The role and mechanism of lncRNA NEAT1 in the fibrosis of pulmonary epithelial cell

Hui Xu1 · YanBo Chen2 · Jinqiang Zhuang1 · Shun Zhu1 · Bing Xu3 · Jiang Hong1

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Abstract
Background Pulmonary fibrosis is a serious clinical fatal disease. Epithelial–mesenchymal transition (EMT) and lncRNA NEAT1 have been implied in its development and progression.
Objective To study the role of lncRNA NEAT1 in the progression of fibrosis in human pulmonary epithelial cells (BEAS-2B). Specifically, BEAS-2B was transfected with NEAT1 and miR-29c, EMT and cell proliferation were measured and the expression level of relevant genes was determined by Western blot.
Result Results showed that NEAT1 promotes fibrosis and proliferation of BEAS-2B cells via the up-regulation of α-SMA, Vimentin, Snail and proliferation-related genes including Cyclin D1 and Cyclin E; miR-29c is a target gene of NEAT1 and through which NEAT1 regulates EMT and expression of proliferation-related genes.
Conclusion This study investigated the mechanism of pulmonary fibrosis progression by elucidating the role of NEAT1/miR-29c in the fibrosis and proliferation of BEAS-2B cells, thus providing a basis for the new therapeutic targets of pulmonary fibrosis.

Keywords lncRNA NEAT1 · Fibrosis · Epithelial–mesenchymal transition · Proliferation · miR-29c

Introduction
Pulmonary fibrosis is a serious clinical fatal disease with limited effective drugs. The mechanism of its pathogenesis and progression is complicated. Nonetheless, it is generally believed that epithelial–mesenchymal transition (EMT) plays an important role in the development and progression of pulmonary fibrosis (Horowitz and Thannickal 2006; Rout-Pitt et al. 2018). Furthermore, the activation of several signaling pathways such as transforming growth factor-beta (TGF-beta) and Wnt/beta-catenin signaling pathways were suggested to play an important role in the EMT process (Willis and Borok 2007; Saito et al. 2018; Juan et al. 2017). Therefore, the investigation of EMT and its participating factors may provide new approaches for targeted treatment of pulmonary fibrosis.

Long non-coding RNAs (lncRNA) are a class of long RNA transcripts without protein-coding ability (Moran et al. 2012; Lee et al. 2019). In recent years, lncRNA has been found to be involved in the biological processes of many diseases by regulating a variety of cell processes such as cell cycle, apoptosis, metabolism, and EMT (Zhang et al. 2019; Guo et al. 2019). Naturally, aberrantly expressed lncRNA was thought to be one of the important biomarkers for various diseases including pulmonary fibrosis. For example, Sun found that overexpressed lncRNA uc.77 and 2700086A05Rik were involved in the pathogenesis of idiopathic pulmonary fibrosis via its regulation on EMT (Sun et al. 2016). And Song explored the role of lncRNA MRAK088388 and MRAK081523 and the corresponding mechanisms involved in pulmonary fibrosis (Song...
et al. 2014). Studies have also shown that lncRNA NEAT1 accelerated the progression of liver fibrosis by modulating miR-122 and Kruppel-like factor 6 (Yu et al. 2017), which, however, has not been validated in the progression of pulmonary fibrosis. Therefore, in this study, we investigated the role of lncRNA NEAT1 in the development and progression of pulmonary fibrosis in vitro, as well as its underlying mechanisms.

Materials and methods

Cell culture and treatment

Human normal pulmonary epithelial cells (BEAS-2B) were purchased from FuHeng Cell Center, Shanghai, China, and cultured using LHC-8 (Gibco) medium at 37 °C with 5% CO2. According to the method used by Liu et al. (2017a), the pulmonary fibrosis cell model was established by inducing the cells with TGF-β1 at a concentration of 5 ng/ml for 48 h.

Transfection

Transfections of the NEAT1 expression plasmid, NEAT1 siRNA, miR-29c mimics and miR-29c inhibitor were performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific). Specifically, the full-length NEAT1 sequence was inserted into the pcDNA3.1 vector for construction of NEAT1 expression plasmid and the target sequence of the NEAT1 siRNA was: 5'-GUG AGA AGU UGC UUA GAA ACU UUC C-3' (Wang et al. 2016).

qRT-PCR

Total RNA was extracted using the RNAiso Plus kit (Takara). Reverse transcription of RNA was performed using TM RT Master Mix (Takara), and qPCR was performed using SYBR Premix Ex Taq II (Takara). The qRT-PCR reaction was performed on an ABI 7900 platform (Applied Biosystems). mRNA expression was determined with β-actin as an internal reference and the relative expression level of the target gene was calculated using the \(2^{-\Delta\Delta CT} \) method. Primer sequences are as follows: NEAT1-F: TTGTTCAG AGCCCATGAT; NEAT1-R: TGAAAACCTTACCACG GA; β-actin-F: GGCTCCGGCATGTGCAAG; β-actin-R: CCTCGGTCAGCAGCACGG.

Cell proliferation measured with CCK-8 assay

BEAS-2B cells were seeded in 96-well plates for treatment and transfection. The cell proliferation was determined with the CCK8 kit (Dojindo, Japan) at three different time points (24 h, 48 h, and 72 h) after the transfection where the absorbance at 450 nm was measured by a microplate reader.

Western blot

The total protein was extracted from cells with RIPA lysis buffer and the protein concentration was determined using a BCA kit. 20 µg of protein was loaded into the SDS-PAGE gel for electrophoresis, and the protein was then electrophoresed into the PVDF membrane for blocking and subsequent primary and secondary antibody incubation. Exposure and capture of images were conducted using a chemiluminescent imaging system (Clinx Science Instruments). Primary antibodies were purchased from Abcam and beta-actin was used as an internal reference. The antibodies used in this experiment were: α-SMA (1:1000, ab32575, Abcam); Vimentin (1:1000, ab92547, Abcam); Snail (1:1000, ab53519, Abcam); Cyclin D1 (1:2000, ab16663, Abcam); Cyclin E (1:500, ab133266, Abcam); β-actin (1:2000, ab8226, Abcam).

Construction of dual-luciferase reporter system

The oligonucleotide (wt) containing the NEAT1 target sequence and its mutant sequence (mut) without the miR-29c-binding site was amplified and cloned into the pmirGLO vector (luciferase reporter plasmid). After that, the luciferase reporter plasmid and miR-29c mimics were co-transfected into BEAS-2B cells. Luciferase activity was then measured using the dual-luciferase reporter assay system (Promega) according to the instructions.

Statistical analysis

Data were analyzed using SPSS 18. 0 software and expressed as mean ± SD. \( p < 0.05 \) was considered statistically significant.

Results

NEAT1 promoted fibrosis and proliferation of BEAS-2B cells

First, we transfected BEAS-2B cells with NEAT1 overexpression plasmid and siRNA separately and verified by qRT-PCR to confirm the validity of overexpression plasmid (Fig. 1a) and siRNA (Fig. 1b). Following that, we then transfected NEAT1 siRNA in TGF-β1-induced BEAS-2B cells, transfected BEAS-2B cells with NEAT1 overexpression plasmid, and cell morphology was observed under a microscope. The results showed that NEAT1 siRNA was able to reverse TGF-β1-induced BEAS-2B
cell fibrosis (Fig. 1c), whereas overexpression of NEAT1 promoted BEAS-2B cell fibrosis (Fig. 1d). At the same time, the CCK8 assay demonstrated that NEAT1 siRNA inhibited the proliferation of TGF-β1-treated BEAS-2B cells \((p < 0.05)\) (Fig. 1e), while overexpression of NEAT1 promoted the proliferation of BEAS-2B cells \((p < 0.05)\) (Fig. 1f). Taken together, these results indicated that NEAT1 promoted the fibrosis and proliferation of BEAS-2B cells.
NEAT1 regulated the expression of EMT- and proliferation-related genes in BEAS-2B cells

With the Western blot analysis, we found that TGF-β1 induced the up-regulation of α-SMA, Vimentin, Snail and proliferation-related genes such as Cyclin D1 and Cyclin E, while NEAT1 siRNA could reverse the up-regulation of these genes (Fig. 2). Not surprisingly, overexpression of NEAT1 promoted the up-regulation of these genes (Fig. 2). These results indicated that NEAT1 promotes the fibrosis and proliferation of BEAS-2B cells by promoting the expression of EMT- and proliferation-related genes.

miR-29c is the target gene of NEAT1

Using the StarBase database, we found that miR-29c is a potential target for NEAT1 (Fig. 3). Luciferase experimental results showed that overexpression of miR-29c inhibited the luciferase activity of the wt reporter plasmid and had no
effect on the luciferase activity of the mut reporter plasmid (Fig. 3), indicating that miR-29c is a target gene of NEAT1.

**NEAT1 regulated BEAS-2B cell fibrosis and proliferation via miR-29c**

Recovery experiments showed that miR-29c inhibitor reversed the inhibition of fibrosis and proliferation induced by NEAT1 siRNA in BEAS-2B cells (Fig. 4a, b) \((p < 0.05)\); on the contrary, miR-29c mimics reversed the fibrosis and proliferation-promoting effects induced by overexpression of NEAT1 (Fig. 4c, d) \((p < 0.05)\). These results indicated that NEAT1 regulated BEAS-2B cell fibrosis and proliferation via miR-29c.

**NEAT1 regulated EMT- and proliferation-related genes through miR-29c**

Similarly, the recovery experiments also showed that miR-29c inhibitor reversed the down-regulation of EMT- and proliferation-related genes induced by NEAT1 siRNA in BEAS-2B cells (Fig. 5a), whereas miR-29c mimics reversed the up-regulation of EMT- and proliferation-related genes caused by overexpression of NEAT1 (Fig. 5b). These lines of evidence indicated that NEAT1 regulated EMT- and proliferation-related genes through miR-29c.

**Discussion**

LncRNA and miRNA are hot topics in recent years. Compared with miRNA, there are relatively few studies on lncRNA and, therefore, many functions are still poorly understood. Nonetheless, studies have shown that lncRNA is actually involved in the progression of many diseases (Neil et al. 2019; Prinz et al. 2019). LncRNA NEAT1 has been implicated in various tumors. For example, HIF-2α-activated lncRNA NEAT1 promoted the invasion and metastasis of hepatocellular carcinoma cells by affecting EMT (Zheng et al. 2018); the RGFR pathway regulated lncRNA NEAT1 and promoted the progression of glioblastoma by regulating EZH2 and Wnt/β-catenin pathways (Chen et al. 2017). Moreover, NEAT1 was reported to be involved in liver fibrosis (Yu et al. 2017) and renal fibrosis (Huang et al. 2019); however, there is only few research on NEAT1 in pulmonary fibrosis. Therefore, in this regard, our research is both innovative and theoretically founded.
Pulmonary fibrosis is a serious disease characterized by fibroblast proliferation, and EMT is one of the main sources of fibroblasts. During this process, epithelial cells lose their epithelial phenotype, acquire fibroblast-like properties, and exhibit decreased cell adhesion and increased motility. At the molecular level, this process is accompanied by an increase of markers for pulmonary fibrosis, such as α-SMA, Vimentin, and Snail (Liu et al. 2017b). The results of this study indicated that NEAT1 promoted fibrosis and proliferation of BEAS-2B cells, which was due to the up-regulation of EMT-related genes including α-SMA, Vimentin, and Snail and proliferation-related genes such as Cyclin D1 and Cyclin E. In other words, we believe that NEAT1 can promote the progression of pulmonary fibrosis.

LncRNA can be involved in related physiological and pathological processes by acting as a ceRNA for microRNA (Cui and Zhao 2019; Huang et al. 2019). In this study, we found that miR-29c is the target gene of NEAT1. Xie et al. (2017) found that miR-29c could prevent pulmonary fibrosis by regulating epithelial cell renewal and apoptosis; a study by Matsushima and Ishiyama (2016) showed that microRNA-29c regulated apoptosis by modulating the cell surface death receptor Fas of lung fibroblasts, which in turn inhibiting pulmonary fibrosis. Therefore, in these studies, miR-29c acted as an inhibitor in the process of pulmonary fibrosis. Our results demonstrated that NEAT1 regulated the expression of EMT- and proliferation-related genes through miR-29c to affect the fibrosis and proliferation of BEAS-2B cells, which substantiated the miR-29c regulation network in pulmonary fibrosis.

In summary, our study showed that NEAT1 affected the fibrosis and proliferation of BEAS-2B cells by regulating the expression of EMT- and proliferation-related genes through its target gene miR-29c, which in turn, rendering NEAT1 as a potential target for the treatment of pulmonary fibrosis.
Author contribution Bing Xu and Jiang Hong designed experiments; Hui Xu and YanBo Chen carried out experiments and wrote the manuscript; Jinqiang Zhuang and Shun Zhu analyzed experimental results.

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Compliance with ethical standards

We confirmed that all methods in our study were performed in accordance with the relevant guidelines of CONSORT 2010.

Conflict of interest The authors declare that they have no conflict of interest.

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