

# lncRNAPCAT29 inhibits pulmonary fibrosis via the TGF- $\beta$ 1-regulated RASAL1/ERK1/2 signal pathway

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Received March 21, 2017; Accepted November 1, 2017

DOI: 10.3892/mmr.2018.8807

**Abstract.** Pulmonary fibrosis is a severe respiratory disease characterized by the aggregation of extracellular matrix components and inflammation-associated injury. Studies have suggested that long non-coding RNAs (lncRNA) may serve a role in the pathophysiological processes of pulmonary fibrosis. However, the potential molecular mechanisms involving the lncRNA, prostate cancer-associated transcript 29 (lncRNAPCAT29) in the progression of pulmonary fibrosis are yet to be determined. In the present study, the role of lncRNAPCAT29 and the potential signaling mechanism in pulmonary fibrosis progression was investigated. Reverse transcription-quantitative polymerase chain reaction and immunohistochemistry revealed that the expression levels of lncRNAPCAT29 were downregulated within interstitial lung cells from mice with silica-induced pulmonary fibrosis. Transfection with lncRNAPCAT29 was associated with upregulated expression of microRNA (miRNA)-221 and downregulated expression of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1); reduced inflammation and fibrotic progression was also associated with lncRNAPCAT29 transfection. TGF- $\beta$ 1 expression levels were inhibited within pulmonary fibroblasts due to lncRNAPCAT29 expression; NEDD4 binding protein 2 and Plexin-A4 expression levels were also suppressed. Analysis of the potential mechanism underlying silica-induced pulmonary fibrosis revealed that the expression levels of RAS protein activator like 1 (RASAL1) and extracellular signal-regulated kinases 1/2 (ERK1/2) were suppressed due to lncRNAPCAT29 expression. The results of the present study

demonstrated that lncRNAPCAT29 induced miRNA-221 upregulation and TGF- $\beta$ 1 downregulation. These observations were associated with reduced inflammation and progression of silica-induced pulmonary fibrosis via the TGF- $\beta$ 1-regulated RASAL1/ERK1/2 signaling pathway, which may serve as a potential target for the treatment of pulmonary fibrosis.

## Introduction

Pulmonary fibrosis is a refractory pulmonary disease that significantly affects lung-associated functions (1). Pulmonary fibrosis is also a type of severe interstitial lung disease that has been associated with a progressive loss of lung function; in addition, relatively higher mortality rates have been observed in clinical settings compared with pulmonary contusion (2). Investigations have revealed that pulmonary fibrosis is caused by various factors, including inflammation and breathing disorders (3). A systematic review and meta-analysis demonstrated the association between the severity of breathing disorders, and the aggregation of extracellular matrix components and inflammation-associated injury (4). A reported increase in the incidence and mortality rates of pulmonary fibrosis has been associated with the development of severe acute respiratory syndrome (5). Therefore, understanding the potential signaling mechanism underlying pulmonary fibrosis is essential to understand the progression of this disease.

Long non-coding RNAs (lncRNAs) are associated with numerous human diseases via the regulation of different signal pathways within cells (6-8). A previous analysis of lncRNA as a competing endogenous RNA and its association with protein-coding genes has indicated potential associations among lncRNAs, microRNAs (miRNAs) and mRNAs in pulmonary fibrosis (9), which may be applied to future investigations into the treatment of this disease. In addition, Wu *et al* (10) reported that miRNA-489 could inhibit silica-induced pulmonary fibrosis by targeting myeloid differentiation response 88 and mothers against decapentaplegic homolog 3, which are negatively regulated by lncRNA-CHRF (10). Studies regarding lncRNA polymorphisms are of increasing interest to scientists and pathologists, and may aid the development of lung disease-associated therapies (11-14). Therefore, investigations into the potential roles of lncRNAs are crucial in understanding human pulmonary diseases.

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**Key words:** pulmonary fibrosis, long non-coding RNA prostate cancer-associated transcript 29, inflammation, transforming growth factor- $\beta$ 1, microRNA-221, RAS protein activator like 1/extracellular signal-regulated kinases 1/2

Evidence has revealed that the lncRNA, prostate cancer-associated transcript 29 (lncRNAPCAT29), constitutes a tumor-suppressive factor within numerous cell types (11); however, the role of lncRNAPCAT29 in the progression of pulmonary fibrosis has yet to be analyzed. In the present study, the role of lncRNAPCAT29 in the progression of pulmonary fibrosis and its underlying mechanism were investigated. Additionally, the involvement of lncRNAPCAT29 in suppressing pulmonary fibroblast proliferation and ameliorating inflammation in silica-induced pulmonary fibrosis were analyzed.

## Materials and methods

**Statement of ethics.** Animal procedures were conducted in accordance with humane animal care standards. Experimental protocols were approved by the Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (Jinan, China).

**Animals.** Specific pathogen-free C57BL/6 male mice (age, 4–6 weeks of age; body weight, 26–32 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Mice were fed under pathogen-free conditions ( $23 \pm 1^\circ\text{C}$ ; relative humidity,  $50 \pm 5\%$ ) and were maintained under a 12-h light/dark cycle with free access to food and water. To establish a mouse model of pulmonary fibrosis, mice were instilled with 50 mg/kg silica (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 0.05 ml sterile saline intratracheally or with 0.05 ml sterile saline intratracheally ( $n=30/\text{group}$ ) (12). Mice were sacrificed, and lungs were harvested and stored at  $-80^\circ\text{C}$  immediately after treatment.

**Cell culture and reagents.** Pulmonary fibroblasts were isolated from experimental mice treated with 50 mg/kg silica or sterile saline. Lung tissues were sectioned to  $\sim 1\text{ mm}^3$  and were digested with 0.25% trypsin for  $\sim 12\text{ h}$  at  $4^\circ\text{C}$ . Cells were then cultured in Minimum Essential Medium (MEM; Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a  $37^\circ\text{C}$  humidified atmosphere containing 5%  $\text{CO}_2$ . Subsequently, cells were filtered via  $100\text{ }\mu\text{m}$  nylon filters to remove undigested tissue.

**Endogenous overexpression of TGF- $\beta$ 1.** Pulmonary fibroblasts ( $1 \times 10^7$ ) were isolated from experimental mice prior to treatment ( $n=5$ ) and were cultured in MEM supplemented with 10% FCS. Cells were grown to 85% confluence and were subsequently transfected with pcdue12.4-TGF- $\beta$ 1 (TGF- $\beta$ 1, 100 pmol; GenBank: GQ338152.1; Invitrogen; Thermo Fisher Scientific, Inc.) or pcdue12.4 (Control, 100 pmol; Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Stable TGF- $\beta$ 1 overexpression within pulmonary fibroblasts was identified using a GS screening system (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol (13).

**Endogenous overexpression of miRNA-221 or lncRNAPCAT29.** Pulmonary fibroblasts were grown to 85% confluence and were subsequently transduced with 100 pmol plentivirus-miRNA-221

(miRNA-221, 5'-GUGUAGUCCACCACUAGCUAGC-3'), or 100 pmol plentivirus-lncRNAPCAT29 (lncRNAPCAT29, 5'-AUCUCGACGUGCGGUUACUCUA-3'), 100 pmol plentivirus-lncRNA vector (5'-UUAGGCUGAGUAGCUUGAA-3') or 100 pmol scramble miRNA (5'-CAUGUAAGCGGAUUGCA-3') using a lentiviral vector system (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's protocol. All miRNA sequences were supplied by Invitrogen; Thermo Fisher Scientific, Inc. After 48 h transduction, stable expression of miRNA-221 and lncRNAPCAT29 within pulmonary fibroblast cells were identified as stated in a previous report (14). In addition, plentivirus-lncRNAPCAT29-transduced cells were then transduced with miRNA-221 using Lipofectamine RNAiMax reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

**Small interfering-RNA (siRNA) for miRNA-221 or TGF- $\beta$ 1 knockdown.** siRNA sequences targeting miRNA-221 or TGF- $\beta$ 1 gene sequences were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The siRNA oligonucleotide sequences were as follows: si-TGF- $\beta$ 1 5'-AGCTTCTGTCCGGATCTAA-3'; si-miRNA-221 5'-GTG TAGTCCACCACTAGCTAGC-3' or si-Vector (Control) 5'-ACGTAGATCCTTCAGCACC-3'. The siRNAs were transfected into pulmonary fibroblast cells for further analysis using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to a recent study (15). lncRNAPCAT29-overexpressed fibroblast cells were also transected with siRNA sequences targeting miRNA-221. lncRNAPCAT29-overexpressed cells were also treated with si-TGF- $\beta$ 1 and pcdue12.4-TGF- $\beta$ 1. After 48 h transfection, cells were used for further analysis.

**Proliferation assay.** Transfected/transduced pulmonary fibroblasts ( $1 \times 10^3$ ) exhibiting stable expression of each condition were seeded in a 96-well plate for 48 h in triplicate. Following incubation at  $37^\circ\text{C}$ , 20  $\mu\text{l}$  MTT (5 mg/ml) in PBS solution was added to each well and incubated for 4 h at  $37^\circ\text{C}$ . The medium (100  $\mu\text{l}$ ) was removed and 100  $\mu\text{l}$  dimethyl sulfoxide was added to the wells to solubilize the crystals. The optical density was measured using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

**Migration assay.** Prior to incubation for 48 h at  $37^\circ\text{C}$  in a Matrigel Invasion Chamber (BD Biosciences), according to the manufacturer's protocol, treated cells were suspended at a density of  $1 \times 10^5$  in 500  $\mu\text{l}$  serum-free MEM (Sigma-Aldrich; Merck KGaA). Migration of transfected pulmonary fibroblasts was analyzed in  $\geq 3$  randomly-selected fields of each membrane via light microscopy using ImageJ software (version 2.2; National Institutes of Health, Bethesda, MD, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was obtained from pulmonary fibroblasts isolated from experimental mice using an RNAeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). Expression levels of PCAT29 in cells were measured via Verso One-Step RT-qPCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) and RT-qPCR conditions were performed as described previously (16). Forward and reverse primers were synthesized by Invitrogen (Thermo Fisher Scientific,

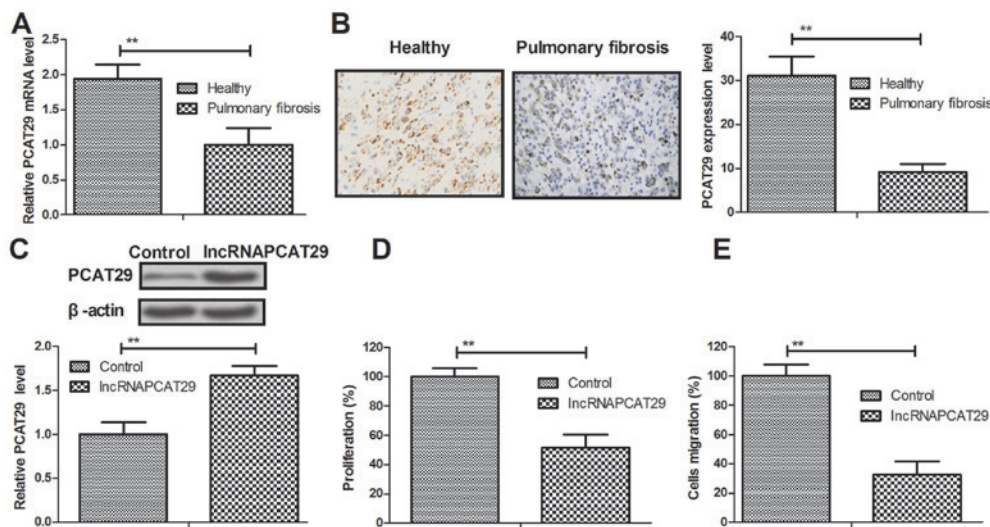


Figure 1. IncRNAPCAT29 inhibits pulmonary fibroblast proliferation and migration. (A) PCAT29 expression levels were significantly downregulated within pulmonary fibroblasts as determined by reverse transcription-quantitative polymerase chain reaction. (B) Immunohistochemistry revealed that PCAT29 was located within the cytoplasm of interstitial lung cells. Magnification,  $\times 20$ . (C) IncRNAPCAT29 transfection was associated with increased PCAT29 protein expression levels within pulmonary fibroblasts. IncRNAPCAT29 transfection significantly inhibited pulmonary fibroblast (D) proliferation and (E) migration. Results were expressed as the mean  $\pm$  standard deviation of three independent experiments. \*\* $P < 0.01$ , vs. the control group (IncRNA vector). IncRNAPCAT29, long non-coding RNA prostate cancer-associated transcript 29.

Inc.). PCAT29 forward, 5'-TTTATGCTTGAGCCTTGA-3' and reverse, 5'-CTTGCCTGAAATACTTGC-3';  $\beta$ -actin (control) forward, 5'-GTGGGCGCCCAGGCACCA-3' and reverse, 5'-CTCCTTAATGTCACGCACGATTT-3'; miRNA-221, 5'-CTCAACTGGTGTCTGTTGAGTCGGCAATTCAGTTGAGTGGGGTATT-3'; and tRNA<sup>thr</sup> 5'-CTCAAC TGGTGTCTGTTGGA-3'. Relative mRNA expression alterations were calculated by  $2^{-\Delta\Delta C_q}$  (17). Results are expressed as the n-fold compared with the control.

**Western blot analysis.** Protein was extracted from treated cells using radioimmunoprecipitation assay buffer (M-PER reagent for cells; Thermo Fisher Scientific, Inc.), followed by homogenization at 4°C for 10 min. Protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 20  $\mu$ g protein was electrophoresed via 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Blocking buffer (5% milk) was applied to membranes for 2 h at 37°C prior to incubation with primary antibodies at 4°C overnight. The primary antibodies used in the immunoblotting assays included: TGF- $\beta$ 1 (1:200; ab92486), matrix metalloproteinase (MMP) 3 (1:1,000; ab53015), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; 1:500; ab6671), interleukin-1 $\beta$  (IL-1 $\beta$ ; 1:500; ab200478), MMP9 (1:500; ab73734), RAS protein activator like 1 (RASAL1; 1:500; ab214321), extracellular signal-regulated kinase 1/2 (ERK 1/2; 1:1,000; ab32537), phosphorylated (p)-Thr202/Tyr204 ERK1/2 (1:500; ab214362), fibronectin (FN; 1:500; ab2413), extracellular matrix collagen I (COL1; 1:500; ab34710), NEDD4 binding protein 2 (N4bp2; 1:500; ab102634), plexin A4 (PLXNA4; 1:500; ab39350) and  $\beta$ -actin (1:500; ab8227; all Abcam, Cambridge, UK). Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG; 1:5,000; cat. no. 1706515; Bio-Rad Laboratories, Inc.) was applied for 2 h at 37°C and bands were detected using WesternBright ECL Chemiluminescent HRP Substrate (Advansta, Inc., Menlo Park, CA, USA).

**Immunohistochemical staining.** Lung tissues were obtained from experimental mice following treatment. Tissues were fixed with 4% paraformaldehyde for 12 h at 4°C, paraffin-embedded lung tissue sections (4  $\mu$ m) were prepared and epitope retrieval was performed using Tris-HCl buffer (AP-9005-050; Thermo Fisher Scientific, Inc.) for 30 min at 37°C for further analysis. The paraffin-embedded sections were treated with hydrogen peroxide (3%) for 15 min and subsequently blocked with a regular blocking solution for 20 min at 37°C. The sections were subsequently incubated with rabbit anti-mouse PCAT29 antibody [1:500; Q7L5N7; Baiqi Biotechnology (Suzhou Co., Ltd., Suzhou, China) at 4°C for 12 h]. Sections were washed three times and incubated with HRP-conjugated anti-rabbit IgG (1:10,000; cat. no. 1706515; Bio-Rad Laboratories, Inc.) for 1 h at 37°C. Tissues sections were observed in six randomly selected fields under a confocal microscope (LSM780; Carl Zeiss AG, Oberkochen, Germany). Densitometric semi-quantification of the immunoblot data was performed using Quantity-One software version 4.2 (Bio-Rad Laboratories, Inc.).

**Statistical methods.** Data are expressed as the mean  $\pm$  standard error of the mean. Unpaired data were analyzed by Student's t-test. Comparisons of data between multiple groups were analyzed using one-way analysis of variance followed by Tukey's honest significant difference test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**IncRNAPCAT29 inhibits pulmonary fibroblast proliferation and migration in vitro.** Expression levels of IncRNAPCAT29 in pulmonary fibroblasts isolated from healthy and silica-induced mouse models of pulmonary fibrosis were analyzed. The results of the present study demonstrated that PCAT29 was significantly downregulated in pulmonary fibroblasts from



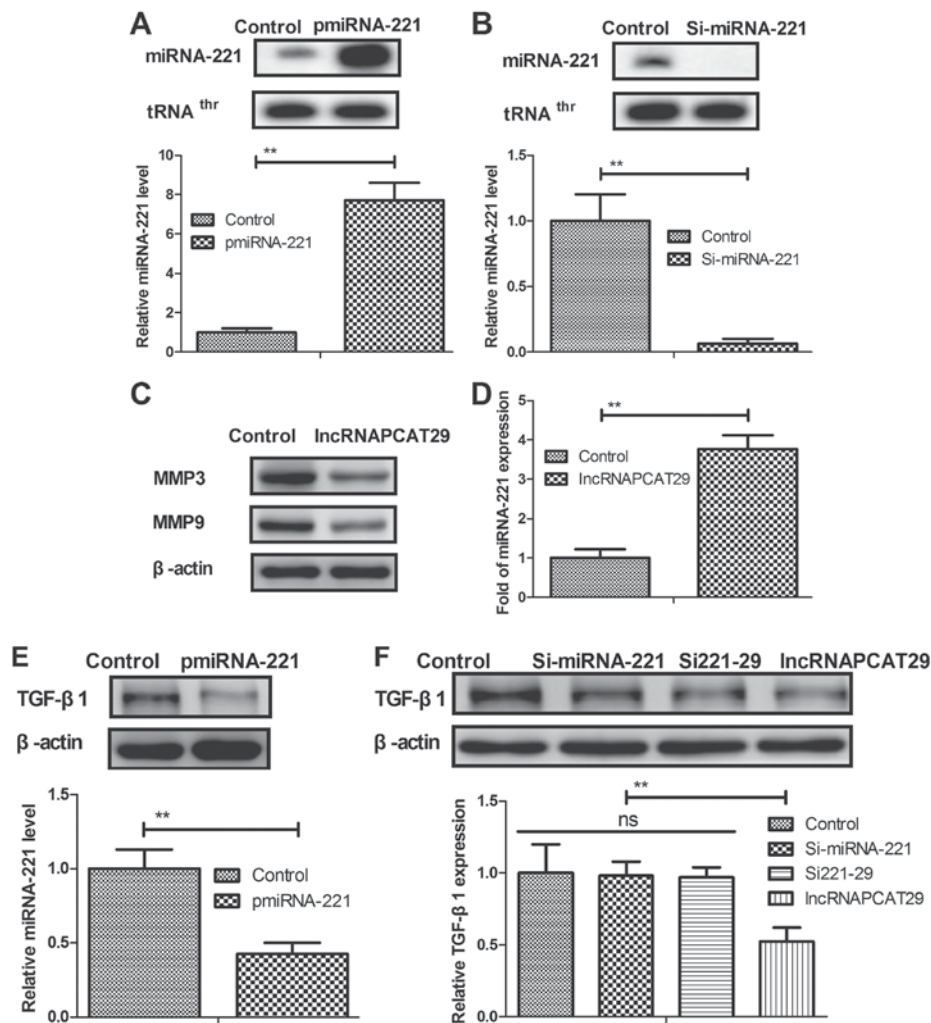


Figure 2. lncRNAPCAT29 inhibits fibroblast differentiation by targeting TGF- $\beta$ 1 within pulmonary fibroblasts. (A) miRNA-221 overexpression increased miRNA-221 expression levels within pulmonary fibroblasts determined by RT-qPCR. (B) miRNA-221 silencing decreased miRNA-221 expression levels within pulmonary fibroblasts determined by RT-qPCR. (C) lncRNAPCAT29 transfection suppressed pulmonary fibroblast differentiation, as determined by decreasing levels of MMP3 and MMP9. (D) miRNA-221 expression levels were significantly upregulated by lncRNAPCAT29 transfection. (E) miRNA-221 overexpression suppressed TGF- $\beta$ 1 expression levels within pulmonary fibroblast cells. (F) lncRNAPCAT29 inhibited TGF- $\beta$ 1 expression levels and miRNA-221 downregulation inhibited this suppression within pulmonary fibroblasts. Results were expressed as the mean  $\pm$  standard deviation of three independent experiments. \*\* $P$ <0.01, vs. the control group (scramble miRNA). lncRNAPCAT29, long non-coding RNA prostate cancer-associated transcript 29; miRNA-221, microRNA-221; MMP, matrix metalloproteinase; pmiRNA-221, miRNA-221 overexpression; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Si-miRNA-221, miRNA-221 silencing; Si221-29, miRNA-221 silencing and lncRNAPCAT29 overexpression; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

the pulmonary fibrosis group, as determined by RT-qPCR and immunohistochemistry (Fig. 1A and B). lncRNAPCAT29 transfection increased PCAT29 protein expression levels in pulmonary fibroblasts (Fig. 1C). In addition, significant inhibition of pulmonary fibroblast cell proliferation was observed (Fig. 1D). The results also suggested that migration of pulmonary fibroblasts was downregulated in response to lncRNAPCAT29 overexpression (Fig. 1E). Collectively, these results indicated that lncRNAPCAT29 is downregulated in pulmonary fibrosis, whereas lncRNAPCAT29 overexpression may inhibit pulmonary fibroblast proliferation and migration *in vitro*.

**lncRNAPCAT29 inhibits differentiation by targeting TGF- $\beta$ 1 in pulmonary fibroblasts.** In the present study, the potential molecular mechanism of lncRNAPCAT29-associated inhibition of pulmonary fibroblast proliferation was analyzed. Overexpression of miRNA-221 (pmiRNA-221) was associated

with increased expression levels of miRNA-221, whereas silenced miRNA-221 (Si-miRNA-221) was associated with reduced expression of miRNA-221 within pulmonary fibroblasts (Fig. 2A and B). lncRNAPCAT29 transfection was observed to suppress differentiation of pulmonary fibroblast cells, as determined by reduced levels of MMP3 and MMP9 (Fig. 2C). miRNA-221 was also significantly upregulated due to lncRNAPCAT29 transfection (Fig. 2D). pmiRNA-221 was associated with reduced TGF- $\beta$ 1 expression levels within pulmonary fibroblasts (Fig. 2E), additionally western blot analysis indicated that lncRNAPCAT29 inhibited TGF- $\beta$ 1 expression; whereas, Si-miRNA-221 expression inhibited lncRNAPCAT29-suppressed (Si221-29) TGF- $\beta$ 1 expression within pulmonary fibroblasts (Fig. 2F). Collectively, these results suggested that lncRNAPCAT29 inhibited fibroblast differentiation via affecting the miRNA-221-regulated TGF- $\beta$ 1 signaling pathway in pulmonary fibroblasts.

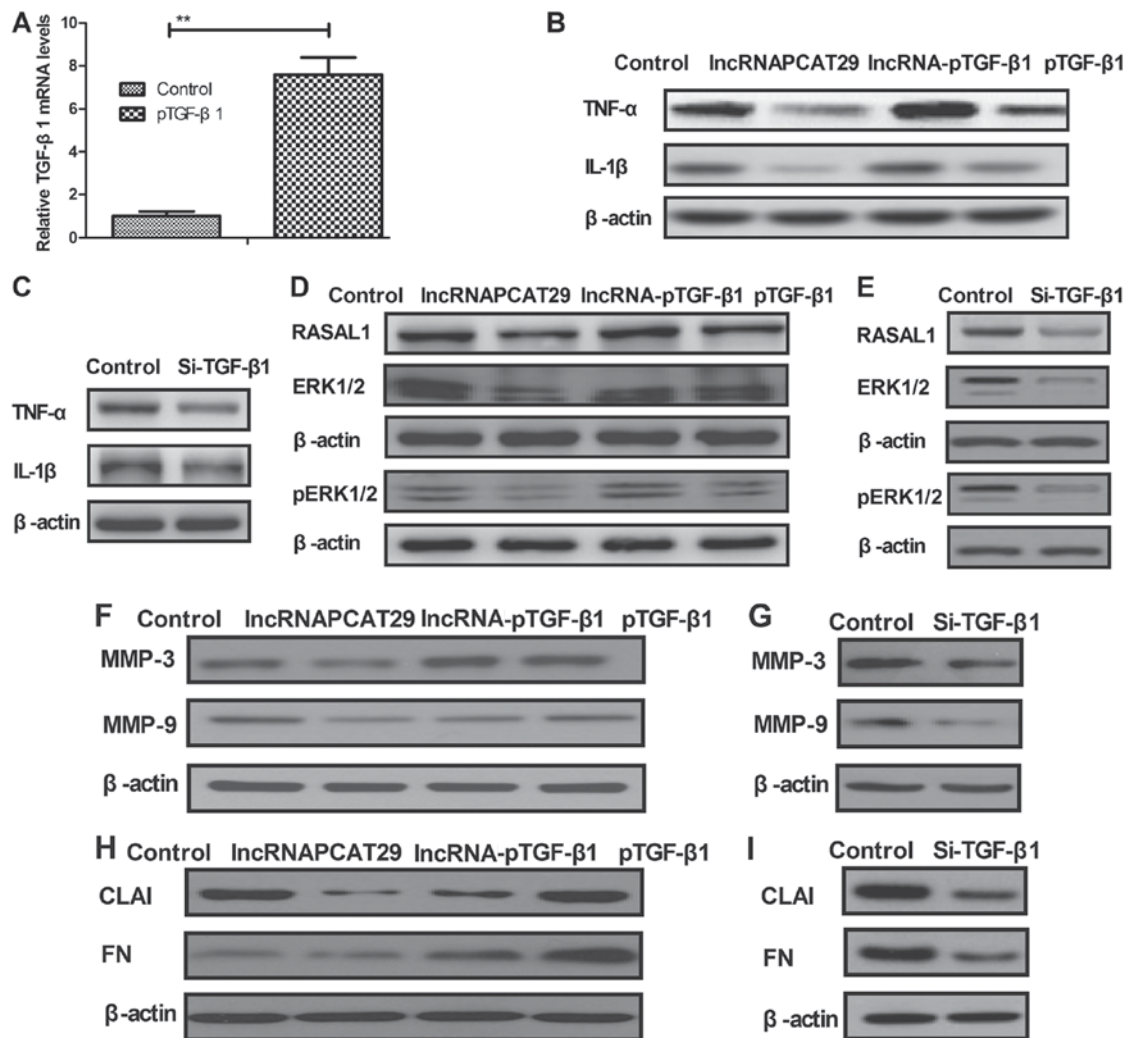


Figure 3. IncRNAPCAT29 inhibits inflammatory cytokine expression by targeting the TGF- $\beta$ 1-mediated RASAL1/ERK1/2 signaling pathway within pulmonary fibroblasts. (A) TGF- $\beta$ 1 transduction upregulated TGF- $\beta$ 1 mRNA expression compared with in the control pulmonary fibroblast cells. Control, pVector. (B) IncRNAPCAT29 inhibited expression of the inflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ ; TGF- $\beta$ 1 overexpression abrogated IncRNAPCAT29-mediated downregulation of TNF- $\alpha$  and IL-1 $\beta$  in pulmonary fibroblasts. Control, pVector. (C) TGF- $\beta$ 1 downregulation inhibited the expression of the inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  within pulmonary fibroblasts. Control, Si-Vector. (D) Effects of TGF- $\beta$ 1 overexpression on IncRNAPCAT29 transfection-inhibited RASAL1, ERK1/2 expression and ERK1/2 phosphorylation. Control, pVector. (E) TGF- $\beta$ 1 downregulation decreased RASAL1 and ERK1/2 expression, and ERK1/2 phosphorylation within pulmonary fibroblasts. Control, Si-Vector. (F) TGF- $\beta$ 1 overexpression blocked IncRNAPCAT29-induced downregulation of MMP3 and MMP9 expression within pulmonary fibroblasts. Control, pVector. (G) TGF- $\beta$ 1 downregulation inhibited MMP3 and MMP9 expression within pulmonary fibroblasts. Control, Si-Vector. (H) TGF- $\beta$ 1 overexpression abolished IncRNAPCAT29-induced downregulation of CLAI and FN expression within pulmonary fibroblasts. Control, pVector. (I) TGF- $\beta$ 1 downregulation inhibited CLAI and FN expression within pulmonary fibroblasts. Control, Si-Vector. \*\* $P < 0.01$  vs. the control group. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments. CLAI, extracellular matrix collagen I; ERK 1/2, extracellular signal-regulated kinases 1/2; FN, fibronectin; IL-1 $\beta$ , interleukin-1 $\beta$ ; IncRNAPCAT29, long non-coding RNA prostate cancer-associated transcript 29; pTGF- $\beta$ 1, transforming growth factor- $\beta$ 1 overexpression; IncRNA-pTGF- $\beta$ 1, IncRNAPCAT29 overexpression and TGF- $\beta$ 1 overexpression; MMP, matrix metalloproteinase; RASAL 1, RAS protein activator like 1; Si-TGF- $\beta$ 1, TGF- $\beta$ 1 downregulation; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

*IncRNAPCAT29 inhibits the expression of inflammatory cytokines by targeting the TGF- $\beta$ 1-mediated RASAL1/ERK1/2 signal pathway.* As shown in Fig. 3A, TGF- $\beta$ 1 overexpression markedly upregulated TGF- $\beta$ 1 mRNA expression compared with in the control (pudu2.4-transduced group) pulmonary fibroblast cells. In addition, it was observed in the present study that inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$  were downregulated within pulmonary fibroblasts following IncRNAPCAT29 transfection; however, this downregulation was abrogated by TGF- $\beta$ 1 overexpression (pTGF- $\beta$ 1) (Fig. 3B). TGF- $\beta$ 1 knockdown (Si-TGF- $\beta$ 1) also inhibited the expression of TNF- $\alpha$  and IL-1 $\beta$  within pulmonary fibroblast

cells (Fig. 3C). Expression levels of RASAL1, ERK1/2 and p-ERK1/2 were downregulated in IncRNAPCAT29-transfected pulmonary fibroblasts, whereas pTGF- $\beta$ 1 eradicated IncRNAPCAT29-inhibited (IncRNA-pTGF- $\beta$ 1) RASAL1 and ERK1/2 expression, as well as ERK1/2 phosphorylation (Fig. 3D). Si-TGF- $\beta$ 1 also decreased RASAL1, ERK1/2 expression and ERK1/2 phosphorylation (Fig. 3E). The results also demonstrated that pTGF- $\beta$ 1 inhibited IncRNA-pTGF- $\beta$ 1-associated downregulation of MMP3 and MMP9 expression, whereas Si-TGF- $\beta$ 1 inhibited MMP3 and MMP9 expression (Fig. 3F and G). In addition, the expression levels of CLAI and FN were significantly decreased due to

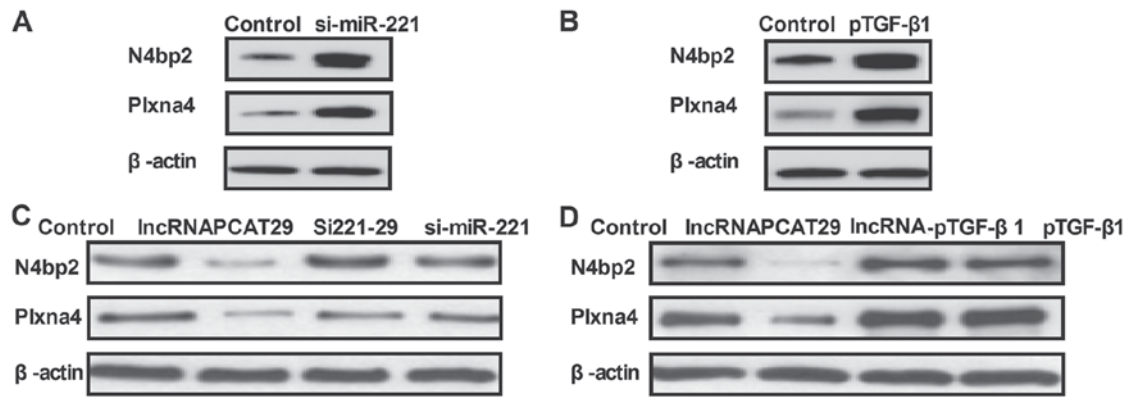


Figure 4. lncRNAPCAT29 inhibits TGF- $\beta$ 1-regulated N4bp2 and Plxna4 expression levels in pulmonary fibroblasts. (A) miR-221 knockdown increased N4bp2 and Plxna4 expression levels within pulmonary fibroblasts. Control, scramble miRNA. (B) TGF- $\beta$ 1 overexpression increased N4bp2 and Plxna4 expression levels within pulmonary fibroblasts. Control, pVector. (C) miR-221 knockdown abolished lncRNAPCAT29-decreased N4bp2 and Plxna4 expression levels within pulmonary fibroblasts. Control, scramble miRNA. (D) TGF- $\beta$ 1 overexpression abolished lncRNAPCAT29-decreased N4bp2 and Plxna4 expression levels within pulmonary fibroblasts. Control, pVector. Results were expressed as the mean  $\pm$  standard deviation of three independent experiments. lncRNAPCAT29, long non-coding RNA prostate cancer-associated transcript 29; Si221-29, miRNA-221 silencing and lncRNAPCAT29 overexpression; lncRNA-pTGF- $\beta$ 1, lncRNAPCAT29 overexpression and TGF- $\beta$ 1 overexpression; si-miR-221, miRNA-221 silencing; N4bp2, NEDD4 binding protein 2; Plxna4, Plexin-A4; pTGF- $\beta$ 1, TGF- $\beta$ 1 overexpression; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

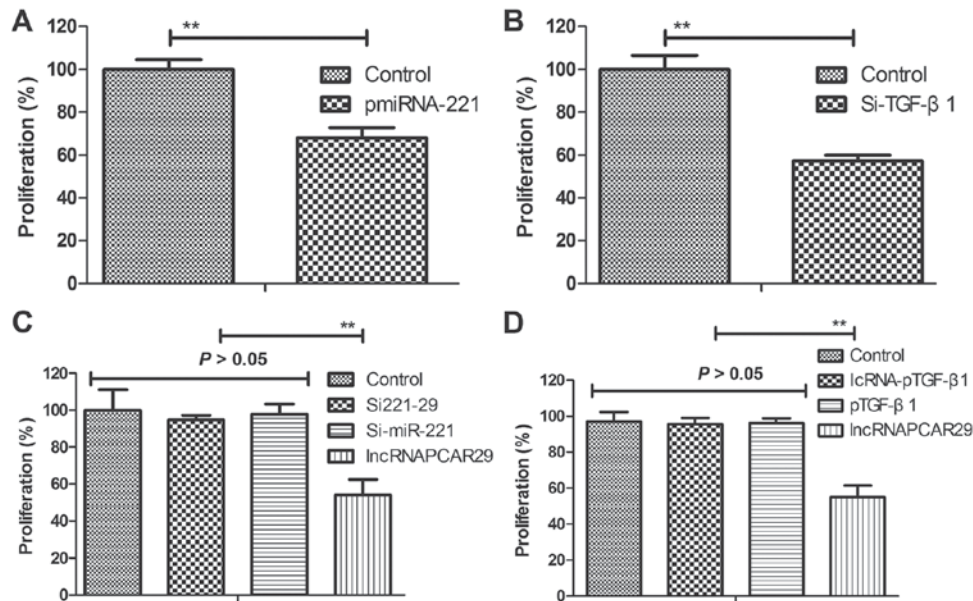


Figure 5. lncRNAPCAT29 regulates the growth of pulmonary fibroblasts via the miRNA-221-mediated TGF- $\beta$ 1 signaling pathway. Proliferation of pulmonary fibroblasts was inhibited by (A) miRNA-221 upregulation compared to scrambling miRNA (Control) and (B) TGF- $\beta$ 1 knockdown compared to pVector (Control). (C) miRNA-221 knockdown abolished lncRNAPCAR29-suppressed proliferation. Control, scramble miRNA. (D) TGF- $\beta$ 1 overexpression abolished lncRNAPCAR29-suppressed proliferation. Control, pVector. Results were expressed as the mean  $\pm$  standard deviation of three independent experiments. \*\*P < 0.01 vs. the control group. lncRNAPCAT29, long non-coding RNA prostate cancer-associated transcript 29; si221-29, lncRNAPCAT29 overexpression and microRNA-221 knockdown; lncRNA-pTGF- $\beta$ 1, lncRNAPCAT29 overexpression and TGF- $\beta$ 1 overexpression; pTGF- $\beta$ 1, TGF- $\beta$ 1 overexpression; Si-miRNA-221, miRNA-221 silencing; Si-TGF- $\beta$ 1, TGF- $\beta$ 1 downregulation; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

lncRNAPCAT29 transfection; however, this inhibition was abrogated by pTGF- $\beta$ 1. Conversely, Si-TGF- $\beta$ 1 suppressed CLAI and FN expression (Fig. 3H and I). Collectively, these results suggested that lncRNAPCAT29 may inhibit inflammatory cytokines expression by targeting the RASAL1/ERK1/2 signal pathway in pulmonary fibroblasts.

*lncRNAPCAT29 inhibits the expression of N4bp2 and Plxna4, which are regulated by TGF- $\beta$ 1 within pulmonary fibroblasts.* The expression levels of N4bp2 and Plxna4 are associated with pulmonary fibrosis; therefore, the effects of lncRNAPCAT29

on N4bp2 and Plxna4 expression in pulmonary fibroblasts were investigated. miR-221 knockdown or TGF- $\beta$ 1 overexpression significantly increased N4bp2 and Plxna4 expression in pulmonary fibroblasts (Fig. 4A and B). As presented in Fig. 4C and D, lncRNAPCAT29 transfection inhibited N4bp2 and Plxna4 expression, which was eliminated by miR-221 overexpression (lncRNA-miR-221) or pTGF- $\beta$ 1 expression. Collectively, these results suggested that lncRNAPCAT29 may inhibit the expression levels of N4bp2 and Plxna4, which are regulated by the miRNA-221-inhibited TGF- $\beta$ 1 pathway within pulmonary fibroblasts.



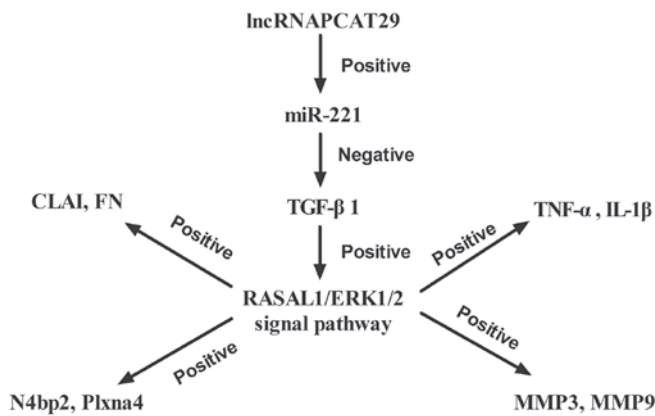


Figure 6. Schematic diagram of the signaling pathways mediated by lncRNAPCAT29. CLAI, extracellular matrix collagen I; ERK 1/2, extracellular signal-regulated kinases 1/2; FN, fibronectin; IL-1 $\beta$ , interleukin-1 $\beta$ ; lncRNAPCAT29, long non-coding RNA prostate cancer-associated transcript 29; N4bp2, NEDD4 binding protein 2; MMP, matrix metalloproteinase; miR-221, microRNA-221; RASAL 1, RAS protein activator like 1; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

*lncRNAPCAT29 regulates the growth of pulmonary fibroblasts via the miRNA-221-mediated TGF- $\beta$ 1 signaling pathway.* In the present study, the effects of miRNA-221 and TGF- $\beta$ 1 on pulmonary fibroblast growth were analyzed. miRNA-221 upregulation (pmiRNA-221) or TGF- $\beta$ 1 knockdown (Si-TGF- $\beta$ 1) inhibited pulmonary fibroblast proliferation (Fig. 5A and B). In addition, the results demonstrated that miRNA-221 downregulation or TGF- $\beta$ 1 overexpression abolished lncRNAPCAT29-suppressed proliferation of pulmonary fibroblasts (Fig. 5C and D). Collectively, these results suggested that lncRNAPCAT29 regulated growth of pulmonary fibroblasts via the miRNA-221-mediated TGF- $\beta$ 1 signal pathway.

## Discussion

Pulmonary fibrosis is an intractable lung disease characterized by the accumulation of collagen, injury to the overlying epithelium and fibroblast differentiation (18). Increasing evidence has suggested that lncRNAs are associated with human fibrotic diseases via regulation of cellular signal pathways (9,19). In addition, a recent study has reported a novel epigenetic cascade of renal fibrogenesis via TGF- $\beta$ 1-induced epigenetic aberrations of miRNAs and DNA methyltransferase (20). In addition, miRNA-221 promoted fibrosis in cystic fibrosis airway epithelial cells (21). In the present study, the associations between lncRNA, miRNA and pulmonary fibrosis were investigated. The results demonstrated that lncRNAPCAT29 expression was reduced within pulmonary fibroblasts isolated from silica-induced mouse models. lncRNAPCAT29 transfection inhibited pulmonary fibroblast differentiation by targeting the TGF- $\beta$ 1-mediated RASAL1/ERK1/2 signal pathway, which is regulated by miR-221.

PCAT29 is a potential target for prostate cancer therapy and is regarded as the first androgen receptor-repressed lncRNA (22). In the present study, it was demonstrated that lncRNAPCAT29 is downregulated within pulmonary fibroblasts of silica-induced pulmonary fibrotic mice, which may regulate the proliferation and differentiation of pulmonary

fibroblasts. A recent study indicated that miRNA-221 expression levels may be elevated within cystic fibrosis airway epithelial cells, which may affect the expression of transcriptional regulators via regulating the expression of activating transcription factor 6 (21). Therefore, the potential target of lncRNAPCAT29 within pulmonary fibroblasts was investigated. Results of the present study indicated that lncRNAPCAT29 transfection increased expression levels of miRNA-221 and decreased TGF- $\beta$ 1 expression within pulmonary fibroblasts.

TGF- $\beta$ 1 overexpression is associated with the progression of pulmonary fibrosis (23). TGF- $\beta$ 1 has been regarded as a therapeutic target for pulmonary fibrosis due to TGF- $\beta$ 1-associated genes or signals that restore extracellular matrix homeostasis (24). In the present study, it was reported that miRNA-221 transduction decreased TGF- $\beta$ 1 expression within pulmonary fibroblasts, which was associated with the suppression of pulmonary fibrosis (25). However, TGF- $\beta$ 1 overexpression eliminated the effects of lncRNAPCAT29-inhibition on differentiation of pulmonary cytokines and associated inflammation. Therefore, lncRNAPCAT29 may serve a role in regulating the growth of pulmonary fibroblasts via the miRNA-221-inhibited TGF- $\beta$ 1 signal pathway.

RASAL1 is a key protein associated with renal fibrosis and hepatic stellate cell proliferation (26,27). Research has revealed that Paridis Rhizoma saponins attenuate liver fibrosis in rats by downregulating expression of the RASAL1/ERK1/2 signaling pathway (28). As reported in the present study, lncRNAPCAT29 inhibited the RASAL1/ERK1/2 signaling pathway within pulmonary fibroblasts; therefore, lncRNAPCAT29 may have contributed to the suppression of pulmonary fibrosis development. Studies have indicated that ERK inhibitors decrease the expression levels of MMP2 and MMP9 in alveolar epithelial cells, which may be a potential target for the treatment of lung fibrosis (29,30). Additionally, upregulated expression and activity of MMP9 has been reported in bleomycin-induced pulmonary fibrosis (31). In the present study, lncRNAPCAT29 transfection was associated with decreased levels of MMP3 and MMP9 expression, which suppressed pulmonary fibroblast cell differentiation. Analysis into the potential mechanism underlying the effects of lncRNAPCAT29 indicated that RASAL1 and ERK1/2 expression levels were reduced, mediated by the miRNA-221-inhibited TGF- $\beta$ 1 signaling pathway. lncRNAPCAT29 was also demonstrated to inhibit the expression of N4bp2 and Plxna4, which was regulated by the miRNA-221-inhibited TGF- $\beta$ 1 pathway within pulmonary fibroblasts. However, further investigation into the numerous molecules within the RASAL1/ERK1/2 pathway is required.

In conclusion, the results of the present study demonstrated the potential role of lncRNAPCAT29 in the progression of pulmonary fibrosis as well as the potential underlying mechanism. Findings revealed that lncRNAPCAT29 overexpression is associated with improvements in pulmonary fibrosis; lncRNAPCAT29 exerted key functions in silica-induced pulmonary fibrosis via the miR-221-TGF- $\beta$ 1-regulated RASAL1/ERK1/2 signal pathway (Fig. 6). The present study has provided novel insights into understanding the complex molecular mechanisms of certain miRNAs and the lncRNA-mediated RASAL1/ERK1/2 signaling pathway in silica-induced pulmonary fibrosis. These findings may

contribute to the development of lncRNA-associated therapy for the treatment of pulmonary fibrosis. However, the *in vivo* efficacy of lncRNAPCAT29 treatment has yet to be investigated.

## Acknowledgements

Not applicable.

## Funding

No funding was received.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XL performed the experiments in this study. SG designed the experiments. HX analyzed the data generated in this study.

## Ethics approval and consent to participate

Experimental protocols were approved by the Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (Jinan, China).

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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