

## Supplementary material

### Title:

LTBP2 is secreted from lung myofibroblasts and is a potential biomarker for idiopathic pulmonary fibrosis

### Authors:

Yasunori Enomoto, Sayomi Matsushima, Kiyoshi Shibata, Yoichiro Aoshima, Haruna Yagi, Shiori Meguro, Hideya Kawasaki, Isao Kosugi, Tomoyuki Fujisawa, Noriyuki Enomoto, Naoki Inui, Yutaro Nakamura, Takafumi Suda, Toshihide Iwashita

### Supplementary Tables

**Table S1.** Antibodies used in FACS

Primary antibody	Target cells	Fluorescence	Company	Dilution
Anti-CD45	Blood cells	APC	BioLegend	1:150
Anti-TER-119	Erythrocytes	APC	eBioscience	1:150
Anti-CD324 (E-cadherin)	Epithelial cells	APC	BioLegend	1:150
Anti-CD31	Vascular endothelial cells	APC	BioLegend	1:150
Anti-CD146	Smooth muscle cells and pericytes	APC	BioLegend	1:150
Anti-LYVE1	Lymphatic endothelial cells	APC	R&D	1:60
Anti-Sca-1	Fibroblasts	PE-Cy7	eBioscience	1:600
Anti-PDGFR $\alpha$ (CD140a)	Fibroblasts	PE	BioLegend	1:50
Streptavidin	(Biotin)	PerCP-Cy5.5	BioLegend	1:300
Anti-CD49e (integrin	Myofibroblasts (cell surface	(Biotin)	Abcam	1:150

$\alpha 5$ )	marker)			
Anti- $\alpha$ -smooth muscle actin	Myofibroblasts (intra-cellular protein)	FITC	Abcam	1:100

**Table S2.** Genes that are highly upregulated in myofibroblasts (MFB) or steady-state fibroblasts (FB) from mouse lungs

<b>Genes upregulated in MFB</b>	<b>Symbol</b>	<b>MFB-signal</b>	<b>FB-signal</b>	<b>Fold change (MFB/FB)</b>
Secreted phosphoprotein 1	<i>Spp1</i>	156374.3	15.1	10351.5
Gremlin 1	<i>Grem1</i>	16794.8	14.6	1153.1
Kinesin family member 26B	<i>Kif26b</i>	17524.0	17.8	984.3
Thrombospondin 4	<i>Thbs4</i>	79551.2	164.4	483.8
Collagen triple helix repeat containing 1	<i>Cthrc1</i>	32474.7	141.2	230.0
Periostin, osteoblast specific factor	<i>Postn</i>	49741.5	224.0	222.0
Tenascin C	<i>Tnc</i>	35515.7	186.9	190.0
Chondrolectin	<i>Chodl</i>	17701.0	105.8	167.4
Desmin	<i>Des</i>	43449.3	442.6	98.2
Inhibin beta-A	<i>Inhba</i>	93682.0	1789.1	52.4
Collagen, type VIII, alpha 1	<i>Col8a1</i>	12495.5	262.7	47.6
Baculoviral IAP repeat-containing 5	<i>Birc5</i>	43879.4	1225.4	35.8
Actin, alpha 2, smooth muscle, aorta	<i>Acta2</i>	106982.3	3203.1	33.4
SHC (Src homology 2 domain containing) family, member 4	<i>Shc4</i>	29557.0	937.9	31.5
Scleraxis	<i>Scx</i>	19402.9	656.0	29.6
Heparin-binding EGF-like growth factor	<i>Hbegf</i>	25328.7	887.9	28.5
Procollagen-proline, 2-oxoglutarate 4-dioxygenase	<i>P4ha3</i>	48170.5	1712.9	28.1
Transforming, acidic coiled-coil containing protein 3	<i>Tacc3</i>	12922.9	495.0	26.1
Latent transforming growth factor beta binding protein 2	<i>Ltbp2</i>	112347.8	4430.0	25.4
Activity regulated cytoskeletal-associated protein	<i>Arc</i>	14632.2	686.5	21.3
Mesenchyme homeobox 1	<i>Meox1</i>	15498.8	727.3	21.3

Actin, gamma 2, smooth muscle, enteric	<i>Actg2</i>	22188.7	1146.0	19.4
Pleiotrophin	<i>Ptn</i>	17711.5	930.7	19.0
Zinc finger, DHHC domain containing 3	<i>Zdhhc3</i>	28057.0	1683.2	16.7
Collagen, type VII, alpha 1	<i>Col7a1</i>	38948.6	2678.5	14.5
2,3-bisphosphoglycerate mutase	<i>Bpgm</i>	111491.4	7866.0	14.2
Collagen, type XII, alpha 1	<i>Col12a1</i>	150876.1	12420.8	12.1
Family with sequence similarity 173, member B	<i>Fam173b</i>	14059.1	1162.7	12.1
Neuronal regeneration related protein	<i>Nrep</i>	90753.0	7789.2	11.7
Tissue inhibitor of metalloproteinase 1	<i>Timp1</i>	51218.5	4468.9	11.5
Discs, large (Drosophila) homolog-associated protein 2	<i>Dlgap2</i>	12334.5	1111.7	11.1
Fibronectin 1	<i>Fn1</i>	108667.5	10496.6	10.4
Transgelin	<i>Tagln</i>	99265.9	9621.9	10.3
Collagen, type V, alpha 3	<i>Col5a3</i>	25504.9	2504.9	10.2
Tropomyosin 2, beta	<i>Tpm2</i>	117555.4	12240.7	9.6
Lysyl oxidase-like 2	<i>Loxl2</i>	27945.0	3375.2	8.3
Elastin	<i>Eln</i>	375373.3	48597.3	7.7
Collagen, type V, alpha 1	<i>Col5a1</i>	97043.5	15209.0	6.4
Sarcosine dehydrogenase	<i>Sardh</i>	13725.2	2195.3	6.3
Collagen, type IV, alpha 2	<i>Col4a2</i>	29962.5	4908.1	6.1
Chemokine (C-C motif) ligand 2	<i>Ccl2</i>	12963.0	2201.0	5.9
SPEG complex locus	<i>Speg</i>	17501.8	2999.8	5.8
Collagen, type VI, alpha 3	<i>Col6a3</i>	16958.0	3189.0	5.3
Secreted acidic cysteine rich glycoprotein	<i>Sparc</i>	554817.5	105624.7	5.3
<b>Genes upregulated in steady-state FB</b>	<b>Symbol</b>	<b>MFB-signal</b>	<b>FB-signal</b>	<b>Fold change (FB/MFB)</b>
Delta/notch-like EGF-related receptor	<i>Dner</i>	319.6	15308.1	47.9
Sodium channel, voltage-gated, type III, alpha	<i>Scn3a</i>	425.7	14025.8	32.9
ABI gene family, member 3 (NESH) binding protein	<i>Abi3bp</i>	465.9	13804.5	29.6
FMS-like tyrosine kinase 3 ligand	<i>Flt3l</i>	2997.3	88458.0	29.5
Retinol binding protein 4, plasma	<i>Rbp4</i>	569.2	16108.8	28.3
Solute carrier family 10 (sodium/bile acid cotransporter family), member 6	<i>Slc10a6</i>	763.1	16598.2	21.8
Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3	<i>Gabra3</i>	2820.6	50110.7	17.8
Adrenergic receptor, beta 3	<i>Adrb3</i>	800.3	13909.5	17.4
Epidermal growth factor-containing fibulin-like extracellular matrix protein 1	<i>Efemp1</i>	4169.3	71505.8	17.2
Cell adhesion molecule-related/down-regulated by oncogenes	<i>Cdon</i>	1643.3	27186.1	16.5
Mitochondrial transcription termination factor 4	<i>Mterf4</i>	1376.0	21849.2	15.9

Dipeptidase 1 (renal)	<i>Dpep1</i>	3009.2	47483.6	15.8
ATP-binding cassette, sub-family A (ABC1), member 8a	<i>Abca8a</i>	1325.0	19997.0	15.1
Fibulin 1	<i>Fbln1</i>	7692.5	108968.4	14.2
Apolipoprotein D	<i>Apod</i>	2072.4	29043.0	14.0
Phosphatidic acid phosphatase type 2B	<i>Ppap2b</i>	3124.7	43473.8	13.9
A kinase (PRKA) anchor protein (gravin) 12	<i>Akap12</i>	10163.1	136662.8	13.4
Growth differentiation factor 10	<i>Gdf10</i>	4296.0	56210.1	13.1
Complement component 7	<i>C7</i>	1347.9	16344.2	12.1
Complement component factor h	<i>Cfh</i>	9081.6	108051.4	11.9
Osteoglycin	<i>Ogn</i>	5302.9	62204.3	11.7
Solute carrier family 38, member 5	<i>Slc38a5</i>	5386.2	59901.3	11.1
Matrix metalloproteinase 3	<i>Mmp3</i>	3321.7	34777.3	10.5
RGD motif, leucine rich repeats, tropomodulin domain and proline-rich containing	<i>Rltpr</i>	1911.5	19913.5	10.4
Coagulation factor III	<i>F3</i>	4354.1	43716.7	10.0
Progesterin and adipoQ receptor family member VI	<i>Paqr6</i>	1668.1	16197.0	9.7
Hedgehog-interacting protein	<i>Hhip</i>	1388.7	13304.8	9.6
Complement factor H-related 2	<i>Cfhr2</i>	4355.1	40274.9	9.2
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	<i>Hmgcs2</i>	2037.9	18390.0	9.0
Gelsolin	<i>Gsn</i>	72027.0	647052.8	9.0
Bone morphogenetic protein 4	<i>Bmp4</i>	3748.1	33637.8	9.0
Hydroxysteroid 11-beta dehydrogenase 1	<i>Hsd11b1</i>	4025.1	35932.1	8.9
Podocan	<i>Podn</i>	1918.9	16890.0	8.8
Glypican 6	<i>Gpc6</i>	2993.9	24704.9	8.3
Integrin, beta-like 1	<i>Itgbl1</i>	1918.1	15445.6	8.1
Angiopoietin 1	<i>Angpt1</i>	4720.3	36485.2	7.7
Platelet derived growth factor receptor, alpha polypeptide	<i>Pdgfra</i>	11852.0	86126.0	7.3
Insulin-like growth factor binding protein 6	<i>Igfbp6</i>	8787.8	63348.0	7.2
Complement component 4B (Chido blood group)	<i>C4b</i>	6995.2	49948.4	7.1
Very low density lipoprotein receptor	<i>Vldlr</i>	2062.4	14483.7	7.0
C-fos induced growth factor	<i>Figf</i>	4176.8	28956.9	6.9
Sulfotransferase family 5A, member 1	<i>Sult5a1</i>	7031.9	48640.0	6.9
Complement component 1, s subcomponent 2	<i>C1s2</i>	9771.5	66757.1	6.8
Decorin	<i>Dcn</i>	24793.5	167281.3	6.7
Integrin alpha 8	<i>Itga8</i>	8519.4	56961.3	6.7
Neuroblastoma, suppression of tumorigenicity 1	<i>Nbl1</i>	7885.3	52238.2	6.6

Peptidase inhibitor 16	<i>Pil6</i>	2352.5	14502.6	6.2
Tumor necrosis factor alpha induced protein 6	<i>Tnfaip6</i>	6374.6	38703.5	6.1
HtrA serine peptidase 3	<i>Htra3</i>	5375.2	31504.5	5.9
Complement component 3	<i>C3</i>	62397.1	363510.2	5.8
Complement component 1, s subcomponent 1	<i>C1s1</i>	9683.9	56265.3	5.8
Collagen, type XIII, alpha 1	<i>Coll3a1</i>	5510.2	31843.6	5.8
Alcohol dehydrogenase 1 (class I)	<i>Adh1</i>	14653.7	79543.6	5.4
Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	<i>Svep1</i>	8957.6	47882.3	5.3
Interleukin 6	<i>Il6</i>	6277.5	33302.5	5.3
NADPH oxidase 4	<i>Nox4</i>	4753.6	24154.9	5.1
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	<i>Sema3d</i>	2641.6	13387.7	5.1
Slit homolog 3 (Drosophila)	<i>Slit3</i>	7279.1	36823.0	5.1
RNA binding motif, single stranded interacting protein	<i>Rbms3</i>	2767.8	13864.8	5.0

Genes that are upregulated in MFB were selected according to the following criteria: (1) five-fold greater than in steady-state FB; (2) five-fold greater than in total lung homogenates; and (3) two-fold greater than the mean value of all genes in MFB. Conversely, genes upregulated in steady-state FB were defined according to the following criteria: (1) five-fold greater than in MFB; (2) five-fold greater than in total lung homogenates; and (3) two-fold greater than the mean value of all genes in steady-state FB. The listed genes are available in the NCBI Reference Sequence.

**Table S3.** Gene ontology profiles of genes that are highly upregulated in myofibroblasts (MFB) or steady-state fibroblasts (FB) from mouse lungs

<b>Gene ontology terms of upregulated genes in MFB</b>	<b>Number (n = 44)</b>	<b>p</b>
Proteinaceous extracellular matrix	17	5.60E-18
Extracellular matrix	15	1.40E-17
Basement membrane	11	1.50E-14
Secreted	22	1.10E-12
Extracellular space	20	2.00E-10
Extracellular region	21	3.20E-10
Collagen	8	3.30E-10
Endodermal cell differentiation	6	4.60E-09
ECM-receptor interaction	8	8.00E-09
Collagen triple helix repeat	7	1.10E-08
Hydroxylation	7	2.10E-08
Signal peptide	25	2.40E-08
Signal	27	2.80E-08
Collagen trimer	7	3.40E-08
Focal adhesion	9	1.70E-07
Protein digestion and absorption	7	2.70E-07
Domain:Fibronectin type-III 9	4	7.40E-06
Disulfide bond	19	1.30E-05
Cell adhesion	9	1.60E-05
Domain:Fibronectin type-III 7	4	3.00E-05
TSPN	4	3.90E-05
Short sequence motif:Cell attachment site	5	4.10E-05
Domain:Fibronectin type-III 6	4	4.80E-05
Glycoprotein	20	5.20E-05
Fibronectin, type III	6	7.60E-05
Region of interest:Nonhelical region (NC2)	3	8.70E-05
PI3K-Akt signaling pathway	8	8.90E-05
Domain:Fibronectin type-III 14	3	1.20E-04
Extracellular matrix structural constituent	4	1.20E-04
Region of interest:Nonhelical region (NC1)	3	1.20E-04
Domain:Fibronectin type-III 5	4	1.30E-04
Extracellular matrix organization	5	1.50E-04
Domain:Fibronectin type-III 2	5	2.30E-04
Domain:Fibronectin type-III 1	5	2.40E-04
Laminin G domain	4	2.40E-04
Domain:Fibronectin type-III 11	3	2.60E-04
Domain:Fibronectin type-III 12	3	2.60E-04
Domain:Fibronectin type-III 10	3	3.20E-04
Domain:Fibronectin type-III 4	4	3.80E-04
Domain:Fibronectin type-III 3	4	8.40E-04
Region of interest:Triple-helical region	3	8.70E-04
Domain:TSP N-terminal	3	9.70E-04
<b>Gene ontology terms of upregulated genes in steady-state FB</b>	<b>Number (n = 59)</b>	<b>p</b>

Disulfide bond	40	3.20E-20
Signal peptide	41	6.10E-19
Signal	44	2.50E-18
Extracellular region	31	3.00E-17
Disulfide bond	36	5.40E-17
Extracellular space	29	6.80E-17
Secreted	29	1.00E-16
EGF-like domain	10	6.20E-09
EGF-like calcium-binding, conserved site	8	1.20E-08
EGF-type aspartate/asparagine hydroxylation site	8	1.20E-08
EGF-like calcium-binding	8	7.10E-08
Insulin-like growth factor binding protein, N-terminal	8	8.80E-08
Complement activation	5	1.10E-07
Proteinaceous extracellular matrix	10	2.90E-07
Complement and coagulation cascades	7	3.00E-07
EGF_CA	8	5.10E-07
Sushi/SCR/CCP	6	5.50E-07
Anaphylatoxin/fibulin	4	7.80E-07
Classical Complement Pathway	5	1.40E-06
CCP	6	2.00E-06
ANATO	4	2.00E-06
Blood microparticle	7	2.30E-06
Epidermal growth factor-like domain	8	5.00E-06
EGF	8	5.80E-06
Complement Pathway	5	8.30E-06
Growth factor	6	2.00E-05
EGF-like, conserved site	7	2.10E-05
Domain:EGF-like 1	6	2.40E-05
Staphylococcus aureus infection	5	3.30E-05
Extracellular matrix	7	3.30E-05
Domain:Anaphylatoxin-like	3	5.70E-05
Complement C3a/C4a/C5a anaphylatoxin	3	8.00E-05
Anaphylatoxin	3	8.00E-05
Lectin Induced Complement Pathway	4	9.80E-05
Growth factor activity	6	1.00E-04
Pertussis	5	1.50E-04
Domain:Sushi 1	4	1.70E-04
Domain:Sushi 2	4	1.70E-04
Sushi	4	2.50E-04
Positive regulation of ERK1 and ERK2 cascade	6	2.90E-04
Cross-link:Isoglutamyl cysteine thioester (Cys-Gln)	3	3.40E-04
Calcium ion binding	10	3.50E-04
Alpha-2-macroglobulin, conserved site	3	3.60E-04
Alpha-2-macroglobulin, thiol-ester bond-forming	3	3.60E-04
A-macroglobulin complement component	3	4.40E-04

Alpha-macroglobulin, receptor-binding	3	4.40E-04
Alpha-2-macroglobulin, N-terminal	3	5.20E-04
Alpha-2-macroglobulin	3	5.20E-04
Alpha-2-macroglobulin, N-terminal 2	3	5.20E-04
Domain:EGF-like 2; calcium-binding	4	5.60E-04
Complement activation, classical pathway	4	6.60E-04
SM01359	3	8.10E-04
SM01361	3	8.10E-04
Terpenoid cyclases/protein prenyltransferase alpha-alpha toroid	3	8.30E-04
Developmental protein	10	8.80E-04
SM01360	3	9.70E-04

Genes listed here were selected in accordance with Table S2.

**Table S4.** Sequences of gene-specific primers used in this study

<b>Mouse</b>	<b>Forward primer</b>	<b>Reverse primer</b>
Glyceraldehyde-3-phosphate dehydrogenase ( <i>Gapdh</i> )	aactttggcattgtggaagg	ggatgcagggatgatgttct
Actin, alpha 2, smooth muscle, aorta ( <i>Acta2</i> )	tgtgctggactctggagatg	gaaggaatagccacgctcag
Latent transforming growth factor beta-binding protein-2 ( <i>Ltbp2</i> )	gcgaatgcaagaacacagaa	aatgctcttccccaacacac
<b>Human</b>	<b>Forward primer</b>	<b>Reverse primer</b>
Glyceraldehyde-3-phosphate dehydrogenase ( <i>GAPDH</i> )	cctgcaccaccaactgctta	gtcaaaggtggaggagtggg
Actin, alpha 2, smooth muscle, aorta ( <i>ACTA2</i> )	aagacagctacgtgggtgac	atcttctcccgggttgccctt
Latent transforming growth factor beta-binding protein-2 ( <i>LTBP2</i> )	ggatggacaacagcaaacagc	catcggaatgacctctcgc

**Table S5.** Comparison of clinical factors between groups with higher levels (>18 ng/mL) and lower levels (≤18 ng/mL) of serum LTBP2 in patients with idiopathic pulmonary fibrosis



	<b>LTBP2-high</b>	<b>LTBP2-low</b>	<b>p</b>
	<b>n = 65</b>	<b>n = 51</b>	
Age, years	73 (68–78)	68 (63–73)	<0.05
Male	57 (88)	44 (86)	NS
Current or former smoker	52 (80)	44 (86)	NS
Smoking dose (pack-year)	34 (10–50)	40 (20–63)	NS
PaO <sub>2</sub> on room air (Torr)	75 (64–84)	78 (71–88)	<0.05
% predicted FVC (%)	67 (54–84)	83 (69–94)	<0.01
% predicted DLCO (%)	58 (48–79)	67 (54–84)	NS
Serum KL-6 (U/mL)	1105 (773–1705)	864 (515–1231)	<0.05
Serum SP-D (ng/mL)	239 (169–369)	211 (127–274)	NS
Serum LDH (U/L)	238 (209–279)	218 (199–262)	NS

Data are described as n (%) or median (interquartile range). All p values were evaluated by Fisher's exact test or the Mann–Whitney U test as appropriate.

Abbreviations: DLCO = diffusion capacity for carbon monoxide; FVC = forced vital capacity; KL-6 = Krebs von den Lungen-6; LDH = lactate dehydrogenase; LTBP2 = latent transforming growth factor- $\beta$  binding protein-2; NS = not significant; SP-D = surfactant protein-D.

Supplementary figure legends

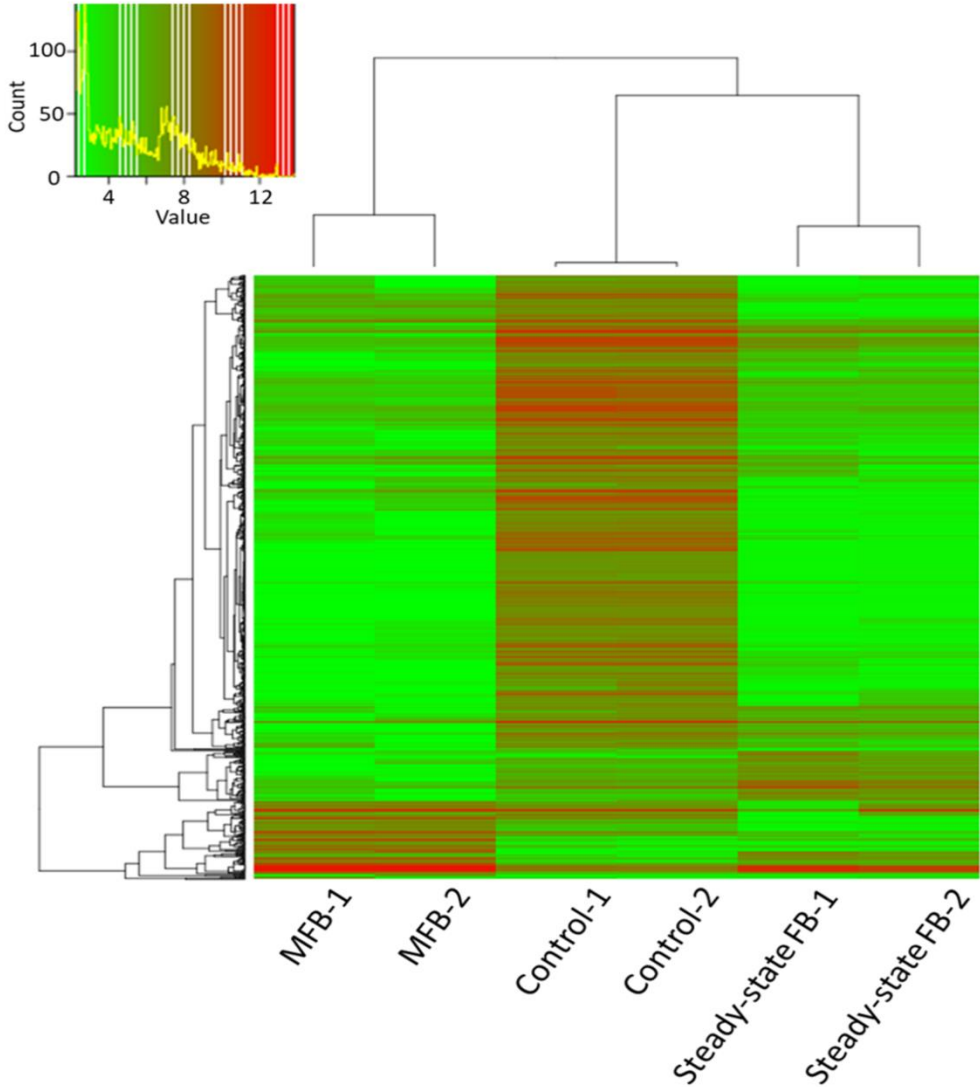
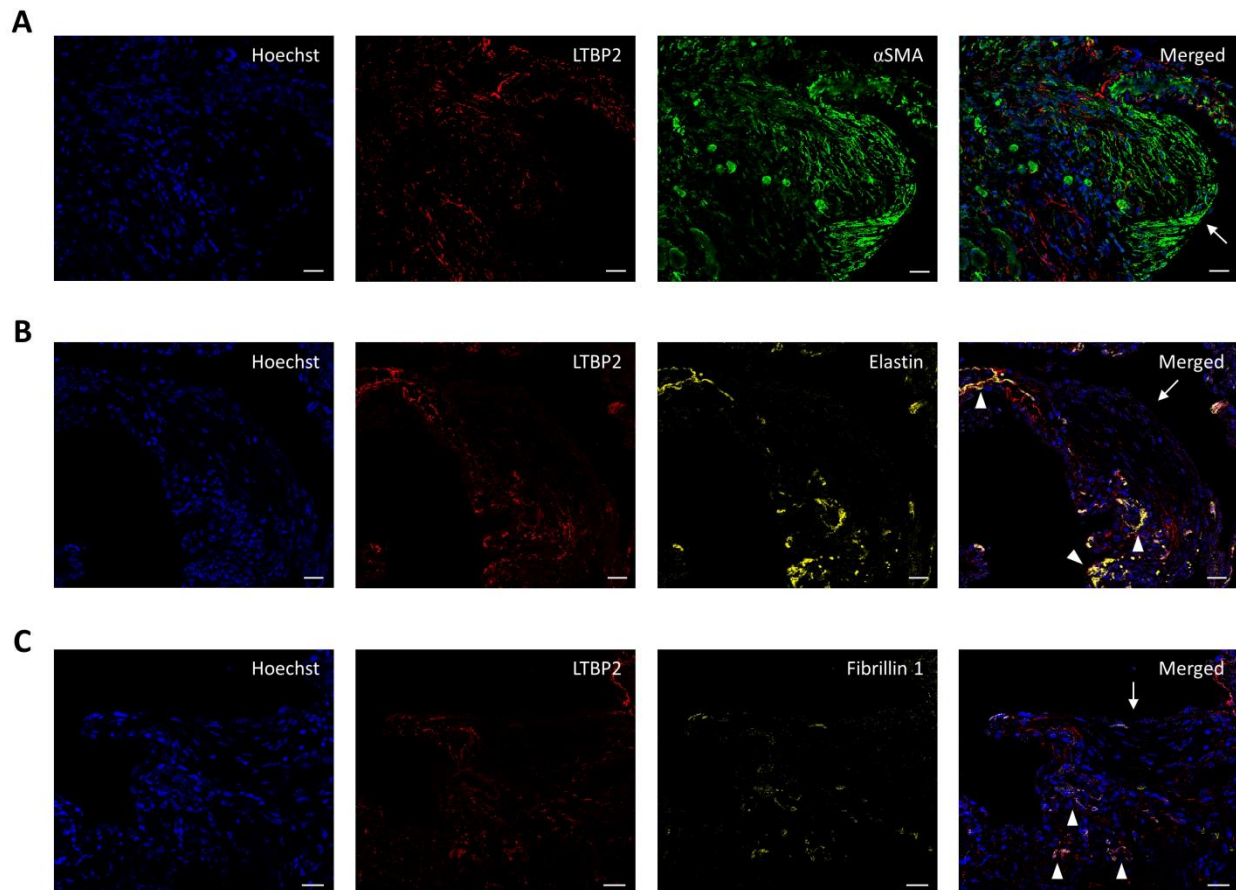


Figure S1. Heatmap of gene expression profiles in each sample

This heatmap is generated using a list of the top 1,000 genes with a large signal gap among all samples (myofibroblasts [MFB], steady-state fibroblasts [FB], and controls). Lung homogenates are used as controls.



**Figure S2.** Immunofluorescence images of fibrotic lesions, including fibroblastic foci, in a biopsied lung of idiopathic pulmonary fibrosis

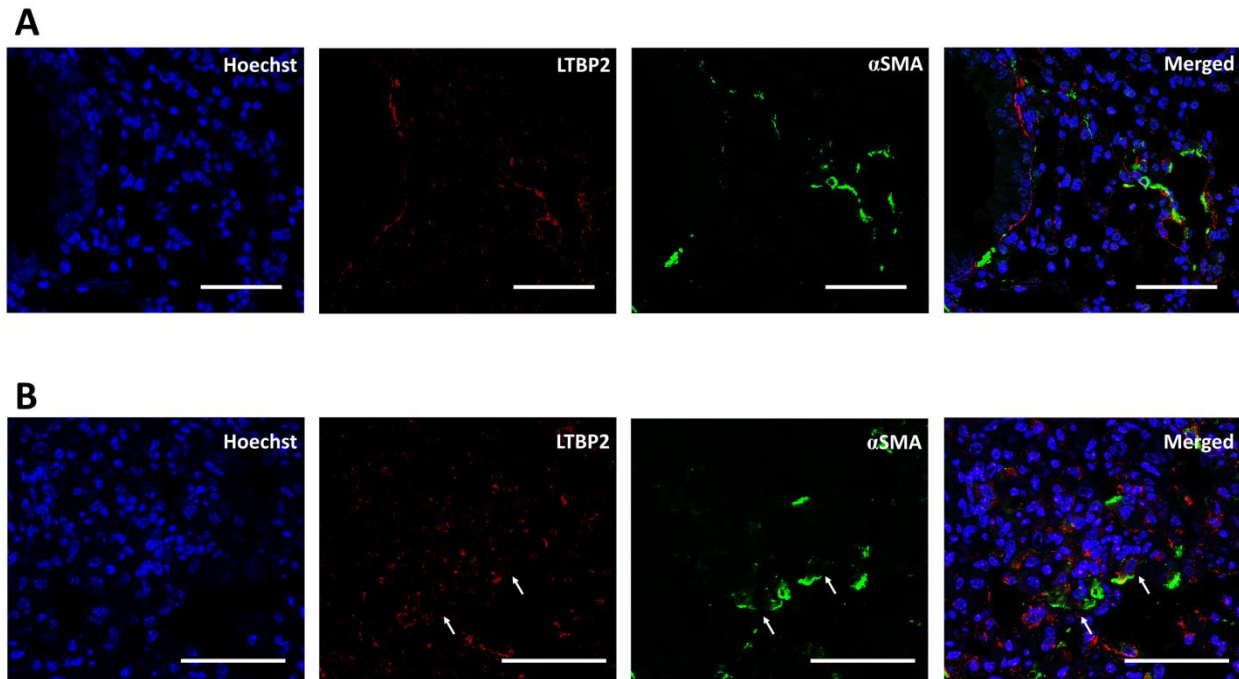
A. Anti-latent transforming growth factor- $\beta$  binding protein-2 (LTBP2) and anti- $\alpha$ -smooth

muscle actin ( $\alpha$ SMA) staining. LTBP2 is broadly upregulated in the fibrotic interstitium but was scarce in the  $\alpha$ SMA-positive fibroblastic focus (arrow).

**B.** Anti-LTBP2 and anti-elastin staining. The positivity of LTBP2 and elastin was partially overlapping (triangles). The fibroblastic focus (arrow) did not show apparent positivity for both proteins.

**C.** Anti-LTBP2 and anti-fibrillin-1 staining. Similar to elastin, the positivity of fibrillin-1 was also partially overlapping with LTBP2 (triangles). The fibroblastic focus (arrow) did not show apparent positivity for both proteins.

All scale bars = 20  $\mu$ m.

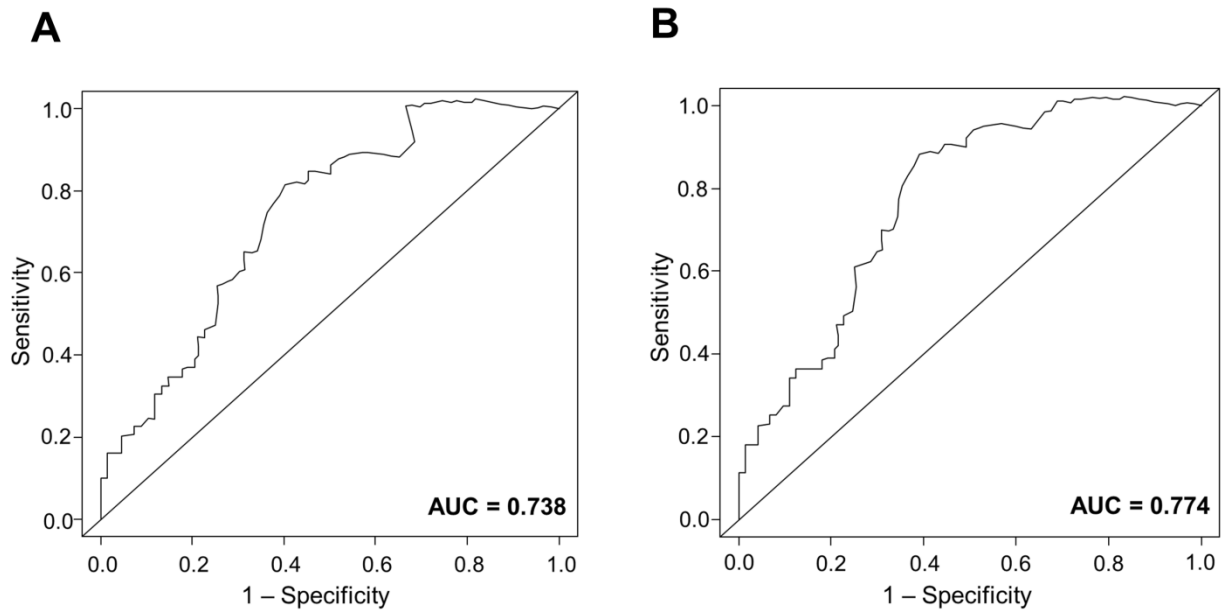


**Figure S3.** Immunofluorescence images of mouse lungs

**A** (Untreated mouse lung). Latent transforming growth factor- $\beta$  binding protein-2 (LTBP2) and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) were observed only around the bronchiole and the blood vessel.

**B** (Bleomycin-treated mouse lung). LTBP2 positivity was increased in the interstitium mainly as an extracellular matrix protein. Some of the proteins colocalized with  $\alpha$ SMA-positive myofibroblasts (arrows).

All scale bars = 50  $\mu$ m.



**Figure S4.** Receiver operating characteristic curves for serum latent transforming growth factor- $\beta$  binding protein-2 concentrations (evaluated at the time of diagnosis of idiopathic pulmonary fibrosis) during the 60-month follow-up

**A.** The endpoint was set at all-cause death.

**B.** The endpoint was set at death by respiratory events.