Original Research

G protein-coupled receptor 56 regulates matrix production and motility of lung fibroblasts

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Abstract

Idiopathic pulmonary fibrosis is a chronic, progressive, and fatal fibrotic lung disease with a poor prognosis, but no effective treatment is available. G protein-coupled receptor 56 (GPR56) plays a role in cell adhesion and tumor progression, but its function in fibrogenesis has not been explored. In this *in vitro* study, we found that GPR56 in IPF fibroblasts was lower than in normal fibroblasts. GPR56 regulated the production of fibronectin and type I collagen, and also changed the migratory and invasive capacity of lung fibroblasts. However, it was not sufficient to activate some classic markers of fibroblast and myofibroblast, such as α-smooth muscle actin and fibroblast specific protein 1. These findings demonstrate that reduced expression of GPR56 in lung fibroblasts may be an important link with pulmonary fibrosis, playing a role in regulating some important fibroblast functions.

Keywords: Pulmonary fibrosis, G protein-coupled receptor 56, extracellular matrix, cell motility

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Introduction

Idiopathic pulmonary fibrosis (IPF), predominantly found in older adults, is considered a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown etiology. The characteristics of IPF include restrictive ventilatory disorder and progressive worsening of lung function. Pulmonary fibrosis is characterized by excessive deposition of extracellular matrix (ECM), accumulation and proliferation of fibroblasts/myofibroblasts, formation of fibroblast foci, and distortion of the alveolar structure.[1–4] Recently, several clinical trials using interferon-γ, pirfenidone, acetylcysteine, bosentan, and etanercept have targeted some specific signal pathways associated with the pathogenesis of IPF.[5–10] Most of these trials have failed.[3] Thus, new drug targets to suppress pulmonary fibrosis are needed.

G protein-coupled receptors (GPCRs) are the largest cell membrane receptor family whose common feature is the structure of seven α-helical transmembrane regions (TM7). GPCRs play a key role in recognizing a variety of extracellular ligands, transducing a number of extracellular signals, and regulating various biological processes, such as cell growth, differentiation, migration, and tumor metastasis.[11,12] Adhesion GPCRs are a subfamily of GPCRs made up of about 30 members that play a role in cell adhesion.[13] G protein-coupled receptor 56 (GPR56; TM7XN1) is an orphan GPCR, whose ligand is still not clear. It is a member of the adhesion GPCR family and a 693 amino acid protein coded by a gene localized on chromosome 16q13. GPR56 was expressed ubiquitously and abundantly in various systems, such as central nervous system, endocrine system, metabolic system, and respiratory system.[14] Recently, mutant GPR56 was shown to be related to a human brain cortical malformation called bilateral frontoparietal polymicrogyria (BFPP), which suggests its involvement in cell adhesion, migration, differentiation, and early development of the human cerebral cortex.[15–17] Moreover, compared to normal cell lines and tissues, the expression of GPR56 is higher in high metastatic melanoma cell lines but lower in many tumor tissues, which demonstrate the role of cell adhesion pathways in cell transformation, tumorigenesis, and tumor metastasis.[18,19] Therefore, GPR56 is associated with physiological and pathological developments of some human diseases.

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Our recent work suggested that β-arrestins play an important role in regulating lung fibrogenesis.[20] We reasoned that some GPCRs may control pulmonary fibrosis. Thus, this *in vitro* study is to investigate whether the expression of GPR56 is different in IPF fibroblasts compared to normal counterparts and to investigate the possible role of GPR56 in lung fibrogenesis. Our findings suggest that GPR56 regulates certain fibroblast functions, particularly when its expression is reduced.

Materials and methods

Ethics statement

All experiments were approved by the Ethics Committee of Beijing Chao-Yang Hospital and were in accordance with the guidelines outlined by the Committee. Written informed consent to participate in this study was obtained from each participant.

Human lung fibroblasts

Human lung fibroblasts were isolated from diagnostic surgical lung biopsies from patients with IPF (n = 3) or lung transplant explants obtained from donors (n = 3). Patient demographic, pathologic, lung function, and treatment information were listed in Table 1. The diagnosis of IPF was arrived at by standard accepted American Thoracic Society Recommendations.[3] The lung tissues were minced and attached to the bottom of culture flask for 4 h and then cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL). After fibroblast confirmations, the cells of passages four to eight were used for Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and Western blotting, and the similar passages were used to compare fibroblasts of normal and IPF.

Fibroblast lines

MRC-5 (American Type Culture Collection (ATCC), Rockefeller, MD), a normal cell line derived from human fetal lung, was cultured in DMEM supplemented with 10% FBS. LL 97A (AlMy; ATCC) and LL 29 (AnHa; ATCC), two IPF cell lines derived from human lungs, were purchased from ATCC and cultured in Ham's F12K medium supplemented with 15% FBS. MRC-5 of passages 14–18, and LL 97A and LL 29 of passages 10–12 were used for the following assays. The similar passages were used to compare fibroblasts of normal and IPF.

mRNA analysis

RNA was extracted from human lung fibroblasts using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and was reverse transcribed using Quantscript RT Kit (Tiangen, Beijing, China). Resultant cDNAs were subjected to real-time PCR using Real Master Mix (SYBR Green; Tiangen). Primers were qSTAR qPCR primer pairs against *Homo sapiens* gene GPR56 NM_005682 (Origene, Rockville, MD) and β-actin (Table 2; Invitrogen, Shanghai, China). qRT-PCR process consisted of 42 repeated cycles of temperature changes, each of which contained three stages as follows: the denaturing stage at a temperature around 95°C, timing 15 s; the annealing stage at a temperature of around 64°C, timing 45 s; the extending stage at a temperature around 68°C, timing 7 min; the melting curve at a temperature of around 60–95°C, 0.5°C/s. For analysis, the expression for GPR56 gene of interest (GOI) was calculated as 2^{-Ct} followed by normalization to β-actin, with the formula $2^{-(Ct\ GOI\ -\ Ct\ \beta\text{-actin})}$. Ultimately, the fold change in normalized gene expression was calculated by comparing values from fibroblasts from the patients with IPF (IPF-Fb) to those from the donors (NOR-Fb) according to the following formula: $2^{-\Delta Ct\ IPF\text{-}Fb/-\Delta Ct\ NOR\text{-}Fb}$. Values were calculated for replicates of three independent experiments, and P values were calculated with independent sample *t*-test analysis.[20]

Table 2 The primer/siRNA sequence table

Gene	Sequence	
β-actin	Forward	5′-AAGACCTGTACGCCAACACAGT-3′
	Reverse	5′-GGACTCGTCATACTCCTGCTTG-3′
GPR56 siRNA-1	Sense	5′-CCAUCAAGGUGCACAUGAATT-3′
	Antisense	5′-UUCAUGUGCACCUUGAUGGTT-3′
GPR56 siRNA-2	Sense	5′-CUGGGAGAUUACAUCUUCUTT-3′
	Antisense	5′-AGAAGAUGUAAUCUCCCAGTT-3′
Negative siRNA	Sense	5′-UUCUCCGAACGUGUCACGUTT-3′
	Antisense	5′-ACGUGACACGUUCGGAGAATT-3′

Table 1 Clinical characteristics of the patients with IPF and the donors

Patient	Age (year)	Sex	Smoking	Pathology	FVC (%)	FEV1 (%)	DLCOc SB (%)	PAP (mmHg)	Previous treatment
IPF-1	62	Male	Yes	IPF/UIP	54.9	55.5	36.8	44 / 17	No
IPF-2	53	Male	Yes	IPF/UIP	87.0	87.9	59.5	–	Antibiotics (unknown)
IPF-3	61	Female	No	IPF/UIP	71.0	75.5	17.8	23 / 10	N-acetylcysteine
Normal-1	55	Male	Yes	–	101.3	83.7	97.4	–	–
Normal-2	56	Female	No	–	98.9	81.8	98.0	–	–
Normal-3	60	Female	No	–	99.3	82.9	97.7	–	–

DLCOc SB, CO diffusion capacity, single breath method; IPF, idiopathic pulmonary fibrosis; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; PAP, pulmonary artery pressure; UIP, usual interstitial pneumonia.

Array query

The expression profile data related to healthy control (n = 6) and early IPF (n = 8) were acquired from the array dataset GSE24206[21] in the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24206).

siRNA transfection

MRC-5 line was transfected with small interfering RNA oligos (siRNA) targeting the GPR56 gene (NM_005682.5) (Table 2; GenePharma, Shanghai, China) with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). Fibroblasts (1.5×10^4 cells) were transfected with 3 pmol siRNA-1 plus 3 pmol siRNA-2 duplex (Table 2) and 1 μL of Lipofectamine RNAiMAX reagent mixture in all the siRNA interference experiments. The cells were starved with FBS-free medium for 24 h and then incubated with medium containing FBS for 48 h until the following assays.

Plasmid construction and transfection

Full-length GPR56 cDNA (NM_005682.5) was amplified by PCR with cDNA from ATCC as template. The resulting PCR product was inserted into BglII–SalI sites of pIRES2-DsRed-Express2 (Clontech, Palo Alto, CA). LL 97A and LL 29 lines were transfected with the plasmid targeting GPR56 gene with Amaxa™ Basic Nucleofector™ Kit for Primary Mammalian Fibroblasts (Amaxa Scientific, Cologne, Germany). Five μg plasmids were transfected into about 1.0×10^6 cells using the Nucleofector™ Program A-024. After 48 h, positive cells were sorted by MoFlo XDP Cell Sorter (Beckman Coulter, Brea, CA) at Peking University and then recultured for another 48 h until the following assays.

Western blotting

Total cellular proteins were extracted from lung fibroblasts using RIPA Lysis Buffer (Beyotime, Shanghai, China) with addition of protease inhibitors and phosphatase inhibitors. After electrophoresis and transferring, membranes were blocked in 5% nonfat dried milk in 1 × Phosphate Buffered Saline (PBS) for at least 4 h at room temperature and then incubated overnight at 4°C with primary antibodies in PBS. Following primary incubation, membranes were washed and then incubated with secondary antibodies for 1 h in dark at room temperature. The following primary antibodies were diluted in 1 × PBS: β-actin (1:5000; California Bioscience, Coachella, CA), GPR56 (1:100; R&D Systems, Minneapolis, MN), fibronectin (FN, 1:200; Abcam, Cambridge, MA), type I collagen (COLI, 1:200; Abcam), matrix metalloproteinase-9 (MMP-9, 1:200; Abcam), MMP-2 (1:100; Abcam), α-smooth muscle actin (α-SMA, 1:400; R&D Systems), and fibroblast-specific protein-1 (FSP1/S100A4, 1:50; Abcam). The secondary antibodies were used: DyLight™ 680-labeled antibody to sheep IgG (H + L) (1:10,000; KPL, Washington, D.C.), IRDye® 680RD Goat anti-mouse IgG (H + L) (1:10,000; LI-COR Biosciences, Lincoln, NE), IRDye® 680RD Goat anti-rabbit IgG (H + L) (1:10,000; LI-COR Biosciences). The quantitative densitometric analysis relative to β-actin was used to aid clarity.

Collagen I direct Enzyme Linked Immunosorbent Assay (ELISA)

Transfected fibroblasts (2.5×10^4 cells) were plated onto 24-well plates and starved for 24 h followed by another 48 h incubation with 1% FBS medium. A total of 500 μL final medium was collected and the concentration of collagen I secreted by fibroblasts was determined with direct ELISA. The wells of a Polyvinyl Chloride Polymer (PVC) microtiter plate (Corning Costar, Cambridge, MA) were coated with the culture medium with PBS. Type I collagen was detected by primary antibody to collagen I (1:1000; Abcam) and secondary antibody peroxidase-conjugated affinipure goat anti-rabbit IgG (H + L) (1:10,000; Proteintech). The absorbance was measured at 450 nm.

Immunofluorescence staining

Transfected fibroblasts (2.5×10^4 cells) were plated in 24-well culture plates and cultured with medium supplemented with FBS or stimulated by medium supplemented with human recombinant Transforming Growth Factor-β1 (TGF-β1) (5 ng/mL; BD Biosciences, Franklin Lakes, NJ) and 0.1% Albumin from Bovine Serum (BSA) (Sigma Aldrich, St. Louis, MO) for 48 h. Then the cells were fixed in 90% methanol for 30 min and stained with the indicated antibodies, including α-SMA (1:400; R&D Systems) and FSP1 (1:100; Abcam), Fluorescein (FITC)-conjugated Affinipure Goat Anti-Mouse IgG (H + L) (1:200; Proteintech, Chicago, IL), Fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (1:200; Proteintech), and Rhodamine (TRITC)-conjugated Goat Anti-Mouse IgG (H + L) (1:200; Proteintech).

Fibroblast migration

Transfected fibroblasts (1.0×10^5 cells) for wound scratch assay were grown to about 70–80% confluency in six-well plates and then wounded with a pipet tip after transfecting and starving for 24 h. After scratched, 0, 24, 36, and 48-h time points were observed at 40 × magnification. The average healing distances were measured at three different points per group with the changes of time. For ECM migration assay, the transwells were coated with collagen, type I solution from rat tail (0.5 mg/mL; Sigma Aldrich). Transfected fibroblasts (0.5×10^4 cells) in serum-free medium containing 0.1% BSA (Sigma Aldrich) were loaded into the top chamber of 24-well transwell plate inserts (8 μm pore size; Corning Costar), and normal medium was loaded into the bottom chamber. After 48 h, fibroblasts that migrated across the collagen filter were stained with gentian violet staining and counted in five randomly chosen fields per group at 100 × magnification.

Fibroblast invasion

After transfection, fibroblasts (0.5×10^4 cells) in serum-free medium containing 0.1% BSA (Sigma Aldrich) were loaded into the top chamber with BD Matrigel™ Basement Membrane Matrix (BD Biosciences), and medium containing twice the usual FBS concentration was loaded into the bottom chamber. After 48 h, the filters were fixed and stained with gentian violet staining. Noninvading cells as well as the basement membrane matrix were removed from

the upper side of the filter by gentle scrubbing with a cotton swab. The number of fibroblasts that invaded through the basement membrane was counted in five randomly chosen fields from duplicate filters per group at 40 × magnification.

Statistical analysis

Differences in measured variables between experimental groups were assessed using Analysis of Variance (ANOVA) or independent samples *t*-test by SPSS 17.0 software. Fisher's Least-Significant Difference (LSD) test was used as the supplementary of ANOVA. Data are expressed as mean ± Standard Error of Mean (SEM) where applicable. A *P* value less than 0.05 was considered a statistically significant difference.

Results

GPR56 is decreased in idiopathic pulmonary fibrosis IPF fibroblasts

Lung fibroblasts were isolated, cultured, and confirmed from diagnostic surgical lung biopsies from three patients with IPF and three normal lungs (Table 1). First, we assessed GPR56 expressions in normal and IPF fibroblasts with qRT-PCR and Western blotting (Figure 1). GPR56 was significantly downregulated in IPF samples compared to normal samples (Figure 1(a)). To confirm these data, we acquired array data from the GEO repository. There were a clear trend of reduction of GPR56 in early IPF than the health controls from the array dataset GSE24206 (Figure 1(b)). The protein levels of GPR56 were decreased in IPF fibroblasts compared to normal samples (Figure 1(c)). Furthermore, GPR56 expression was lower in LL 97A and LL 29 lines compared to that in MRC-5 line (Figure 1(d)). These findings indicate that the expression of GPR56 appears insufficient in IPF fibroblasts and imply that GPR56 may be a link with in pulmonary fibrosis.

GPR56 regulates the production of ECM proteins

Next, we wanted to see whether expression of GPR56 regulates some important fibroblast functions by gain-of-function and loss-of-function experiments. We reasoned that reduced expression of GPR56 in normal fibroblasts may

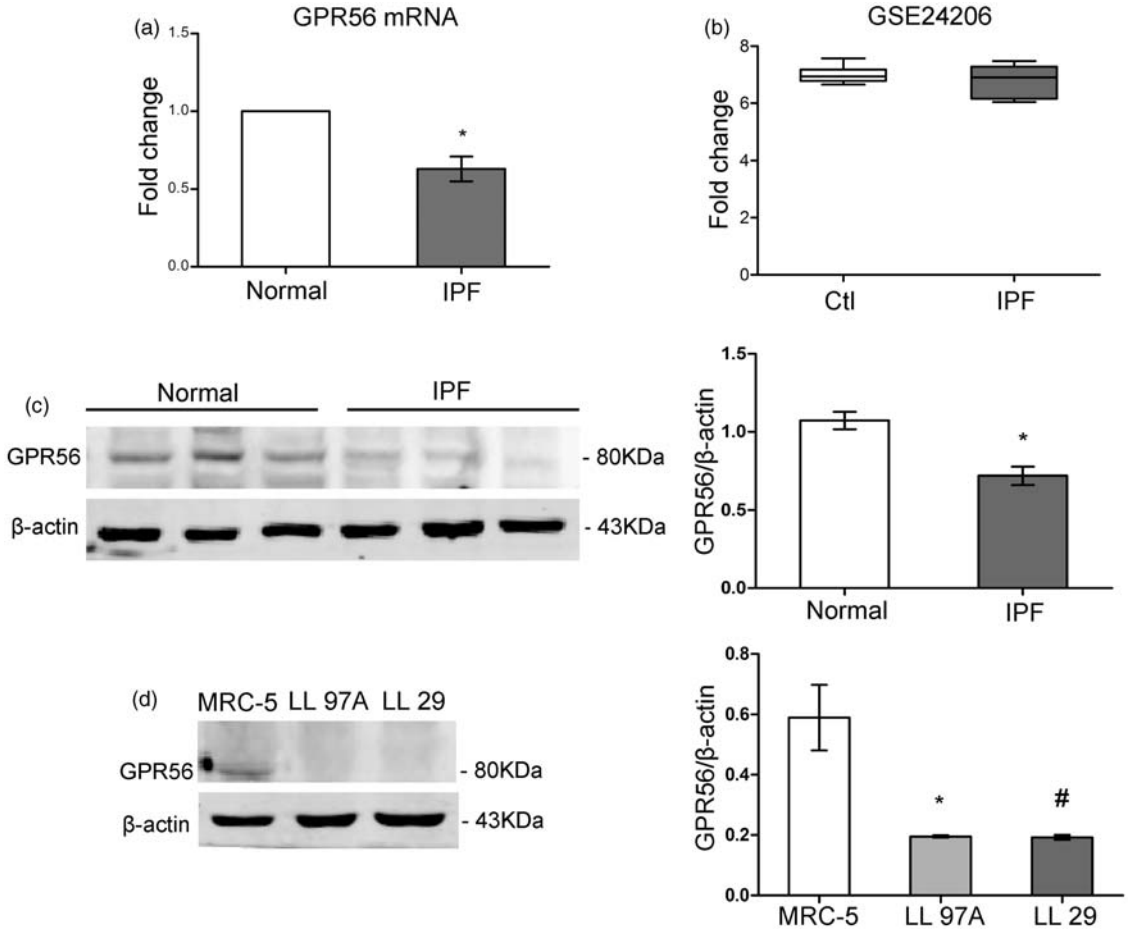

Figure 1 The expression of GPR56 in lung fibroblasts. (a) The mRNA levels of GPR56 were assessed by qRT-PCR. Results were derived from three patients with IPF and three donors. The data were analyzed by $2^{-\Delta\Delta Ct}$ method, $^{*}P < 0.05$. (b) The fold change of early IPF (n = 8) relative to healthy control (n = 6) was assessed according to the array dataset GSE24206 from the GEO repository. The fold change of early IPF was decreased compared to healthy control, $P = 0.303$. (c) and (d) Western blotting was used to analyze the protein levels of GPR56 in lung fibroblasts. Blotting of β-actin served as loading controls. (c) The protein levels of GPR56 in primary lung fibroblasts of the patients with IPF and the donors, $^{*}P < 0.05$. (d) The protein levels of GPR56 in MRC-5, LL 97A and LL 29 lines, LL 97A compared to MRC-5: $^{*}P < 0.05$; LL 29 compared to MRC-5: $^{\#}P < 0.05$. The data (except b) were presented as mean ± SEM and the experiments (except b) were repeated three times

force them to gain some properties of fibrotic fibroblasts. On the other hand, overexpression of GPR56 in fibrotic fibroblasts may allow them to reduce their fibrotic characteristics. Both siRNA interference and plasmid overexpression of GPR56 (Figure 2) were used to compare some major indicators. GPR56 was successfully knocked down with specific siRNAs to GPR56 in MRC-5 line (Figure 2(a)). We also successfully overexpressed GPR56 in two IPF fibroblast lines (Figure 2(b)).

The expressions of ECM production, including FN and type I collagen in IPF and normal fibroblasts, were first assessed (Figure 3). The intracellular FN and type I collagen were increased in MRC-5 line with reduced GPR56 (Figure 3(a)). Similarly, the extracellular type I collagen was increased in MRC-5 line with reduced GPR56 (Figure 3(b)). Furthermore, the intracellular FN and type I collagen decreased in LL 97A and LL 29 lines when GPR56 was overexpressed (Figure 3(c)). Moreover, the extracellular type I collagen was decreased in LL 97A and LL 29 lines overexpressing GPR56 (Figure 3(d)). These data indicate that downregulated GPR56 may increase the production of ECM proteins in fibroblasts, and vice versa.

GPR56 is not sufficient to regulate the classic marker expressions of fibroblast and myofibroblast

One of the key processes in the development of pulmonary fibrosis is myofibroblast differentiation. Both siRNA interference and plasmid overexpression were used to assess whether alteration of GPR56 expression changes the expressions of fibroblast marker FSP1 and myofibroblast marker α-SMA (Figure S1–S3). The expressions of α-SMA and FSP1 were not changed in MRC-5 line with reduced GPR56, independent of TGF-β1 stimulation (Figure S1(a)). Similarly, the protein levels of α-SMA and FSP1 were also not altered in MRC-5 line with GPR56 interference regardless of TGF-β1 stimulation (Figure S1(b)). Furthermore, the expressions of α-SMA and FSP1 were not changed in LL 97A (Figure S2(a)) and LL 29 (Figure S2(b)) lines when overexpressing GPR56, independent of TGF-β1 stimulation. Moreover, the protein levels of α-SMA and FSP1 were not altered in LL 97A (Figure S3(a)) and LL 29 (Figure S3(b)) lines overexpressing GPR56 regardless of TGF-β1 stimulation. These findings suggest that GPR56 alone may not be sufficient to regulate the classic marker expressions of fibroblast and myofibroblast.

Reduced GPR56 changes fibroblast motility

The capacity of fibroblasts to migrate to and invade injured tissue may be dysregulated in pulmonary fibrosis. We sought to determine whether GPR56 could affect fibroblast motility. Wound scratch assay, transwell assay, and Western blotting were used to assess the motility of fibroblasts (Figures 4 to 6).

Knocking down GPR56 significantly accelerated wound healing at 48-h time point according to wound scratch assay (Figure 4(a)). Similarly, reduced GPR56 significantly increased the migratory capacity of MRC-5 line (Figure 4(b)). However, overexpressing GPR56 in

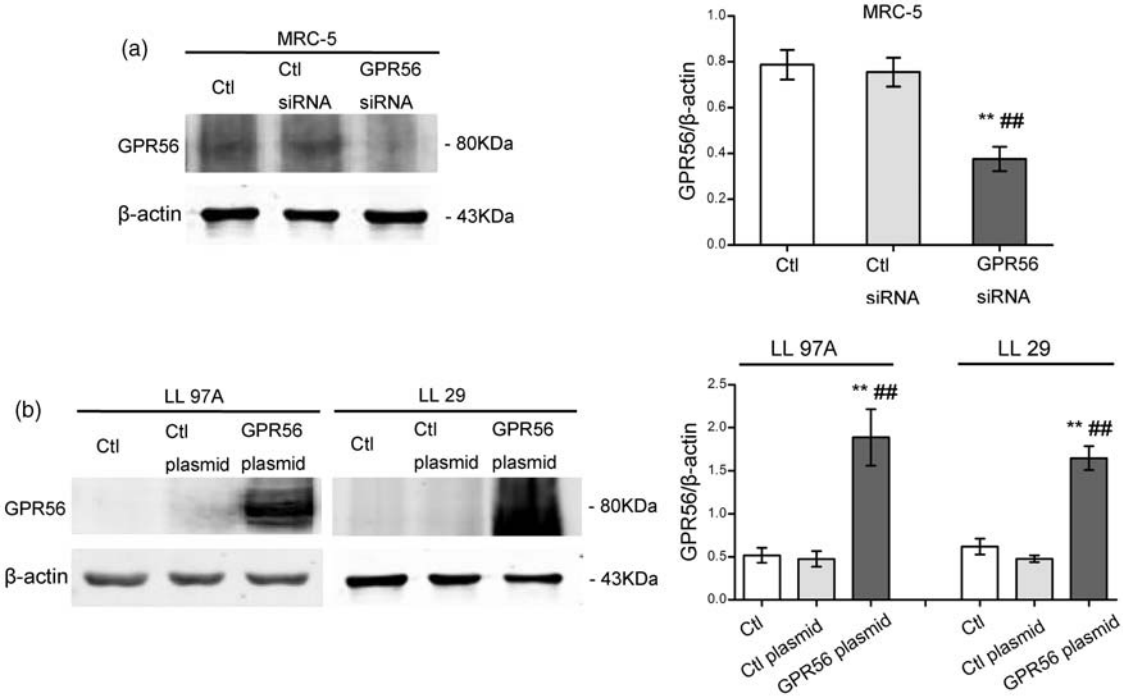

Figure 2 The expressions of GPR56 in three fibroblast lines after transfections *in vitro*. MRC-5 lines were transfected with Lipofectamine RNAiMAX only *(Ctl)*, negative siRNA *(Ctl siRNA)*, and siRNAs targeting GPR56 gene *(GPR56 siRNA)*. Both LL97A and LL29 lines were transfected with nucleofector solution only *(Ctl)*, negative reconstituted plasmid *(Ctl plasmid)*, and reconstituted plasmid targeting GPR56 gene *(GPR56 plasmid)*. Western blotting was used to assess the expressions of GPR56 in MRC-5 line after siRNA interference (a) and LL 97A and LL 29 lines after plasmid overexpression (b). GPR56 siRNA (plasmid) compared to Ctl: $^{**}P < 0.01$; GPR56 siRNA (plasmid) compared to Ctl siRNA (plasmid): $^{\#\#}P < 0.01$. The data were presented as mean ± SEM and the experiments were repeated three times

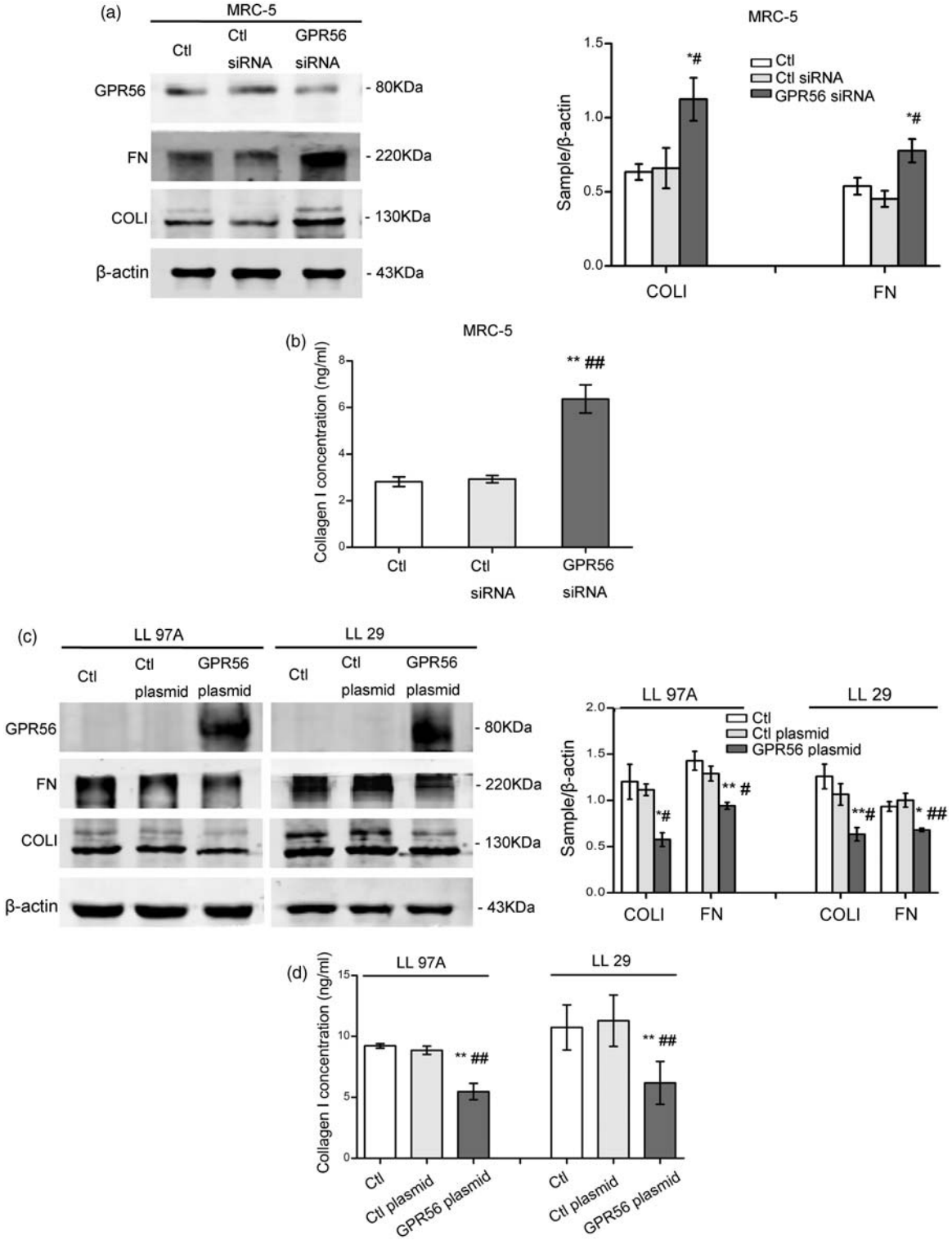

Figure 3 The effects of GPR56 on fibronectin and type I collagen production *in vitro*. (a) and (c) After recultured for 48 h, the expressions of fibronectin and type I collagen in three fibroblast lines were assessed by Western blotting under the different levels of GPR56. GPR56 siRNA (plasmid) compared to Ctl: $^{*}P < 0.05$, $^{**}P < 0.01$; GPR56 siRNA (plasmid) compared to Ctl siRNA (plasmid): $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. (b) and (d) Collagen I direct ELISA was used to measured type I collagen production of fibroblasts. Transfected fibroblasts were plated onto 24-well plates and starved for 24 h. Another 48-h culture media were collected and type I collagen was determined with direct ELISA. GPR56 siRNA (plasmid) compared to Ctl: $^{**}P < 0.01$; GPR56 siRNA (plasmid) compared to Ctl siRNA (plasmid): $^{\#\#}P < 0.01$. The data were presented as mean ± SEM and the experiments were repeated three times

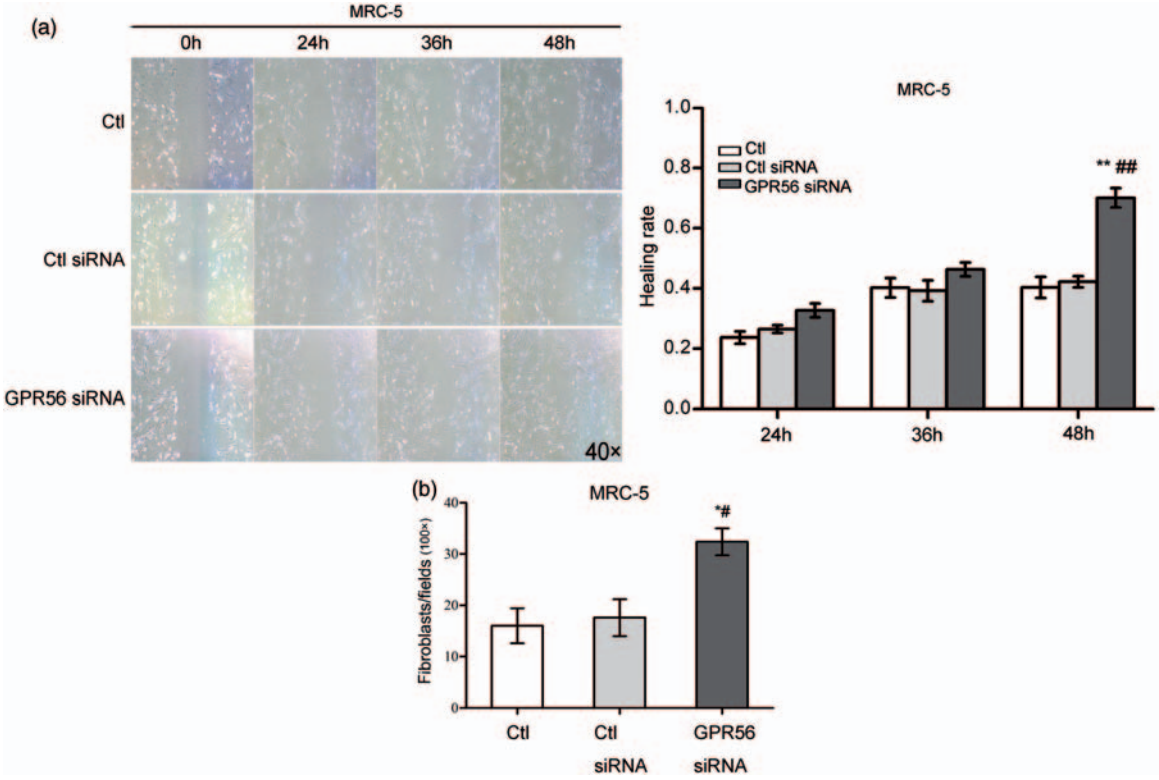

Figure 4 Reduced GPR56 stimulates fibroblast migration *in vitro*. (a) Wound scratch assay is used to indirectly measure the migratory capacity of MRC-5 line in six-well plates. Time points 0, 24, 36, 48 h were observed at 40 × magnification. The average healing distances were measured at three different points per group with the changes of time. (b) 24-well transwell plate inserts coated with collagen to directly measure the migratory capacity of MRC-5 line toward FBS. Transfected fibroblasts that migrated across the collagen filter were stained with gentian violet staining and counted in five randomly chosen fields per group at 100 × magnification at 48-h time point. GPR56 siRNA compared to Ctl: $^{*}P < 0.05$, $^{**}P < 0.01$; GPR56 siRNA compared to Ctl siRNA: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. The data were presented as mean ± SEM and the experiments were repeated three times.

LL 97A and LL 29 lines did not affect fibroblast migrations from both wound scratch assays (Figure 5(a)) and ECM migration assays (Figure 5(b)). We recently reported that β-arrestins regulate fibroblast invasiveness.[20] We next investigate the impact of GPR56 on fibroblast invasion (Figure 6). Knocking down GPR56 significantly increased the invasive capacity of MRC-5 line (Figure 6(a)). However, overexpression of GPR56 in LL 97A and LL 29 lines did not altered fibroblast invasion (Figure 6(b)). Finally, we wanted to test whether the regulation of invasion by GPR56 was related to the expression of metalloproteases (Figure 6(c) and (d)). Knocking down GPR56 markedly increased the expression of MMP-9 in MRC-5 line. MMP-2 expression was not altered by GPR56 knock down (Figure 6(c)). On the other hand, overexpression of GPR56 in LL 97A and LL 29 lines did not change MMP-9 and MMP-2 levels (Figure 6(d)).

Taken together, these data indicate that GPR56 regulates certain fibrotic functions of fibroblasts, such as matrix production and motility.

Discussion

The pathogenesis of progressive pulmonary fibrosis is unclear. The current *in vitro* study was to investigate the role of GPR56 in lung fibroblasts. Normal lung tissues, even modified airway epithelium, may be the derivation of the isolated fibroblasts. Therefore, after necessary fibroblast confirmations, we demonstrate that GPR56 is significantly decreased in IPF fibroblasts. With gain-of-function and loss-of-function experiments, we show that downregulated GPR56 regulates certain fibroblast functions, including matrix production, fibroblast migration, and invasion through MMPs. Therefore, we suggest that GPR56 may be a link with IPF.

Our previous study demonstrated that β-arrestins regulate pulmonary fibrosis by modulating fibroblast invasiveness.[20] Several GPCRs have been demonstrated to have a role in fibrogenesis. For example, we reported that CXCR3, the chemokine receptor, regulates pulmonary fibrosis through interferon-γ.[22] GPR56 is demonstrated to be reduced in many tumors, while IPF is a disease similar to cancer.[23] Therefore, we reasoned that some other GPCR families may also modulate fibrosis. In this study, we found that GPR56, the orphan GPCR, was downregulated in IPF fibroblasts. As previous studies shown, GPR56 mutations have been identified in BFPP patients. Recent report has demonstrated that collagen III could be the ligand of GPR56 in the developing brain. BFPP could be caused by abolishing the ability of GPR56 to bind to collagen III because of four disease-associated mutations of GPR56,[24,25] which are not studied in IPF.

One of the hallmarks of pulmonary fibrosis is the accumulation of ECM, including FN and type I collagen.[26,27]

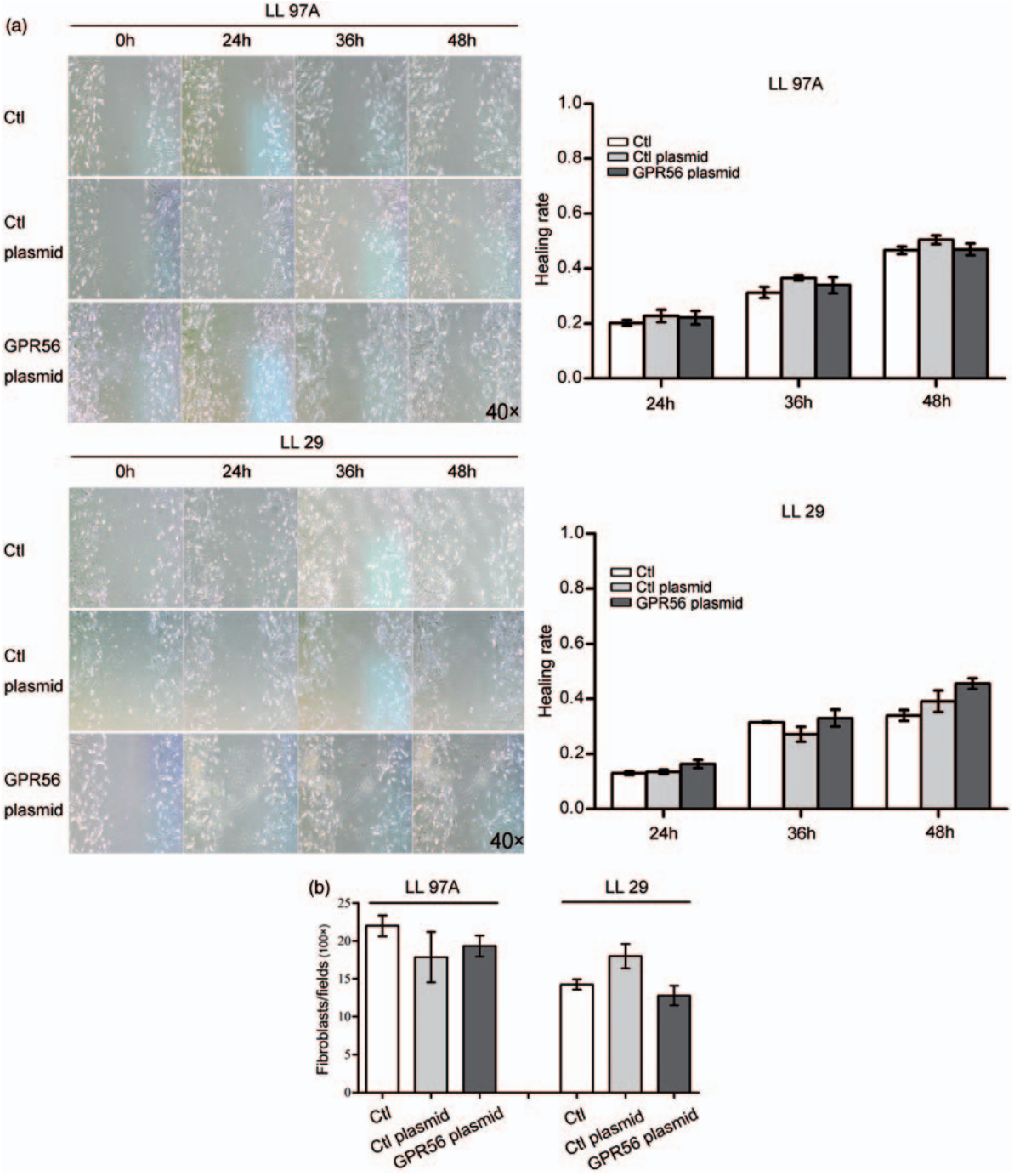

Figure 5 Overexpressed GPR56 does not change the migratory capacity *in vitro*. (a) Wound scratch assay is used to indirectly measure the migratory capacity of LL 97A and LL 29 lines in six-well plates. Time points 0, 24, 36, and 48 h were observed at 40 × magnification. The average healing distances were measured at three different points per group with the changes of time. (b) 24-well transwell plate inserts coated with collagen to directly measure the migratory capacity of LL 97A and LL 29 lines toward FBS. Transfected fibroblasts that migrated across the collagen filter were stained with gentian violet staining and counted in five randomly chosen fields per group at 100 × magnification at 48-h time point. The data were presented as mean ± SEM and the experiments were repeated three times.

Therefore, we hypothesized that GPR56 may regulate ECM production. In this study, we observed that GPR56 significantly regulated the production of both FN and type I collagen through gain-of-function and loss-of-function experiments. It is not clear how GPR56 regulates ECM production. Lysophosphatidic acid (LPA) might play a possible role in this process. LPA is a family of bioactive lyso-phospholipid that mediates most of its biological effects through GPCRs. Recently, LPA and its receptor 2 (LPA2) were investigated in pulmonary fibrosis. LPA2 deficiency can protect from bleomycin-induced lung injury; attenuate bleomycin-induced expression of FN, α-SMA, and collagen in lung tissue; reduce LPA-induced expression of TGF-β1; and inhibit the differentiation of lung fibroblasts to myofibroblasts leading to decreased expression of FN, α-SMA, and collagn.[28] In addition, integrins and tissue transglutaminase

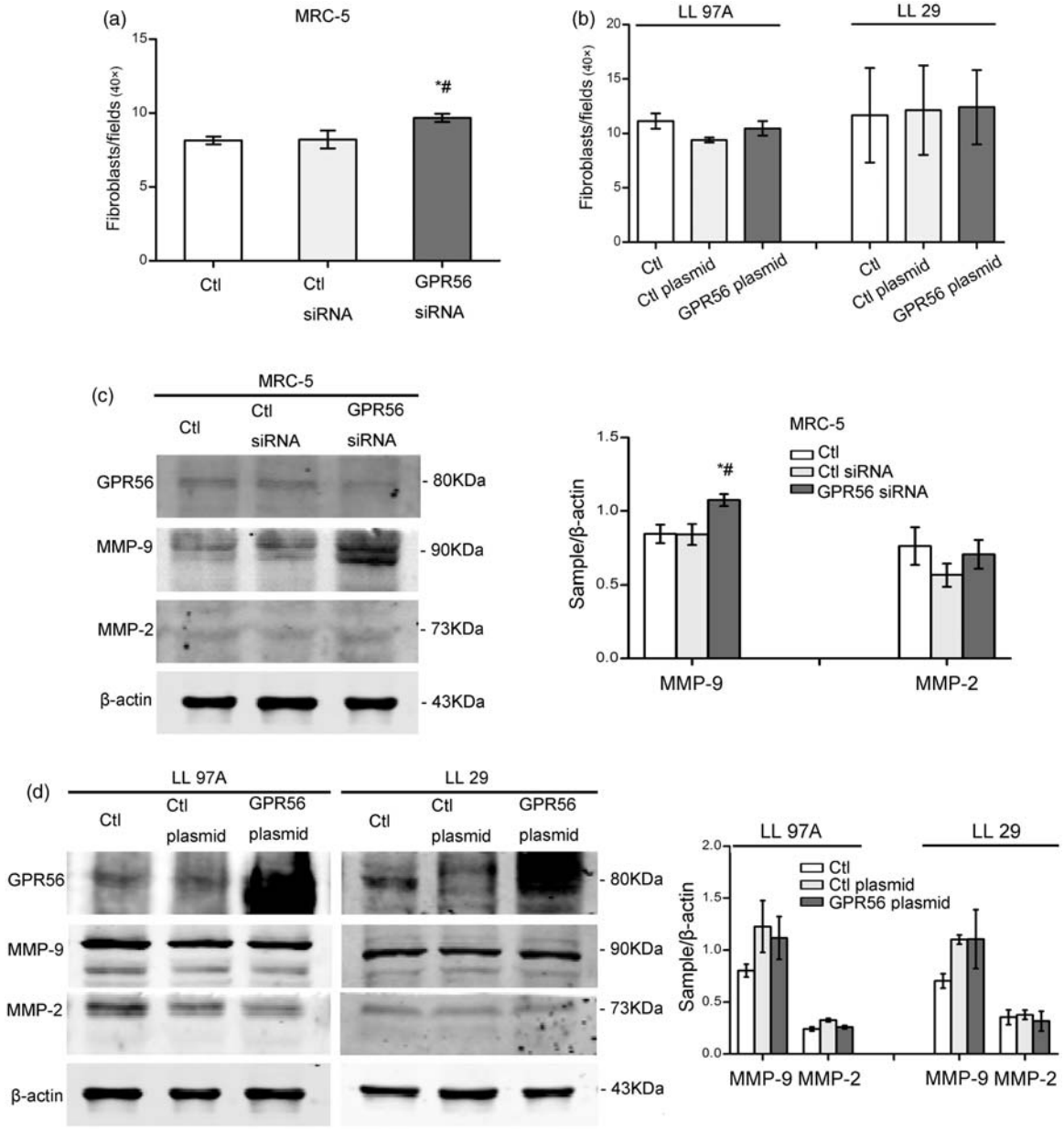

Figure 6 Reduced GPR56 stimulates fibroblast invasion and increases MMP-9 level *in vitro*. (a) and (b) 24-well transwell plate inserts with Matrigel to assess the invasive capacity of three fibroblast lines toward FBS. Transfected fibroblasts that invaded through the basement membrane were stained with gentian violet staining and counted in five randomly chosen fields from duplicate filters per group at 40 × magnification at 48-h time point. (c) and (d) After recultured for 48 h, the expressions of MMP-9 and MMP-2 in three fibroblast lines were assessed by Western blotting under the different levels of GPR56. GPR56 siRNA compared to Ctl: $^{*}P < 0.05$; GPR56 siRNA compared to Ctl siRNA: $^{\#}P < 0.05$. The data were presented as mean ± SEM and the experiments were repeated three times

(TG2) might involve in regulating the function of ECM by interacting with GPR56.[29–34] TG2 has been demonstrated to be increased in IPF and plays an important role in pulmonary fibrogenesis. In this report, knockdown of TG2 in primary human lung fibroblasts leads to functional defects.[35] The interactions between GPR56 and TG2 might be focused in the next studies.

Recent observations have demonstrated that the cell migration is different between normal and IPF fibroblasts. IPF fibroblasts have an increased migratory capacity compared to normal counterparts.[36,37] GPR56 is found to suppress tumor progression by regulating cell adhesion, migration, and tumor metastasis.[19,33,38] We thought that GPR56 may have an effect on fibroblast motility. We found that GPR56 interference promoted fibroblast migration and invasion; however, GPR56 overexpression in fibrotic fibroblasts did not. These data suggest that GPR56 may regulate fibroblast motility at a basal level, so that elevated levels of GPR56 would not increase cell motility. Our previous study demonstrated that the loss of β-arrestin 1 and β-arrestin 2 has no influence on fibroblast migration, but makes fibroblasts invade less in a bleomycin model compared to normal counterparts.[20] According to our data, β-arrestin-independent pathway might be important in enhancing fibroblast motility after GPR56 interference.

An invading cell must be able to alter cell-to-cell and cell-to-ECM adhesion, degrade the ECM, and change its cytoskeleton.[39] The action of MMPs is one meaningful mechanism for allowing fibroblasts to invade into the alveolar space for proliferating, forming foci, and producing collagen and has been studied in ECM remodeling and basement membrane disruption.[4] We speculated that the expressions of MMP-9 and MMP-2 may be associated with regulating fibroblast motility with GPR56 expression. We found that GPR56 interference had an impact on MMP-9, while GPR56 overexpression did not change metalloprotease MMP-2 and MMP-9. Similar to the regulation of fibroblast motility, MMP-9 levels were regulated by GPR56 at a basal level. MMP-9 and MMP-2 associate to fibrocyte migration through basement membrane-like proteins.[40] Thy-1(–) and TGF-β1 stimulation might contribute to the production of MMP-9.[41] The receptor for advanced glycation end-products (RAGE) has been suggested to maintain lung homeostasis by mediating cell adhesion. RAGE is expressed at a high basal level in lung. Downregulation of RAGE expression may lead to a decreased binding of the basement membrane and an increased susceptibility to alveolar injury and/or prevent the proper re-epithelialization of alveoli during IPF pathogenesis. RAGE expression was diminished with releasing its soluble isoform through MMP-9-dependent pathway after cytokine stimulation.[42] How reduced GPR56 is related to MMP-9 induction is still to be further studied.

The activation of fibroblasts and the differentiation of myofibroblasts accompanied with the classic marker expressions of FSP1 and α-SMA are commonly considered to be the hallmarks of pulmonary fibrosis.[26,27] We assumed that fibroblast marker FSP1 and myofibroblast marker α-SMA would be influenced by GPR56. However, in contrast to the effects of GPR56 in fibroblasts, we found that the expression changes of GPR56 are insufficient to affect FSP1 and α-SMA expressions in a basal level or under differentiation stimulus. Recent studies showed that GPR56 plays a potential role in neural development and differentiation.[43] But whether it affects fibroblast differentiation should be further validated.

IPF is a fatal disease with no effective treatment. Recent reports have emphasized that some GPCRs and β-arrestins play an important role in the development of IPF.[20,22] Our *in vitro* studies demonstrate that GPR56 is decreased in IPF fibroblasts. Furthermore, manipulation of GPR56 expression alters certain pro-fibrotic aspects of characteristics of fibroblasts. The experiments reported here were mainly based on *in vitro* studies that may not be fully representative of the developing process of IPF. *In vivo* studies that investigate the effect of GPR56 in an experimental animal model of IPF and with genetically modified mice would be critical. Nevertheless, our findings suggest that GPR56 may play a role in the pathogenesis of pulmonary fibrosis.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; DJ and CW conceived the study, JY, ZW, JL, DJ, and CW conducted the experiments, DL, HD, JW, DJ, and CW supplied critical reagents, materials, and analysis tools; JY, DJ, and CW wrote the manuscript.

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REFERENCES

1. Meltzer EB, Noble PW. Idiopathic pulmonary fibrosis. *Orphanet J Rare Dis* 2008;**3**:8
2. Noble PW, Barkauskas CE, Jiang D. Pulmonary fibrosis: Patterns and perpetrators. *J Clin Invest* 2012;**122**:2756–62
3. Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, Colby TV, Cordier JF, Flaherty KR, Lasky JA, Lynch DA, Ryu JH, Swigris JJ, Wells AU, Ancochea J, Bouros D, Carvalho C, Costabel U, Ebina M, Hansell DM, Johkoh T, Kim DS, King TE Jr, Kondoh Y, Myers J, Muller NL, Nicholson AG, Richeldi L, Selman M, Dudden RF, Griss BS, Protzko SL, Schunemann HJ. An official ATS/ERS/JRS/ALAT statement: Idiopathic pulmonary fibrosis: Evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med* 2011;**183**:788–824
4. Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001;**134**:136–51
5. Azuma A, Nukiwa T, Tsuboi E, Suga M, Abe S, Nakata K, Taguchi Y, Nagai S, Itoh H, Ohi M, Sato A, Kudoh S. Double-blind, placebo-controlled trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2005;**171**:1040–7
6. Demedts M, Behr J, Buhl R, Costabel U, Dekhuijzen R, Jansen HM, MacNee W, Thomeer M, Wallaert B, Laurent F, Nicholson AG, Verbeken EK, Verschakelen J, Flower CD, Capron F, Petruzzelli S, De Vuyst P, van den Bosch JM, Rodriguez-Becerra E, Corvasce G, Lankhorst I, Sardina M, Montanari M. High-dose acetylcysteine in idiopathic pulmonary fibrosis. *N Engl J Med* 2005;**353**:2229–42
7. King TE Jr, Albera C, Bradford WZ, Costabel U, Hormel P, Lancaster L, Noble PW, Sahn SA, Szwarcberg J, Thomeer M, Valeyre D, du Bois RM. Effect of interferon gamma-1b on survival in patients with idiopathic pulmonary fibrosis (INSPIRE): A multicentre, randomised, placebo-controlled trial. *Lancet* 2009;**374**:222–8
8. King TE Jr., Behr J, Brown KK, du Bois RM, Lancaster L, de Andrade JA, Stahler G, Leconte I, Roux S, Raghu G. BUILD-1: A randomized placebo-controlled trial of bosentan in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2008;**177**:75–81
9. Raghu G, Brown KK, Bradford WZ, Starko K, Noble PW, Schwartz DA, King TE Jr. A placebo-controlled trial of interferon gamma-1b in patients with idiopathic pulmonary fibrosis. *N Engl J Med* 2004;**350**:125–33
10. Raghu G, Brown KK, Costabel U, Cottin V, du Bois RM, Lasky JA, Thomeer M, Utz JP, Khandker RK, McDermott L, Fatenejad S. Treatment of idiopathic pulmonary fibrosis with etanercept: an exploratory, placebo-controlled trial. *Am J Respir Crit Care Med* 2008;**178**:948–55
11. Muller DJ, Wu N, Palczewski K. Vertebrate membrane proteins: Structure, function, and insights from biophysical approaches. *Pharmacol Rev* 2008;**60**:43–78
12. Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 2002;**3**:639–50
13. Yona S, Lin HH, Siu WO, Gordon S, Stacey M. Adhesion-GPCRs: Emerging roles for novel receptors. *Trends Biochem Sci* 2008;**33**:491–500

14. Regard JB, Sato IT, Coughlin SR. Anatomical profiling of G protein-coupled receptor expression. *Cell* 2008;**135**:561–71
15. Koirala S, Jin Z, Piao X, Corfas G. GPR56-regulated granule cell adhesion is essential for rostral cerebellar development. *J Neurosci* 2009;**29**:7439–49
16. Li S, Jin Z, Koirala S, Bu L, Xu L, Hynes RO, Walsh CA, Corfas G, Piao X. GPR56 regulates pial basement membrane integrity and cortical lamination. *J Neurosci* 2008;**28**:5817–26
17. Piao X, Hill RS, Bodell A, Chang BS, Basel-Vanagaite L, Straussberg R, Dobyns WB, Qasrawi B, Winter RM, Innes AM, Voit T, Ross ME, Michaud JL, Descarie JC, Barkovich AJ, Walsh CA. G protein-coupled receptor-dependent development of human frontal cortex. *Science* 2004;**303**:2033–6
18. Ke N, Sundaram R, Liu G, Chionis J, Fan W, Rogers C, Awad T, Grifman M, Yu D, Wong-Staal F, Li QX. Orphan G protein-coupled receptor GPR56 plays a role in cell transformation and tumorigenesis involving the cell adhesion pathway. *Mol Cancer Ther* 2007;**6**:1840–50
19. Zendman AJ, Cornelissen IM, Weidle UH, Ruiter DJ, van Muijen GN. TM7XN1, a novel human EGF-TM7-like cDNA, detected with mRNA differential display using human melanoma cell lines with different metastatic potential. *FEBS Lett* 1999;**446**:292–8
20. Lovgren AK, Kovacs JJ, Xie T, Potts EN, Li Y, Foster WM, Liang J, Meltzer EB, Jiang D, Lefkowitz RJ, Noble PW. Beta-arrestin deficiency protects against pulmonary fibrosis in mice and prevents fibroblast invasion of extracellular matrix. *Sci Transl Med* 2011;**3**:74ra23
21. Meltzer EB, Barry WT, D'Amico TA, Davis RD, Lin SS, Onaitis MW, Morrison LD, Sporn TA, Steele MP, Noble PW. Bayesian probit regression model for the diagnosis of pulmonary fibrosis: Proof-of-principle. *BMC Med Genomics* 2011;**4**:70
22. Jiang D, Liang J, Hodge J, Lu B, Zhu Z, Yu S, Fan J, Gao Y, Yin Z, Homer R, Gerard C, Noble PW. Regulation of pulmonary fibrosis by chemokine receptor CXCR3. *J Clin Invest* 2004;**114**:291–9
23. Vancheri C, Failla M, Crimi N, Raghu G. Idiopathic pulmonary fibrosis: A disease with similarities and links to cancer biology. *Eur Respir J* 2010;**35**:496–504
24. Luo R, Jeong SJ, Jin Z, Strokes N, Li S, Piao X. G protein-coupled receptor 56 and collagen III, a receptor-ligand pair, regulates cortical development and lamination. *Proc Natl Acad Sci USA* 2011;**108**:12925–30
25. Luo R, Jin Z, Deng Y, Strokes N, Piao X. Disease-associated mutations prevent GPR56-collagen III interaction. *PLoS One* 2012;**7**:e29818
26. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: One function, multiple origins. *Am J Pathol* 2007;**170**:1807–16
27. Phan SH. Biology of fibroblasts and myofibroblasts. *Proc Am Thorac Soc* 2008;**5**:334–7
28. Huang LS, Fu P, Patel P, Harijith A, Sun T, Zhao Y, Garcia JG, Chun J, Natarajan V. Lysophosphatidic acid receptor 2 deficiency confers protection against bleomycin-induced lung injury and fibrosis in mice. *Am J Respir Cell Mol Biol* 2013;**49**:912–22
29. Akimov SS, Belkin AM. Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. *Blood* 2001;**98**:1567–76
30. Akimov SS, Krylov D, Fleischman LF, Belkin AM. Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. *J Cell Biol* 2000;**148**:825–38
31. Hang J, Zemskov EA, Lorand L, Belkin AM. Identification of a novel recognition sequence for fibronectin within the NH2-terminal beta-sandwich domain of tissue transglutaminase. *J Biol Chem* 2005;**280**:23675–83
32. Velling T, Risteli J, Wennerberg K, Mosher DF, Johansson S. Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins alpha 11beta 1 and alpha 2beta 1. *J Biol Chem* 2002;**277**:37377–81
33. Xu L, Begum S, Hearn JD, Hynes RO. GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. *Proc Natl Acad Sci USA* 2006;**103**:9023–8
34. Xu L, Hynes RO. GPR56 and TG2: Possible roles in suppression of tumor growth by the microenvironment. *Cell Cycle* 2007;**6**:160–5
35. Olsen KC, Sapinoro RE, Kottmann RM, Kulkarni AA, Iismaa SE, Johnson GV, Thatcher TH, Phipps RP, Sime PJ. Transglutaminase 2 and its role in pulmonary fibrosis. *Am J Respir Crit Care Med* 2011;**184**:699–707
36. Cai GQ, Zheng A, Tang Q, White ES, Chou CF, Gladson CL, Olman MA, Ding Q. Downregulation of FAK-related non-kinase mediates the migratory phenotype of human fibrotic lung fibroblasts. *Exp Cell Res* 2010;**316**:1600–9
37. Ding Q, Cai GQ, Hu M, Yang Y, Zheng A, Tang Q, Gladson CL, Hayasaka H, Wu H, You Z, Southern BD, Grove LM, Rahaman SO, Fang H, Olman MA. FAK-related nonkinase is a multifunctional negative regulator of pulmonary fibrosis. *Am J Pathol* 2013;**182**:1572–84
38. Shashidhar S, Lorente G, Nagavarapu U, Nelson A, Kuo J, Cummins J, Nikolich K, Urfer R, Foehr ED. GPR56 is a GPCR that is overexpressed in gliomas and functions in tumor cell adhesion. *Oncogene* 2005;**24**:1673–82
39. Woodhouse EC, Chuaqui RF, Liotta LA. General mechanisms of metastasis. *Cancer* 1997;**80**:1529–37
40. Garcia-de-Alba C, Becerril C, Ruiz V, Gonzalez Y, Reyes S, Garcia-Alvarez J, Selman M, Pardo A. Expression of matrix metalloproteases by fibrocytes: Possible role in migration and homing. *Am J Respir Crit Care Med* 2010;**182**:1144–52
41. Ramirez G, Hagood JS, Sanders Y, Ramirez R, Becerril C, Segura L, Barrera L, Selman M, Pardo A. Absence of Thy-1 results in TGF-beta induced MMP-9 expression and confers a profibrotic phenotype to human lung fibroblasts. *Lab Invest* 2011;**91**:1206–18
42. Buckley ST, Medina C, Kasper M, Ehrhardt C. Interplay between RAGE, CD44, and focal adhesion molecules in epithelial-mesenchymal transition of alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2011;**300**:L548–59
43. Bai Y, Du L, Shen L, Zhang Y, Zhang L. GPR56 is highly expressed in neural stem cells but downregulated during differentiation. *Neuroreport* 2009;**20**:918–22

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