The Cross-Talk Between TGF-β and Periostin Can Be Targeted for Pulmonary Fibrosis

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ONLINE DATA SUPPLEMENT

Online Supplemental Information

Cell culture

WI-26 VA4 cells (SV-40 virus transformed human lung fibroblast cell line, Japanese Collection of Research Bioresources Cell Bank, Ibaraki, Japan), MRC-5 cells (normal embryonic lung fibroblast cells, Riken BioResource Center, Tsukuba, Japan), mock- and β_3-transfected SW480 cells were maintained in Dulbecco's modified Eagle's medium (MilliporeSigma, Burlington, MA) supplied with 10% fetal bovine serum. NHLFs were purchased from Lonza (Basel, Swizerland) and were maintained according to the manufacturer's instructions. SW480 cells and SW480 cells transfected with β_3 integrin were prepared as previously described (1).

RNAi-mediated knockdown

ON-TARGET plus siRNAs for periostin, integrin α_V, integrin β_3, integrin β_5, Smad2 or Smad3 and control RNA were purchased from Dharmacon/GE Healthcare (Lafayette, CO): Periostin (J-020118-06), ITGAV (L-004565-00), ITGB3 (L-004124-00), ITGB5 (L-004125-00), Smad2 (J-003561-06), Smad3 (J-020067-06), Control, Non-targeting Pool (D-001810) siRNAs were transfected into cells using Lipofectamine RNAiMax (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

DNA microarray analysis

NHLFs were transfected with 5 nM periostin siRNA for 24 h followed by stimulation with 1 ng/mL of TGF-β for 24 h. Total RNA with an RNA integrity number greater than 9.0 was applied to Agilent Expression Array (SurePrint G3 Human GE8x60K v3 Microarray, Takara Bio, Kusatsu, Japan). The calculated relative signal intensity values were subjected to MultiExperiment Viewer (MeV) v4.9 software (Dana-Farber Cancer Institute) and presented on a heat map. The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE 124782. For gene ontology analysis, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (National Cancer Institute) was used.

Quantitative real-time PCR (qRT PCR)

For qRT-PCR analysis, total RNA was isolated using RNAiso PLUS (Takara Bio) and reverse-transcribed with the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The PCR reactions were performed on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using the THUNDERBIRD SYBR qPCR Mix (Toyobo). Threshold cycles of primer probes were normalised to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and relative values were calculated.

ELISA

ELISA for human periostin was performed using two rat anti-human periostin mAbs, SS18A and SS17B (Shino-test, Tokyo, Japan), as previously reported (2). ELISAs for IGFBP3 and IL-11 were performed using an IGFBP3 and IL-11 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Transient transfection

For transient overexpression of periostin, the *POSTN* gene previously subcloned (3) was inserted into the expression vector pME18S (4). Then the pME18S-*POSTN* plasmid was transfected using Lipofectamine 2000 (Thermo Fisher Scientific) or Fugene6 reagent (Promega, Madison, WI) according to the manufacturer's instructions.

Western blotting

Western blotting was performed as previously described (3). The following Abs were used for the Western blotting: rabbit anti-phospho Smad2 Ab (138D4, Cell Signaling Technology, Danvers, MA), rabbit anti-phospho Smad3 Ab (C25A9, Cell Signaling Technology), and rabbit anti-Smad2/3 Ab (D7G7, Cell Signaling Technology). The chemiluminescence signal was detected by a LAS 3000 (GE Healthcare, Chicago, IL) camera system and quantified by ImageJ software (National Institute of Health).

Generation of stable TGF-β reporter cell lines and luciferase assay

WI-26 VA4 cells were transfected with pGL4.48 vector containing three copies of an SBE that drives transcription of the luciferase reporter gene luc2P (Promega) and were incubated for 48 h. Stably transfected cells were selected with 200 μg/mL hygromycin.

Flow cytometry

Cells were resuspended in ice-cold phosphate-buffered saline (PBS) containing 5% (vol/vol) FBS and stained with the following Abs against integrin—α_V (BioLegend, San Diego, CA), $\alpha_V\beta_3$ (MilliporeSigma), and $\alpha_V\beta_5$ (MilliporeSigma)—followed by BB515-probed goat anti-mouse Ig Ab (BD Biosciences, Franklin Lakes, NJ). Flow cytometric analyses were conducted using FACSCalibur (BD Biosciences) and FlowJo software.

Cell adhesion assay

Ninty six-well plates were coated with 10 μg/mL recombinant human periostin (Bio-Techne, Minneapolis, MN) at 4 °C for 16 h. Five × 10^4 SW480 cells or SW480 cells transfected with β_3 integrin in 100 μL of serum-free DMEM containing 0.5% BSA and 0.25 mM $MnCl_2$ were incubated with indicated concentrations of integrin inhibitors for 15 min at room temperature and then were seeded onto each well. Plates were centrifuged at 10 × g for 5 min followed by incubation for 1 h at 37 °C. Unbound cells were washed away, and the adherent cells were fixed with 1% formaldehyde and were stained with 0.5% crystal violet. The stain was

extracted with 2% Triton X-100 and was quantified by measuring the absorbance at 595 nm by a microplate reader (Perkin Elmer, Waltham, MA).

Immunohistochemistry

Lung tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial sections of deparaffinized, rehydrated lung tissues were washed with PBS, and treated with 0.3% H_2O_2 in methanol for 30 min to eliminate endogenous peroxidase activity. Lung sections were subjected to immunostaining using mouse anti-periostin (1:500; SS19C), rabbit anti–IGFBP-3 (1:100; LS-12492, LS Bio, Seattle WA), rabbit anti–IL-11 (1:100; bs-1827R, Bioss, Woburn, MA), rabbit anti-CTGF (1:200; bs-0743R, Bioss), rabbit anti-SERPINE1 (1:100; 13801, Proteintech, Rosemont, IL), and rabbit anti-phosphorylated Smad3 (1:100; ab52903, Abcam, Cambridge, UK) Abs. To unmask antigenic epitopes, sections for IGFBP-3, IL-11, and SERPINE1, and phosphorylated Smad3 were heated at 120 °C for 15 min with 10 mM citrate buffer (pH 6.0). Sections were then incubated with peroxidase for 30 min with Histofine Simple Stain Kits (Nichirei Biosciences, Tokyo, Japan) as the secondary Ab. Peroxidase activity was detected with a solution of 3,3'-diaminobenzidine and H_2O_2, with Mayer's hematoxylin as the counterstain.

Confocal microscopy

Lung sections were incubated with mouse anti-periostin Ab (1:500) and rabbit anti-phosphorylated Smad3 Ab (1:100) as the primary Ab for 24 h at 4 °C and then incubated with

Alexa Fluor 594-labeled anti-mouse IgG Ab or Alexa Fluor 488-labeled anti-rabbit IgG Ab (1:500, Thermo Fisher Scientific) as the secondary Ab, respectively, for 0.5 h at room temperature. Autofluorescence was removed by Vector TrueVIEW Autofluorescence Quenching Kit (VECTOR, Burlingame, CA). Sections were mounted in VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (VECTOR) and then examined by confocal laser scanning microscopy (LSM880, Zeiss, Germany).

Mice

Nine-week-old C57BL/6 male mice (Japan SLC, Hamamatsu, Japan) and periostin-deficient (*Postn*$^{-/-}$) male mice backcrossed to C57BL/6 for 10 generations or their sex-matched heterozygous littermates (*Postn*$^{+/-}$) whose weight were less than 28.5 g were used. *Postn*$^{-/-}$ mice were prepared as previously described (5). Murine lung fibrosis was induced by single administration of BLM (3 mg/kg, Nihon Kayaku, Tokyo, Japan) via the *oropharyngeal* aspiration route on day 0. For drug delivery, two osmotic pumps (model 2002, Alzet, Cupertino, CA) filled with 200 μL CP4715 (50 mg/mL) or equal volume vehicle (50% DMSO) were subcutaneously implanted into C57BL/6 mice at five days before BLM instillation. The release speed of CP4715 was set at 0.5 μL/h. After BLM administration, we monitored the mice every day to evaluate lung injury-induced mortality and measured body weight up to day 21 or 14, respectively. For hydroxyproline measurement and histological examination, murine lung tissues were harvested on day 10.

Hydroxyproline measurement

Left lobes of mouse lungs were freeze-dried for 12 h. The dried lungs were hydrolyzed in 2 mL of 6 N hydrochloric acid at 116 °C for 2 h. An aliquot (600 μL) of the hydrolysate was mixed with 1-butanol (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) and then concentrated by evaporation. The concentrates were adjusted to 1 mL by distilled water. A 250 μL *aliquot* of the final products was reacted with an equal volume of 0.05 M chloramine T (MilliporeSigma) solution for 20 min. We used hydroxy-L-proline (Nacalai Tesque, Kyoto, Japan) as a hydroxyproline standard. Equal volumes of 3.15 M perchloric acid solution and 5% p-dimethylamino benzaldehyde (MilliporeSigma) solution were sequentially added to the reaction mixture and incubated at 60 °C for 20 min. The concentration of hydroxyproline was evaluated by optical absorbance at 557 nm.

References

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Supplemental Figures

Figure E1 Requirement of periostin for expression of the signature molecules of pulmonary fibrosis in MRC-5 cells

Effects of periostin knockdown on expression at mRNA levels of *SERPINE1*, *CTGF*, *IGFBP3*, *IL11*, *POSTN*, *FN1* and *COL1A1* at MRC-5 cells (A) and at protein levels of IGFBP3, IL-11, and periostin estimated by ELISA at NHLFs (B). MRC-5 cells or NHLFs were stimulated with or without 3 ng/mL of TGF-β for 24 h in the presence of control or periostin siRNA (n=3). (C) Effects of periostin overexpression on protein expression of IGFBP3 and IL-11. MRC-5 cells were transiently transfected with the expression plasmid encoding periostin and then treated with or without 3 ng/mL of TGF-β for 24 h. Expression of IGFBP3 and IL-11 was evaluated by ELISA. The same experiments were performed three times. The values of mRNA were adjusted by *GAPDH* expression. **P* < 0.05, ***P* < 0.01, NS: not significant.

Figure E2 Activation of Smad3 by the cross-talk between TGF-β and periostin in lung fibroblasts

Effects of periostin overexpression on Smad2 and Smad3 phosphorylation. SBE/luciferase-harboring WI-26 VA4 cells were transiently transfected with the expression plasmid encoding periostin and then treated with or without 3 ng/mL of TGF-β for 24 h. Densitometric quantification of Smad2 and Smad3 phosphorylation. The data were

normalized to total Smad2 or Smad3 and expressed as the fold change compared to the first group at time 0 min. The same experiments were performed three times.

Figure E3 Expression of $\alpha_V\beta_3/\beta_5$ integrin on MRC-5 and WI-26 VA4 cells

Expression of integrin α_V, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ on MRC-5 and WI-26 VA4 cells is depicted.

Figure E4 Expression of $\alpha_V\beta_3/\beta_5$ integrin on SW480 cells and SW480 cells transfected with integrin β_3

Expression of integrin α_V, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ on mock-transfected SW480 cells and integrin β_3-transfected SW480 cells is depicted.

Figure E5 Photograph of the osmotic pump implanted in the back of a mouse

Procedures of implanting the osmotic pump into the back of a mouse are sequentially shown.

Figure E6 Gross appearance of the lungs of CP4715-treated mice

CP4715-treated mice were prepared as shown in Figure 8. Gross appearances of the lungs and the whole pictures of Masson trichrome staining of four lobes of PBS-administered CP4715-untreated (left), BLM-administered CP4715-untreated (middle), and BLM-administered CP4715-treated (right) mice are depicted. It is of note that the lungs of PBS-administered CP4715-untreated and BLM-administered CP4715-untreated mice show up as dark and light brown, respectively and the lungs of BLM-administered CP4715-treated mice show an intermediate color.

Figure E7 Effect of CP4715 on TGF-β signals in lung fibroblasts derived from IPF patients

Effects of CP4715 on phosphorylation of Smad2 and Smad3. Three clones of lung fibroblasts derived from four IPF patients (#IPD32, #IPF38, and #IPF172) were stimulated with or without 1 ng/mL of TGF-β for 24 h in the presence of 1 μM CP4715 from 24 h prior to the TGF-β stimulation.

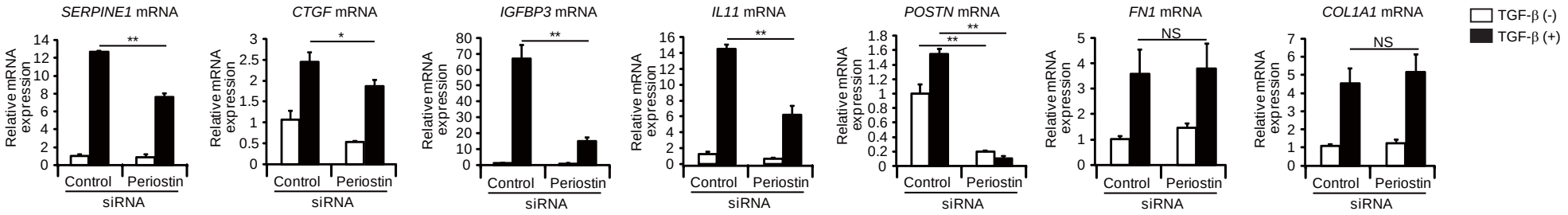
Figure E8 Schematic model of the cross-talk between TGF-β and periostin for pulmonary fibrosis

p:phosphorylated

Target cDNA	Forward sequence	Reverse sequence
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'
SERPINE1	5'-TCCAGCAGCTGAATTCCTG-3'	5'-GCTGGAGACATCTGCATCCT-3'
CTGF	5'-CCTGCAGGCTAGAGAAGCAG-3'	5'-TGGAGATTTTGGGAGTACGG-3'
IGFBP3	5'-CCAACTGTGACAAGAAGGGATT-3'	5'-CCTTCCCCTTGGTGGTGTA-3'
IL11	5'-CTGTGGGGACATGAACTGTG-3'	5'-AGGGTCTGGGGAAACTCG-3'
Periostin	5'-CAGAGAAATCCCTCCATGAAA-3'	5'-CAGGAGCTCTTTCAAGTCTGC-3'
FN1	5'-GGGAGAATAAGCTGTACCATCG-3'	5'-TCCATTACCAAGACACACACACT-3'
COL1A1	5'-CTGGACCTAAAGGTGCTGCT-3'	5'-GCTCCAGCCTCTCCATCTT-3'
Smad2	5'-GGAATTTGCTGCTCTTCTGG-3'	5'-TCTGCCTTCGGTATTCTGCT-3'
Smad3	5'-TGCTGGTGACTGGATAGCAG-3'	5'-GCTGCAAGGTGAAGATGTCA-3'
Smad7	5'-CCAACTGCAGACTGTCCAGA-3'	5'-CAGGCTCCAGAAGAAGTTGG-3'
ITGAV	5'-GCACCCTCCTTCTGATCCT-3'	5'-GAGGACCTGCCCTCCTTC-3'
ITGB3	5'-GCCCTGCTCATCTGGAAAC-3'	5'-TACAGTGGGTTGTTGGCTGT-3'
ITGB5	5'-AGTCCAACCTGACCGTCCT-3'	5'-GAGGATGCTACCGACCACAG-3'

Nanri *et al.* Table E1

A



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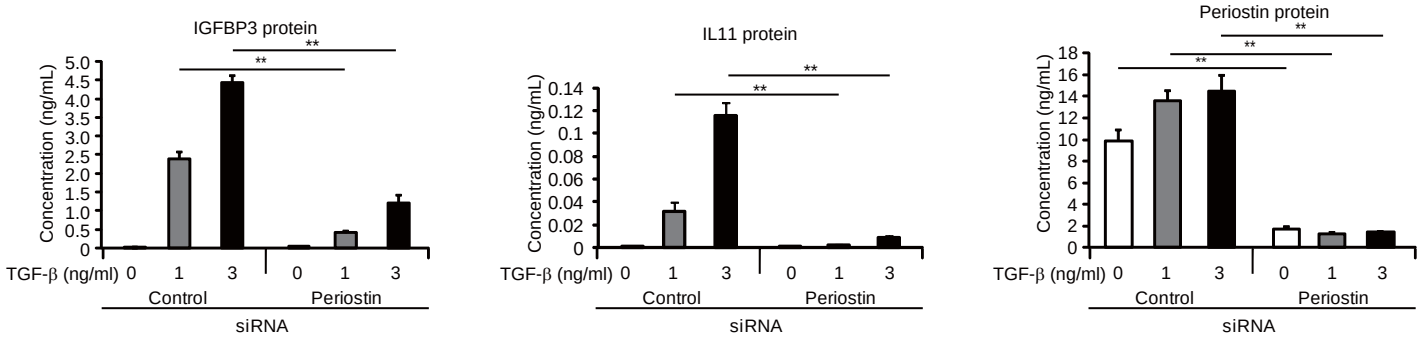

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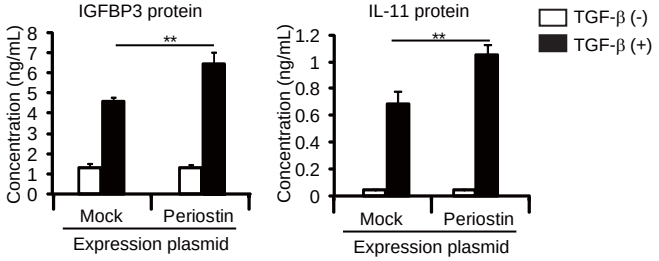

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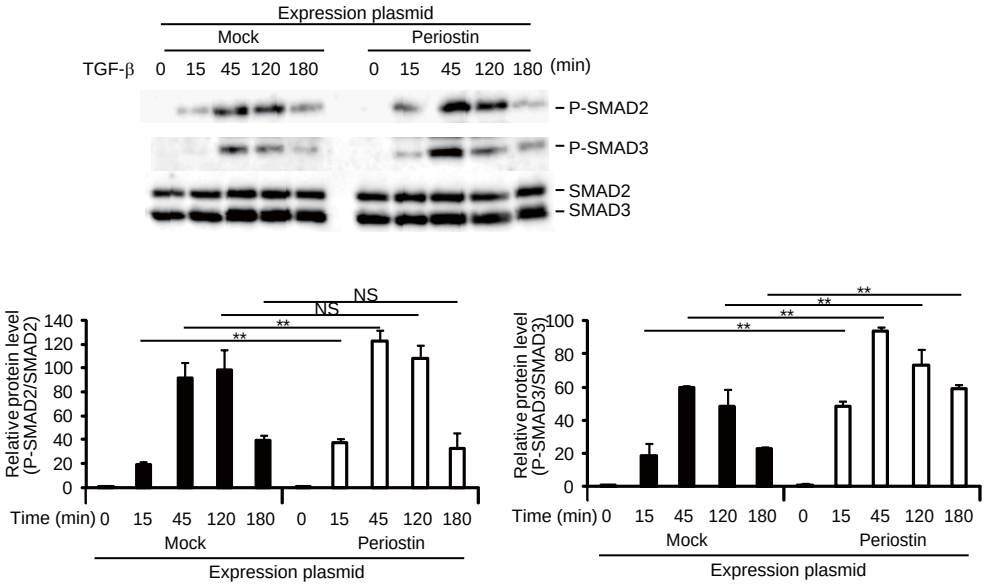

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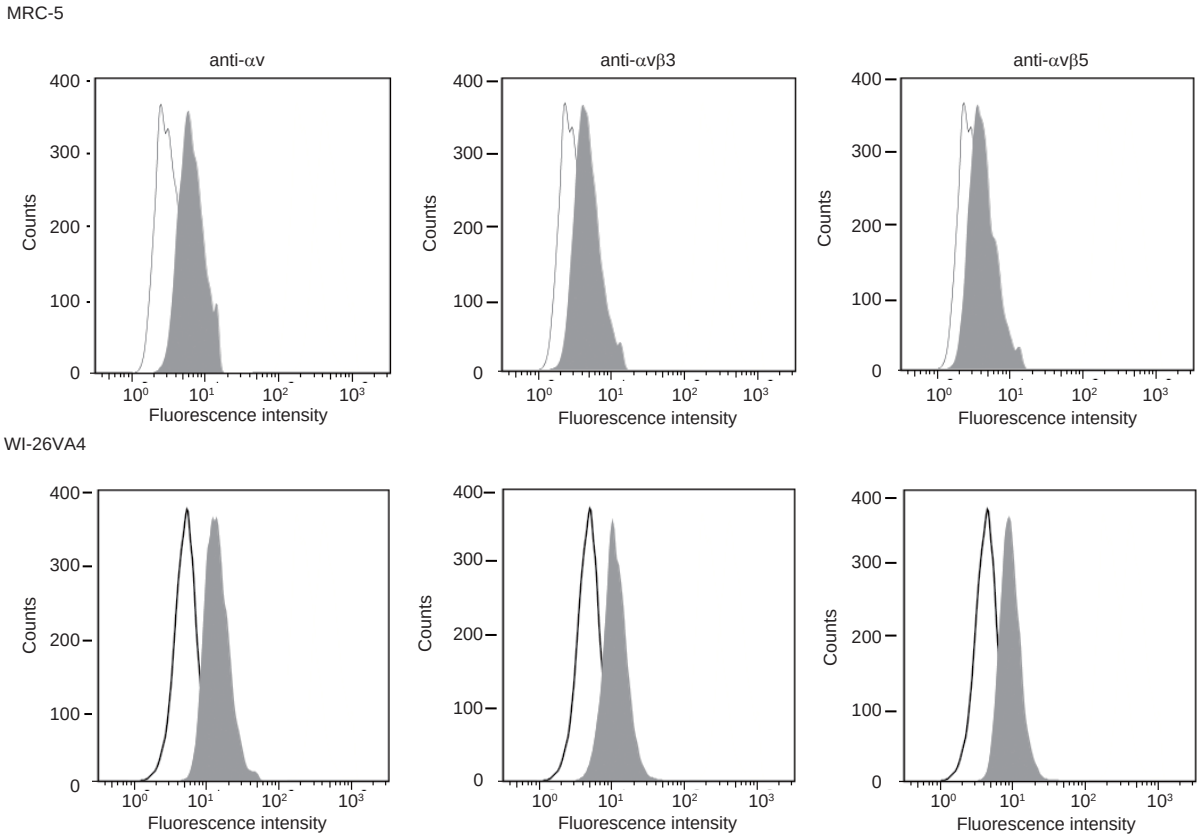

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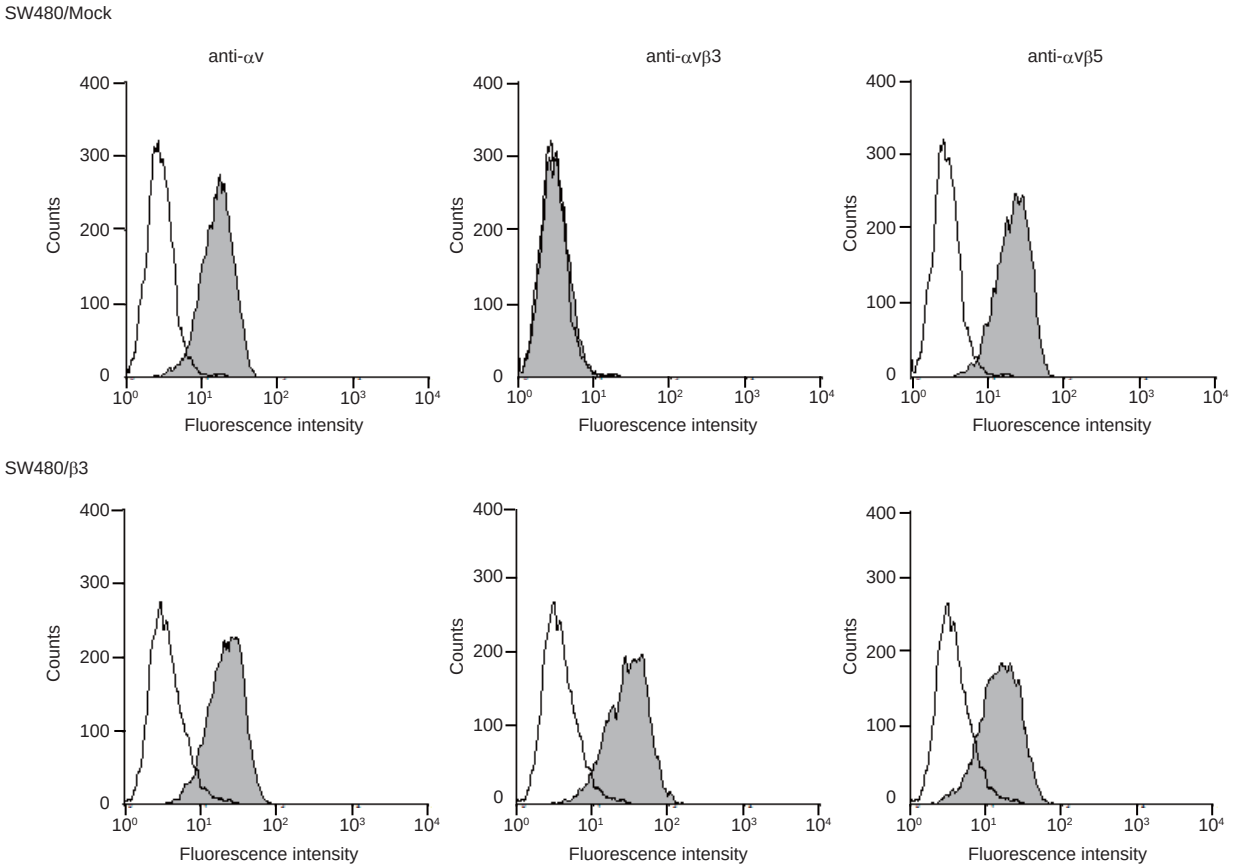

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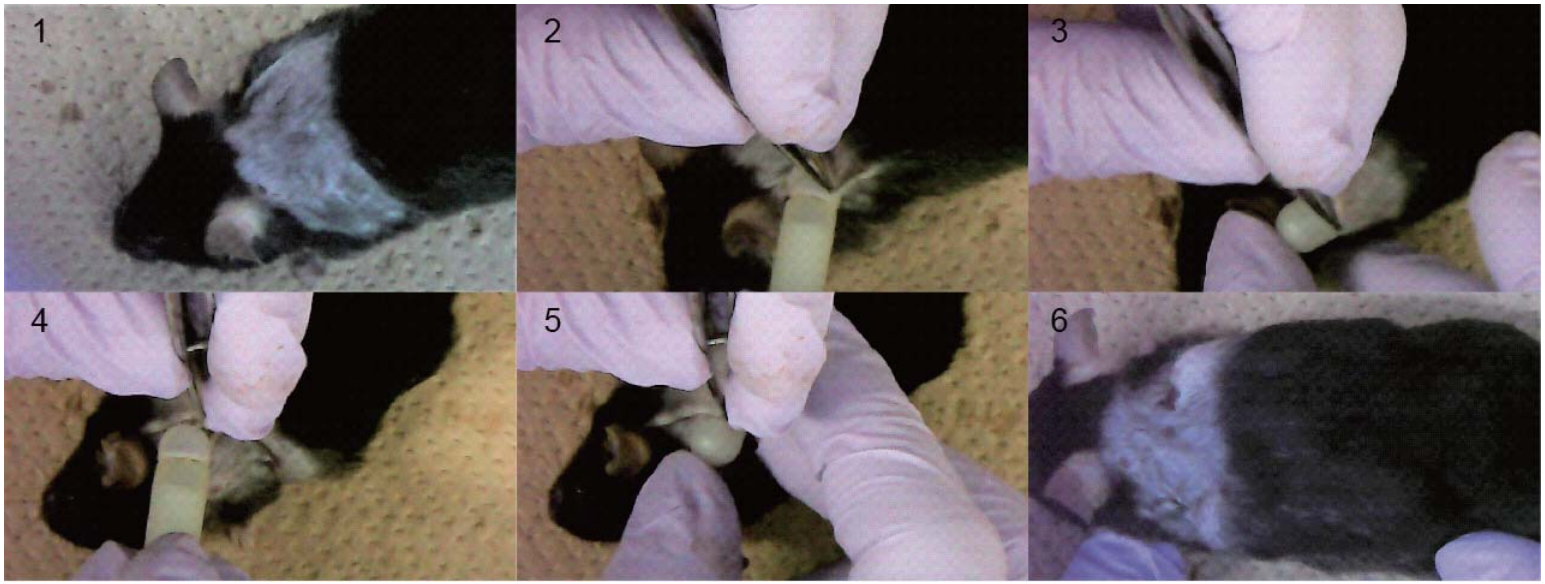

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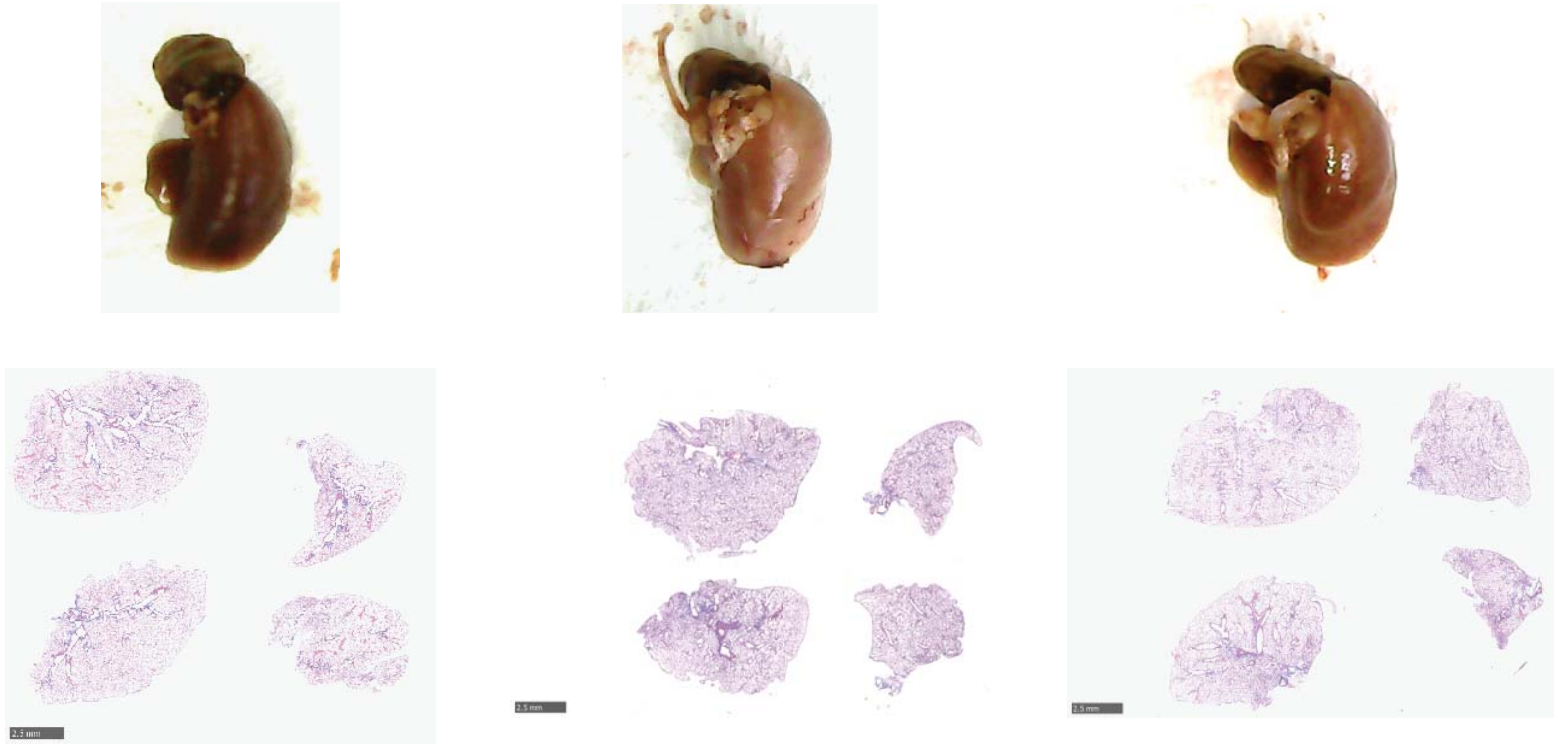
PBS + CP4715 (-)

BLM + CP4715 (-)

BLM + CP4715 (+)



Scale bar; 2500 μm

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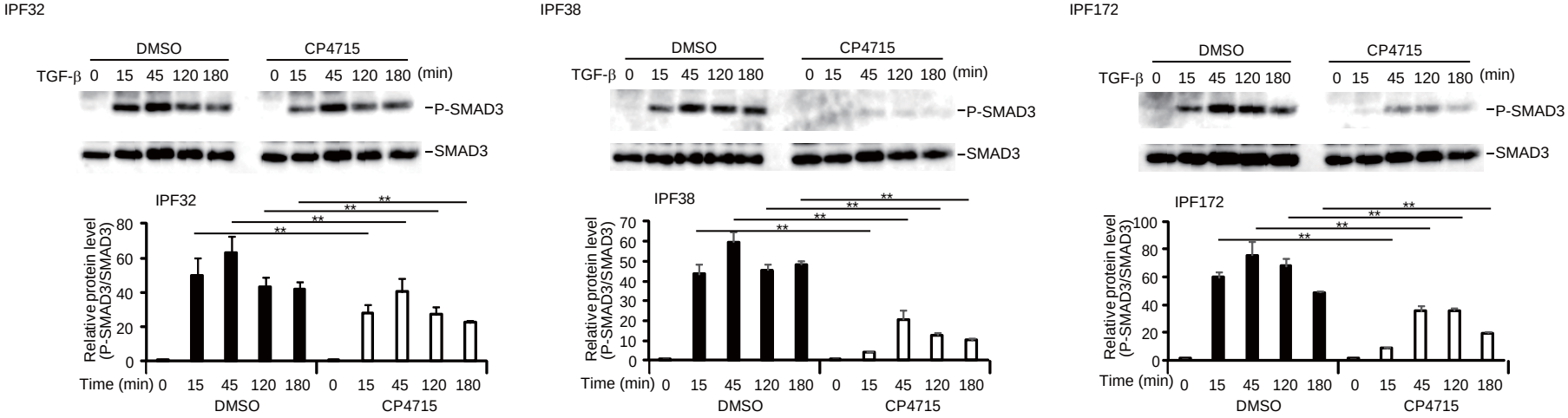

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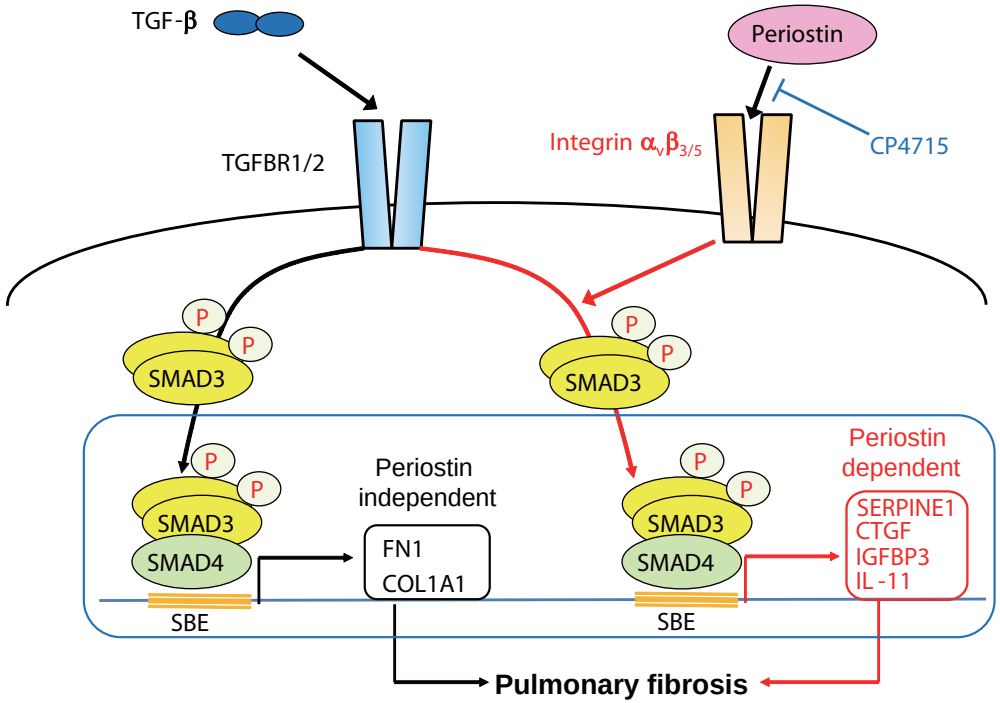

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