

# Long non-coding RNA HOTTIP enhances the fibrosis of lung tissues by regulating the miR-744-5p/PTBP1 signaling axis

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**Abstract.** Fibrosis of lung tissue can induce the occurrence and development of numerous types of lung disease. The expression levels of the long non-coding RNA (lncRNA) HOXA distal transcript antisense RNA (HOTTIP) have been reported to be upregulated during the development of fibrosis in liver tissues, which subsequently activated hepatic stellate cells. However, whether the lncRNA HOTTIP participates in the occurrence and development of lung fibrosis remains unknown. The present study aimed to investigate the role of lncRNA HOTTIP in lung fibrosis and its potential mechanism. In the present study, A549 cells were stimulated with TGF- $\beta$ 1 to induce lung fibrosis *in vitro*. A549 was transfected with short hairpin RNA-HOTTIP, overexpression-polypyrimidine tract binding protein 1 (PTBP1), microRNA (miR)-744-5p mimic or miR-744-5p to regulate gene expression. Cell proliferation and migration were determined using 5'-ethynyl-2'-deoxyuridine and wound healing assays, respectively. The expression levels of  $\alpha$ -smooth muscle actin, collagen I, collagen III and fibronectin 1 were analyzed using western blotting. starBase was used to identify molecules that may interact with the lncRNA HOTTIP and dual luciferase reporter assays were used to validate the findings. Moreover, an *in vivo* lung fibrosis model was established by bleomycin induction in mice. Histological injury was observed using hematoxylin and eosin and masson staining. The results of the present study revealed that the proliferation and migration of A549 cells were both suppressed following the knockdown of HOTTIP. The lncRNA HOTTIP was found to target and downregulate the expression levels of miR-744-5p. The overexpression of miR-744-5p inhibited the proliferation and migration of A549 cells. Furthermore,

miR-744-5p targeted and downregulated the expression levels of PTBP1. It was subsequently demonstrated that the overexpression of PTBP1 rescued miR-744-5p-induced suppression of the proliferation and migration of A549 cells. The knockdown of lncRNA HOTTIP expression also relieved the fibrosis of the lung tissues of mice. In conclusion, the results of the present study suggested that the lncRNA HOTTIP may promote the fibrosis of lung tissues by downregulating the expression levels of miR-744-5p and upregulating the expression levels of PTBP1.

## Introduction

Pulmonary fibrosis is a disease induced by interstitial pneumonia; however, to the best of our knowledge, the mechanism underlying the occurrence of this disease is yet to be elucidated (1,2). At present, injury to epithelial cells, cellular senescence and an abnormal immune response are considered to contribute to lung fibrosis development (3-5). Moreover, the excessive accumulation of fibroblasts and the increased secretion of extracellular matrix promotes the fibrosis of lung tissues (6). However, the specific molecular mechanism underlying lung fibrosis requires further investigations.

Non-coding RNAs are types of RNA that cannot encode proteins, and previous studies have reported that non-coding RNA could participate in, and regulate various physiological processes, such as cell differentiation, aging and the cell cycle (7). Long non-coding RNAs (lncRNAs) are a type of non-coding RNA that are >200 nucleotides in length, and the lncRNAs have been reported to regulate the proliferation of multiple types of cells (8). The upregulated lncRNA HOXA distal transcript antisense (HOTTIP) was revealed to promote the development of prostate cancer and lung cancer (9,10). A previous study reported that the expression levels of HOTTIP were upregulated during the development of hepatic fibrosis, and higher levels of HOTTIP induced the fibrosis of hepatic tissue by downregulating the expression levels of microRNA (miRNA/miR)-148a (11). Furthermore, the upregulated expression level of the lncRNA HOTTIP enhanced the fibrosis of liver tissues by inducing the activation of hepatic stellate cells (12). However, to the best of our knowledge, whether lncRNA HOTTIP can affect the occurrence and development of fibrosis of lung tissues remains unknown.

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starBase was used to predict targets of lncRNA HOTTIP. Previous studies have revealed that the upregulated expression levels of miR-744-5p promoted the proliferation and metastasis of ovarian cancer and non-small cell lung cancer cells (13,14). In addition, analysis using starBase revealed that miR-744-5p could target and regulate the expression of polypyrimidine tract binding protein 1 (PTBP1). It has been observed that the expression levels of PTBP1 are upregulated in pulmonary fibrosis tissues from mice (15). However, to the best of our knowledge, whether the lncRNA HOTTIP could promote the fibrosis of lung tissues by regulating the miR-744-5p/PTBP1 axis is yet to be elucidated.

The present study established *in vitro* and *in vivo* pulmonary fibrosis models to investigate the role of HOTTIP in pulmonary fibrosis. Moreover, the binding relationship between miR-774-5p and lncRNA HOTTIP or PTBP1 was determined. The current results revealed that the molecular functions of the lncRNA HOTTIP were achieved by regulating the miR-774-5p/PTBP1 signaling axis, which suggested the importance of the lncRNA HOTTIP/miR-774-5p/PTBP1 signaling pathway during the development of lung fibrosis.

## Materials and methods

**Cell lines and culture.** A549 cells were obtained from the American Type Culture Collection and cultured in RPMI-1640 medium (Hyclone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. A total of 2 ng/ml TGF-β1 (cat. no. PHG9214; Gibco; Thermo Fisher Scientific, Inc.) was used to treat A549 cells at 37°C for 48 h to induce fibrosis. Cells incubated with normal medium were used as the control.

**Animal studies.** A total of 24 male mice (weight, 18-20 g; age, 8 weeks) were purchased from the Shanghai Animal Experiment Center of the Chinese Academy of Sciences. All mice were housed under controlled temperature (~22°C) and humidity (~55%) with 12/12 h light cycle and free access to water and food. Bleomycin (BLM; Thermo Fisher Scientific, Inc.) was used to induce the fibrosis of lung tissues in mice. After accommodation for 1 week, mice were divided into the following experimental groups: i) Control group, in which mice were intravenously injected with 1 ml normal saline; ii) BLM group, in which mice were intravenously injected with BLM (5 mg/kg/day); iii) BLM + short hairpin (sh)RNA-negative control (NC) group, in which mice were intravenously injected with BLM and shRNA-NC (Shanghai GeneChem Co., Ltd.); and iv) BLM + shRNA-HOTTIP-1 group, in which mice were injected with BLM and shRNA-HOTTIP-1 (Shanghai GeneChem Co., Ltd.). After 2 weeks, mice were euthanized by inhalation of 5% isoflurane (cat. no. HR135327; Hairui Chemical) for 30 sec prior to cervical dislocation, as previously described (16,17); death of mice was verified by the lack of heartbeat and a cold body. Experimental protocols were approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China).

Lung tissues were subsequently collected and fixed in 10% formaldehyde solution (Sigma-Aldrich; Merck KGaA) for 24 h at room temperature, embedded in paraffin and cut

into 5-μm sections. Then, the sections were stained with hematoxylin and eosin (H&E; Thermo Fisher Scientific, Inc.) at room temperature for 5 min for observation of histological injury or Masson's trichrome dye (Thermo Fisher Scientific, Inc.) at room temperature for 8 min for observation of lung fibrosis at room temperature. The images were captured by a light microscope (magnification, x400; Olympus Corporation).

**Cell transfection.** Short hairpin (sh)RNAs targeting HOTTIP (shRNA-HOTTIP-1/2) and its negative control (shRNA-NC), miR-774-5p mimic, mimic-NC, overexpression (oe)-PTBP1 plasmid, oe-NC plasmid, miR-744-5p inhibitor and inhibitor-NC were obtained from Shanghai GeneChem Co., Ltd. PTBP1 overexpression plasmid (oe-PTBP1) pcDNA3.1-LINC00885 was commercially constructed by Shanghai GenePharma Co., Ltd.; empty pcDNA 3.1 vector (oe-NC) was used as the control. A549 cells were transfected with shRNA-HOTTIP-1/2 (500 ng/μl), shRNA-NC plasmid (500 ng/μl), miR-774-5p mimic (40 nM), mimic-NC (40 nM), oe-PTBP1 (15 nM), oe-NC (15 nM), miR-744-5p inhibitor (40 nM) and inhibitor-NC (40 nM) using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) at 37°C according to the manufacturer's protocol. At 48 h post transfection, cells were harvested for subsequent experiments.

**5'-ethynyl-2'-deoxyuridine (EdU) assay.** The EdU assay was performed using a Click-iT EdU Imaging kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Fluorescence was detected using a fluorescence microscope (magnification, x100; Olympus Corporation).

**Wound healing assay.** Cells were plated into six-well plates (1x10<sup>5</sup> cells/well) before the experiments and allowed to growth to 90% confluence in RPMI-160 medium with 10% FBS at 37°C. Then a scratch was made using a 10-μl sterile pipette tip in the cell monolayer. The cells were washed twice to remove debris and incubated with RPMI-160 medium without FBS for 48 h at 37°C. The scratch was imaged with an inverted light microscope (magnification, x100; Olympus Corporation) at 0 h, and again following 48 h of incubation. The width of the scratch was calculated using ImageJ software (version 1.46; National Institutes of Health).

**Dual luciferase reporter assay.** starBase (starbase.sysu.edu.cn) was used to predict the potential binding sites between lncRNA HOTTIP and miR-744-5p, which were verified by dual luciferase reporter assay. Briefly, the wild-type (WT) and mutant (MUT) 3'-untranslated region (UTR) of HOTTIP was obtained from Shanghai GeneChem Co., Ltd and cloned into a pGL3-promoter (Promega Corporation). Point mutations in the binding site was generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies) according to the manufacturer's protocol. A549 cells were co-transfected with 50 ng WT-HOTTIP or MUT-HOTTIP and 20 μM miR-744-5p mimic or mimic NC using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.). Following 48-h transfection, the relative luciferase activity was measured using a Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'→3')
Long non-coding HOXA distal transcript antisense RNA	F: CCTAAAGCCACGCTTCTTTG R: TGCAGGCTGGAGATCCTACT
MicroRNA-774-5p	F: CTGTTGCCACTAACCTCAACCT R: TGCGGGGCTAGGGCTAACAGCA
Polypyrimidine tract binding protein 1	F: AGCGCTGCGTCGCTGCGCACGTGGGAAG R: AACAAATGGAATCTGGGGATGGACTATTC
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
GAPDH	F: ATGACATCAAGAAGGTGGTG R: CATACCAGGAAATGAGCTTG

**Immunofluorescence assay.** Paraffin sections (5- $\mu$ m thick) were de-waxed with xylene, rehydrated with ethanol and microwaved for 15 min for antigen retrieval. After cooling, the sections were blocked with H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature. After blocking with 5% goat serum (cat. no. 16210072; Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 30 min, sections were incubated with the following primary antibodies at 4°C overnight: Anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; 1:200; cat. no. ab5694; Abcam) and anti-E-cadherin (1:200; cat. no. ab231303; Abcam). Afterwards, the sections were incubated with FITC-labeled goat anti-mouse secondary antibody (1:200; cat. no. IF-0051; Beijing Dingguo Changsheng Biotechnology Co., Ltd.) for 1 h at 37°C. Cell nuclei were stained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 2 min at room temperature. Fluorescence was detected using a fluorescence microscope (magnification, x200; Olympus Corporation).

**Western blotting.** Total protein from A549 cells was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using a BCA assay. The proteins (30  $\mu$ g/lane) were subsequently separated via 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto the PVDF membranes (EMD Millipore). After blocking in 10% non-fat milk for 1 h at 37°C, the membranes were then incubated with the following primary antibodies at 4°C overnight: Anti- $\alpha$ -SMA (1:1,000; cat. no. ab5694; Abcam), anti-collagen I (1:1,000; cat. no. ab34710; Abcam), anti-collagen III (1:1,000; cat. no. ab6310; Abcam), anti-fibronectin 1 (FN1; 1:1,000; cat. no. ab2413; Abcam), anti-E-cadherin (1:1,000; cat. no. ab231303; Abcam) and anti-GAPDH (1:1,000; cat. no. ab9485; Abcam). Following the primary antibody incubation, the membranes were incubated with a polyclonal goat anti-rabbit (1:5,000; cat. no. ab98512; Abcam) or anti-mouse (1:5,000; cat. no. ab97040; Abcam) HRP-conjugated secondary antibody for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). ImageJ software (1.46; National Institutes of Health) was used to quantify the protein bands.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA from A549 cells was extracted using TRIzol® (Invitrogen;

Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was subsequently performed on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR Premix Ex Taq reagent (Takara Bio, Inc.). qPCR was performed as follows: 60°C for 2 min, 95°C for 25 sec and 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The primers sequences used for the qPCR are listed in Table I. The expression levels of target genes were analyzed using the 2<sup>- $\Delta\Delta C_q$</sup>  method (18).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc.). All experiments were repeated in triplicate and data are presented as the mean  $\pm$  SD. Statistical differences between groups were determined using a one-way ANOVA followed by a Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Knockdown of lncRNA HOTTIP suppresses the TGF- $\beta$ 1-induced fibrosis of A549 cells.** TGF- $\beta$ 1 was used to induce the fibrosis of A549 cells. The expression levels of the lncRNA HOTTIP were significantly upregulated in A549 cells following stimulation with TGF- $\beta$ 1 compared with the control group (Fig. 1A). The expression of HOTTIP was knocked down in A549 cells via transfection with shRNAs. RT-qPCR analysis revealed that the expression levels of the lncRNA HOTTIP were significantly downregulated following the transfection with shRNA-HOTTIP-1/2 compared with the shRNA-NC group (Fig. 1B), and the knockdown efficiency of shRNA-HOTTIP-1 was more significant compared with shRNA-HOTTIP-2. Therefore, cells transfected with shRNA-HOTTIP-1 were selected for use in subsequent assays.

EdU and wound healing assays were performed to detect the proliferation and migration of A549 cells, respectively. As shown in Fig. 1C-E, the proliferation and migration of A549 cells were both significantly increased following stimulation with TGF- $\beta$ 1 compared with the control group. However, the proliferation and migration of A549 cells were



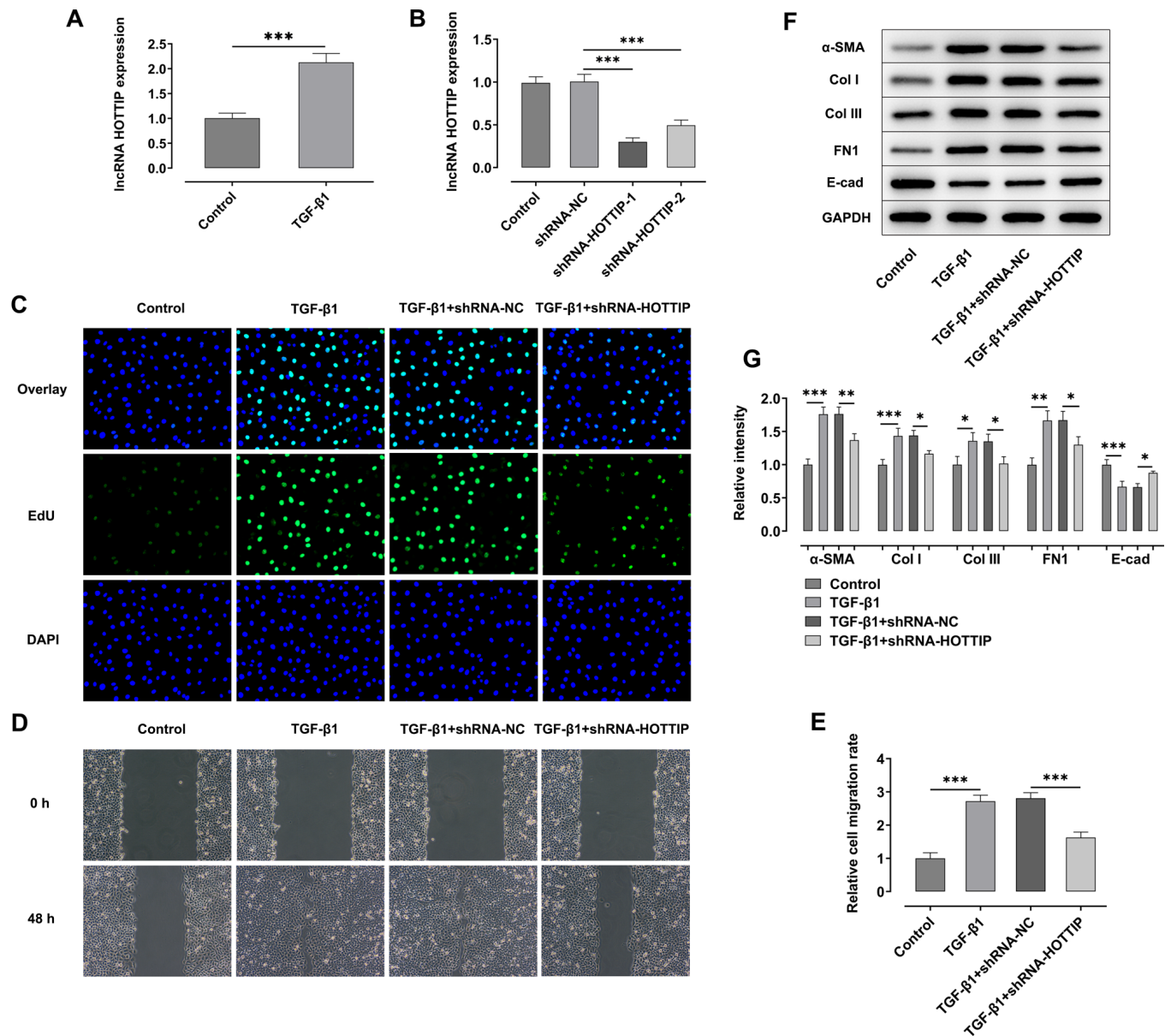


Figure 1. Knockdown of lncRNA HOTTIP relieves the TGF-β1 induced fibrosis of A549 cells. (A) RT-qPCR was used to analyze the expression levels of lncRNA HOTTIP in A549 cells stimulated with TGF-β1. (B) Expression levels of lncRNA HOTTIP were analyzed by RT-qPCR following transfection with shRNA-HOTTIP-1/2. (C) EdU assay was performed to determine the proliferation of A549 cells following stimulation with TGF-β1 with or without shRNA-HOTTIP transfection. Magnification, x100. (D) Wound healing assay was performed to detect the migration of A549 cells following stimulation with TGF-β1 with or without shRNA-HOTTIP transfection. Magnification, x100. (E) Semi-quantification of results from wound healing assay. (F) Western blotting was performed to analyze the expression levels of α-SMA, Col I, Col III, FN1 and E-cad in A549 cells following stimulation with TGF-β1 with or without shRNA-HOTTIP transfection. (G) Protein expression was quantified. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. lncRNA, long non-coding RNA; HOTTIP, HOXA distal transcript antisense RNA; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; NC, negative control; α-SMA, α-smooth muscle actin; Col I, collagen I; Col III, collagen III; E-cad, E-cadherin; FN1, fibronectin 1; EdU, 5-ethynyl-2'-deoxyuridine.

suppressed following the concomitant knockdown of HOTTIP in the TGF-β1 + shRNA-HOTTIP group compared with the TGF-β1 + shRNA-NC group. Moreover, the expression levels of α-SMA, collagen I (Col I), collagen III (Col III) and FN1 were significantly upregulated, while expression levels of E-cadherin (E-cad) were significantly downregulated in TGF-β1 group, compared with the control group. However, the expression levels of α-SMA, collagen I, collagen III and FN1 were downregulated, while the expression level of E-cadherin was significantly upregulated following the knockdown of HOTTIP in TGF-β1 stimulated cells compared with the TGF-β1 + shRNA-NC group (Fig. 1F-G).

*HOTTIP targets and downregulates the expression levels of miR-744-5p.* Using the starBase database, it was predicted that HOTTIP had the potential to target miR-744-5p. The expression levels of miR-744-5p were significantly downregulated following stimulation with TGF-β1 compared with the control group (Fig. 2A). Moreover, the expression levels of miR-744-5p were significantly upregulated following the knockdown of HOTTIP compared with the shRNA-NC group (Fig. 2B). Subsequently, miR-744-5p was overexpressed in A549 cells. As shown in Fig. 2C, the expression levels of miR-744-5p in cells transfected with the miR-744-5p mimic were significantly upregulated compared with the mimic-NC group. The



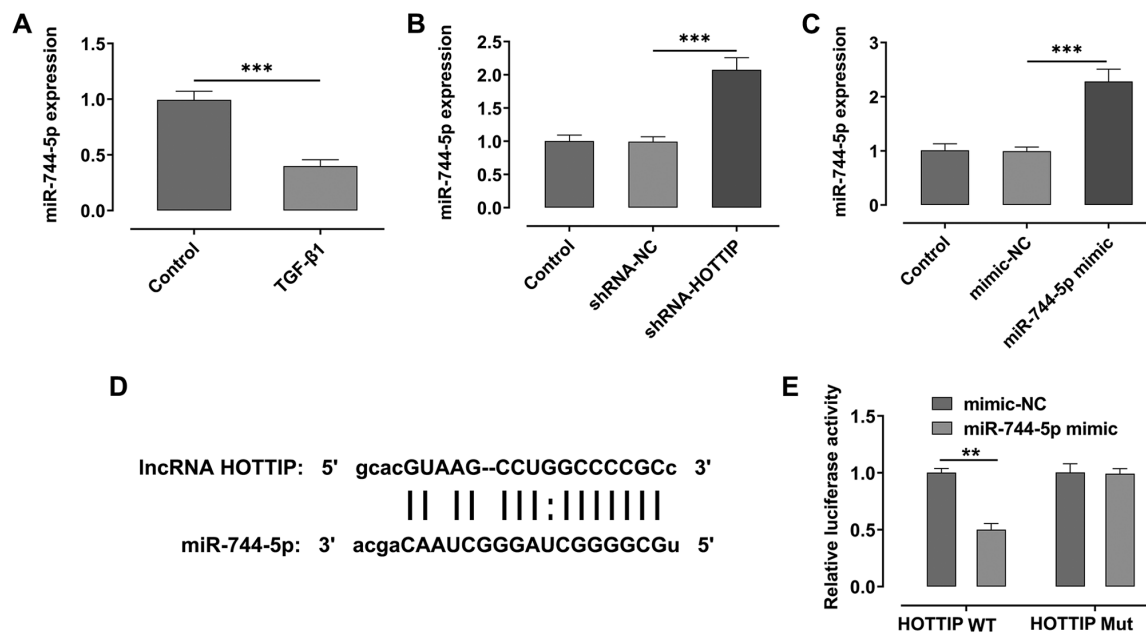


Figure 2. IncRNA HOTTIP downregulates the expression levels of miR-744-5p. (A) Expression levels of miR-744-5p in A549 cells stimulated with TGF- $\beta$ 1 were analyzed using RT-qPCR. (B) Expression levels of miR-744-5p in A549 cells transfected with shRNA-HOTTIP were analyzed using RT-qPCR. (C) Transfection efficiency of miR-744-5p mimic was analyzed using RT-qPCR. (D) Potential binding sites between miR-744-5p and IncRNA HOTTIP are shown. (E) Dual luciferase reporter assay was performed to determine the relationship between miR-744-5p and IncRNA HOTTIP. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . miR, microRNA; HOTTIP, HOXA distal transcript antisense RNA; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; NC, negative control; IncRNA, long non-coding RNA; WT, wild-type; Mut, mutant.

potential binding sites between the IncRNA HOTTIP and miR-744-5p were then identified (Fig. 2D). The results of the dual luciferase reporter assay indicated that the relative luciferase activity was suppressed in the IncRNA HOTTIP wild-type (WT) and miR-744-5p mimic group, compared with HOTTIP WT and mimic-NC group (Fig. 2E).

*Knockdown of HOTTIP suppresses the TGF- $\beta$ 1-induced fibrosis of A549 cells by regulating the expression of miR-744-5p.* In the following experiments, the expression of miR-744-5p in HOTTIP-knockdown A549 cells was also successfully knocked down using a miR-744-5p inhibitor (Fig. 3A). The results demonstrated that the combined TGF- $\beta$ 1 and shRNA-HOTTIP-induced inhibition of proliferation and migration of A549 cells were both partially rescued following the concurrent knockdown of miR-744-5p (Fig. 3B-D). Furthermore, the expression levels of  $\alpha$ -SMA, Col I, Col III and FN1 were upregulated, while the expression levels of E-cad were downregulated, in the TGF- $\beta$ 1 + shRNA-HOTTIP + miR-744-5p inhibitor group compared with the TGF- $\beta$ 1 + shRNA-HOTTIP + inhibitor-NC group (Fig. 3E).

*miR-744-5p regulates the expression levels of PTBP1.* RT-qPCR analysis demonstrated that the expression levels of PTBP1 were significantly upregulated after A549 cells were stimulated with TGF- $\beta$ 1 compared with the control group (Fig. 4A). However, the expression levels of PTBP1 were significantly downregulated following the overexpression of miR-744-5p compared with the mimic-NC group (Fig. 4B). Using starBase, miR-744-5p was predicted to share a binding site with PTBP1 (Fig. 4C), and the results of the dual luciferase reporter assay demonstrated that the relative luciferase activity

was suppressed in the PTBP1 WT and miR-744-5p mimic group, compared with PTBP1 WT and mimic-NC group (Fig. 4D). Moreover, the expression levels of PTBP1 were downregulated in HOTTIP-knockdown A549 cells, compared with the control; however, the expression levels of PTBP1 were rescued upon the concurrent knockdown of miR-744-5p in the cells (Fig. 4E).

*HOTTIP regulates TGF- $\beta$ 1-induced fibrosis of A549 cells by modulating the miR-744-5p/PTBP1 signaling axis.* To determine the effect of PTBP1 on the development of fibrosis in A549 cells, PTBP1 was overexpressed in A549 cells. As shown in Fig. 5A, the expression levels of PTBP1 were significantly upregulated in A549 cells in the oe-PTBP1 group compared with the oe-NC group. The results of EdU and wound healing assays demonstrated that the proliferation and migration of A549 cells were suppressed following the overexpression of miR-744-5p in TGF- $\beta$ 1-induced A549 cells compared with the TGF- $\beta$ 1 + mimic-NC group (Fig. 5B-D). However, the proliferation and migration of these cells were rescued following the concurrent overexpression of PTBP1. Similarly, the expression levels of  $\alpha$ -SMA, Col I, Col III and FN1 were upregulated, while the expression levels of E-cad were downregulated, in the TGF- $\beta$ 1 + miR-744-5p mimic + oe-PTBP1 group compared with the TGF- $\beta$ 1 + miR-744-5p mimic + oe-NC group (Fig. 5E).

*Knockdown of HOTTIP relieves the BLM-induced lung fibrosis of mice.* BLM was used to induce fibrosis in mice. Next, shRNA-NC and shRNA-HOTTIP were injected into the mice to determine the effect of IncRNA HOTTIP on the development of lung fibrosis. After the euthanasia of these

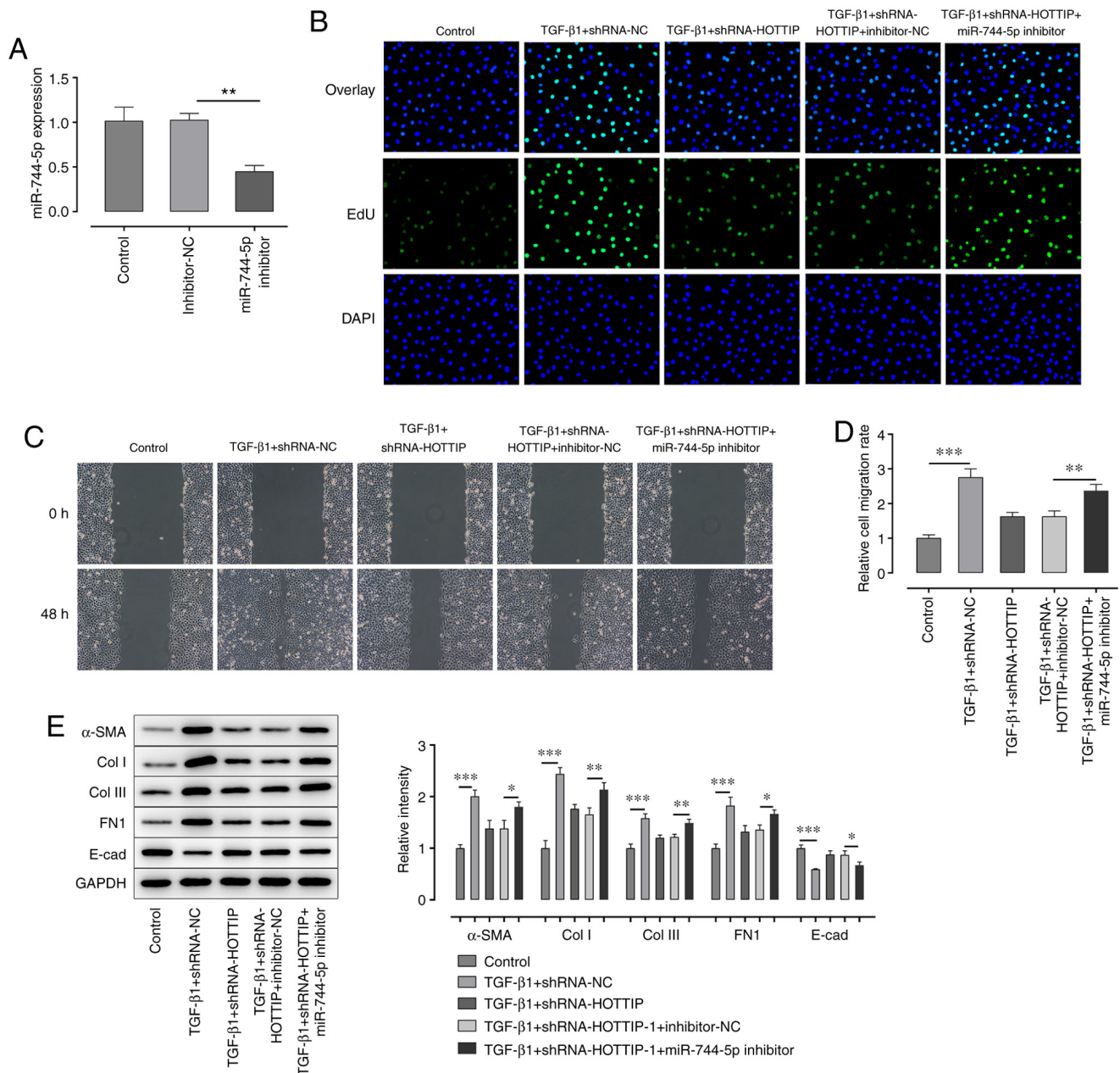


Figure 3. Knockdown of miR-744-5p expression rescues the long non-coding RNA HOTTIP-induced suppression of proliferation and migration. (A) Expression levels of miR-744-5p in A549 cells following transfection with the miR-744-5p inhibitor were analyzed using reverse transcription-quantitative PCR. (B) EdU assay was performed to detect the proliferation of A549 cells following stimulation with TGF- $\beta$ 1 and transfection with shRNA-HOTTIP and miR-744-5p inhibitor. Magnification, x100. (C) Wound healing assays were performed to determine the migration of A549 cells following stimulation with TGF- $\beta$ 1 and transfection with shRNA-HOTTIP and miR-744-5p inhibitor. Magnification, x100. (D) Semi-quantification of the results of the wound healing assay. (E) Western blotting was performed to analyze the expression levels of  $\alpha$ -SMA, Col I, Col III, FN1 and E-cad in A549 cells following stimulation with TGF- $\beta$ 1 and transfection with shRNA-HOTTIP and miR-744-5p inhibitor. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001. miR, microRNA; EdU, EdU, 5-ethynyl-2'-deoxyuridine;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; Col I, collagen I; Col III, collagen III; E-cad, E-cadherin; FN1, fibronectin I; shRNA, short hairpin RNA; NC, negative control.

mice, H&E and Masson's trichrome staining were performed to analyze the fibrosis in the lung tissues of mice. The results demonstrated that the number of nodules was decreased, and the fibrosis of lung tissues was relieved after the knockdown of HOTTIP (Fig. 6A). Compared with the control group, BLM induced increased expression of HOTTIP and PTBP1 and decreased expression of miR-744-5p (Fig. 6B-D). In addition, the expression levels of HOTTIP and PTBP1 were significantly downregulated, while the expression levels of miR-744-5p were significantly upregulated in the lung tissues of the BLM + shRNA-HOTTIP group compared with the

BLM + shRNA-NC group. Immunofluorescence analysis exhibited upregulated expression of  $\alpha$ -SMA and downregulated expression of E-cad in BLM group, compared with the control group. In addition, the results also illustrated that the expression of  $\alpha$ -SMA was downregulated, while the expression of E-cad was upregulated, in the lung tissues from the BLM + shRNA-HOTTIP group compared with the BLM + shRNA-NC group (Fig. 6E and F). Furthermore, BLM increased expression of  $\alpha$ -SMA, Col I, Col III and FN1, and decreased expression of E-cad, compared with the control group. Additionally, the expression levels of  $\alpha$ -SMA, Col I,

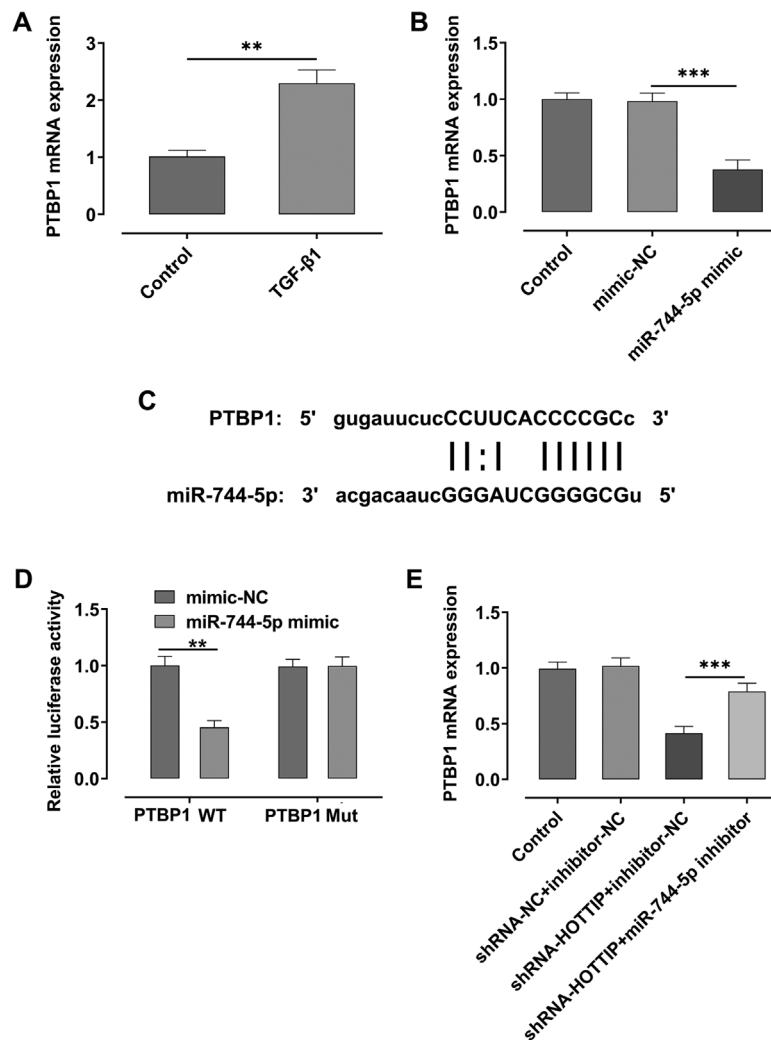


Figure 4. miR-744-5p targets and downregulates the expression levels of PTBP1. (A) Expression levels of PTBP1 in A549 cells following stimulation with TGF-β1 were analyzed using RT-qPCR. (B) Expression levels of PTBP1 in A549 cells following transfection with the miR-744-5p mimic were analyzed using RT-qPCR. (C) Predicted binding sites between miR-744-5p and PTBP1 are shown. (D) Dual luciferase reporter assay was performed to determine the relationship between miR-744-5p and PTBP1. (E) Expression levels of PTBP1 were analyzed in A549 cells following transfection with shRNA-HOTTIP and miR-744-5p inhibitor using RT-qPCR. \*\*P<0.01, \*\*\*P<0.001. miR, microRNA; PTBP1, polypyrimidine tract binding protein 1; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; shRNA, short hairpin RNA; WT, wild-type; MUT, mutant.

Col III and FN1 were significantly downregulated, while the expression levels of E-cadherin were significantly upregulated in the lung tissues from the BLM + shRNA-HOTTIP group compared with the BLM + shRNA-NC group (Fig. 6G and H).

## Discussion

Pulmonary fibrosis is a progressive and irreversible disease characterized by lung tissue remodeling and collagen deposition (19). The development of lung fibrosis has also been found to eventually lead to the loss of lung function (20). The incidence and mortality rates of pulmonary fibrosis have gradually increased (21). However, to the best of our knowledge, the molecular mechanism underlying the pathogenesis of pulmonary fibrosis remains unknown (19). Therefore, further investigating the molecular mechanisms underlying the occurrence and development of pulmonary fibrosis has great significance for the clinical treatment of pulmonary fibrosis.

Previous studies have revealed that the expression of lncRNA was associated with the development of fibrosis in

lung tissues (22,23). The expression levels of lncRNA HOTTIP were found to be upregulated during the development of hepatic fibrosis (12). The upregulated expression levels of lncRNA HOTTIP induced the fibrosis of liver tissues by downregulating the expression levels of miR-148a and upregulating the expression levels of TGF-β receptor (TGFBR)1 and TGFBR2 (11). Moreover, the upregulated expression levels of HOTTIP were discovered to activate hepatic stellate cells by upregulating the expression of serum response factor (12). The knockdown of lncRNA HOTTIP also relieved high glucose-induced inflammation and fibrosis in mice (24). However, whether lncRNA HOTTIP could influence the development of lung fibrosis is yet to be elucidated. A549 cells have been widely used as model human respiratory epithelial cells that can be stimulated with TGF-β1 (25,26); thus, TGF-β1-induced A549 cells were used as the *in vitro* pulmonary fibrosis model in the present study. The present study revealed that the knockdown of the lncRNA HOTTIP suppressed the proliferation and migration of A549 cells and downregulated the expression levels of fibrosis-related proteins (α-SMA, Col I, Col III and FN1).



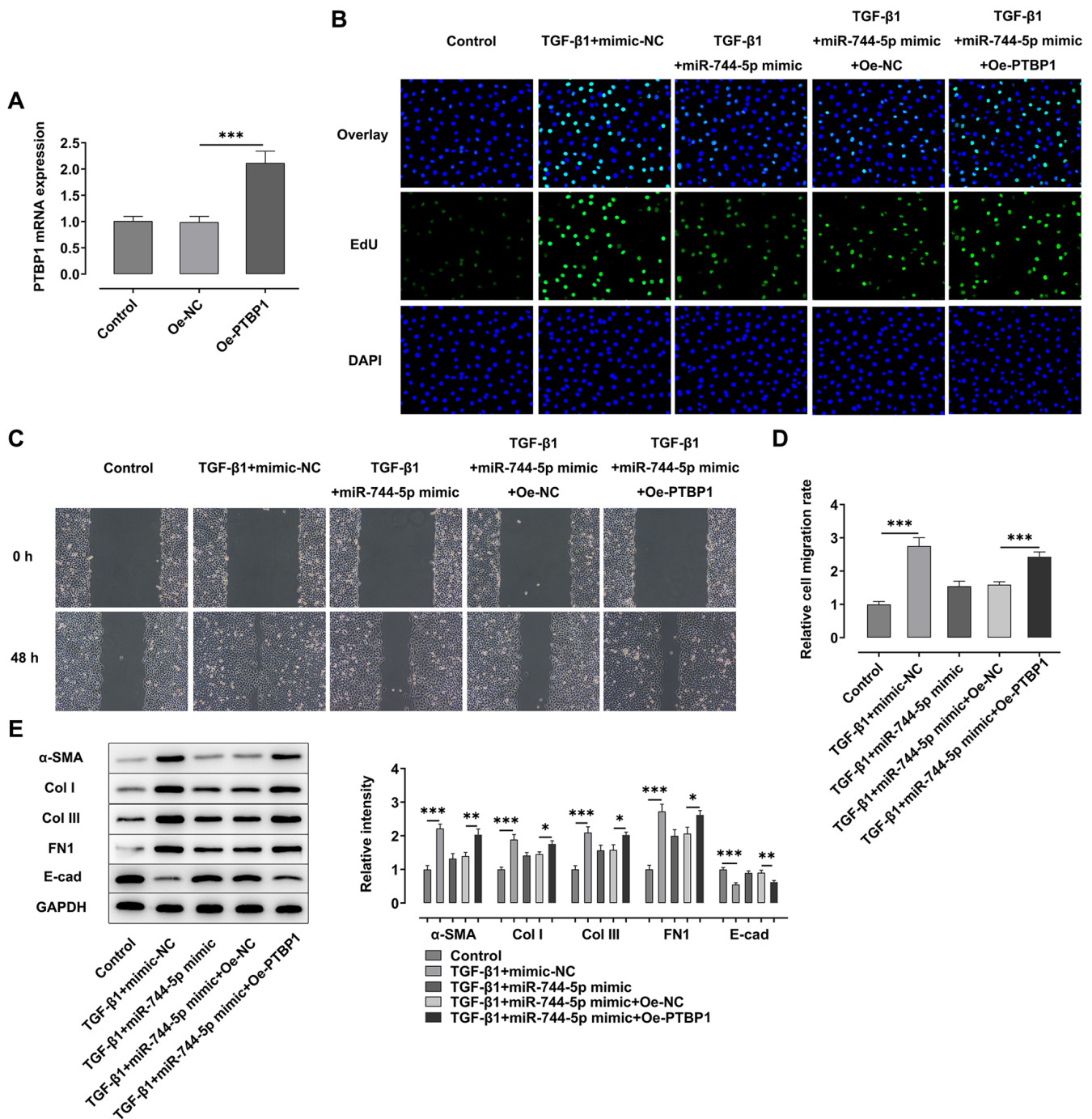


Figure 5. Long non-coding RNA HOTTIP regulates TGF-β1-induced fibrosis of A549 cells by modulating the miR-744-5p/PTBP1 signaling axis. (A) RT-qPCR was performed to analyze the expression levels of PTBP1 in A549 cells transfected with oe-PTBP1. (B) EdU assay was performed to detect the proliferation of A549 cells following stimulation with TGF-β1 and transfection with miR-744-5p mimic and oe-PTBP1. Magnification, x100. (C) Wound healing assay was performed to analyze the migration of A549 cells following stimulation with TGF-β1 and transfection with miR-744-5p mimic and oe-PTBP1. Magnification, x100. (D) Semi-quantification of the results of the wound healing assay. (E) Western blotting was performed to analyze the expression levels of α-SMA, Col I, Col III, FN1 and E-cad in A549 cells following stimulation with TGF-β1 and transfection with miR-744-5p mimic and oe-PTBP1. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. HOTTIP, HOXA distal transcript antisense RNA; miR, microRNA; PTBP1, polypyrimidine tract binding protein 1; oe, overexpression; α-SMA, α-smooth muscle actin; Col I, collagen I; Col III, collagen III; E-cad, E-cadherin; FN1, fibronectin 1; NC, negative control.

The *in vivo* assay results also demonstrated that knockdown of the lncRNA HOTTIP relieved lung fibrosis in mice. These results suggested that knockdown of the lncRNA HOTTIP may alleviate the fibrosis of A549 cells.

The present findings also suggested that the lncRNA HOTTIP may exert its effects by regulating the miR-744-5p/PTBP1 signaling axis. Previous studies have revealed that the expression

levels of miR-744-5p were associated with the occurrence and development of multiple types of cancer (4,27). For instance, the upregulated expression of miR-744-5p inhibited the proliferation and migration of ovarian cancer cells (28), and higher levels of miR-744-5p also repressed the proliferation and invasion of lung cancer cells by regulating the expression of paired box 2 (13). However, the present study reported that the lncRNA HOTTIP

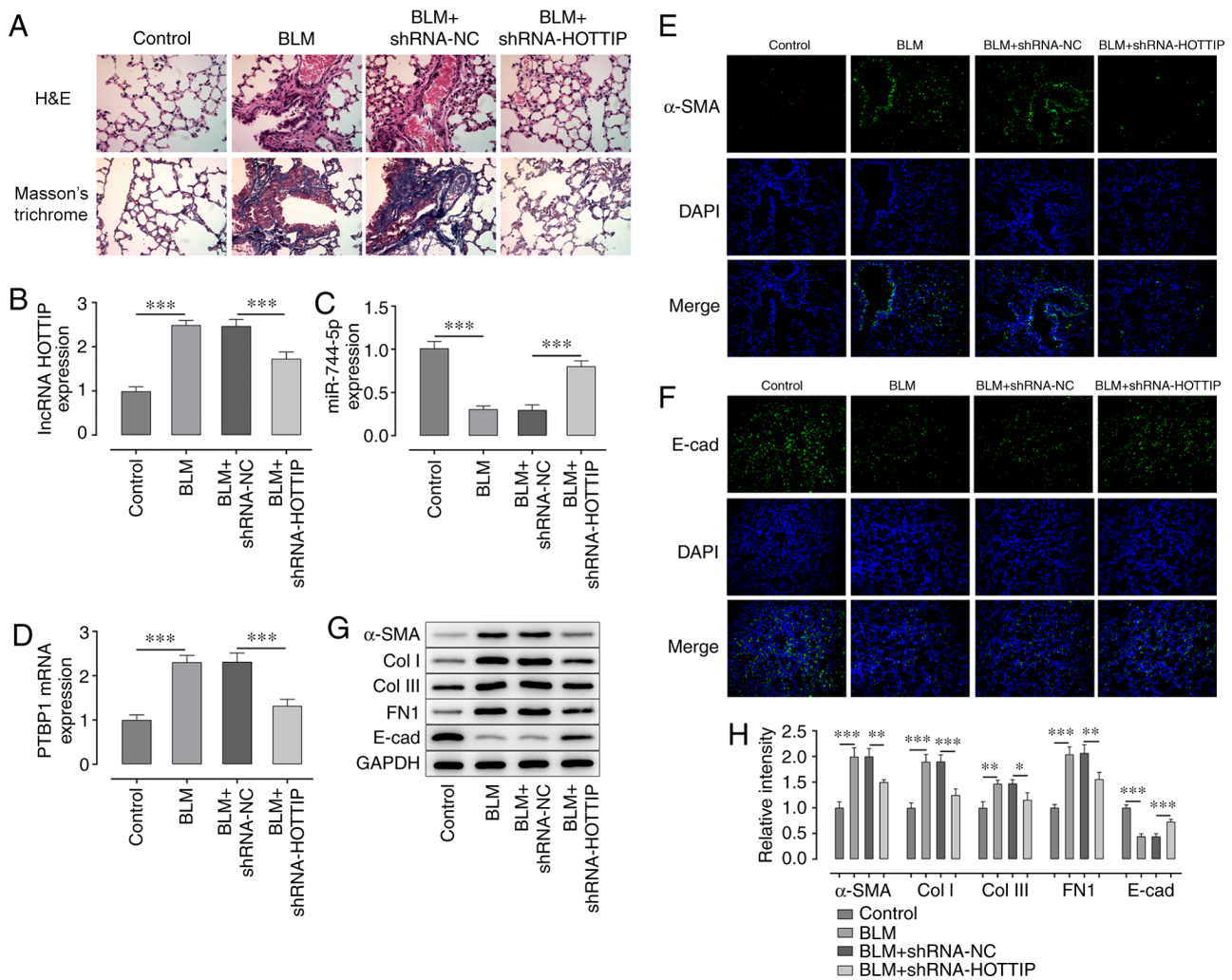


Figure 6. Knockdown of lncRNA HOTTIP relieves lung tissue fibrosis in mice. (A) Fibrosis of lung tissues of mice was detected using H&E and Masson's trichrome staining. Magnification, x400. Expression levels of (B) lncRNA HOTTIP and (C) miR-744-5p in lung tissues of mice treated with BLM with or without shRNA-HOTTIP transfection were analyzed using RT-qPCR. (D) RT-qPCR was performed to analyze the expression levels of PTBP1 in lung tissues of mice treated with BLM with or without shRNA-HOTTIP transfection. Immunofluorescence was performed to analyze the expression levels of (E) α-SMA and (F) E-cadherin in lung tissues of mice treated with BLM with or without shRNA-HOTTIP transfection. Magnification, x200. (G) Western blotting was used to determine the expression levels of α-SMA, Col I, Col III, FN1 and E-cad in lung tissues of mice treated with BLM with or without shRNA-HOTTIP transfection. (H) Semi-quantification of western blotting results from part (G) \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . lncRNA, long non-coding RNA; HOTTIP, HOXA distal transcript antisense RNA; H&E, hematoxylin and eosin; miR, microRNA; BLM, bleomycin; shRNA, short hairpin RNA; NC, negative control; PTBP1, polypyrimidine tract binding protein 1; SMA, α-smooth muscle actin; Col I, collagen I; Col III, collagen III; E-cad, E-cadherin; FN1, fibronectin 1.

targeted and negatively regulated miR-744-5p, and the knock-down of miR-744-5p partially reversed the HOTTIP-induced inhibitory effect on the proliferation and migration of A549 cells. As enhanced cell proliferation and migration suggest the occurrence and development of fibrosis (29), the present results indicated that the lncRNA HOTTIP enhanced the fibrosis of A549 cells by downregulating the expression of miR-744-5p. Furthermore, the expression levels of PTBP1 have been associated with the development of pulmonary fibrosis (15). The present results revealed that miR-744-5p targeted and downregulated the expression of PTBP1. It was also found that the overexpression of PTBP1 partially reversed the inhibitory effects of miR-744-5p on the proliferation and migration of A549 cells.

However, the present study has several limitations. First, even though A549 cells have been extensively used to establish *in vitro* pulmonary fibrosis models, further *in vitro* studies should be

performed with other appropriate cell lines, such as the human lung fibroblast cell line HLF, to validate the present results, which will be taken into consideration in future studies. Second, the current study mainly focused on the role and the molecular mechanism of HOTTIP on the cellular level and animal level; however, the expression of lncRNA HOTTIP in clinical cases of pulmonary fibrosis needs to be analyzed in future studies to support the present findings from cell line and animal studies.

In conclusion, the findings of the present study suggested that the lncRNA HOTTIP may enhance the fibrosis of lung tissues by downregulating the expression of miR-744-5p and upregulating the expression of PTBP1. The current results suggested that the lncRNA HOTTIP/miR-744-5p/PTBP1 signaling pathway may serve a crucial role during the development of lung fibrosis. The results of the current study also provided a potential novel target and strategy for the clinical treatment of lung fibrosis.

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## 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

QW conceptualized and designed the experiments. JL, WC and ZZ conducted the experiments and obtained the data. JL and YZ analyzed the data and interpreted the results. JL and WC wrote the manuscript. QW revised the manuscript. All authors read the final version of the manuscript. QW and JL confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

Experimental protocols were approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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