ORIGINAL

Functional analysis of human fibrocytes derived from monocytes reveals their profibrotic phenotype through paracrine effects

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Abstract : Fibrocytes, which are bone marrow-derived collagen-producing cells, were reported to play a role in the pathogenesis of pulmonary fibrosis. However, their function in pulmonary fibrosis is unclear. We analyzed their function compared with that of monocytes and localization in fibrotic tissues in patients with idiopathic pulmonary fibrosis (IPF). We compared the gene expression profile of monocyte-derived fibrocytes with that of monocytes by microarray analysis. Proliferation and differentiation into myofibroblasts were examined by ^{3}H-thymidine incorporation assay and Western blotting. We measured the level of growth factors in the culture supernatant of fibrocytes by ELISA. The localization of fibrocytes in lung tissues of patients with IPF was determined by immunofluorescence staining. Compared with monocytes, fibrocytes had higher expression of extracellular matrix- and growth factor-encoding genes, including PDGF-B, FGF-2 and VEGF-B. Although fibrocytes did not proliferate in response to PDGF, co-culture of fibrocytes stimulated the growth of lung fibroblasts through the production of PDGF-BB. In the lung of IPF patients, CD45^{+}Collagen-I^{+}FSP-1^{+} cells, which have a similar phenotype to fibrocytes, were detected and co-stained with anti-PDGF antibody. This study suggested that fibrocytes function in pulmonary fibrosis partly by producing PDGF in the lungs of IPF patients. J. Med. Invest. 67 : 102-112, February, 2020

Keywords **:** *fibrocyte, fibroblast, idiopathic pulmonary fibrosis, PDGF*

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and lethal disease of the lungs, characterized by the proliferation of fibroblasts and deposition of extracellular matrix (1, 2). Recent studies on the molecular pathogenesis in IPF demonstrated novel lung fibroblasts from other origins, including bone-marrow cells, alveolar epithelial cells, endothelial cells, pleural cells and pericytes, in addition to resident fibroblasts (3-9).

Fibrocytes are defined as bone marrow-derived and collagen-expressing cells, which constitute approximately 0.5% of the peripheral blood leukocyte population, exhibiting an adherent spindle shape in culture (10, 11). Therefore, fibrocytes express markers of hematopoietic cells (CD34), leukocytes (CD11b, CD13 and CD45) and the extracellular matrix (collagens I and III, and fibronectin) (11-13). A number of studies have reported the potential for bone marrow-derived circulating fibrocytes to enter tissues following injury, leading to lung fibrosis (3, 4, 14-19). In addition, a clinical trial of pentraxin-2, for IPF, which inhibits the generation of fibrocytes, was performed and reported significant anti-fibrotic effects (20, 21).

However, the role of fibrocytes in tissues remains unclear. Kleaveland *et al*. reported that the production of collagen by fibrocytes did not lead to pulmonary fibrosis in the Vav-Cre transgenic mice with specific deletion of the collagen Ia1 gene in hematopoietic cells (22), although fibrocytes are expected to be a source of lung fibroblasts.

We previously reported that fibrocytes have the ability to produce growth factors, and their functions are regulated by antifibrotic agents such as imatinib and nintedanib (23, 24). Garcia *et al*. found that co-culture of fibrocytes with lung fibroblasts increased the expression of collagen Ia1 and alfa-smooth muscle actin (SMA), and levels of chemokines and growth factors in fibroblasts (25). In the present study, to characterize the function of fibrocytes, we compared the gene expression profile of human monocyte-derived fibrocytes with that of monocytes, demonstrating the pro-fibrotic phenotype of fibrocytes. We also examined several functions, including growth and differentiation to myofibroblasts, and the production of growth factors *in vitro* and in lung tissues of patients with IPF.

MATERIALS AND METHODS

Ethics statement

This study was approved by the institutional review board of Tokushima University Hospital (Approval Numbers : 2919, 2920), and conformed to the Declaration of Helsinki.

Isolation of human monocytes and fibrocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood using Ficoll density centrifugation. Human peripheral blood monocytes were purified from PBMCs by magnetic beads for CD14 (23). PBMCs from the same donor were used to isolate fibrocytes according to previously published methods (23), and were then cultured in the fibronectin-coated flasks including DMEM (Dulbecco's Modified, Eagle Medium) (Grand Island Biological Co. Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) for 7 days.

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The adherent cells were harvested using 0.125% trypsin. We used immunomagnetic selection to deplete B lymphocytes (anti-CD19 ; Miltenyi Biotech, Auburn, CA, USA), T lymphocytes (anti-CD3 ; Miltenyi Biotech) and monocytes/macrophages (anti-CD14 ; Miltenyi Biotech) from our crude fibrocyte preparation. The trypsinized cells were stained with anti-CD19, CD3 and CD14 antibodies coupled to magnetic beads. Labeled cells were then sorted by binding the cell population to positive-selection columns using Auto Macs (Miltenyi Biotech).

Total RNA isolation and qPCR

Total cellular RNA was isolated using RNeasy Mini kits and RNase-free DNase kits (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. RNA was reversely transcribed on a Takara PCR thermal cycler Dice (Takara Bio, Inc., Kyoto, Japan) using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) and SYBR® *Premix Ex Taq*™ (Perfect Real Time) (Takara Bio Inc.) following the manufacturer's instructions. The expression of TGF-β1, PDGF-B, FGF-2 and the housekeeping gene human RPL27 was measured by quantitative PCR analysis on an ABI 7700 Sequence Detection system (Applied Biosystems) using the following commercially available sets of primers and fluorogenic probes : (TaqMan®Gene Expression AssayGFs products) : TGF-β1, Ec03469540_ml ; PDGF-BB, Hs00966522_ml ; FGF-2, Hs0026645_ml. The quantitative PCR experiments were performed in triplicate, and the relative expression levels were calculated based on the comparative Ct method.

Microarray analysis

The relative purity of the RNA was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) (26). One hundred and thirty ng of total RNA was amplified and labeled using the Agilent Low Input Quick Amp Labeling Kit, One-Color (5190-2305), and labeled RNA was hybridized with the Human Gene expression 4x44K v2 Microarray Kit (G4845A). Agilent Feature Extraction Image Analysis Software (Version 10.7.3) was used to extract data from raw microarray image files. Data visualization and analysis were performed using GeneSpring GX (Version 12.1) software.

Cell cultures and fibroblast isolation

MRC5 fibroblasts were purchased from ATCC (Masassas, VA, USA). Human IPF-fibroblast cells were generated from biopsy samples from patients with IPF (24).

^{3}H-Thymidine uptake assay

The lung fibroblasts or fibrocytes were added to a 96-well plate at $2x10^4$ cells per well. The cells (50-60% confluent) were cultured in DMEM supplemented with 10% FBS overnight, and then rendered quiescent in DMEM containing 0.1% FBS overnight. Next, the cells (60-70% confluent) were treated with PDGF-BB (0, 1 or 10 ng/ml) for 48 hours with or without irradiated fibroblasts (50 Gy) or fibrocytes (35 Gy) at the indicated cell number. These cells were labeled with ^{3}H-thymidine deoxyribose (TdR) (6.7 Ci/mmol ; Amersham Corp., Arlington Heights, IL, USA) at 1 μCi/well for the final 16 hours. The cells were harvested on glass fibers in a cell harvester, MASHII (Labo Science Co., Tokyo, Japan), and the incorporation of ^{3}H-TdR was measured using a liquid scintillation counter (27). In some experiments, the supernatant of fibrocytes was added and neutralizing antibodies (2 μg/ml) for FGF-2, PDGF-BB, and TGF-β (R&D Systems, Minneapolis, MN, USA) was used. Cell proliferation was evaluated by assaying the incorporation of ^{3}H-TdR. The experiments were performed in triplicate cultures.

ELISA

The human fibrocytes ($1x10^6$) and monocytes ($1x10^6$) from same donor were cultured in 1 ml of fresh culture medium (DMEM with 0.1% FBS). After incubation for 48 h, the culture media were harvested and centrifuged, and the supernatants were stored at -80°C until used as the culture supernatant. For human growth factors, their concentrations were quantified using ELISA kits (R&D Systems) following the manufacturers' instructions. The minimum and maximum detectable protein levels were TGF-β1 (31.2-2000 pg/mL), PDGF-BB (31.2-2000 pg/mL) and FGF-2 (10-640 pg/mL). Data are presented as the mean ± SEM in each group. Data are representative of three separate experiments.

Western blot

Human lung fibroblasts and fibrocytes were cultured in DMEM supplemented with 0.1% FBS with or without 5 ng/ml of TGF-β1. The cells were transferred quickly to ice and lysed in RIPA buffer (10 mM TrisHCl pH 7.4, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 mM NaCl, 1 mM EDTA and 10 μg/ml of aprotinin) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The protein concentration was measured by protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of total cell extract protein was suspended in 2 x Laemmli sample buffer (Bio-Rad Laboratories). These samples were boiled for 5 minutes and electrophoresed on 4-10% NuPAGE Bis-Tris Mini gels (at 200 Voltage for 40 min). Then, proteins were electroblotted onto iBlot™ gel Transfer Stacker polyvinylidene difluoride (PVDF) membranes (Life Technologies, Carlsbad, CA, USA). The membranes were blocked with 0.2% non-fat milk/Tris-buffered saline-Tween 20® solution in blot holders (SNAP i.d. TM Protein Detection System, Millipore, Billerica, MA, USA). Transferred membranes were incubated with anti-alpha-smooth muscle actin antibody (Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight, followed by incubation for 60 min at room temperature with goat-anti mouse IgG horseradish peroxidase-conjugated IgG (Cayman Chemical, Ann Arbor, MI, USA). Transferred membranes were incubated with biotin-conjugated anti-collagen-type 1 antibody (Rockland, Gilbertsville, PA, USA) at 4°C overnight, followed by incubation for 60 min at room temperature with streptavidin peroxidase-conjugated secondary antibody (Rockland). Transferred membranes were incubated with anti-β-actin antibody (GenScript, Piscataway, NJ, USA) at 4°C overnight, followed by incubation for 60 min at room temperature with goat-anti mouse IgG horse radish peroxidase (Cayman Chemical). Protein-antibody conjugates were then developed using enhanced chemiluminescent substrate (Thermo Scientific Inc. ; Rockford, IL). The immunoreactive bands were read under a luminescent image analyzer (Las-4000 mini ; Fuji Film, Tokyo, Japan).

Immunofluorescence staining

The lungs were fixed in 10% buffered formalin and embedded in paraffin. Sections (3 to 4 μm) were stained with hematoxylin and eosin. Paraffin-embedded lung sections were stained to detect fibrocytes using the following antibodies : biotin-conjugated anti-CD45 antibody (Cell Signaling Tech, Danvers, MA, USA), secondary antibody : streptavidin-FITC (eBioscience, Ireland, UK) ; anti-collagen-type 1 antibody (Abcam ; Cambridge, UK) and anti-FSP-1 antibody (Thermo Fisher scientific Inc.), and secondary antibodies conjugated with Alexa Flour 594 or 647 (Thermo Fisher scientific Inc.), respectively.

For triple staining to detect PDGF-BB in fibrocytes, the following antibodies were used : anti-human FSP-1 antibody, (Thermo Fisher scientific Inc.), anti-human CXCR4 antibody

(Abcam) and anti-human PDGF-BB antibody (Novus biological, Littleton, CO, USA), and the fluorescent secondary antibodies conjugated with Alexa Flour 488, 594 or 647 (Thermo Fisher scientific Inc.), respectively. To detect proliferating cells, the section was first stained with anti-Ki-67 antibody (Agilent Technologies) followed by the secondary antibody conjugated with Alexa Flour 594 were used. Images were taken using a confocal laser scanning microscope, A1 system (Nikon, Tokyo, Japan).

Statistical Analysis

The results were analyzed using the Mann-Whitney test for comparison between any two groups, and by nonparametric equivalents of ANOVA for multiple comparisons. $p < 0.05$ was considered to indicate significance.

RESULTS

Comparison of the gene expression profile of monocyte-derived fibrocytes and monocytes

First, we compared the gene expression profile of monocyte-derived fibrocytes and monocytes in paired samples from the same donor by microarray analysis. When we analyzed 9668 genes, we found 4062 genes that were upregulated in fibrocytes compared with monocytes, whereas 5606 genes were down-regulated in fibrocytes compared with monocytes (Figure 1A). As shown in the heat-map in Figure 1B, gene expression in fibrocytes from four donors was homogenous and different from that in monocytes.

Focusing on several groups of genes, among cytokines, genes for those, such as colony stimulating factor (CSF) 2, interleukin (IL) 13, CSF1, IL-1 and IFN-γ, were strongly up-regulated in fibrocytes compared with monocytes, whereas the CSF3R gene was down-regulated in fibrocytes (Table 1). Among growth factors, genes for those, such as platelet-derived growth factor (PDGF) RA, fibroblast growth factor (FGF) R1 and vascular endothelial growth factor (VEGF) B, were strongly up-regulated in fibrocytes, whereas the hepatocyte growth factor (HGF) gene was significantly down-regulated in fibrocytes (Table 2). Among chemokines, several CC chemokine ligand (CCL) genes, such as CCL18, CCL7, CCL2 and CCL13, were up-regulated in fibrocytes (Table 3). Regarding the extra-cellular matrix (ECM), several collagen genes, such as collagen VIa1, Ia1 and VIa2, and integrins such as a3, b5, b8 and a6, were strongly up-regulated in fibrocytes (Table 4). In addition, many matrix-metallopeptidases (MMPs), such as MMP 7, 9, 2 and 14, were significantly up-regulated in fibrocytes (Table 5).

Higher expression of growth factors in fibrocytes than in monocytes

Gene expression analysis revealed the profibrotic phenotype of fibrocytes because genes for growth factors, ECM and MMPs were strongly up-regulated in fibrocytes. Therefore, we focused on the production of growth factors by fibrocytes. As shown in Figure 2, fibrocytes had higher expression of PDGF-B and FGF-2, but not TGF-β, at the mRNA and protein levels.

Ability of fibrocytes to grow and differentiate into myofibroblasts

Next, we examined the growth ability of fibrocytes in response to PDGF-BB and co-culture with fibroblasts. To assess the ^{3}H-TdR uptake of fibrocytes, fibroblasts were irradiated and added into fibrocyte cultures (Figure 3A). As a result, the fibrocytes did not proliferate in response to PDGF-BB even in the co-culture with fibroblasts. On the other hand, fibrocytes were irradiated and co-cultured with fibroblasts. As shown in Figure 3B, fibroblasts had the ability to proliferate after stimulation by PDGF-BB, and co-culture with fibrocytes significantly increased their proliferation in a fibrocyte-dependent manner. These

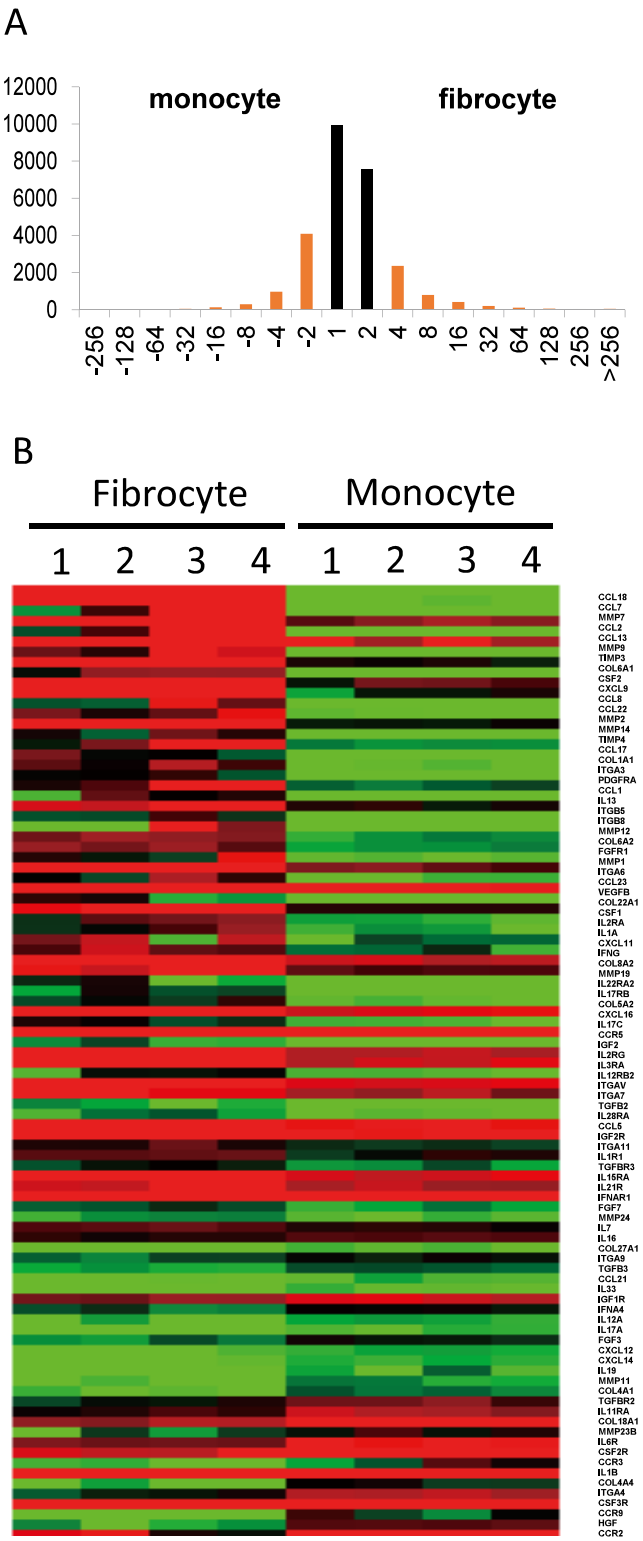

Figure 1 Comparison of gene expression profiles of fibrocytes and monocytes. A semilogarithmic plot showing that 4062 genes were upregulated in fibrocytes compared with in monocytes, whereas 5606 genes were down-regulated in fibrocytes compared with in monocytes among 9668 genes (A). The heat-map of the representative genes showing that gene expression in fibrocytes from four donors was homogenous and different from that in monocytes (B).

Table 1 Selected up- and down-regulated genes related to cytokines and their receptors in fibrocytes in comparison with monocytes

Gene symbol	Probe name	Gene name	P	Fold change
(1)Up				
CSF2	A_23_P133408	colony stimulating factor 2	6E-05	101.4
IL13	A_23_P251031	interleukin 13	0.004	20.88
CSF1	A_33_P3354935	colony stimulating factor 1	4E-06	13.67
IL2RA	A_24_P230563	interleukin 2 receptor, alpha	0.002	13.67
IL1A	A_23_P72096	interleukin 1, alpha	0.011	12.24
IFNG	A_23_P151294	interferon, gamma	0.002	11.12
IL22RA2	A_33_P3422124	interleukin 22 receptor, alpha 2	0.024	8.402
IL17RB	A_23_P167479	interleukin 17 receptor B	0.002	8.086
IL17C	A_33_P3339625	interleukin 17C	0.001	7.402
IL2RG	A_23_P148473	interleukin 2 receptor, gamma	6E-06	5,238
(2)Down				
CSF3R	A_33_P3381262	colony stimulating factor 3 receptor	2E-05	-11.2
IL1B	A_23_P79518	interleukin 1, beta	0.04	-7.9
CSF2RA	A_33_P3232688	colony stimulating factor 2 receptor, alpha	7E-04	-5.46
IL6R	A_33_P3288844	interleukin 6 receptor	1E-06	-4.82
IL11RA	A_23_P71867	interleukin 11 receptor, alpha	7E-04	-4.48
IL19	A_23_P35092	interleukin 19	0.027	-3.25
IL17A	A_23_P332820	interleukin 17A	0.002	-2.53
IL12A	A_23_P91943	interleukin 12A	0.039	-2.52
IFNA4	A_24_P403459	interferon, alpha 4	0.006	-2.39
IL33	A_23_P31945	interleukin 33	0.001	-2.32

Table 2 Selected up- and down-regulated genes related to growth factors and their receptors in fibrocytes in comparison with monocytes

Gene symbol	Probe name	Gene name	P	Fold change
(1)Up				
PDGFRA	A_23_P300033	platelet-derived growth factor receptor, alpha polypeptide	4E-04	27.73
FGFR1	A_23_P372923	fibroblast growth factor receptor 1	2E-04	17.24
VEGFB	A_23_P1594	vascular endothelial growth factor B	3E-05	13.94
IGF2	A_23_P150609	insulin-like growth factor 2	0.001	6.378
TGFB2	A_24_P402438	transforming growth factor, beta 2	0.034	3.601
PDGFB	A_24_P339944	platelet-derived growth factor beta polypeptide	0.172	3.558
IGFR2	A_23_P334021	insulin-like growth factor 2 receptor	8E-04	3.078
TGFRB3	A_23_P200780	transforming growth factor, beta receptor III	0.025	2.523
FGF7	A_33_P3257027	fibroblast growth factor 7	0.005	2.187
PDGFRB	A_23_P421401	platelet-derived growth factor receptor, beta polypeptide	0.017	1.925
(2)Down				
HGF	A_33_P3276713	Hepatocyte growth factor	3E-04	-16
TGFBR2	A_33_P3313825	transforming growth factor, beta receptor II	0.009	-4.01
FGF3	A_33_P3380797	fibroblast growth factor 3	0.004	-2.67
IGF1R	A_23_P417282	insulin-like growth factor 2 receptor	0.003	-2.38
TGFB3	A_23_P88404	transforming growth factor, beta	0.002	-2.14

Table 3 Selected up- and down-regulated genes related to chemokines and their receptors in fibrocytes in comparison with monocytes

Gene symbol	Probe name	Gene name	P	Fold change
(1)Up				
CCL18	A_23_P55270	chemokine (C-C motif) ligand 18	5E-06	3659
CCL7	A_23_P78037	chemokine (C-C motif) ligand 7	3E-06	969.7
CCL2	A_23_P89431	chemokine (C-C motif) ligand2	3E-05	252.9
CCL13	A_24_P125335	chemokine (C-C motif) ligand 13	0.005	248.8
CXCL9	A_24_P18452	chemokine (C-X-C motif) ligand 9	0.001	69.63
CCL8	A_23_P207456	chemokine (C-C motif) ligand 8	2E-04	68.64
CCL22	A_24_P313418	chemokine (C-C motif) ligand 22	0.005	47.8
CCL17	A_23_P26325	chemokine (C-C motif) ligand 17	0.015	36.59
CCL1	A_23_P49759	chemokine (C-C motif) ligand 1	0.019	22.96
CCL23	A_24_P133905	chemokine (C-C motif) ligand 23	0.006	15.28
CXCL11	A_23_P125278	chemokine (C-X-C motif) ligand 11	0.082	11.56
CXCL16	A_33_P3351249	chemokine (C-X-C motif) ligand 16	9E-06	7.065
CCR5	A_33_P3395595	chemokine (C-C motif) receptor 5	0.003	6,583
CCL5	A_23_P152838	chemokine (C-C motif) ligand 5	0.019	3.097
(2)Down				
CCR2	A_23_P212354	chemokine (C-C motif) receptor 2	0.006	-69.02
CCR9	A_33_P3395595	chemokine (C-C motif) receptor 9	0.001	-12.17
CCR3	A_23_P250302	chemokine (C-C motif) receptor 3	0.019	-7.036
CXCL14	A_33_P3590259	chemokine (C-X-C motif) ligand 14	8E-04	-2.784
CXCL12	A_33_P3372840	chemokine (C-X-C motif) ligand 12	0.004	-2.691
CCL21	A_33_P3273623	chemokine (C-C motif) ligand 21	0.01	-2.291

Table 4 Selected up- and down-regulated genes related to the extracellular matrix and cell adhesion molecules in fibrocytes in comparison with monocytes

Gene symbol	Probe name	Gene name	P	Fold change
(1)Up				
COL6A1	A_32_P32254	collagen, type VI, alpha 1	0	118
COL1A1	A_33_P3304668	collagen, type I, alpha 1	3E-04	34.28
ITGA3	A_23_P55251	integrin, alpha 3	3E-04	32.18
ITGB5	A_23_P166633	integrin, beta 5	0.002	20.26
ITGB8	A_24_P759477	integrin, beta 8	2E-04	19.91
COL6A2	A_23_P310956	collagen, type VI, alpha 2	3E-06	19.64
IITGA6	A_33_P3231447	integrin, alpha 6	4E-04	16.02
COL22A1	A_32_P405759	collagen, type XXII, alpha 1	0.004	13.76
COL8A2	A_33_P3297930	collagen, type VIII, alpha 2	7E-06	10.24
COL5A2	A_23_P33196	collagen, type V, alpha 2	8E-04	7.948
ITGAV	A_23_P50907	integrin, alpha V	2E-05	4.295
ITGA7	A_23_P128084	integrin, alpha 7	0.009	3.892
ITGA11	A_23_P206022	integrin, alpha 11	0.004	2.922
(2)Down				
ITGA4	A_33_P3224878	integrin, alpha 4	5E-05	-10.38
COL4A4	A_33_P3227400	collagen, type IV, alpha 4	0.001	-9.755
COL18A1	A_23_P211212	collagen, type XVIII, alpha 1	1E-04	-4.695
COL4A1	A_23_P65240	collagen, type IV, alpha 1	0.002	-3.795
ITGA9	A_23_P252193	integrin, alpha 9	0.005	-2.113
COL27A1	A_33_P3413168	collagen, type XXVII, alpha 1	0.004	-2.021

Table 5 Selected up- and down-regulated genes related to matrix metalloproteases and their inhibitors in fibrocytes in comparison with monocytes

Gene symbol	Probe name	Gene name	P	Fold change
(1)Up				
MMP7	A_23_P52761	matrix metallopeptidase 7	0.04	331.3
MMP9	A_23_P40174	matrix metallopeptidase 9	3E-04	239.4
TIMP3	A_23_P399078	TIMP metallopeptidase inhibitor 3	3E-04	154.4
MMP2	A_33_P3237135	matrix metallopeptidase 2	0.002	47.49
MMP14	A_24_P82106	matrix metallopeptidase 14	4E-08	47.15
TIMP4	A_32_P70315	TIMP metallopeptidase inhibitor 4	2E-04	41.67
MMP12	A_23_P150316	matrix metallopeptidase 12	0.087	19.89
MMP1	A_23_P1691	matrix metallopeptidase 1	0.01	16.63
MMP19	A_23_P203888	matrix metallopeptidase 19	0.001	9.483
MMP24	A_33_P3398331	matrix metallopeptidase 24	0.016	2.042
(2)Down				
MMP23B	A_23_P74088	matrix metallopeptidase 23B	0.032	-4.739
MMP11	A_23_P57417	matrix metallopeptidase 11	0.006	-3.633

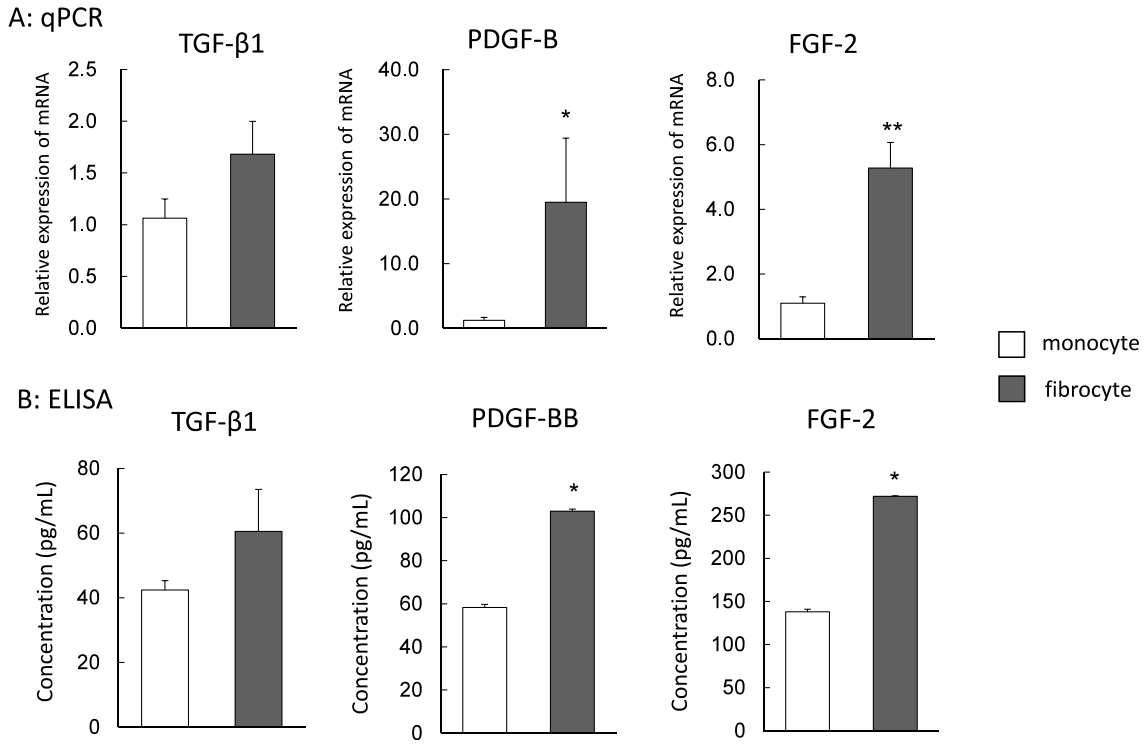

Figure 2 Expression of growth factors in fibrocytes compared with that in monocytes. Expression of TGF-β, PDGF-B and FGF-2 was examined by qPCR (A) and ELISA (B). The experiments were performed as described in Materials and Methods. Expression levels of PDGF-B and FGF-2 were significantly higher in fibrocytes than in monocytes. Similar results were obtained in at least three independent experiments. *$p < 0.01$ vs monocytes. **$p < 0.005$ vs monocytes.

results suggest that fibrocytes can increase the proliferation of fibroblasts.

The supernatant of fibrocytes also stimulated ^{3}H-TdR uptake by fibroblasts (Figure 3C and 3D). Treatment with anti-PDGF-BB antibody significantly reduced the fibrocyte-mediated increase in the growth of lung fibroblasts from MRC5 and IPF patients (Figure 3C and 3D). These data suggest that fibrocytes can stimulate the growth of fibroblasts through PDGF-BB production.

Next we examined the ability of fibrocytes to differentiate into myofibroblasts, which are believed to be activated fibroblasts (Figure 4). In this experiment, MRC5 fibroblasts and fibrocytes were cultured in the medium containing transforming growth factor-β (TGF-β) for three weeks. As shown in Figure 4, fibroblasts highly expressed both α-SMA and collagen I, and their expression further increased during the culture. However, α-SMA was not detected in fibrocytes and its expression did not increase during the culture (Figure 4A). In addition, the expression of collagen I was significantly lower in fibrocytes than in fibroblasts, and it also did not increase during the culture (Figure 4B).

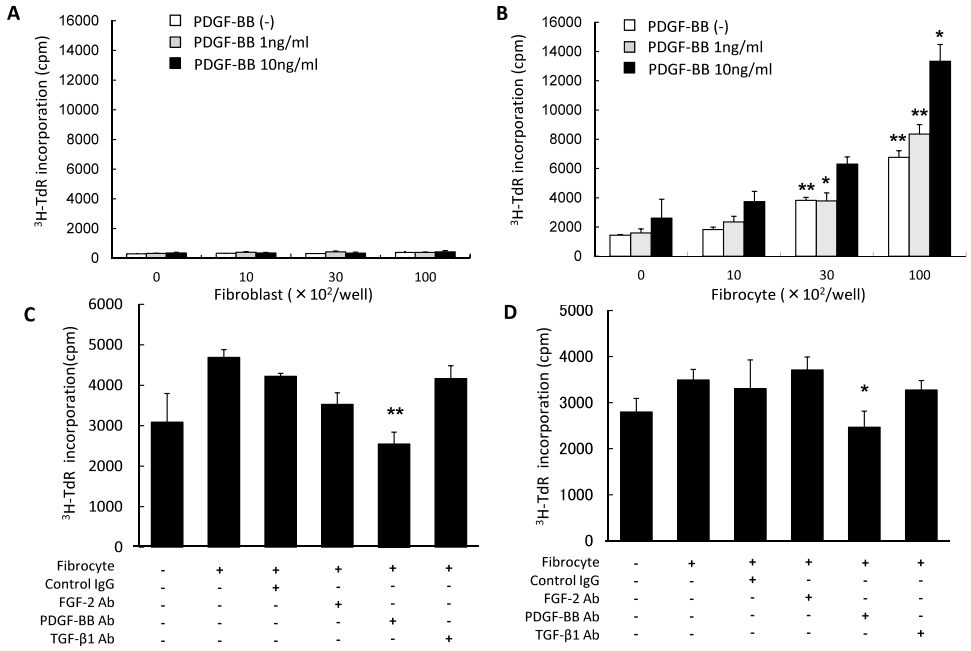

Figure 3 Fibrocytes increase the growth of lung fibroblasts through PDGF-BB production. The proliferation of human fibrocytes (A) or lung fibroblasts MRC5 (B) was examined by ^{3}H-TdR uptake assay. A : Fibrocytes were co-cultured with the indicated number of irradiated fibroblasts with or without PDGF-BB. B : MRC5 cells were co-cultured with the indicated number of irradiated fibrocytes with or without PDGF-BB. $*p < 0.05$ vs no fibrocytes. $**p < 0.01$ vs no fibrocytes. C : The supernatant of fibrocytes was harvested and added to the culture of MRC5 cells in the presence (2 μg/ml) of anti-FGF-2, PDGF-BB and TGF-β neutralizing antibodies or control IgG. $*p < 0.01$ vs control IgG. D : The supernatant of fibrocytes was harvested and added to the culture of IPF fibroblasts in the presence of anti-FGF-2, PDGF-BB and TGF-β neutralizing antibodies or control IgG. Similar results were obtained in at least three independent experiments. $*p < 0.05$ vs control IgG. $**p < 0.01$ vs control IgG.

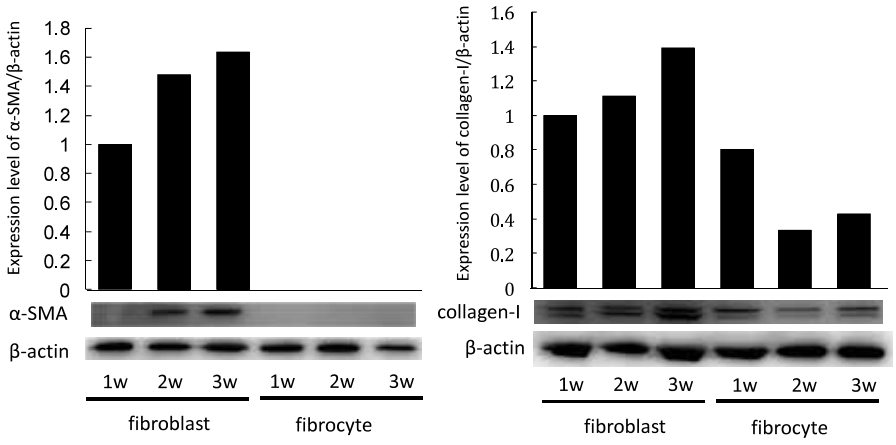

Figure 4 Fibrocytes have lower ability to proliferate and differentiate into myofibroblasts. MRC5 cells and human fibrocytes were cultured in medium containing 5 ng/ml of TGF-β until three weeks. Their cell lysates were harvested weekly and the expression of α-SMA and collagen-I was examined by Western blotting as described in Materials and Methods. The data is the representative of three independent experiments which show the similar results.

Immunostaining of fibrocytes in lung tissues of patients with IPF

To detect fibrocytes in lung tissues of patients with IPF, we performed double and triple staining using several fibrocyte markers such as CD45, collagen I, CXCR4 and FSP-1. As shown in Figure 5A, fibrocytes positive for CD45, collagen I and FSP-1 were detected in the fibrotic areas in lung tissues of patients with IPF. In addition, fibrocytes were found to express PDGF-BB (Figure 5B). On analysis of proliferating cells using anti-Ki67 antibody, most of the positive cells were negative for CD45, although a few cells were positive for both CD45 and Ki67 (Figure 6).

DISCUSSION

In the present study, we examined the gene expression profile of monocyte-derived fibrocytes in comparison with that of monocytes. The fibrocytes were found to strongly express genes related to pulmonary fibrosis such as those for ECM, MMPs, profibrotic growth factors and cytokines. Although fibrocytes did not proliferate in response to PDGF-BB or differentiate into myofibroblasts in response to TGF-β, they have the ability to stimulate the growth of fibroblasts through PDGF-BB production. In lung tissues of patients with IPF, fibrocytes expressed PDGF-BB.

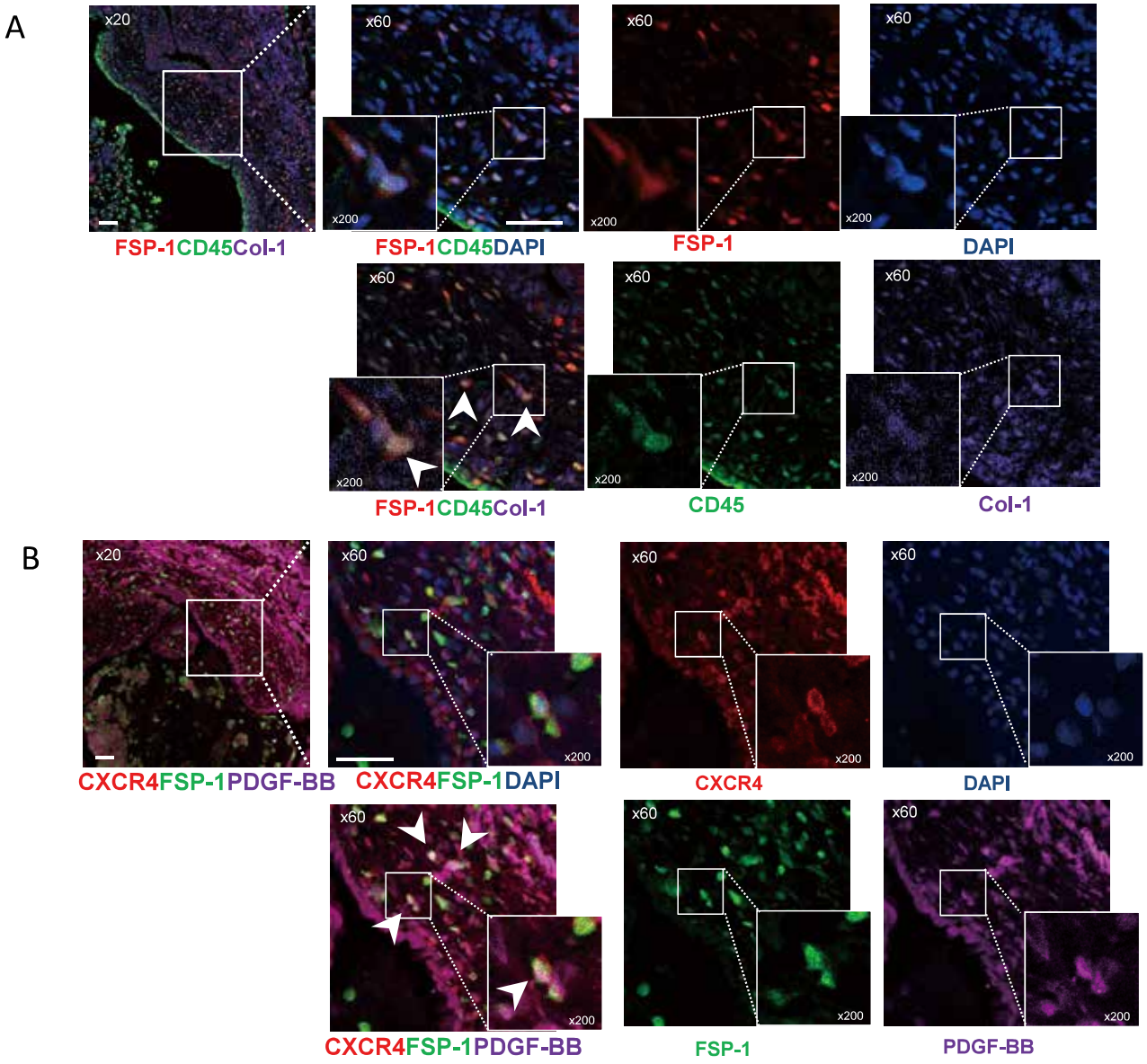

Figure 5 Immunofluorescence analysis of human fibrocytes in lung tissues from IPF patients. A : Triple staining using antibodies against CD45 (green), FSP-1 (red) and collagen-I (purple). The triple-positive cells were detected in the fibrotic area in the lung tissues of patients with IPF (arrowhead). DAPI : blue. B : Triple staining using antibodies against FSP-1 (green), CXCR4 (red) and PDGF-BB (purple). The triple-positive cells were detected in the fibrotic area in the lung tissues of patients with IPF (arrowhead). DAPI : blue. Scale bars: 50 μm.

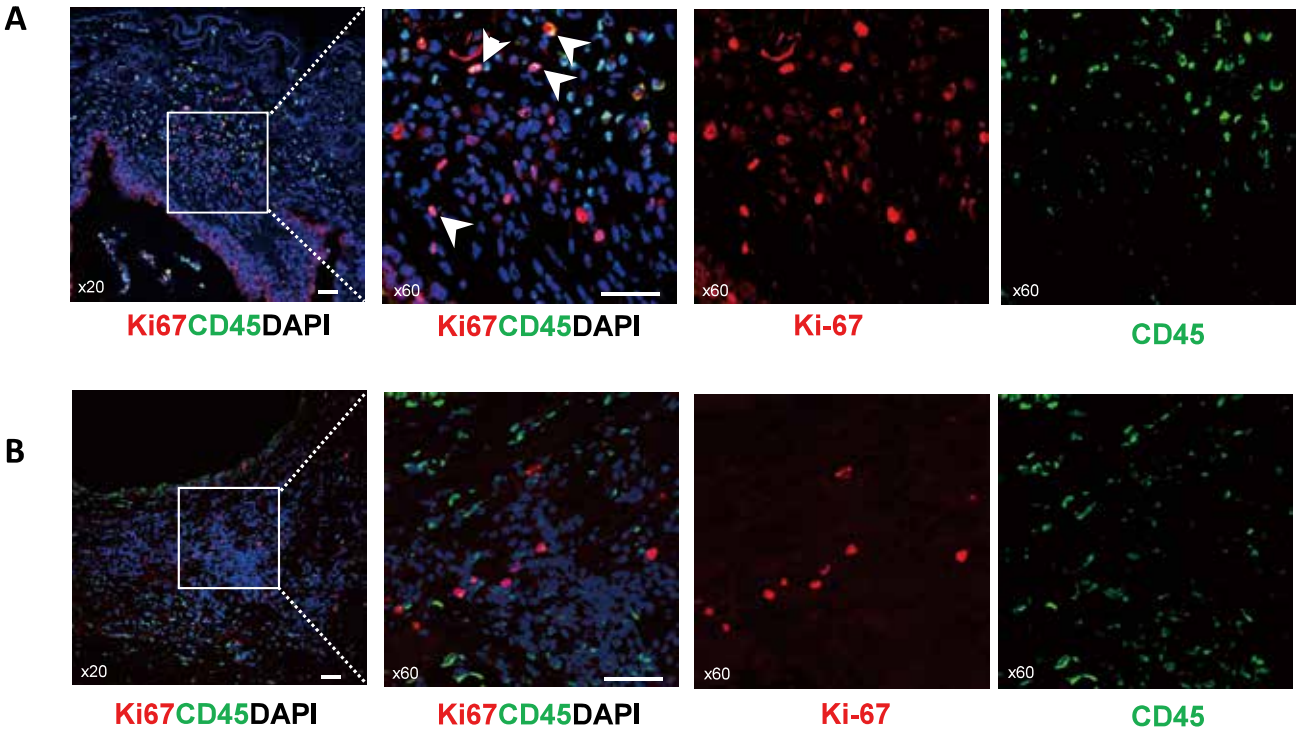

Figure 6 Analysis of the proliferating cells in the lung tissues of patients with IPF. To analyze the proliferating cells, anti-Ki67 antibody (red) was used for a double staining with anti-CD45 antibody (green). A few double-positive cells were observed in the fibrotic area of the lung tissues of IPF patients (arrowhead). The pictures A and B indicate the different area in the lung tissues. DAPI: blue. Scale bars: 50 μm.

Fibrocytes were originally thought to be one of the origins of fibroblasts (3, 4). However, the expression level of collagen was much lower than that in fibroblasts (12, 22, 28). In addition, it has been demonstrated that the production of collagen by bone marrow-derived cells, including fibrocytes, does not lead to pulmonary fibrosis (22, 29). Therefore, other roles of fibrocytes except ECM production have been expected.

In the present study, we demonstrated the characteristic gene expression profile of fibrocytes, which included the preferential expression of genes related to fibrogenesis, such as ECM, MMPs, profibrotic growth factors and cytokines. These data are partly consistent with previous reports (24, 25, 30, 31), and they comprehensively confirmed the pro-fibrotic phenotype of fibrocytes. Among the genes increased in fibrocytes, we focused on the genes encoding growth factors. As one role of fibrocytes, Garcia *et al*. reported that co-culture of fibrocytes from patients with hypersensitivity pneumonitis (HP) increased the expression of collagen Ia1 and α-SMA by lung fibroblasts, possibly through the production of TGF-β (25). This study suggested that fibrocytes promote the differentiation of fibroblasts into myofibroblasts via paracrine effects. In the present study, we found that fibrocytes promoted the growth of fibroblasts through PDGF-BB production. In our experiments, the supernatant of fibrocyte culture did not increase the expression of collagen or α-SMA (data not shown). The reason for the discrepancy is not clear, but of fibroblasts it may be due to the different experimental conditions.

It remains difficult to clearly detect fibrocytes in human lung tissues because several markers, including CD45 and collagen type I, are required to identify them. In the present study, we found fibrocytes in lung tissues of IPF patients by double or triple staining. In addition, growth factor, including PDGF-BB, were co-stained with other fibrocyte markers, suggesting that fibrocytes existing in fibrotic lung tissues produce PDGF-BB.

Human fibrocytes did not differentiate into myofibroblasts in response to TGF-β in the present study. Phillips *et al*. reported α-SMA expression in up to 30% of human fibrocytes after culturing for 28-35 days (4). Several previous reports stated that murine bone marrow-derived fibrocytes do not express α-SMA (5, 32). These studies suggested the poor ability of fibrocytes to differentiate into myofibroblasts. Regarding the proliferative activity of fibrocytes, Madala *et al*. reported the marked proliferation of murine fibrocytes harvested from TGF-α transgenic mice (30). In the present study, human fibrocytes did not exhibit growth by stimulation with PDGF-BB. The poor growth activity compared with that of fibroblasts is consistent with the previous report (14). However, recent studies reported the proliferative activity of fibrocytes in response to CCL2, IL-33 and IL-34, although the levels were low (33-35). Further studies regarding the growth of fibrocytes are required.

On the other hand, fibrocytes increased the proliferation of fibroblasts through PDGF-BB production *in vitro*. Furthermore, the Ki67-positive proliferating cells in IPF lung tissues were mainly found among the CD45-negative cell population, which likely included fibroblasts, and epithelial and endothelial cells. This suggests that fibrocytes play a role in stimulating the growth of resident fibroblasts in the fibrotic lung tissues.

Targeted therapy of fibrocytes and fibrotic macrophages using pentraxin-2 has been investigated in a clinical trial for patients with IPF (20, 21). However, further analysis of the function of fibrocytes is needed to develop novel therapies for IPF by targeting them.

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