

Role of long non-coding RNA MALAT1 in chronic obstructive pulmonary disease

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Abstract. Chronic obstructive pulmonary disease (COPD) is a pathological inflammatory condition of the lungs that is associated with high rates of mortality. Although long non-coding RNAs (lncRNAs) serve a role in lung diseases, their functions in COPD pathogenesis are relatively unknown. The present study aimed to assess the role of differentially expressed lncRNAs in COPD. Expression profile analysis of six lncRNAs in age-matched COPD and non-COPD tissues were conducted. Among the six tested lncRNAs, metastasis-associated in lung adenocarcinoma transcript 1 (MALAT1) was the most consistently overexpressed in COPD lung tissue specimens. To model COPD *in vitro*, human lung fibroblasts were treated with transforming growth factor- β (TGF- β) and MALAT1 was knocked down by small interfering RNA. This promoted cell viability and concurrently inhibited the expression of mesenchymal proteins, fibronectin and α -smooth muscle actin. In COPD, cell senescence is linked to the activation of mammalian target of rapamycin complex 1 (mTORC1). Upon gene silencing of MALAT1 in non-TGF- β -treated cells, cells demonstrated constitutive activation of mTORC1, which was assessed by the protein expression levels of mTORC1 substrate S6 kinase (S6K1). By contrast, upon MALAT1 silencing in the TGF- β -treated cells, mTORC1 activation was not suppressed, despite the mesenchymal cell markers protein expression levels being downregulated. Thus, lncRNA MALAT1 may represent a potent biomarker in COPD patients and may act as a target for both diagnostic and therapeutic purposes.

Introduction

Chronic obstructive pulmonary disease (COPD) is a pathological inflammatory reaction to particle and gas irritants within the lungs (1-3). There are several reasons for the development of COPD, including the inspiration of noxious particles such as cigarette smoke and destruction of the balance between proteolytic and antiproteolytic molecules (2). It is estimated that COPD has caused >2,500,000 mortalities every year across the globe, and it is the one of the top causes of chronic morbidity and mortality in the United States of America (4,5). Current therapeutic strategies against COPD are largely ineffective; this is partly due to its late detection owing to the absence of *bona fide* diagnostic and prognostic molecular biomarkers (6). Because of this, it is imperative to discover and validate such markers in COPD that may be effective in therapeutic design.

Transforming growth factor- β (TGF- β) is an effective profibrotic cytokine, which promotes the expression of fibronectin and α -smooth muscle actin (α -SMA). Treatment of lung fibroblasts with TGF- β is used to induce a model for studying COPD *in vitro* (7). In addition, TGF- β could activate S6 kinase (S6K1) through mTOR complex 1 (mTORC1). Furthermore, in COPD, lung cell senescence is linked to mTOR activity (8).

Non-coding RNAs (ncRNAs), of two types, small (<200 kb) and long (lncRNAs; >200 kb), and are associated with tumor oncogenic and suppressive pathways (9-12). lncRNAs serve numerous functional roles, from regulatory roles in chromatin structure and function, to regulating gene expression and genomic rearrangement (13-16). The abnormal expression of lncRNAs has been reported in numerous human diseases and cancers (17-26). One previous study identified the lncRNA taurine upregulated gene 1 (TUG1) as a potential biomarker in COPD (27); however, the role of lncRNAs in COPD development remain largely unknown. The present study aimed to assess the differential expression of previously identified lncRNAs associated with lung diseases, in COPD and non-COPD lung tissues, and to investigate their potential role in COPD pathogenesis. The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was demonstrated to be consistently upregulated in COPD lung tissues, and it was revealed to affect *in vitro* viability and the expression of fibronectin and α -SMA in HFL1 lung fibroblast cells.

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The results indicated that MALAT1 may serve a significant role in COPD pathogenesis.

Materials and methods

Patient studies. Ethics approval was obtained from the Institutional Review Board of the Hua Mei Hospital, University of Chinese Academy of Sciences (Ningbo, China). Written informed consent was obtained from each participant. Ten age-matched pairs of lung tissue biopsy samples were obtained from patients with COPD (8 males and 2 females; age, 60.7±21.65 years) and healthy controls (4 males and 6 females; age range, 55.7±24.60 years) at the Respiratory Department of Hwa Mei Hospital from January 2018 to December 2018. Freshly harvested tissues were submerged in RNAlater™ Stabilization Solution (Ambion; Thermo Fisher Scientific, Inc.), and then were snap frozen within 30 mins of obtaining the biopsy sample. All samples were stored frozen in liquid nitrogen until required for experimentation purposes.

Cell culture, treatment and transfection. Human fetal lung fibroblasts (HFL1; American Type Culture Collection) were cultured in F-12K medium (Thermo Fisher Scientific, Inc.) with 10% FBS (Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Thermo Fisher Scientific, Inc.), and incubated at 37°C in a 5%CO₂ incubator. HFL1 cells were pre-treated with TGF-β (5 ng/ml; R&D Systems, Inc.) for 48 h. A total of 4x10⁴ HFL1 cells/well were transiently transfected 1 day post-seeding with non-targeting small interfering (si)RNA scrambled control (siControl; 50 nM) or with siRNA against MALAT1 (siMALAT1; 50 nM; Sigma-Aldrich; Merck KGaA) using Lipofectamine® LTX Transfection reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. siMALAT1 sequences were as follows: Sense: 5'-GAGCUUGACUUG AUUGUAUtt-3' and antisense: 5'-AUACAAUCAAGUCA GCUCt-3', where lowercase letters were used to denote the protected region; HFL1 cells were transfected for 48 h before subsequent experimentation.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the human lung tissues and HFL1 cells was isolated using the RNeasy MinElute Cleanup kit (Qiagen GmbH), according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed into cDNA and qPCR was subsequently performed using the KAPA SYBR® FAST One-Step qRT-PCR kit. The following thermocycling conditions were used: Initial denaturation at 95°C for 5 min; 42 cycles of 95°C for 5 sec and 60°C for 30 sec; and a final extension at 72°C for 5 min. The lncRNA targets were selected based on a literature search for lncRNAs that were associated with lung disease (27-32) and included the following targets: Highly upregulated in liver cancer (HULC), nuclear enriched abundant transcript 1 (NEAT1), HOX transcript antisense RNA (HOTAIR), maternally expressed gene 3 (MEG3), MALAT1 and urothelial cancer associated 1 (UCA1). The primer pairs used for amplification in qPCR are provided in Table I. Triplicates of the assays were repeated for ≥5 times, and the relative lncRNA expression levels were quantified using the

Table I. List of primers used to amplify long non-coding RNAs.

Gene	Primer sequence (5'→3')
MALAT1	F: TAGGAAGACAGCAGCAGACAGG R: TTGCTCGCTTGCTCCTCAGT
HULC	F: TCATGATGGAATTGGAGCCTT R: CTCTTCTGGCTTGCAGATTG
MEG3	F: GCCAAGCTTCTTGAAGGCC R: TTCCACGGAGTAGAGCGAGTC
NEAT1	F: TGGCTAGCTCAGGGCTTCAG R: TCTCCTTGCCAAGCTTCCTTC
UCA1	F: CATGCTTGACACTTGGTGCC R: GGTGCGAGGTGGATCTCTTC
HOTAIR	F: CAGTGGGGAAGCTCTGACTCG R: GTGCCTGGTGCTCTCTTACC

F, forward; HOTAIR, HOX transcript antisense RNA; HULC, highly upregulated in liver cancer; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MEG3, maternally expressed 3; NEAT1, nuclear enriched abundant transcript 1; R, reverse; UCA1, urothelial cancer-associated 1.

2- $\Delta\Delta C_q$ method. The expression levels were normalized to the internal reference gene, 18S ribosomal RNA, and are represented as the mean ± SD of the relative fold change of lncRNA in primary COPD over non-COPD lung tissue specimens.

Cell viability assay. Using a previously reported protocol, cell viability was quantified using an MTT assay (Sigma-Aldrich; Merck KGaA) according to the manufacturer's recommendations at 48 h after transfection (33). Results from three independent experiments were represented as mean ± SD.

Immunofluorescence. Immunofluorescence was performed according to a previously published protocol (34). Briefly, 1x10⁶/ml HFL1 cells were seeded in 35-mm cell culture dishes, the cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.5% Triton X-100 for 5 min at room temperature, before being subsequently blocked with 4% BSA (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were then incubated with the primary antibody against SMA (1:200; cat. no. ab5694; Abcam) at 4°C for 12 h. Following the primary incubation, the slides were incubated with 2-mg/ml solution of the goat anti-mouse IgG H&L (Alexa Flour® 488) secondary antibody (cat. no. ab150117; Abcam) at 37°C for 1 h. The nuclei were counterstained with DAPI for 5 min. Images were captured using a Zeiss LSM 510 inverted confocal microscope.

Protein extraction and western blotting. Total protein was extracted from 1x10⁷ HFL1 cells using RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1% protease cocktail inhibitor (cat. no. 78425; Pierce; Thermo Fisher Scientific, Ltd.), 1 mM PMSF (cat. no. 36978; Pierce; Thermo Fisher Scientific, Ltd.), 1 mM Na₃VO₄ (cat. no. S6508; Sigma-Aldrich; Merck KGaA), and 50 mM NaF (cat. no. S7920; Sigma-Aldrich; Merck KGaA)]. Total protein was quantified using a BCA protein

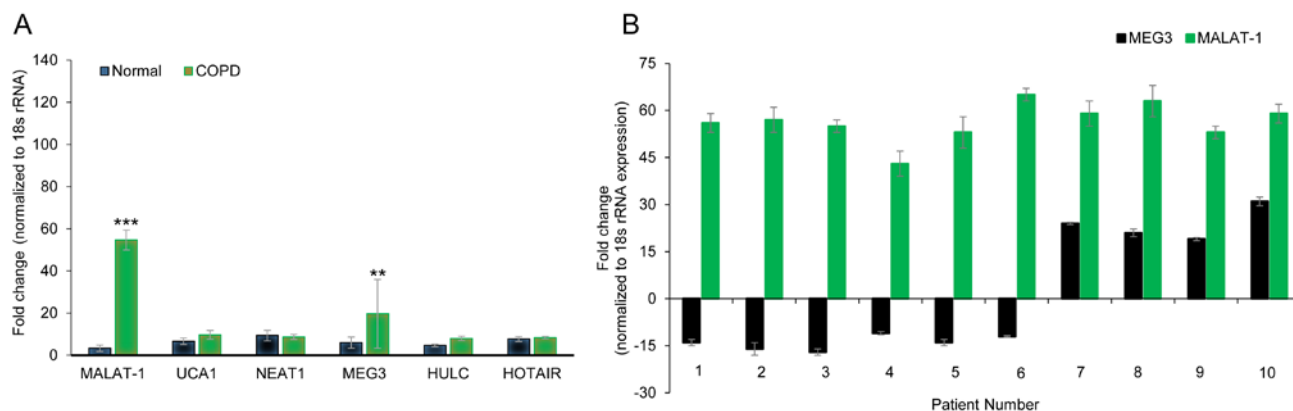


Figure 1. lncRNA MALAT1 is upregulated in the lung tissue of patients with COPD. (A) Gene expression profiles of lncRNAs in age-matched COPD and non-COPD lung tissues. RT-qPCR was performed on six lncRNAs in three pairs of age-matched COPD and non-COPD lung tissues. Data was normalized to 18S rRNA expression and presented as the mean \pm SD. (B) Expression of MALAT1 and MEG3 lncRNAs in 10 pairs of age-matched COPD and non-COPD lung tissues, assessed by RT-qPCR. ** $P < 0.01$ and *** $P < 0.001$ vs. non-COPD group. COPD, chronic obstructive pulmonary disorder; HOTAIR, HOX transcript antisense RNA; HULC, highly upregulated in liver cancer; lncRNA, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MEG3, maternally expressed 3; NEAT1, nuclear enriched abundant transcript 1; rRNA, ribosomal RNA; RT-qPCR, reverse transcription-quantitative PCR; UCA1, urothelial cancer associated 1.

assay kit (Pierce; Thermo Fisher Scientific, Ltd.), according to the manufacturer's protocol. To the loading buffer, 40 μ g of protein was added and placed in a 100°C water bath for 5 min to promote denaturation. The samples were separated by 5% SDS-PAGE. The separated proteins were transferred to PVDF membranes using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc.). Subsequently, the PVDF membranes were blocked for 0.5 h in 5% dry fat-free milk in TBS + 0.1% Tween 20 on ice. The membranes were incubated with primary antibodies against α -SMA (cat. no. 19245), phosphorylated (p) ribosomal S6K1 on threonine 389 (p-S6K1; cat. no. 97596; 1:1,000; Cell Signaling Technology, Inc.); S6K1 (cat. no. 2708; 1:1,000; Cell Signaling Technology, Inc.), fibronectin (cat. no. ab32419; 1:1,000; Abcam) and GAPDH (cat. no. ab181602; 1:1,000; Abcam) at room temperature for 1.5 h and were subsequently washed 3 times in 5% TBST for 5 mins. This was followed by incubation with an HRP-conjugated secondary antibody anti-rabbit IgG (cat. no. ZB-2301; 1:2,000; OriGene Technologies) at room temperature for 1 h. Following 3 washes in 5% TBST, protein bands were visualized with Pierce™ ECL Western Blotting substrate (Thermo Fisher Scientific, Inc.) and analyzed by the Image Lab™ Software (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analyses were performed using SPSS 22.0 (IBM Corp.) and figures were produced using GraphPad Prism 7.0 (GraphPad Software, Inc.). Statistical significance was determined using an ANOVA followed by Tukey's post hoc test for the differences in lncRNA expression between paired tissue samples. All experimental data are presented as the mean \pm SD of at least three independent experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

lncRNA expression in primary lung disease and matched lymph node metastatic tissues. The expression levels of six

lncRNAs (HULC, NEAT1, HOTAIR, MEG3, MALAT1, UCA1), which were previously reported to serve a role in a variety of lung diseases (29-33,35), were determined by RT-qPCR in three pairs of age-matched primary lung tissue obtained from COPD or non-COPD subjects. Of the six lncRNAs examined, MALAT1 (54.67 \pm 4.72 vs. 3.33 \pm 1.52 fold change in COPD and non-COPD, respectively; $P < 0.001$) and MEG3 (19.67 \pm 16.29 vs. 6.00 \pm 2.64 fold change in COPD and non-COPD, respectively; $P < 0.01$) were overexpressed in the COPD lung tissues (Fig. 1A), which suggested that the expression of these lncRNAs may be associated with COPD pathogenesis. Of note, the SD of MEG3 expression was high, suggesting highly varied expression levels in the three tissues examined. To further confirm these results, the expression levels of MEG3 and MALAT1 were explored in 10 age-matched COPD and non-COPD subjects. MALAT1 expression was consistently higher in COPD lung tissues compared to non-COPD tissue (Fig. 1A), whereas MEG3 expression varied and even demonstrated reduced expression levels in a number of COPD patients (Fig. 1B). These data indicated that MALAT1 may be a robust biomarker for COPD.

Gene silencing of MALAT1 affects the viability of HFL1 cells following TGF- β pretreatment. siRNA-mediated silencing of MALAT1 expression was used to determine whether MALAT1 can alter the viability of HFL1 cells. Transient transfection of HFL1 with siMALAT1 decreased MALAT1 expression by 80% 48 h post-transfection compared with the siControl ($P < 0.001$; Fig. 2A). In addition, to determine whether TGF- β treatment could affect cell viability, HFL1 cells were exposed to TGF- β (5 ng/ml) pretreatment for 48 h. The viability of HFL1 cells treated with TGF- β and transfected with siMALAT1 was significantly increased compared with HFL1 cells treated with TGF- β + siControl ($P < 0.05$; Fig. 2B). The above results showed that MALAT1 silencing could improve the viability of HFL1 cells following TGF- β pretreatment.

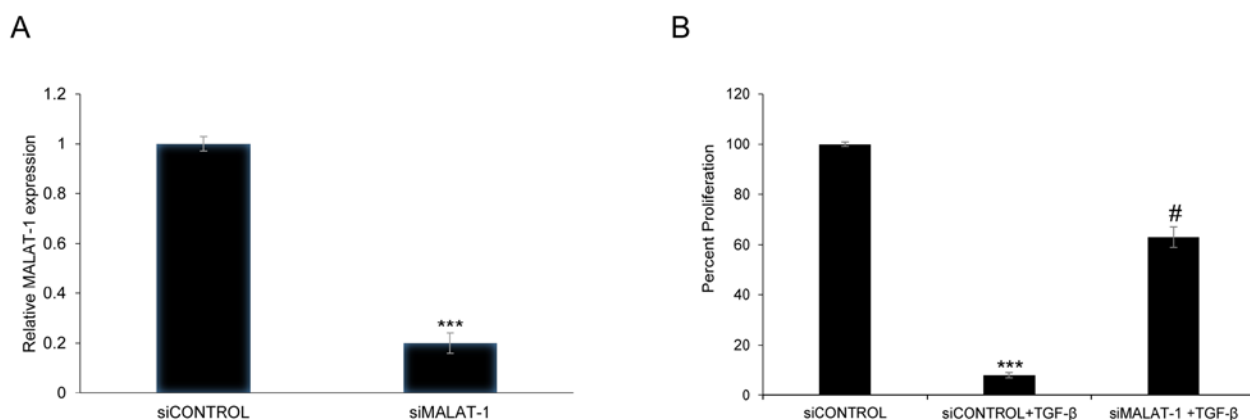


Figure 2. Gene silencing of lncRNA MALAT1 affects the viability of HFL1 cells following TGF- β pretreatment. (A) Relative expression levels of lncRNA MALAT1 in HFL1 cells transfected with siControl or siMALAT1 were measured by reverse transcription-quantitative PCR. (B) Viability of HFL1 cells transfected with siControl or siMALAT1 with or without TGF- β pretreatment were detected with the MTT assay. Data were normalized to 18S rRNA expression and presented as the mean \pm SD. *** P <0.001 vs. siControl; # P <0.05 vs. siControl + TGF- β group. HFL1, human fetal lung fibroblast 1; lncRNA, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; rRNA, ribosomal RNA; si, small interfering RNA; TGF- β , transforming growth factor- β .

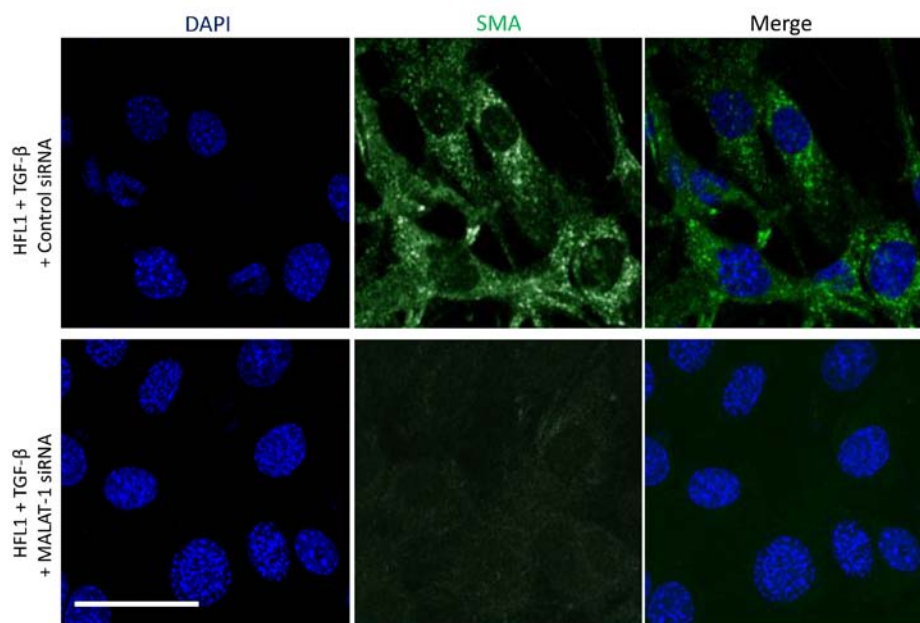


Figure 3. Silencing MALAT1 decreases α -SMA protein expression following TGF- β treatment of HFL1 cells. Immunofluorescent staining to detect the relative protein expression of α -SMA in HFL1 cells treated with TGF- β for 48 h and transfected with siControl or siMALAT1. Nuclei were stained with DAPI. Scale bar, 100 μ m. Images are representative of ≥ 10 different images obtained within the experimental set. α -SMA, α -smooth muscle actin; HFL1, human fetal lung fibroblast 1; MALAT1, metastasis associated lung adenocarcinoma transcript 1; siRNA, small interfering RNA; TGF- β , transforming growth factor- β .

Downregulation of MALAT1 expression attenuates α -SMA and fibronectin protein expression in HFL1 cells. The protein expression levels of α -SMA and fibronectin in siControl- or siMALAT1-transfected HFL1 cells co-treated with or without TGF- β was determined. Immunofluorescence revealed that silencing MALAT1 gene expression markedly decreased α -SMA protein expression following TGF- β treatment of HFL1 cells (Fig. 3). TGF- β treatment induced the protein expression of α -SMA and fibronectin in HFL1 cells, which was attenuated following silencing of MALAT1 gene expression (Fig. 4). Since TGF- β -mediated induction of fibronectin and α -SMA is driven through mTORC1 (36),

the levels of p-S6K1, a marker of mTORC1 activation, were evaluated. P-S6K1 (T389) protein expression markedly increased in HFL1 cells treated with TGF- β ; however, in HFL1 cells transfected with siMALAT1, no change was observed in p-S6K1 protein expression compared with HFL1 cells transfected with siMALAT1 + TGF- β (Fig. 4). These data suggested that MALAT1 gene silencing downregulated α -SMA and fibronectin without inhibiting mTORC1. Notably, p-S6K1 protein expression was detected in HFL1 cells transfected with siMALAT1, but untreated with TGF- β , which indicated that MALAT1 gene silencing may induce mTORC1 activation. The reason for decreased

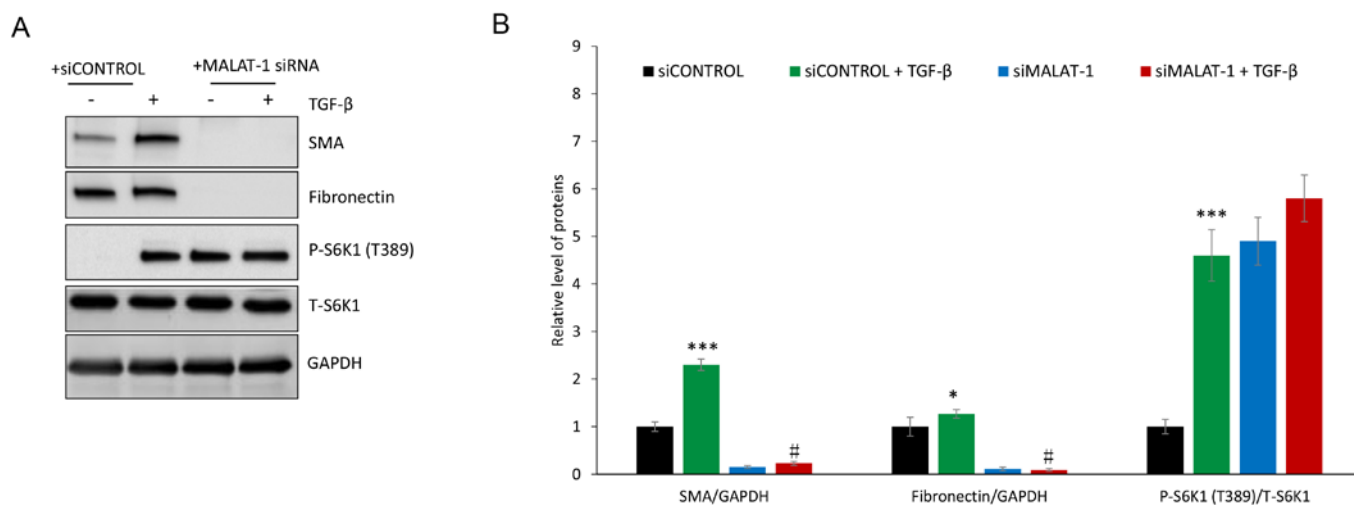


Figure 4. Gene silencing of MALAT1 suppresses the protein expression levels of α -SMA and fibronectin in HFL1 cells. (A) siMALAT1 transfection inhibited the expression levels of α -SMA and fibronectin protein, whereas TGF- β promoted the expression levels of α -SMA and fibronectin protein. (B) The relative expression levels of α -SMA and fibronectin normalized to GAPDH, and the ratio of P-S6K1 (T389) to total S6K1 formalized to GAPDH. GAPDH was used as a loading control and for normalization. * $P < 0.05$ and *** $P < 0.001$ vs. siControl; # $P < 0.05$ vs. siControl + TGF- β group. α -SMA, α -smooth muscle actin; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; p, phosphorylated; S6K1, mTOR complex 1 S6 kinase; si, small interfering RNA; T, total; TGF- β , transforming growth factor- β .

α -SMA and fibronectin protein expression remains to be determined.

Discussion

Previous studies have demonstrated that lncRNAs serve a vital role in gene regulatory processes, and that they can affect normal and transformed cellular functions (36,37). Although lncRNAs do not encode proteins, numerous studies have revealed that they regulate the process of transcription (38,39), indicating that differences detected in the expression of lncRNAs between normal and diseased tissues are not a secondary readout for physiological changes. Given the symptomatic exacerbation observed in COPD patients, and the high cost-of-burden of the disease, it is of vital importance to gain a better understanding of the underlying mechanisms of COPD pathogenesis (40-43). ncRNAs can negatively regulate gene expression at the post-transcriptional level and serve crucial regulatory roles in a number of biological processes, and the altered expression of these ncRNAs has been demonstrated to lead to inflammation and COPD (44). However, there is inadequate data on the expression profile of lncRNAs in COPD (26). A previous study reported that the lncRNAs AJ005396 and S69206 are overexpressed in fibrotic lung tissue (45), and that lncRNAs, such as X inactive specific transcript, HOTAIR and MALAT1, are aberrantly expressed in the bronchial epithelium of cystic fibrosis patients (46). MALAT1 is reportedly overexpressed in, and contributes to the poor prognosis of, non-small cell lung cancer (47). Bi *et al* (48) compared the lncRNA expression profile in lung tissue from non-smokers and smokers with or without COPD; 120 lncRNAs were revealed to be upregulated and 43 downregulated in smokers with COPD compared with smokers without COPD. However, the study is limited owing to a small sample size and the use of male subjects only. Thus, additional larger studies with both sexes are warranted. lncRNAs associated with COPD are largely

unknown; thus, the present study profiled the expression of six lncRNAs (HULC, NEAT1, HOTAIR, MEG3, MALAT1, and UCA1) based on those that were previously demonstrated to be associated with a variety of lung diseases (30-35), and MALAT1 was identified as a potential biomarker of COPD.

The mechanism by which MALAT1 facilitates the pathogenesis of COPD with TGF- β induction may be similar to a previous study that examined the lncRNA TUG1 in COPD patients (28). Thus, it would be worthwhile to determine the effect of MALAT1 in combination with TUG1 and its effect on COPD diagnosis. In addition, previous studies revealed that the aggressive malignant characteristics of lung cancer are attributed to lncRNAs, such as MALAT1 (24,47). Thus, these studies clearly link MALAT1 with lung disease.

TGF- β signaling participates in COPD pathogenesis (49). α -SMA and fibronectin are both mesenchymal markers that are stimulated by TGF- β *in vitro* (27), and were demonstrated in the present study to decrease in HFL1 cells when MALAT1 gene expression was silenced. Notably, the results indicated that the TGF- β -mediated induction of these proteins occurred alongside mTORC1 activation. However, the downregulation of α -SMA and fibronectin that was observed following MALAT1 silencing occurred independently of mTORC1, because p-S6K1 protein expression was not affected. In fact, the silencing of MALAT1 induced the phosphorylation of S6K1 in the HFL1 cells which were not treated with TGF- β , suggesting that the loss of MALAT1 expression might permit mTORC1 signaling in the absence of TGF- β . This indicated the potential existence of a complicated upstream interaction between TGF- β signaling, MALAT1 regulation and mTORC1 activation. This mechanism warrants further investigation in the setting of COPD, because there is evidence in other diseases that MALAT1 is a crucial player in disease processes; for example, in bladder cancer, MALAT1 downregulation inhibits the viability and invasive properties of bladder cancer cells by attenuating

autophagy via the regulation of the AMP-activated protein kinase/mTOR pathway (50).

In conclusion, lncRNA MALAT1 is upregulated in patients with COPD, and the downregulation of MALAT1 induced cellular viability following TGF- β stimulation in HFL1 cells. MALAT1 was indicated to serve a regulatory role in the mTOR pathway, and is related to the mesenchymal protein expression. Together, these data suggested that MALAT1 may serve as a target for understanding the pathogenesis of COPD. Although further investigations are required to assess the function of MALAT1, and its target genes and associated regulatory mechanism in COPD, the present study provides a foundation for further exploration into the role of this lncRNA in COPD.

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Availability of data and materials

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

Authors' contributions

TJH and ZHY designed the experiments; HBH and WC performed the experiments; and HBS collected the samples used in the studies. The manuscript was prepared by TJH. The final manuscript was read and approved by all the authors.

Ethics approval and consent to participate

Ethics approval was obtained from the Institutional Review Board of the Hua Mei Hospital, University of Chinese Academy of Sciences (Ningbo, China). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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