,h**) Effects of various inhibitors on collagen production by $PGF_{2\alpha}$ in IMR90 cells. Cells were treated with 1 μM $PGF_{2\alpha}$ in the presence of 5 μM SB202190, a p38 inhibitor, 10 μM SP600125, a JNK inhibitor, 50 μM PD98059, a MEK inhibitor, 25 μM BAPTA-AM, a Ca^{2+} chelator or 10 μM Y-27632, a ROCK inhibitor (n = 7-16) (**g**), or cells were treated with indicated concentrations of Y-27632 (n = 3-10) (**h**). Inhibitors were added 1 h before stimulation of $PGF_{2\alpha}$, and incubations were continued for 48 h. (**i**) Effects of Y-27632 and SIS3 on collagen production by $PGF_{2\alpha}$ and TGF−β1. IMR90 cells were stimulated with TGF−β1 or $PGF_{2\alpha}$ in the presence or absence of 10 μM Y-27632 or 3 μM SIS3 (n = 3-10). *P < 0.05 for indicated comparison.