

Screening of long non-coding RNA and TUG1 inhibits proliferation with TGF- β induction in patients with COPD

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Objective: To evaluate differentially expressed long noncoding RNAs (lncRNAs) and the potential role of lncRNA TUG1 in patients with chronic obstructive pulmonary disease (COPD).

Methods: Total RNA was extracted from both COPD and non-COPD lung tissues, and microarray analysis was performed with 25,628 lncRNA probes and 20,106 mRNA probes. In addition, five up-regulated and five down-regulated lncRNAs were selected for identification using quantitative real-time polymerase chain reaction. COPD cell model was established by transforming growth factor β (TGF- β) treatment. Cell Counting Kit-8 assay was used to detect BEAS-2B and HFL1 cell proliferation after TUG-siRNA transfection with TGF- β treatment. In addition, the expression levels of α -SMA and fibronectin proteins were determined using Western blot in BEAS-2B and HFL1 cells after TUG-siRNA transfection with TGF- β treatment.

Results: There were 8,376 (32.7%) differentially expressed lncRNAs and 5,094 (25.3%) differentially expressed mRNAs in COPD lung tissues compared with non-COPD lung tissues. Five of the analyzed lncRNAs (BC038205, BC130595, TUG1, MEG3, and LOC646329) were markedly increased, while five lncRNAs (LOC729178, PLAC2, LOC339529, LINC00229, and SNHG5) were significantly decreased in COPD lung tissues compared with non-COPD lung tissues (n=20) (** $P < 0.001$). Knockdown of lncRNA TUG1 promotes BEAS-2B and HFL1 cell proliferation after TGF- β treatment through inhibiting the expression levels of α -SMA and fibronectin.

Conclusion: Abundant, differentially expressed lncRNAs and mRNAs were identified by microarray analysis and these might play a partial or key role in the diagnosis of patients with COPD. LncRNA TUG1 may become a very important class of biomarker and may act as a potential diagnostic and therapeutic target for patients with COPD.

Keywords: COPD, microarray analysis, long noncoding RNA, lncRNA TUG1, TGF- β

Introduction

Lung diseases mainly include chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), asthma, cystic fibrosis (CF), and non-small-cell lung cancer (NSCLC). COPD is an abnormal inflammatory reaction of lungs to harmful particles or gases (primarily cigarette smoke).¹ COPD has become a primary public health problem and is the fourth main reason of chronic morbidity and mortality in the US.² It has been the cause of more than 2,500,000 deaths every year worldwide, according to the 2002 report of the World Bank/World Health Organization.³ There are several reasons for the development of COPD, including the inspiration of noxious particles such as cigarette smoke, the destruction of the balance between proteolytic and antiproteolytic molecules,⁴ and oxidative stress.⁵ However, there are difficulties in selecting

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an appropriate therapeutic method for COPD, especially with regard to the lack of classical studies.⁶ Therefore, it is also urgent to find specific and effective markers for the diagnosis of COPD. IPF presents as a common, chronic, and progressive lung disease with poor prognosis.⁷ Asthma is characterized by periods of reversible airflow obstruction. CF presents as an autosomal recessive disease and is characterized by chronic endobronchial infection, progressive airway obstruction, and abnormal airway secretions.⁸

Long noncoding RNAs (lncRNAs), a group of noncoding RNAs (ncRNAs) which are non-protein coding transcripts transcribed by RNA polymerase II,⁹ have a length of more than 200 nucleotides.^{10,11} With regard to their biological functions, lncRNAs play a significant role in chromatin structure and function,¹² promoting numerous biological functions including gene transcription, regulating splicing and participating in epigenetics,^{13,14} genomic rearrangement,¹⁵ etc. Studies indicated that lncRNAs were connected with various diseases⁹ and resulted in abnormal expression of abundant gene products which may, in turn, generate biological processes involving growth,¹⁶ differentiation,¹⁷ and carcinogenesis.¹⁸ They have also been implicated in numerous human diseases^{19–21} such as neurodegeneration diseases,²² cardiovascular diseases,²³ and cancers.²⁴ Previously, differentially expressed lncRNAs and mRNAs between fibrotic lung and normal lung tissues were selected for micro-array analysis. Among them, AJ005396 and S69206 were found to be increased in the fibrotic lung tissues.²⁵ MALAT-1 is highly expressed in NSCLC and is involved in poor prognosis.²⁶ One study has reported the aberrant expression of lncRNAs in the CF bronchial epithelium.²⁷ However, the lncRNAs involved in the development of COPD or asthma have not been studied.

Genomic screening techniques revealed that taurine treatment of mouse retinal cells resulted in the up-regulation of lncRNA TUG1 (7.1 kb in length).²⁸ LncRNA TUG1 is highly conservative, and its inactivation results in damage to the formation of photoreceptors in the developing rodent retina.²⁸ Recently, researchers have found that TUG1 was significantly decreased in NSCLC.²⁹ However, some studies showed that TUG1 was increased in gastric cancer, osteosarcoma, and bladder cancer,^{30–32} probably because lncRNAs showed predominantly tissue-specific expression,^{33,34} whereas the function and potential mechanism of TUG1 in patients with COPD are still unknown. Therefore, in our study, we explored the possible function and mechanism of TUG1 in patients with COPD.

In our study, we detected differentially expressed lncRNAs and mRNAs in COPD and non-COPD lung tissues using

human microarray analysis. We conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and gene ontology (GO) enrichment analysis of the differentially expressed mRNAs. Then, we selected five up-regulated and five down-regulated lncRNAs, which were identified using quantitative real-time polymerase chain reaction (qRT-PCR), from 20 COPD and 20 non-COPD lung tissues. Furthermore, we selected lncRNA TUG1 and analyzed its effect on proliferation and expression levels of α -SMA and fibronectin proteins by knocking out lncRNA TUG1 through transforming growth factor β (TGF- β) treatment. The knocking out of lncRNA TUG1 inhibited the expression levels of α -SMA and fibronectin in BEAS-2B and HFL1 cells. Our results manifested that differentially expressed lncRNAs can act as potential molecular biomarkers for the diagnosis of COPD and that lncRNA TUG1, in particular, may play an important role as a diagnostic marker in the COPD patients.

Materials and methods

Patients and clinical specimens

The study was approved by the Institutional Review Board of Third Xiangya Hospital, Central South University. Lung tissues from human COPD and non-COPD patients were collected from the Third Xiangya Hospital in 2015. Written informed consent to participate in the study was acquired from all the patients. The tissue samples were immediately placed in the refrigerator at -80°C until further analysis.

Cell culture and transfection

BEAS-2B (human lung epithelial cells) and HFL1 (human lung fibroblasts) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). BEAS-2B was cultured in LHC-8 medium without gentamicin (Thermo Fisher Scientific, Waltham, MA, USA) and HFL1 was cultured in F-12K medium (Thermo Fisher Scientific). Penicillin (100 U/mL), streptomycin (100 $\mu\text{g}/\text{mL}$), and 10% fetal bovine serum (FBS; Thermo Fisher Scientific) were added to all the culture media. Cell lines were cultured with 5% CO_2 at 37°C . For cell treatments, we used 2 ng/mL TGF- β (R&D Systems, Inc., Minneapolis, MN, USA) for 48 h. Transient transfection was assayed using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's protocol. NC (negative control) siRNA and TUG1-siRNA were synthesized. The siRNA sequences used to target TUG1 were 5'-GCU UGG CUU CUA UUC UGA AUC CUU U-3' (sense) and 5'-AAA GGA UUC AGA AUA GAA GCC AAG C-3' (antisense).²⁹ BEAS-2B and HFL1 cells were transfected with Lipofectamine 3000 (Thermo Fisher

Scientific). Cells were cultured for 48 h after transfection and then were analyzed by cell viability assay and Western blot analysis.

Arraystar lncRNA array

Tissues were cryopulverized using the BioPulverizer™ (Biospec Products, Inc., Bartlesville, OK, USA) and homogenized using the Mini-Bead-Beater-16 (Biospec). The homogenized samples were separated. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, and the precipitate was washed with 75% ethanol and redissolved with RNase-free water. Total RNA was extracted and RNA quality was determined. Labeling reaction was carried out using Quick Amp Labeling Kit, One-Color (Agilent p/n 5190-0442), then the labeled/amplified RNA was purified and labeled cRNA quality was determined. Hybridization was performed using Agilent Gene Expression Hybridization Kit (Agilent p/n 5188-5242) according to manufacturer's instructions and the microarray was washed. Agilent microarray scanner (Agilent p/n G2565BA) was used and data were extracted using Agilent Feature Extraction software (version 11.0.1.1).

RNA preparation, reverse transcription, and qRT-PCR analysis

Total RNA was extracted from human lung cells and tissues by using TRIzol reagent according to the manufacturer's instructions. RevertAid™ First Strand cDNA Synthesis Kit (no K1622; Thermo Fisher Scientific) was used to synthesize the cDNA with random primers and 1.0 μ g RNA was included in each reverse transcription reaction. qPCR was performed with SYBR Premix Ex Taq (Takara Bio Inc., Kusatsu, Japan). The primers of objective genes and GAPDH (internal loading control) were designed and are shown in

Table 1. The ABI 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK) was used for PCR reactions. All the experiments were repeated at least three times and the results are displayed as the mean \pm standard deviation.

Cell viability assay

First, 50 μ M NC siRNAs or TUG1 siRNAs (si-TUG1) were transfected into BEAS-2B and HFL1 cells for 48 h. Second, the cells were treated with TGF- β (2 ng/mL) for 48 h. BEAS-2B, BEAS-2B/TGF- β , BEAS-2B/TGF- β /NC-siRNA, and BEAS-2B/TGF- β /TUG1-siRNA cells were seeded in 96-well plates at 1×10^4 cells per well at 37°C. At 24, 48, and 72 h, 10 μ L of cholecystokinin octapeptide (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated for 4 h. The absorbance was detected at a wavelength of 450 nm. The methods used for the treatment and detection of HFL1 cells were identical with those used for BEAS-2B cells.

Western blot analysis

The total proteins of BEAS-2B and HFL1 cells were extracted using a lysis buffer including 10% protease inhibitor. BCA Protein Assay kit (QCbio Science Technologies Co., Ltd., Shanghai, People's Republic of China) was used to detect the concentrations of total protein. Approximately, 30 μ g of heat-denatured proteins were added to sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA), blocked with 5% skim milk (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and incubated with primary antibody overnight at 4°C. Next day, the PVDF membranes with proteins were incubated with the secondary antibody for 2 h. Special antibodies were detected by using enhanced

Table 1 Sequence of the primers used for validation of selected lncRNAs

LncRNA	Primer sequence (5'-3')	Product length (bp)
BC038205	Forward: GAAATCCAACAACAGAGTGCC	161
BC130595	Forward: TCATTCTGTCCATCCCCT	148
TUG1	Forward: TCCTGCTTTCCTGACCCTCT	191
MEG3	Forward: CGCCCTCTGACTGATGGAC	114
LOC729178	Forward: GTGGAGCCGGTGGAGATAAT	122
PLAC2	Forward: CATCGCAAGAAGAGAAGCAC	127
LOC646329	Forward: TTACGAGTTAGAAGAGTTGGGATA	240
LOC339529	Forward: CCCACCTCCTTCAAACCTCG	223
LINC00229	Forward: GTTTGCTGGATGGCTGTAGG	172
SNHG5	Forward: TGTGGACGAGTAGCCAGTGAAG	108
GAPDH	Forward: TGTCGTCATGGGTGTGAAC	154

Abbreviation: lncRNA, long noncoding RNA.

chemiluminescence (ECL) reagents (Amersham Biosciences, Inc., Little Chalfont, UK). The ImageJ software program was used to analyze the expression levels of special proteins. The primary antibodies, anti- α -SMA and anti-fibronectin, were purchased from Abcam (Cambridge, UK).

Statistical analysis

Statistical Product and Service Solutions (SPSS) software 21 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA USA) were used to analyze the data of all experiments by Student's *t*-tests. *P*-values less than 0.05 were considered to be statistically significant.

Results

Expression of lncRNAs and mRNAs in COPD lung tissues

Six patient samples with or without COPD were randomly selected for this study. Histopathology was assessed by a pathologist and patient characteristics are shown in Table 2. lncRNA array (Kangcheng, Shanghai, People's Republic of China) was employed to measure the differentially expressed lncRNAs in COPD and non-COPD lung tissues. We detected the expression of 25,628 lncRNAs and 20,106 mRNAs using microarray analysis. According to fold-change (COPD vs non-COPD lung tissues) and *P*-value, as shown in Table 3, we screened out significantly differentially expressed lncRNAs (8,376 [32.7%]) and mRNAs (5,049 [25.1%]) in three COPD lung tissue samples and compared with non-COPD lung tissue samples (fold-change >2.0 or <0.5; *P*<0.05). Among the differentially expressed lncRNAs, 3,939 (15.4%) were identified to be up-regulated, whereas 4,437 (17.3%) were identified to be down-regulated in the COPD lung tissue groups. Furthermore, 396 (1.6%) lncRNAs were up-regulated, whereas 438 (1.7%) were down-regulated when the fold-change was >5 (Figure 1A). Among the differentially expressed mRNAs, 3,623 (18.0%) were up-regulated,

Table 2 Clinical characteristics of the subjects involved in the studies

Characteristics	No COPD	COPD
Number of subjects	20	20
Age (years)	52.5±10.8	69.3±5.6
Sex, male (female)	13 (7)	15 (5)
Pack-years	0	35.8±13.1
FEV ₁ (% predicted)	90.8±8.3	56.2±9.4**
FEV ₁ /FVC (%)	74.3±5.7	53.5±9.7*

Notes: Values are expressed as mean ± SD. **P*<0.05, ***P*<0.01.

Abbreviations: FEV₁ (% predicted), forced expiratory volume in 1 second as percentage of predicted value; FVC, forced vital capacity; SD, standard deviation.

Table 3 Analysis of differentially expressed lncRNAs and mRNAs between COPD and non-COPD lung tissues

Probe class	Differentially expressed RNA	Up-regulated	Down-regulated
lncRNA	8,376 (25,628)	3,939 (47.0%)	4,437 (53.0%)
mRNA	5,094 (20,106)	3,623 (71.1%)	1,471 (28.9%)

Abbreviation: lncRNA, long noncoding RNA.

whereas 1,471 (7.3%) were down-regulated in the COPD lung tissue groups. Also, 305 (1.5%) mRNAs were up-regulated, whereas 71 (0.4%) mRNAs were down-regulated when the fold-change was >5 (Figure 1B). Hierarchical clustering also displayed variations in the expression of lncRNAs (Figure 1C) and mRNAs (Figure 1D) between COPD and non-COPD lung tissues.

Analysis of genomic locus among differentially expressed lncRNAs

According to the relationship of lncRNA with its nearby coding gene and the coordinate of the coding gene, as shown in Table 4, among the up-regulated lncRNAs, 785 (19.9%) were exon sense-overlapping lncRNAs (the lncRNA's exon overlaps a coding transcript exon on the same genomic strand), 215 (5.5%) intron sense-overlapping lncRNAs (the lncRNA overlaps the intron of a coding transcript on the same genomic strand), 972 (24.7%) natural antisense lncRNAs (the lncRNA is transcribed from the antisense strand and overlaps with a coding transcript), 195 (5.0%) bidirectional lncRNAs (the lncRNA is oriented head to head to a coding transcript within 1,000 bp), 1,701 (43.2%) intergenic lncRNAs (there are no overlapping or bidirectional coding transcripts nearby the lncRNA), and 71 (1.8%) nonoverlapping antisense lncRNAs (the lncRNA is transcribed from the antisense strand without sharing overlapping exons). Among the down-regulated lncRNAs, 274 (6.2%) were exon sense-overlapping lncRNAs, 160 (3.6%) intron sense-overlapping lncRNAs, 1,267 (28.6%) natural antisense lncRNAs, 176 (4.0%) bidirectional lncRNAs, 1,548 (34.9%) intergenic lncRNAs, 974 (22.0%) intronic antisense lncRNAs, and 38 (0.9%) nonoverlapping antisense lncRNAs.

KEGG pathway analysis of the differentially expressed mRNAs

Pathway analysis is a functional analysis mapping genes to KEGG pathways.³⁵ KEGG pathway analysis demonstrated numerous enrichment-related pathways including 46 up-regulated and 8 down-regulated pathways. Among up-regulated pathways, the top 10 pathways in humans

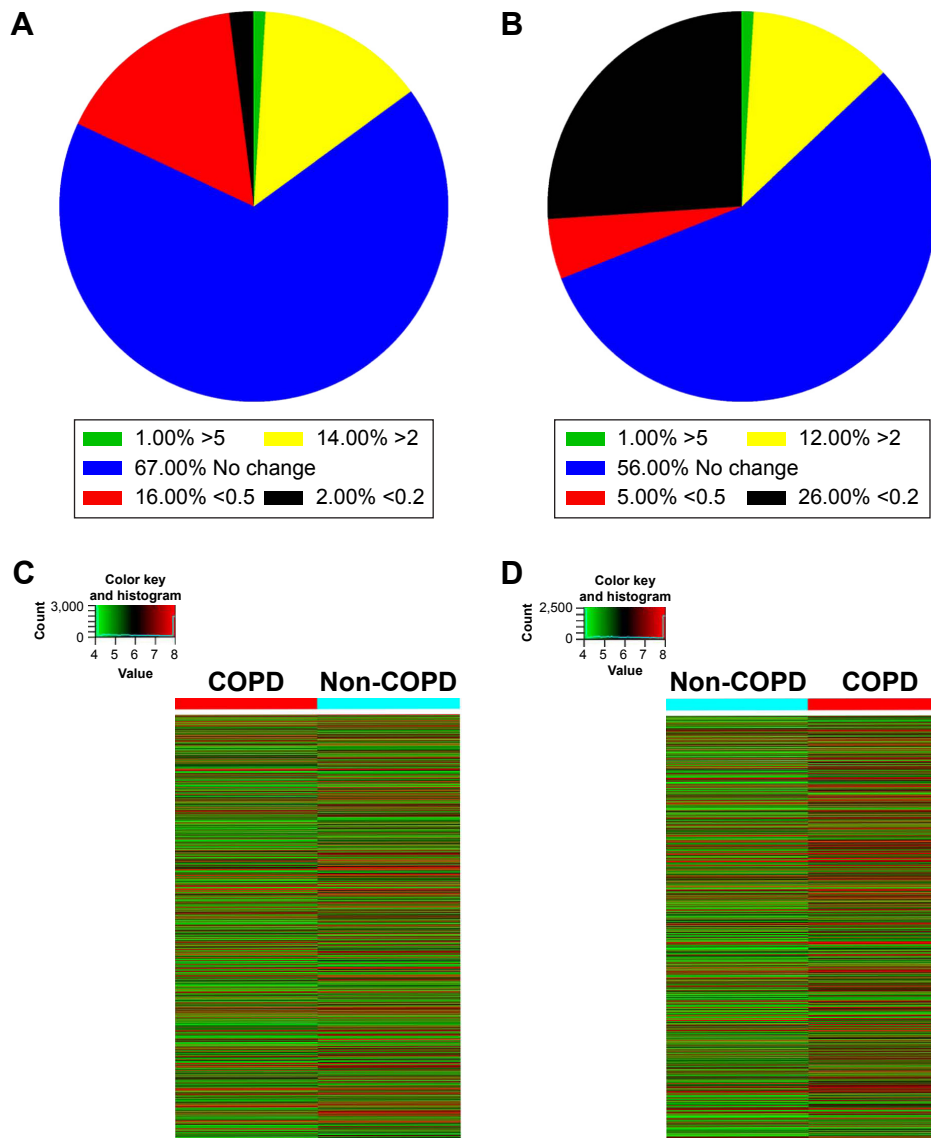


Figure 1 The expression of lncRNAs and mRNAs in COPD lung tissues. **(A)** The percentage of differentially expressed lncRNAs in COPD lung tissues compared with non-COPD lung tissues. **(B)** The percentage of differentially expressed mRNAs in COPD lung tissues relative to non-COPD lung tissues. **(C)** The hierarchical clustering of differentially expressed lncRNAs, which are displayed on a scale from green (low) to red (high), between COPD and non-COPD lung tissues. **(D)** Heat map of distinguishable mRNA expression amid lung tissues samples; red shows relatively high expression and green shows relatively low expression. **Abbreviation:** lncRNAs, long noncoding RNAs.

(humans) are listed in Figure 2A, which include lysosome, *Staphylococcus aureus* infection, osteoclast differentiation, leishmaniasis, B-cell receptor signaling pathway, rheumatoid arthritis, tuberculosis, other glycan degradation, inflammatory bowel disease, and hematopoietic cell lineage.

Simultaneously, the down-regulated pathways include dilated cardiomyopathy, rap1 signaling pathway, hypertrophic cardiomyopathy, PPAR signaling pathway, bladder cancer, bile secretion, regulation of actin cytoskeleton, and adrenergic signaling in cardiomyocytes (Figure 2B).

Table 4 The proportion of differentially expressed lncRNAs in COPD lung tissues based on genomic locus position

Gene regulation levels	Exon-sense overlapping	Intron-sense overlapping	Natural antisense	Bidirectional	Intergenic	Intronic antisense	Nonoverlapping antisense
Up-regulation	785 (19.9%)	215 (5.5%)	972 (24.7%)	195 (5.0%)	1,701 (43.2%)	0	71 (1.8%)
Down-regulation	274 (6.2%)	160 (3.6%)	1,267 (28.6%)	176 (4.0%)	1,548 (34.9%)	974 (22.0%)	38 (0.9%)

Abbreviation: lncRNAs, long noncoding RNAs.

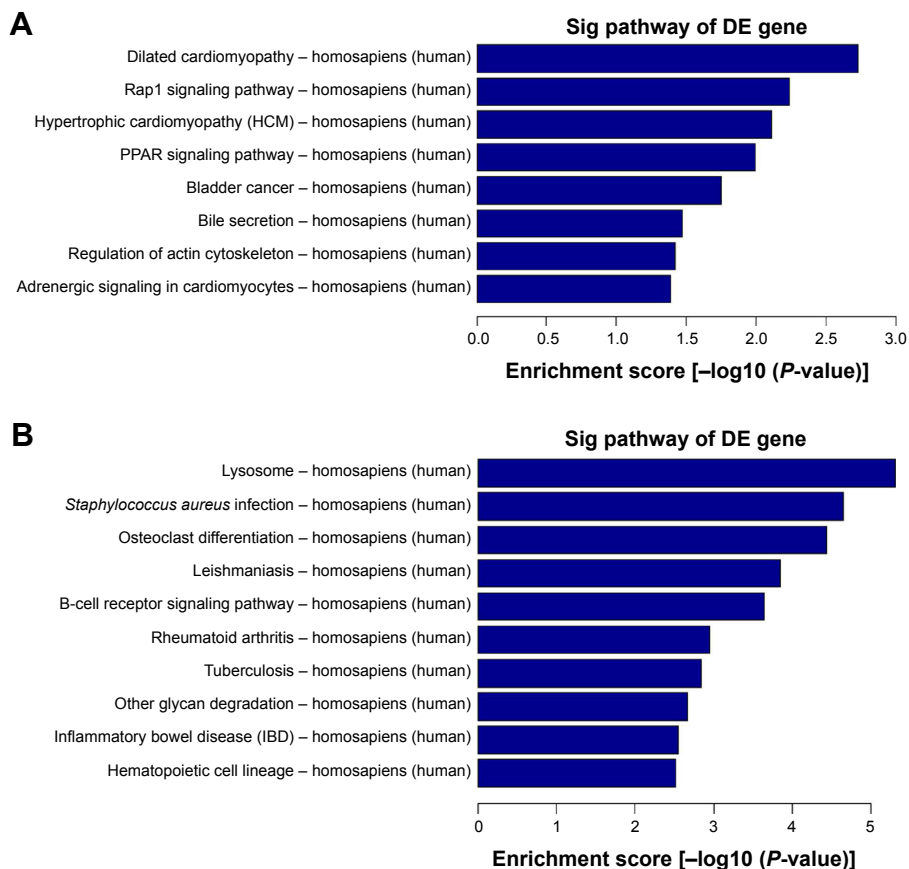


Figure 2 KEGG pathway analysis of the differentially expressed mRNAs. **(A)** Pathway analysis demonstrates the significant pathways of differentially down-regulated mRNAs in model vs normal group. The *P*-value (EASE-score, Fisher's *P*-value, or hypergeometric *P*-value) denotes the significance of the pathway correlated to the conditions. The lower the *P*-value, more significant is the pathway (the recommended *P*-value cutoff is 0.05). **(B)** Differentially up-regulated mRNAs in model vs normal group.

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; Sig, significant.

Gene ontology enrichment analysis of the differentially expressed mRNAs

The Gene Ontology project provides a controlled vocabulary to describe gene and gene product attributes in any organism (<http://www.geneontology.org>). There are three domains in ontology: biological process, cellular component, and molecular function. Fisher's exact test was used to find out if, by chance, there was more overlap between the differentially expressed list and the GO annotation list than would be expected. The *P*-value denotes the significance of GO terms enrichment in the differentially expressed genes. The lower the *P*-value, the more significant is the GO term (*P*-value ≤ 0.05 is recommended). According to the GO analysis results, there were 1,115 up-regulated and 346 down-regulated GO terms in the biological process (COPD vs non-COPD lung tissues). Figures 3 and 4 show the GO classification for the biological process of down-regulated (Figure 3A) and up-regulated mRNAs (Figure 4A). The bar plot shows the top 10 enrichment score values of the significant enrichment terms. Biological processes of down-regulated mRNAs (Figure 3D) mainly include synaptic transmission, regulation of biological

quality, growth, etc., while those of up-regulated mRNAs (Figure 4D) mainly include cellular response to chemical stimulus, regulation of response to stimulus, cell death, etc. Similarly, there were 34 down-regulated (Figure 3B and E) and 127 up-regulated GO terms (Figure 4B and E) in the cellular component (COPD vs non-COPD lung tissues). There were 53 down-regulated (Figure 3C and F) and 115 up-regulated GO terms (Figure 4C and F) in molecular function (COPD vs non-COPD lung tissues).

Identification of 10 differentially expressed lncRNAs

Based on the previously mentioned studies, we selected five up-regulated and five down-regulated lncRNAs. According to the qRT-PCR results, lncRNAs BC038205, BC130595, TUG1, MEG3, and LOC646329 were markedly increased in COPD lung tissues compared to non-COPD lung tissues (n=20) ($***P < 0.001$; Figure 5A). Simultaneously, lncRNAs LOC729178, PLAC2, LOC339529, LINC00229, and SNHG5 were significantly decreased in COPD lung tissues compared to non-COPD lung tissues (n=20) ($***P < 0.001$; Figure 5B).

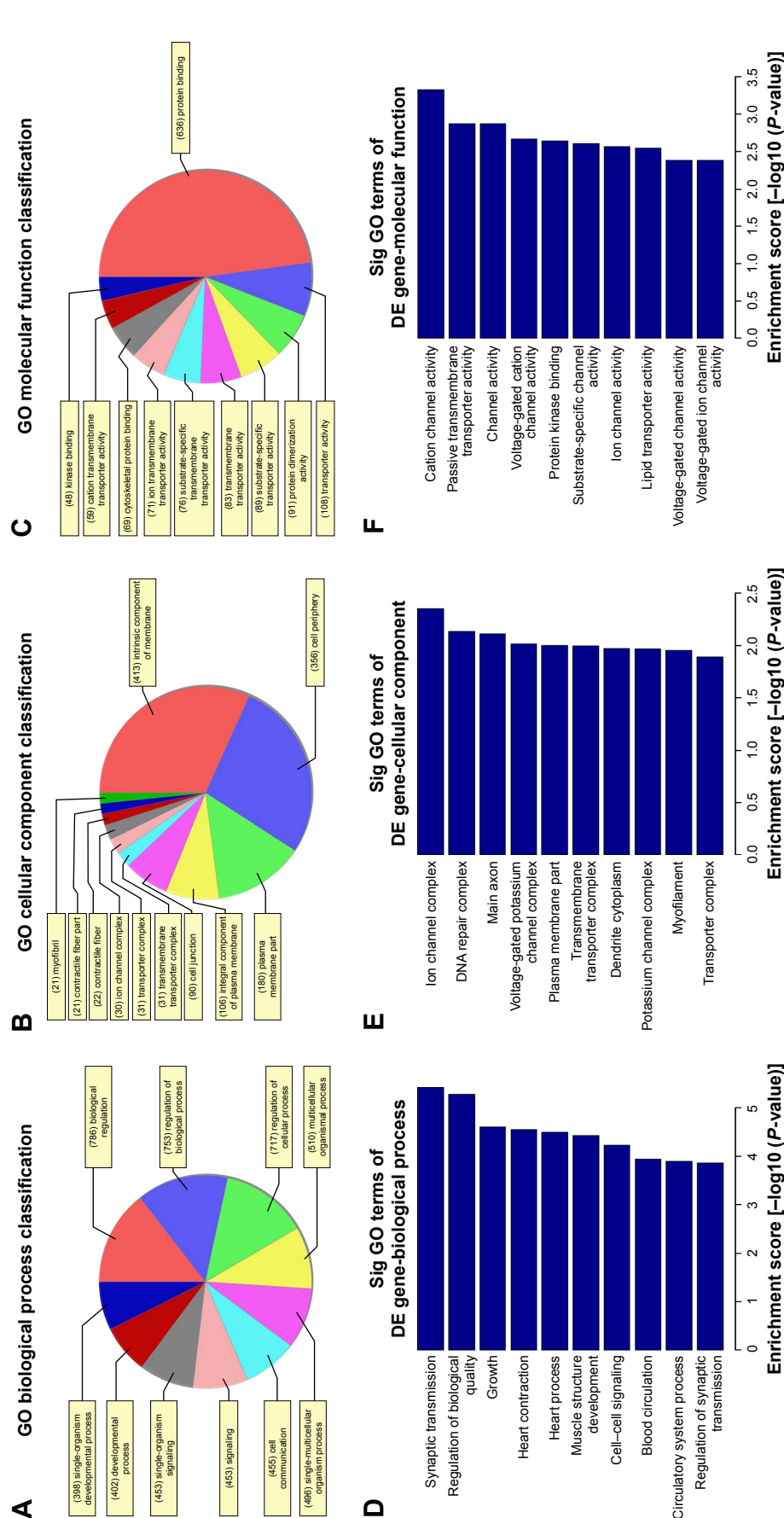


Figure 3 Gene ontology (GO) enrichment analysis of the differentially down-expressed mRNAs. (A) GO biological process classification for cellular components of differentially down-expressed mRNAs. (B) GO biological process classification for molecular function of differentially down-expressed mRNAs. (C) GO biological process classification for cellular components of differentially down-expressed mRNAs. (D) GO analysis indicates the top 10 counts of enrichment score in the biological process of down-regulated mRNAs. (E) GO analysis indicates the top 10 counts of enrichment score in cellular components of down-regulated mRNAs. (F) GO analysis indicates the top 10 counts of enrichment score in molecular function of down-regulated mRNAs.

Abbreviation: Sig, significant.

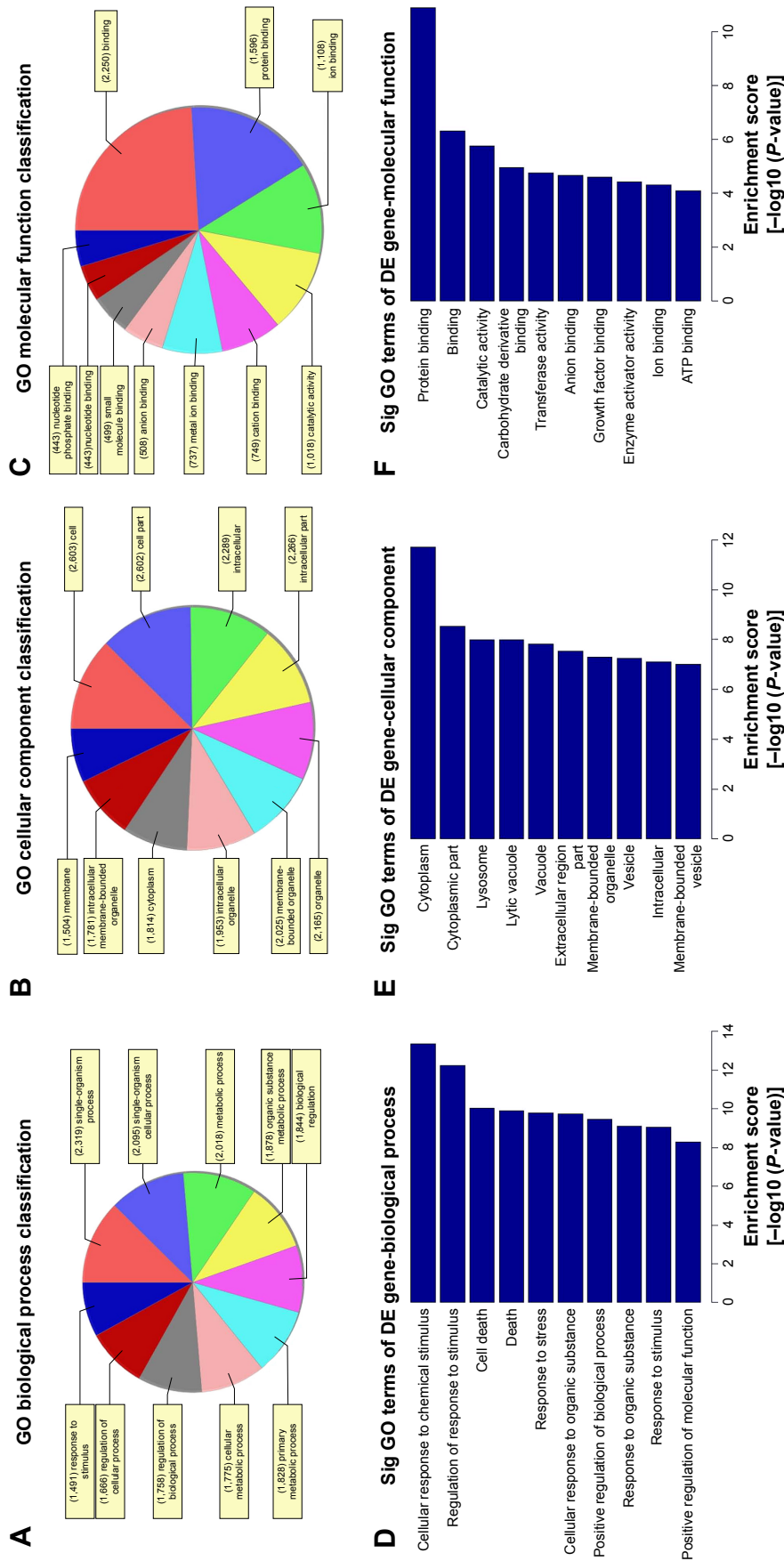


Figure 4 Gene ontology (GO) enrichment analysis of the differentially up-expressed mRNAs. (A) GO biological process classification for biological process of up-regulated mRNAs. (B) GO biological process classification for cellular components of differentially up-expressed mRNAs. (C) GO biological process classification for molecular function of differentially up-expressed mRNAs. (D) GO analysis indicates the top 10 counts of enrichment score in biological process of up-regulated mRNAs. (E) GO analysis indicates the top 10 counts of enrichment score in cellular components of up-regulated mRNAs. (F) GO analysis indicates the top 10 counts of enrichment score in molecular function of up-regulated mRNAs. **Abbreviation:** Sig, significant.

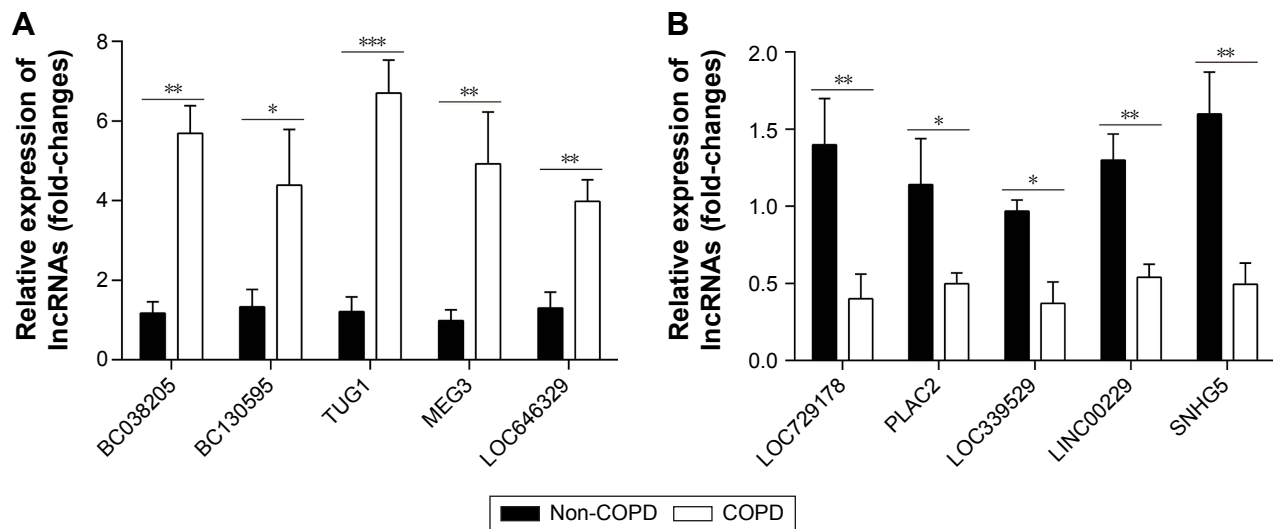


Figure 5 Ten differentially expressed lncRNAs are identified. **(A)** The relative expression levels of 10 differentially up-regulated lncRNAs in COPD lung tissues compared with non-COPD lung tissues (n=20) were measured by qRT-PCR. **(B)** The relative expression levels of 10 differentially down-regulated lncRNAs between COPD and non-COPD lung tissues (n=20) were detected by qRT-PCR. GAPDH served as the control for RNA loading (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Abbreviations: lncRNA, long noncoding RNA; qRT-PCR, quantitative real-time polymerase chain reaction.

Table 5 presents detailed information about significant differentially expressed lncRNAs in different diseases.

Silencing of TUG1 affects the proliferation ability in BEAS-2B and HFL1 cells after TGF- β pretreatment

To determine whether silencing of TUG1 expression by siRNA affected the proliferation of human lung cells, the cells were cultured with 50 μ M NC siRNAs or TUG1 siRNAs (si-TUG1) for 48 h. Transfection effect was detected by qRT-PCR. The expression level of lncRNA TUG1 decreased in BEAS-2B (Figure 6A) and HFL1 cells (Figure 6B) with TUG1 siRNAs. Additionally, to testify whether TGF- β affected the proliferation of human lung cells, cells were

treated with 2 ng/mL TGF- β for 48 h. Then growth rates were determined by Cell Counting Kit-8 (CCK-8) assay. The proliferation of BEAS-2B cells with TGF- β and si-TUG1 was markedly increased relative to BEAS-2B cells with TGF- β ($P < 0.05$). The proliferation of BEAS-2B cells with TGF- β was markedly suppressed compared with BEAS-2B cells ($*P < 0.05$) (Figure 6C). Similar results were observed in HFL1 cells (Figure 6D).

Silencing of TUG1 suppresses the expression levels of α -SMA and fibronectin proteins in BEAS-2B and HFL1 cells

Finally, to explore the expression levels of α -SMA and fibronectin proteins, we cultured BEAS-2B and HFL1

Table 5 Detailed information of significant differentially expressed lncRNAs in diseases

lncRNA	Chrom: txStart-txEnd	Disease types	References
BC038205	Chr11: 88157548–88161279	Glioblastoma multiforme, Alzheimer	56, 57
BC130595	Chr2: 211097415–211098702	Pancreatic cancer	58
TUG1	Chr22: 31365633–31375380	Non-small-cell lung cancer, bladder cancer, esophageal squamous cell carcinoma	29, 31, 59
MEG3	Chr14: 101323960–101327258	Hepatocellular cancer, cervical carcinoma, bladder cancer, gliomas	60–63
LOC646329	Chr7: 130565750–130598069		64
LOC729178	Chr6: 147182941–147453122		65
PLAC2	Chr19: 5558177–5568005		
LOC339529	Chr1: 244080703–244210619		66
LINC00229	Chr22: 45002207–45021299		
SNHG5	Chr6: 86386724–86388451	Gastric cancer, malignant melanoma, endometrial cancer	67–69

Abbreviations: lncRNA, long noncoding RNA; Chrom, chromosome.

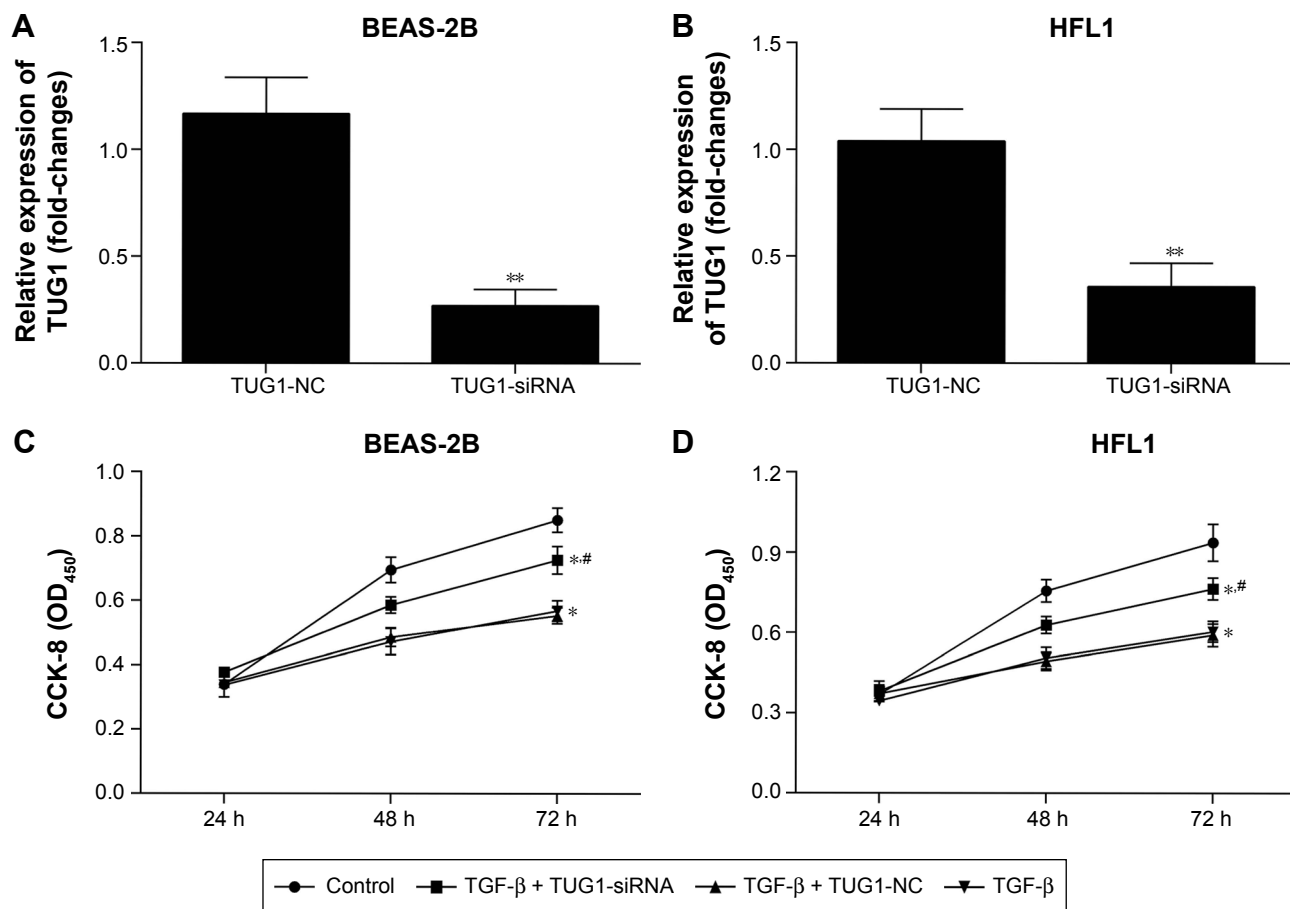


Figure 6 Silencing of TUG1 affects the proliferation ability of BEAS-2B and HFL1 cells after TGF- β pretreatment. **(A)** The relative expression levels of lncRNAs TUG1 in BEAS-2B cells were measured by qRT-PCR (** $P < 0.01$). **(B)** The relative expression levels of lncRNAs TUG1 in HFL1 cells were measured by qRT-PCR (** $P < 0.01$). **(C)** The capacity of proliferation was detected with CCK-8. About 50 μ M of NC siRNAs or TUG1 siRNAs (si-TUG1) were transfected into BEAS-2B cells for 48 h. Silencing of TUG1 expression by siRNA promoted the capacity to proliferate in BEAS-2B cells. TGF- β (2 ng/mL) was treated for 48 h. TGF- β inhibited the capacity to proliferate. # $P < 0.05$ as compared with BEAS-2B cells treated with 50 μ M NC siRNAs and TGF- β (2 ng/mL); * $P < 0.05$ as compared with BEAS-2B cells. **(D)** The capacity to proliferate was also detected using CCK-8 assay. Silencing of TUG1 expression by siRNA promoted the capacity of proliferation. TGF- β inhibited the capacity of proliferation in HFL1 cells. # $P < 0.05$ as compared with HFL1 cells treated with 50 μ M NC siRNAs and TGF- β (2 ng/mL); * $P < 0.05$ as compared with HFL1 cells.

Abbreviations: TGF- β , transforming growth factor β ; lncRNA, long noncoding RNA; qRT-PCR, quantitative real-time polymerase chain reaction; CCK, Cell Counting Kit; NC, negative control; OD, optical density.

cells. Some cells were treated with 50 μ M NC siRNAs or TUG1 siRNAs (si-TUG1) and 2 ng/mL TGF- β for 48 h, while others were treated with 2 ng/mL TGF- β for 48 h. Western blot assay was performed to detect the expression of α -SMA and fibronectin proteins, which was increased in TGF- β BEAS-2B cells compared with BEAS-2B cells. When BEAS-2B cells were transfected with TUG1 siRNAs and TGF- β for 48 h, the expression of α -SMA and fibronectin proteins was decreased relative to BEAS-2B cells with TGF- β and NC siRNAs. TGF- β promoted the expression of α -SMA and fibronectin proteins in BEAS-2B cells (Figure 7A). Similar results were obtained with HFL1 cells (Figure 7C). Relative quantities were defined as the ratio of the grayscale of α -SMA and fibronectin to that of GAPDH (Figures 7B and 7D).

Discussion

Currently, COPD is a primary cause of morbidity around the world and is associated with individualized development of chronic symptoms, airflow obstruction,^{36,37} and undermined health.³⁸ Even worse, it presents with acute episodes of symptom exacerbation.³⁹ Although studies related to clinical diagnoses, causes of development, and fundamental science have been conducted, the molecular mechanism underlying the development of COPD is still not fully known and effective treatment methods for COPD are urgently required. Therefore, in our study, the aim was to find potential and available biomarkers for the diagnosis and treatment of COPD.

lncRNAs are involved in various biological processes and participate in the process of RNA transcription, mRNA splicing, translation,^{11,40,41} and in the pathogenesis of numerous diseases.⁴²⁻⁴⁴ However, lncRNAs that regulate the pathogenetic

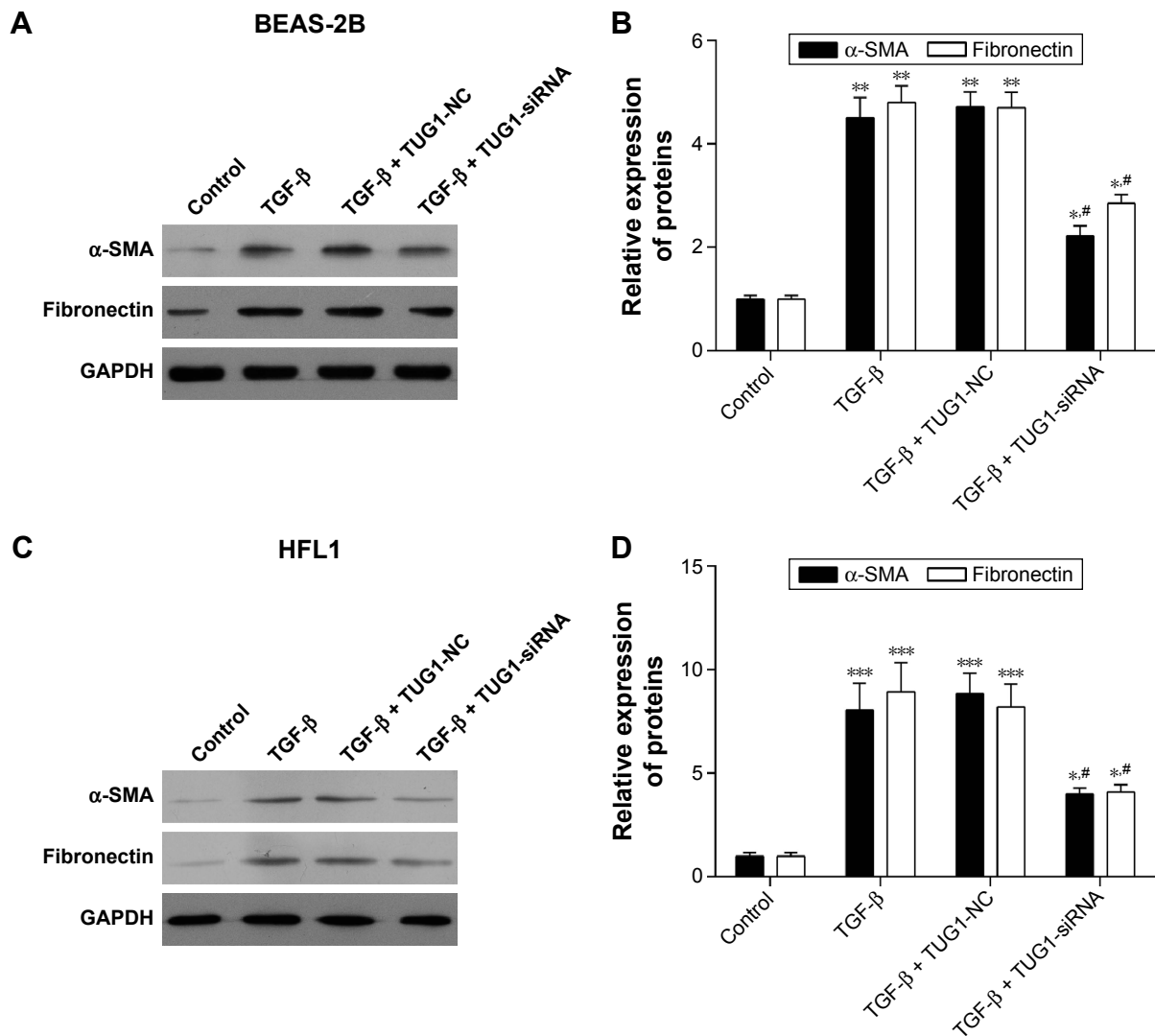


Figure 7 Silencing of TUG1 suppresses the expression levels of α -SMA and fibronectin proteins in BEAS-2B and HFL1 cells. **(A)** Silencing of TUG1 expression by siRNA inhibited the expression levels of α -SMA and fibronectin proteins, while TGF- β promoted the expression levels of α -SMA and fibronectin proteins. About 50 μ M negative control (NC) siRNAs or TUG1 siRNAs (si-TUG1) and 2 ng/mL TGF- β were transfected into BEAS-2B cells for 48 h. The expression levels of α -SMA and fibronectin proteins were detected by Western blot in BEAS-2B cells. **(B)** Gray statistical analysis of the expression levels of α -SMA and fibronectin proteins in BEAS-2B cells. $^{\#}P < 0.05$ as compared with BEAS-2B cells treated with 50 μ M NC siRNAs and TGF- β (2 ng/mL); $^*P < 0.05$ as compared with BEAS-2B cells. $^{**}P < 0.01$. **(C)** The expression levels of α -SMA and fibronectin proteins were detected by Western blot in HFL1 cells treated as mentioned earlier. GAPDH was used for internal control. **(D)** Gray statistical analysis of the expression levels of α -SMA and fibronectin proteins in HFL1 cells. $^{\#}P < 0.05$ as compared with HFL1 cells treated with 50 μ M NC siRNAs and TGF- β (2 ng/mL); $^*P < 0.05$ as compared with HFL1 cells. $^{***}P < 0.001$.

mechanism of COPD are unclear at present. Microarray analysis has been suggested as an available and useful tool to detect molecular mechanisms and find potential biomarkers. Therefore, we studied the differential expression of lncRNAs between COPD and non-COPD lung tissues. According to the results of our microarray analysis, we detected the differential expression of 25,628 lncRNAs and 20,106 mRNAs between COPD and non-COPD lung tissues. Among them, the expression of 8,376 (32.7%) lncRNAs and 5,049 (25.1%) mRNAs was significant (fold-change > 2.0 or < 0.5 ; $P < 0.05$).

Based on the analysis of genomic locus and the correlation with protein-coding genes among differentially expressed lncRNAs, we found 3,249 (38.8%) intergenic lncRNAs,

2,239 (26.7%) natural antisense lncRNAs, 1,059 (12.6%) exon sense-overlapping lncRNAs, 974 (11.6%) intronic antisense lncRNAs, 375 (4.5%) intron sense-overlapping lncRNAs, 371 (4.4%) bidirectional lncRNAs, and 109 (1.3%) nonoverlapping antisense lncRNAs. Therefore, most lncRNAs are intergenic (no overlapping or bidirectional coding transcripts nearby the lncRNA), and TUG1 also belongs to the class of intergenic lncRNAs.

Moreover, microarray assay also includes the pathway analysis and GO enrichment analysis based on differentially expressed mRNAs. Previous studies have shown that COPD is related to lysosome,⁴⁵ *Staphylococcus aureus* infection,⁴⁶ osteoclast differentiation,⁴⁷ leishmaniasis,⁴⁸

dilated cardiomyopathy,⁴⁹ rap1 signaling pathway,⁵⁰ and PPAR signaling pathway,⁵¹ which are concordant with our KEGG pathway results in patients with COPD. In addition, we found 1,115 up-regulated and 346 down-regulated GO terms in biological process, 127 up-regulated and 34 down-regulated GO terms in cellular component, and 115 up-regulated and 53 down-regulated GO terms in molecular function between COPD lung tissues and non-COPD lung tissues according to the GO analysis results. Data regarding other COPD-related pathways and genes correlated with biological process, cellular component, and molecular function can provide a lot of information for the study of COPD.

Furthermore, we selected up-regulated lncRNAs (BC038205, BC130595, TUG1, MEG3, and LOC646329) and down-regulated lncRNAs (LOC729178, PLAC2, LOC339529, LINC00229, and SNHG5) which were identified by qRT-PCR. The results were consistent with those obtained by microarray analysis. Specifically, studies have shown that lncRNA TUG1, induced by p53, inhibited specific genes related to cell-cycle regulation;⁵² silencing of lncRNA TUG1 suppressed retinal development in a developing mouse eye.²⁸ Studies have shown that lncRNA TUG1 was highly expressed in hepatocellular carcinoma⁵³ and urothelial carcinoma of the bladder.³¹ In our study, silencing of TUG1 by siRNA markedly increased the ability of proliferation in BEAS-2B and HFL1 cells. Results indicated that TUG1 deletion decreased the expression levels of α -SMA and fibronectin proteins which are mesenchymal markers in BEAS-2B and HFL1 cells.

TGF- β induces various cellular responses, including differentiation, inflammation, proliferation, and apoptosis, and plays a vital role in COPD, asthma, cancer, and other pathological processes.⁵⁴ Study showed that TGF- β enhanced extracellular matrix synthesis to influence expression of the fibrogenic cytokine in numerous tissues.⁵⁵ In our study, BEAS-2B and HFL1 cells were induced by TGF- β for 48 h. The ability of proliferation and expression of α -SMA and fibronectin proteins were analyzed. Results showed that TGF- β markedly inhibited the ability to proliferate and clearly promoted the expression of α -SMA and fibronectin proteins in BEAS-2B and HFL1 cells.

Conclusion

In summary, our study involving microarray analysis comprehensively indicated the expression levels of lncRNAs in COPD and non-COPD lung tissues. The target prediction of lncRNA and potential functional characteristics will help in exploring the potential effect in patients with COPD. KEGG pathway and GO enrichment analysis may help in exploring potential genomic regulatory networks and offer more new

potential therapeutic targets. Furthermore, relevant research with regard to function and mechanism could make lncRNA TUG1 a potential diagnostic marker in patients with COPD.

Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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