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LncRNA NEAT1 regulates pulmonary fibrosis through miR-9-5p and TGF-β signaling pathway

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Abstract. – OBJECTIVE: Pulmonary fibrosis (PF) is a chronic lung disease with complex pathogenesis and poor prognosis. Studies had demonstrated that long non-coding RNAs (lncRNAs) play an important role in the development of fibrosis. We explored the roles of NEAT1 in PF progression in this study.

PATIENTS AND METHODS: PF tissues and TGF-β1-induced cells were analyzed for the function of NEAT1 in PF progression. qRT-PCR or Western blot was applied to detect NEAT1, miR-9-5p or protein expressions. PF mice model assay was used to detect the effects of NEAT1 on PF in vivo. Luciferase reporter assay was applied to confirm target relationship between NEAT1 and miR-9-5p. Correlation of NEAT1 and miR-9-5p was analyzed by Spearman's method.

RESULTS: We observed that NEAT1 was significantly upregulated while miR-9-5p was downregulated in PF tissues and TGF-β1-induced cells. A negative correlation was exhibited of NEAT1 and miR-9-5p expression in PF tissues. Protein level of p-Smad2 was increased in TGF-β1 induced cells. Furthermore, NEAT1 knockdown increased E-cadherin expression, while decreased N-cadherin, Vimentin, Collagen I, Collagen III and α-smooth muscle actin (α-SMA) expressions in TGF-β1-induced cells. Moreover, NEAT1 could directly target miR-9-5p to regulate the PF induced by TGF-β1. The miR-9-5p overexpression inhibited TGF-β1 and p-Smad2 expression, while NEAT1 overexpression attenuated this effect. In addition, NEAT1 inhibition enhanced E-cadherin expression, and reduced TGF-β1, p-Smad2, N-cadherin, Collagen I, Collagen III, α-SMA and Vimentin expression after BLM treatment.

CONCLUSIONS: Taken together, our findings showed that NEAT1 knockdown attenuated PF via the regulatory of miR-9-5p and TGF-β signaling to repress EMT and might provide new therapeutic targets for PF patients.

Key Words:
NEAT1, MiR-9-5p, TGF-β signaling pathway, Pulmonary fibrosis.

Introduction

Pulmonary fibrosis (PF) is a large group of end-stage changes of lung diseases characterized by fibroblast proliferation and accumulation of a large number of extracellular matrix accompanied by inflammatory damage and tissue structure destruction[1,2]. Most patients with PF are idiopathic in nature, thus the idiopathic pulmonary fibrosis (IPF) which known as "tumor-like disease" with a poor prognosis is considered to be the most common and severe form of PF[3]. The incidence and mortality of IPF are increasing year by year, and the average survival time after diagnosis is less than 3 years[2]. The common treatments (steroid and immunosuppressive therapy) of PF are not very effective, and there is no effective treatment of PF by the reason of our limited understanding of pathology in the fibrosis disease[4]. Therefore, it is urgent to investigate the potential molecular mechanisms of PF progression to afford new effective treatments for patients.

In recent years, lncRNAs have become the star molecules in the study of many diseases. LncRNAs are a class of RNA with more than 200 nucleotides without protein-coding function, and participate in regulating diverse processes in cells, including gene expression and various biological processes[5]. LncRNA was closely related to fibrosis disease in lung, liver,

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kidney and myocardium[6] and they are considered to be new therapeutic targets in fibrosis because of functional regulation in the fibrosis pathology[6,7]. Several lncRNAs were associated with PF. Cao et al[8] analyzed the differentially expressed lncRNAs in bleomycin-induced PF of rat, and a total of 568 differentially expressed lncRNAs were found using microarrays. In the same way, two lncRNAs MRAK088388 and MRAK081523 located in the interstitial lung cell cytoplasm could function as ceRNAs of miR-29b-3p and let-7i-5p to regulate expressions of N4bp2 and Plxna4[9]. Other lncRNAs, such as CHRF[10], H19[11], MALAT1[12] and DNM3[13] had been confirmed to be related to PF and they may become the potential therapeutic targets in the future.

NEAT1, a novel cancer-related lncRNA, is required for paraspeckle formation[14,15]. NEAT1 has been considered as biomarkers of cancer diagnosis and prognosis[14]. NEAT1 upregulation could promote cell proliferation and invasion, and suppress apoptosis, thus contributing to tumorigenesis[14]. High NEAT1 expression level was involved in lung cancer progression and poor prognosis[16]. Nevertheless, the function of NEAT1 in PF has not been reported and needs to be explored. Here, we aimed to investigate NEAT1 roles in PF progression.

Patients and Methods

Tissue Specimens

20 cases of human PF tissue and 20 cases of normal lung tissue were gathered from pathology department of Xuzhou Municipal Hospital. The Ethics Committee of the Xuzhou Municipal Hospital approved this study. Informed consent was written by all participants. This investigation was conducted in accordance with the Declaration of Helsinki.

Cell Culture, Transfection and the Construction of PF Cell Model

Human lung adenocarcinoma cells A549 and human bronchial epithelium (HBE) cells which obtained from Chinese Academy of Sciences in Shanghai were cultured as specified. The si-NEAT1, miR-9-5p mimic and corresponding controls were obtained from GenePharma Company (Shanghai, China). All oligonucleotides were transfected by Lipofectamine 2000 Reagent (Invitrogen; Carlsbad, CA, USA). After transfection, cells were treated with 5 ng/ml TGF-β1 (10 ng/ml, PeproTech, Rocky Hill, NJ, USA) 48 h to induce fibrosis. Then, each group of cells was used in subsequent experiments.

Establishment of Animal PF Model

A total of 35 male C57BL/6 mice (aged 7-8 weeks, 20-22 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd and kept in strict specific pathogen-free conditions. Establishment of animal PF model was performed with 1.5 U/kg bleomycin (BLM, Sigma Chemicals, St. Louis, MO, USA) by single intratracheal instillation as previously described[17]. Briefly, the mice were randomly divided into Control group (the mice were treated with 0.05 ml sterile saline), BLM group (the mice were given 0.05 ml sterile saline containing BLM), BLM+sh-NC group (after BLM treatment, sh-NC was injected into mice) and BLM+sh-NEAT1 group (after BLM treatment, sh-NEAT1 was injected into mice). All mice were euthanized after 4 weeks, and samples including blood and tissue were collected for analyzing of subsequent experiments. All animal procedures were approved by the Ethics Committee of the Xuzhou Municipal Hospital and conducted in accordance with the Declaration of Helsinki.

Enzyme-Linked Immunosorbent Assay

Samples were collected and lysed with lysis buffer, and then there were centrifuged. The protein level of TGF-β1 (mice serum) were determined using ELISA kit (Sigma-Aldrich, St. Louis, MO, USA) according to instruction. The absorbance at 492 nm was measured by a microplate reader.

Haematoxylin And Eosin Staining

The lung tissues were fixed with 10% paraformaldehyde solution and embedded in paraffin, and then were cut into 4 μm sections. Sections were baked at 60°C for 2 hours. Then, sections were dewaxed in xylene for twice, and followed by immersion successively in gradient of ethanol for dehydration. Subsequently, sections were stained with haematoxylin, differentiated in 1% hydrochloric acid-ethanol, and counterstained with eosin after dehydration. Finally, dehydrated and mounted with neutral resin were performed. Pathomorphological observation was applied with light microscope (Olympus Corporation, Tokyo, Japan).

Masson Staining

Lung tissues were made into paraffin section and treated (including baking, dewaxing and dehydration) as previously described in HE staining. The sections were stained with Weiger hematoxylin for 10 min and differentiated in 1% hydrochloric acid-ethanol for 1 min and then dehydrated. Subsequently, the sections were stained with acid fuchsin reagent, and differentiated with 1% phosphomolybdic acid. Then, they were counterstained with aniline blue or green liquor. Sections were dehydrated and mounted with neutral resin. Finally, we observed the sections with a microscope.

Luciferase Reporter Assay

Luciferase reporter vector of NEAT1 contained the wild-type and mutated-type (NEAT1-wt and NEAT1-mut) were obtained from Shanghai GeneChem (Shanghai, China). Cell lines were co-transfected with reporter vector and miR-9-5p mimics or NC. Luciferase activity was measured by the Dual-Luciferase Reporter Assay kit (Promega Corporation, Madison, WI, USA) at 48 h after transfection.

Quantitative Real Time PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed with reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). The qRT-PCR was performed on LightCycler 480 (Roche Applied Science, Mannheim, Germany). The relative levels of RNAs were assessed by $2^{-\Delta\Delta CT}$ method. U6 or GAPDH as internal control. All primers are listed as follows in Table I.

Western Blot

Total proteins were extracted using radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) with PMSF and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The bands were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, membranes were probed with primary antibodies (TGF-β1, Smad2, p-Smad2, E-cadherin, N-cadherin, α-SMA, Vimentin, Collagen I, Collagen III and β-actin antibodies) at 4°C overnight after blocked. Then, they were incubated with secondary antibodies. Bands were visualized after incubation with a chromogenic substrate using ECL prime kit (Invitrogen, Carlsbad, CA, USA) with Image Lab analysis software (Bio-Rad, Hercules, CA, UAS).

Table I. Primer sequences for fluorescence quantification Real-Time PCR.

Gene name	Primer sequences (5′-3′)
GAPDH	F ACGCTGCATGTGTCCTTAG
	R GAGCCTCTTATAGCTGTTTG
U6	F CTCGCTTCGGCAGCACA
	R AACGCTTCACGAATTTGCGT
NEAT1	F CAGGGTGTCCTCCACCTTTA
	R AAACCAGCAGACCCTTTTT
miR-9-5p	F GTGCAGGGTCCGAGGT
	R GCGCTCTTTGGTTATCTAGC
TGF-β1	F GAAACCCACAACGAAATCTATGAC
	R ACGTGCTGCTCCACTTTTAACT

Statistical Analysis

Data were expressed as means ± SD. The correlation of MALAT1 and miR-9-5p was assessed using Spearman's method. The comparisons between groups were performed by Student's *t*-test or Tukey-Kramer Post-Hoc test after one-way analysis of variance (ANOVA). The $p < 0.05$ were considered statistically significant.

Results

NEAT1 and MiR-9-5p Expressions in PF Tissues

20 pulmonary fibrotic tissues and 20 normal lung tissues were introduced to analyze NEAT1 and miR-9-5p expressions by qRT-PCR (Figure 1A). We observed that NEAT1 was upregulated while miR-9-5p was downregulated in PF tissues *vs.* normal tissues (Figure 1B and C). Moreover, Spearman's correlation analysis indicated that NEAT1 and miR-9-5p levels exhibited an inverse correlation in PF tissues (Figure 1D).

NEAT1 and MiR-9-5p Expressions in Cells After Treated by TGF-β1

After HBE and A549 cells exposed in TGF-β1 which could induce fibrosis *in vitro* for 48 h, NEAT1 as well as miR-9-5p expressions were analyzed by qRT-PCR in those cells. Either in HBE or A549 cells, TGF-β1 treatment could enhance NEAT1 level while reducing miR-9-5p level (Figure 2A and B). Furthermore, in which way TGF-β1 treatment affects Smad2 expression was detected by Western blot assays. It is observed that TGF-β1 treatment could increase p-Smad2 expression level while had no effect on Smad2 expression both in HBE and A549 cells

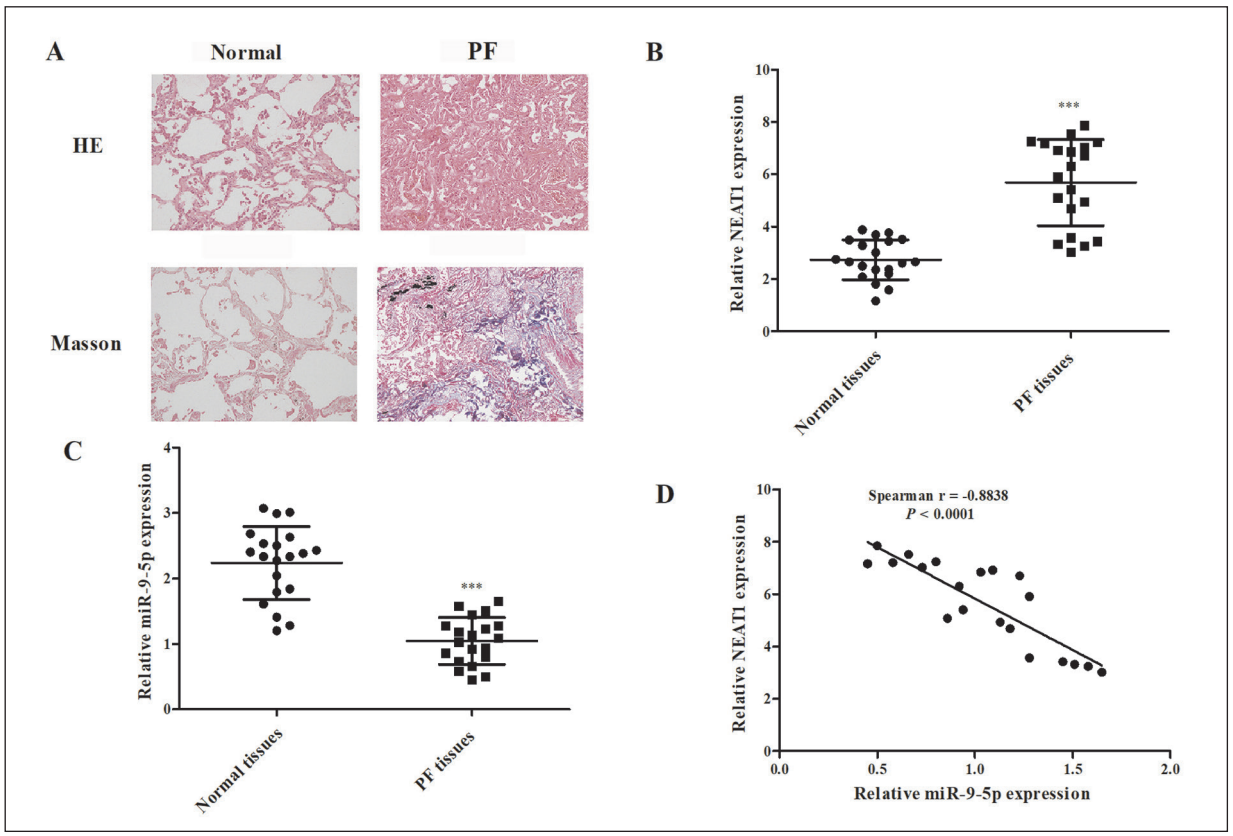

Figure 1. Expressions of NEAT1 and miR-9-5p were examined in PF tissues. **A,** HE and Masson staining of normal and PF tissues (200X). **B,** Expression of NEAT1 in tissues samples. **C,** MiR-9-5p expression in PF tissues. **D,** The correlation between NEAT1 and miR-9-5p expression in PF tissues. ***$p < 0.001$, compared with Normal tissues.

(Figure 2C). Collectively, findings proposed that NEAT1 and miR-9-5p might be associated with PF *in vitro*.

Knockdown of NEAT1 Inhibits PF in Vitro

To further explore NEAT1 roles in PF, si-NC or si-NEAT1 was transfected into cells to reduce the expression of NEAT1 (Figure 3A). Epithelial-mesenchymal transition (EMT) which is manifested as transformation of epithelial cells into stromal cells is considered to be a common cause of PF, so we further explored the effects of NEAT1 knockdown on PF by the detection of the epithelial cell marker E-cadherin, and interstitial and fibrosis markers including N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III expressions[18]. Results showed that NEAT1 knockdown decreased TGF-β1-induced N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III expression while E-cadherin expression increased both in cells (Figure 3B and C). Collectively, above results suggest that NEAT1 knockdown suppresses PF *in vitro*.

MiR-9-5p Directly Binds to NEAT1

To explore the regulator mechanisms of NEAT1 and miR-9-5p, StarBase v3.0 was used to predict details, and the binding sites were shown in Figure 4A. Luciferase reporter plasmids which contained NEAT1 sequence binding sites of miR-9-5p were constructed and then transfected into cells with miR-NC or miR-9-5p mimics respectively. Luciferase activity in NEAT1-wt group was notably induced by miR-9-5p mimics versus other groups (Figure 4B and C). Furthermore, miR-9-5p was down-regulated by NEAT1 overexpression, while up-regulated by NEAT1 inhibition (Figure 4D and E). Results proposed that miR-9-5p directly interacted with NEAT1 and negatively regulated by NEAT1. To explore whether NEAT1 affected PF progression through targeting miR-9-5p, pc-NEAT1 or pc-NC was transfected into cells with miR-NC or miR-9-5p mimics. After all cells were

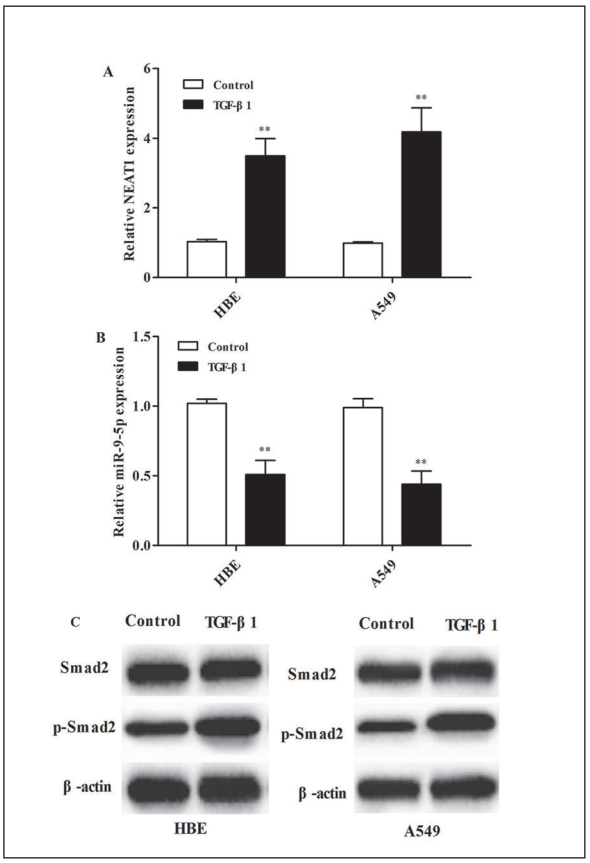

Figure 2. NEAT1, miR-9-5p and Smad2 expression in TGF-β1-induced cells. **A,** NEAT1 expression in cells after TGF-β1 treated. **B,** MiR-9-5p expression in cells after TGF-β1 treated. **C,** Smad2 expression in cells after TGF-β1 treated. $^{**}p < 0.01$, compared with Control group.

treated by TGF-β1 (10 ng/ml) 48 h, E-cadherin, N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III expressions were measured. It observed that the miR-9-5p mimics could increase E-cadherin expression and suppress N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III expression, while pc-NEAT1 attenuated these effects (Figure 4F and G). Combined with these results, we proposed NEAT1 could contribute to PF by targeting miR-9-5p *in vitro*.

NEAT1 Regulates TGF-β Pathway

PF could be ameliorated through negative regulation of TGF-β signaling pathway by miR-9-5p, so we further investigated whether NEAT1 could regulate the TGF-β pathway in PF cells[19]. We investigated the effects of alteration in NEAT1 and miR-9-5p expression on TGF-β1 and p-Smad2, which are considered as important factors in the TGF-β pathway. Of note, miR-9-5p mimics markedly decreased TGF-β1 expression, while pc-NEAT1 relieved the effects (Figure 5A-D). Besides, the effects of NEAT1 and miR-9-5p on protein expression of p-Smad2 were similar to TGF-β1 (Figure 5C and D). In general, NEAT1 targeted miR-9-5p and regulated the TGF-β pathway in PF.

NEAT1 Knockdown Inhibits PF Via the TGF-β Pathway In Vivo

Finally, we sought to further function of NEAT1 in PF, PF mice models were established

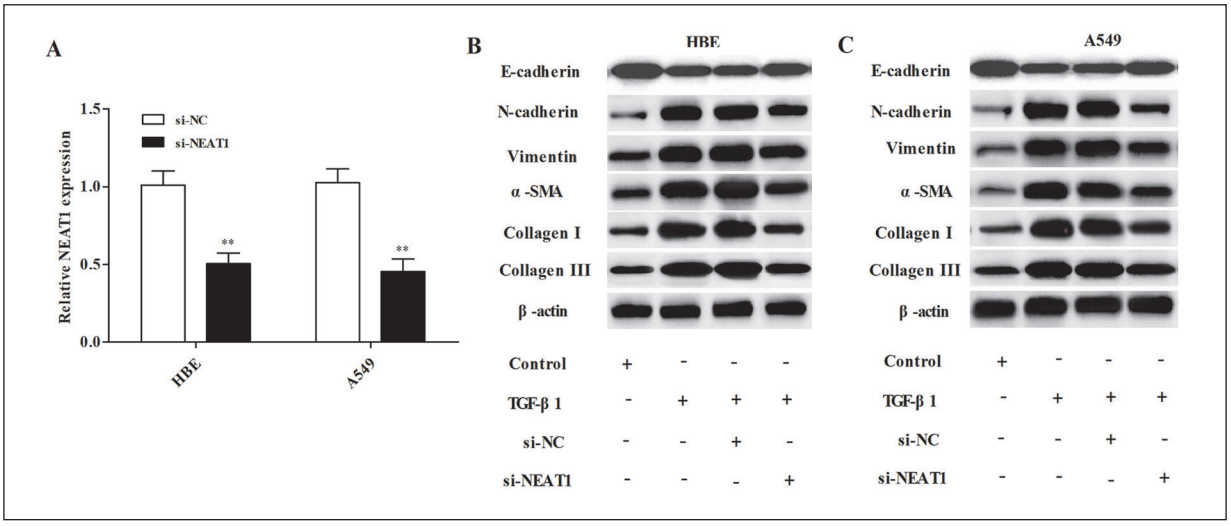

Figure 3. NEAT1 knockdown ameliorates PF *in vitro*. **A,** NEAT1 expression in TGF-β1-induced cells after si-NEAT1 transfected. **B, C,** The effect of NEAT1 knockdown on expressions of epithelial cell marker E-cadherin, and interstitial and fibrosis markers including N-cadherin, Vimentin, α-smooth muscle actin (α-SMA), Collagen I and Collagen III in TGF-β1-induced cells. $^{**}p < 0.01$, compared with si-NC group.

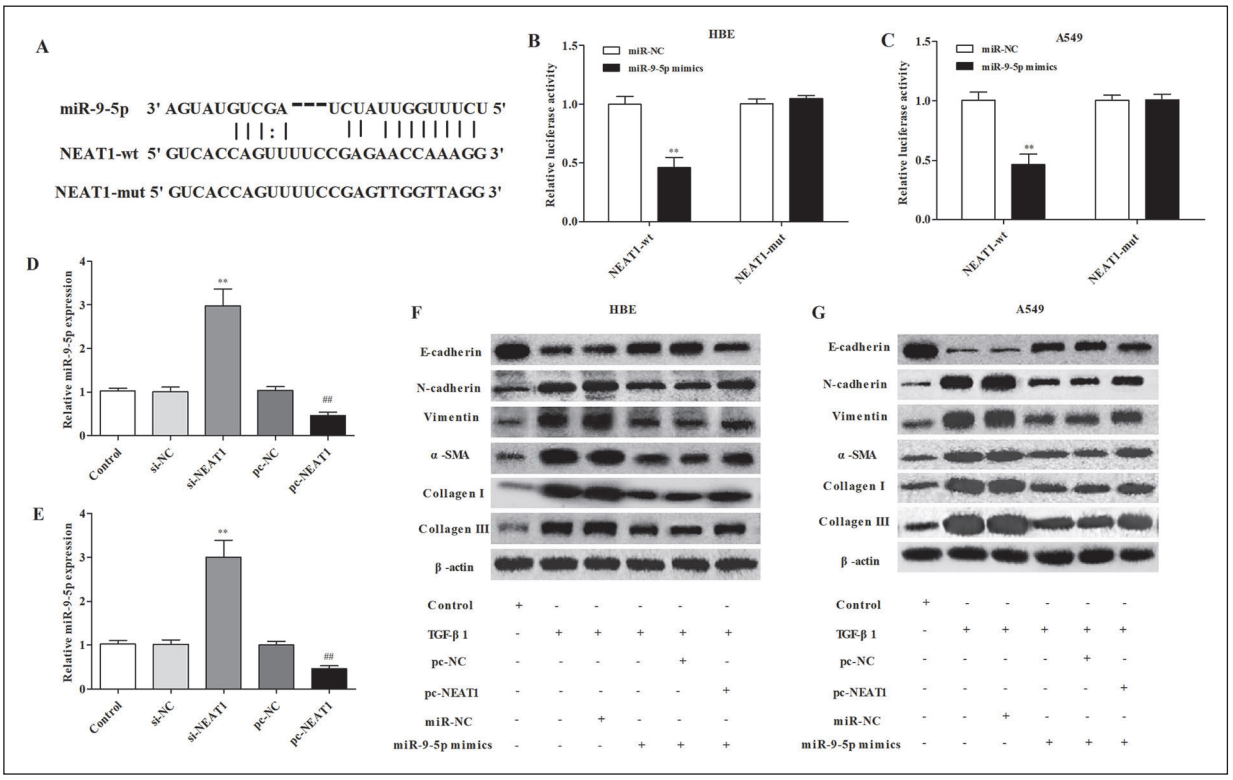

Figure 4. NEAT1 regulates miR-9-5p by directly targeting it. **A,** Predicted binding site between miR-9-5p and NEAT1. **B, C,** The Luciferase activity in HBE and A549 cells. **D, E,** Expression of miR-9-5p in cells transfected with si-NEAT1 or pc-NEAT1. **F, G,** The effect of miR-9-5p on expressions of epithelial cell marker E-cadherin, and interstitial and fibrosis markers including N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III in TGF-β1-induced cells. $^{**}p < 0.01$, compared with miR-NC or si-NC group; $^{\#\#}p < 0.01$, compared with pc-NC group.

by BLM treatment and verified by HE and Masson staining assays. We observed that NEAT1 inhibition significantly attenuated the PF induced by BLM (Figure 6A). To ensure the sh-NEAT1 efficiency in PF mice models, NEAT1 expression was detected after transfection with sh-NEAT1. sh-NEAT1 could downregulate the expression of NEAT1 in BLM-induced PF mice models (Figure 6B). Serum TGF-β1 level in mice was detected by ELISA. Notably, TGF-β1 level increased in BLM group versus control group, while decreased in the presence of sh-NEAT1 (Figure 6C). Also, the expression of TGF-β1, p-Smad2, E-cadherin, N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III were detected in lung tissues. E-cadherin expression was decreased, and TGF-β1, p-Smad2, N-cadherin, α-SMA, Vimentin, Collagen I and Collagen III expression were increased in BLM group versus control (Figure 6D). However, sh-NEAT1 enhanced E-cadherin expression, and reduced TGF-β1, p-Smad2, N-cadherin, α-SMA, Vimentin, Collagen I and Collagen III expression after BLM treatment (Figure 6D). Overall, NEAT1 inhibition represses PF by regulating the TGF-β pathway to inhibit EMT *in vivo*.

Discussion

Plenty of lncRNAs were reported to associate with fibrosis and may serve as new potential therapeutic targets in fibrosis[7]. However, the specific potential mechanism of them in fibrosis still remains unclear and waits to be elucidated. We observed an increasing expression of NEAT1 in PF tissues and cells. Mechanically, we detected that NEAT1 directly targets miR-9-5p. In addition, our results suggested that NEAT1 promotes PF *via* regulating miR-9-5p and TGF-β signaling to facilitate EMT. Collectively, we proposed NEAT1 might be a novel target for PF diagnosis and treatment.

NEAT1 was involved in the process of fibrosis[20-22]. Yu et al[20] identified that NEAT1 promoted liver fibrosis progression *via* regulating miR-122 and KLF6 expression and suggested

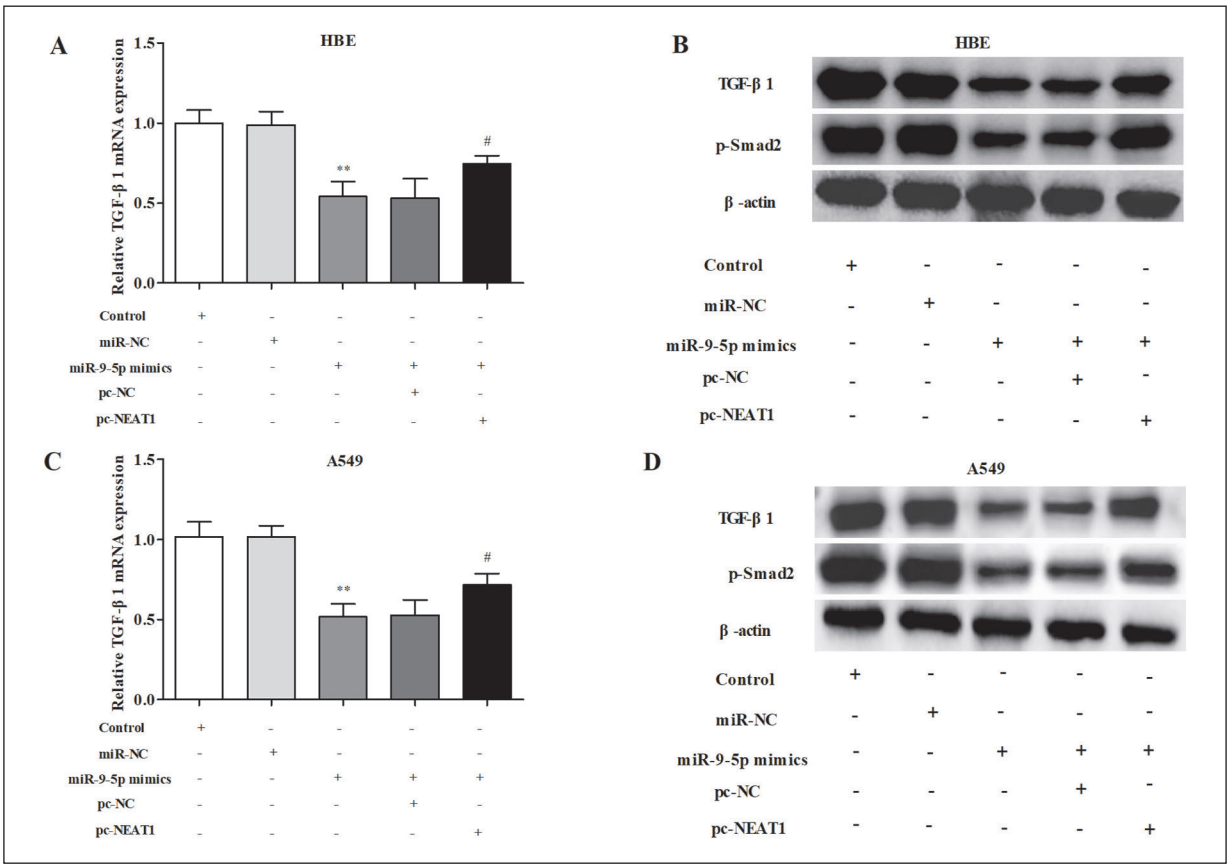

Figure 5. NEAT1 regulates the TGF-β pathway. **A, C,** The effect of miR-9-5p and NEAT1 overexpression on TGF-β1 mRNA levels in cells. **B, D,** The effect of miR-9-5p and NEAT1 overexpression on TGF-β1 and p-Smad2 protein expressions. $^{**}p < 0.01$, compared with miR-NC group; $\#\ p < 0.05$, compared with pc-NC group.

to be a diagnostic marker in liver fibrosis progression. Huang et al[21] suggested that NEAT1 contribute to the fibrosis progression of diabetic nephropathy by regulating Akt/mTOR pathway. Ma et al[22] found that NEAT1 knockdown inhibited fibrosis in high glucose-induced mouse mesangial cells diabetic nephropathy cellular model. Nevertheless, the role of NEAT1 in PF has not been studied. Here, we firstly confirmed NEAT1 was highly expressed in PF tissues and TGF-β1-induced cells. We found that NEAT1 knockdown prevented PF in BLM-induced PF mice model. Therefore, to reveal the potential roles of NEAT1 in PF, further experiments were carried out.

Correlation of lncRNAs and miRNAs play a vital role in PF progression[11,23,24]. Jiang et al[23] demonstrated that lncRNA PFRL inhibition could attenuate PF by regulating miR-26a to prevent fibroblast proliferation and differentiation. Similarly, another research suggested that lncRNA PFAL promoted lung fibrosis by regulating CTGF as a ceRNA of miR-18a[24]. Also, Lu et al[11] revealed that H19 promoted PF by sponging miR-196a. In our study, NEAT1 was indicated as a target of miR-9-5p. In addition, our results showed that miR-9-5p was decreased in PF tissues and TGF-β1-induced cells. Of note, knockdown of NEAT1 upregulated miR-9-5p expression. Overall, we proposed that NEAT1 promoted PF by negatively regulating miR-9-5p which is a miRNA that has been demonstrated to associate with the regulation of EMT in colorectal carcinoma and prostate cancer[25,26].

EMT is the biological process by which epithelial cells are transformed into mesenchymal phenotypic cells through a specific procedure, and it plays an important role in tissue reconstruction, cancer progression and fibrosis, including PF[27,28]. TGF-β/Smad signaling pathway has clarified to benefit to occurrence of EMT in

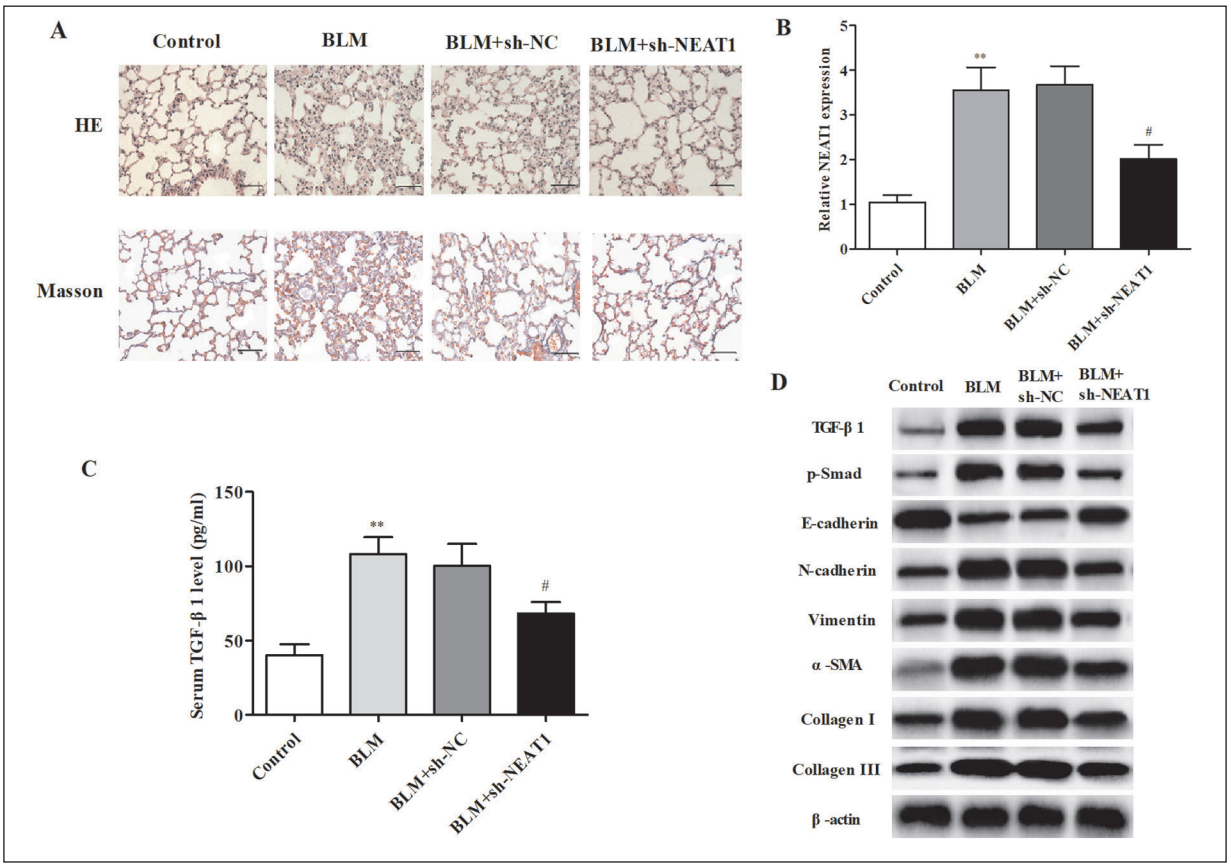

Figure 6. NEAT1 knockdown represses PF *in vivo*. **A,** HE and Masson staining in mice of each group (200X). **B,** NEAT1 expression in mice of each group. **C,** Serum TGF-β1 level in mice of each group. **D,** TGF-β1, p-Smad2, E-cadherin, N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III expressions in mice of each group. $^{**}p < 0.01$, compared Control group; $\#\, p < 0.05$, compared with BLM+sh-NC group.

PF[28]. Subsequently, a series of experiments were conducted to explore whether NEAT1 affected PF by miR-9-5p and TGF-β pathway. We found that p-Smad2 expression was significantly increased in TGF-β1-induced cells and repressed by miR-9-5p overexpression, as well as TGF-β1, while NEAT1 overexpression reversed this phenomenon. In addition, E-cadherin expression (epithelial cell marker) was increased and N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III expression (interstitial and fibrosis markers) were suppressed by miR-9-5p overexpression, while NEAT1 overexpression attenuated these effects. These results suggested that NEAT1 might promote PF by targeting miR-9-5p and regulating TGF-β pathway. To further detect NEAT1 potential role in PF, PF mice models induced by BLM were established and verified by HE and Masson staining assays. NEAT1 knockdown could attenuate BLM-induced PF. Furthermore, NEAT1 knockdown decreased TGF-β1, p-Smad3, N-cadherin, Vimentin, α-SMA, Collagen I and Collagen III expressions, and increased E-cadherin expression in BLM-induced PF mice models. Taken together, we suggested that NEAT1 knockdown repressed PF by regulating miR-9-5p and TGF-β pathway to inhibit EMT.

In conclusion, we demonstrated that NEAT1 promoted PF by regulating miR-9-5p and TGF-β signaling to inhibit EMT. This may provide new insights for the underlying mechanisms of PF. However, this study still has some limitations. In fact, the detailed relationship between miR-9-5p and TGF-β signaling pathway, and the roles of miR-9-5p in PF was not thoroughly explored. Moreover, there may exist other possible miRNAs or/and signaling pathways and mechanisms of NEAT1 in PF. Hence, more explorations need to be performed to further understand the mechanisms of NEAT1 in PF.

Conclusions

To sum up, results showed that NEAT1 was significantly upregulated while miR-9-5p was markedly reduced in PF tissues and cells (TGF-β1-induced). NEAT1 knockdown attenuated PF via regulating miR-9-5p and TGF-β signaling to inhibit EMT, and this was a novel mechanism for NEAT1 in PF. So far as we know, we first proposed that NEAT1 knockdown inhibited PF *in vitro* and *in vivo*. We first identified the regulatory network of NEAT1/miR-9-5p-TGF-β signaling/EMT in PF progression. These findings provide new understanding into the potential mechanisms, and novel therapeutic strategy for PF.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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