Long non-coding RNA NEAT1 promotes pulmonary fibrosis by regulating the microRNA-455-3p/SMAD3 axis

YUAN LIU[1], FU-AI LU[2], LE WANG[1], YONG-FU WANG[2] and CHUN-FENG WU[1]

[1]Department of Rheumatology, Liuzhou People's Hospital, Liuzhou, Guangxi Autonomous Region 545006; [2]Department of Rheumatology, First Affiliated Hospital of Baotou Medical College, Inner Mongolia University of Science and Technology, Baotou, Inner Mongolia 014010, P.R. China

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Abstract. Pulmonary fibrosis is an excessive repair response to tissue damage, triggering hyperplasia of fibrotic connective tissues; however, there is no effective treatment in a clinical setting. The purpose of the present study was to investigate the roles of long non-coding RNA nuclear enriched abundant transcript 1 (NEAT1) and microRNA-455-3p (miR-455-3p) were investigated in pulmonary fibrosis. In this study, the mRNA expression levels of NEAT1, miR-455-3p and SMAD3 in the HPAEpiC alveolar and BEAS-2B bronchial epithelial cell lines were determined using reverse transcription-quantitative PCR, while the markers of epithelial-mesenchymal transformation (EMT) and collagen production were determined using western blot analysis. A wound healing assay was performed to evaluate the migratory ability of the HPAEpiC and BEAS-2B cell lines. The interactions between NEAT1 and miR-455-3p or SMAD3 and miR-455-3p were validated using a luciferase reporter gene assay. The results showed that the mRNA expression levels of NEAT1 and SMAD3 were upregulated in the TGF-β1-treated HPAEpiC and BEAS-2B cell lines, while the mRNA expression level of miR-455-3p was significantly decreased. In addition, silencing NEAT1 effectively alleviated the migratory ability, EMT and collagen generation of the epithelial cells. Following these experiments, NEAT1 was identified as a sponge for miR-455-3p, and SMAD3 was a target gene of miR-455-3p. NEAT1 downregulation or miR-455-3p mimic inhibited the migratory ability, EMT and collagen production of the epithelial cells; however, the effects were reversed by the overexpression of SMAD3. Furthermore, NEAT1 knockdown reduced the expression level of SMAD3 by increasing the expression level of miR-455-3p to further inhibit the migratory ability, EMT and collagen production of epithelial cells.

Correspondence to: Dr Chun-Feng Wu, Department of Rheumatology, Liuzhou People's Hospital, 8 Wenchang Road, Chengzhong, Liuzhou, Guangxi Autonomous Region 545006, P.R. China
E-mail: wuchunfeng99@163.com

Dr Yong-Fu Wang, Department of Rheumatology, First Affiliated Hospital of Baotou Medical College, Inner Mongolia University of Science and Technology, 41 Linyin Road, Kundulun, Baotou, Inner Mongolia 014010, P.R. China
E-mail: wangyongfu238@126.com

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Introduction

Pulmonary fibrosis is the end result of a major category of pulmonary diseases characterized by fibroblast proliferation, extracellular matrix (ECM) deposition and tissue structure destruction. It is also characterized by scar formation caused by abnormal repair of damaged alveolar tissue (1,2). Fibroblasts are derived from epithelial cells that have undergone epithelial-mesenchymal transformation (EMT), in which the markers of the epithelial cells are depleted and the abilities of adhesion, proliferation and migration are enhanced (3). The phenotypic dysregulation of the alveolar epithelial cells, accompanied by excessive ECM deposition, is a crucial component in the progression of pulmonary fibrosis (4). Studies investigating fibrogenesis have made progress in recent years (5,6); however, further investigation is required to elucidate the pathogenesis of pulmonary fibrosis to facilitate the development of effective therapeutic strategies.

Numerous studies have demonstrated that microRNAs (miRNA/miR) act as a class of non-coding single-stranded RNA molecules, which are ~22 nucleotides in length and encoded by endogenous genes, and are involved in a series of biological and pathological processes by binding and degrading the target mRNAs (7,8). Recently, the regulatory functions of miRNAs in pulmonary fibrosis have been increasingly discovered. Using Affymetrix miRNA microarrays, miR-455-3p was found to be downregulated in the lung tissues of patients with idiopathic pulmonary fibrosis (9). Wei *et al* (10) reported that miR-455-3p was decreased in the fibrotic liver, and overexpression of miR-455-3p could inhibit the expression levels of profibrotic markers in hepatic stellate cells. Furthermore, miR-455-3p was also involved in the accumulation of ECM and the expression of fibrosis-related proteins in diabetic nephropathy (11).

In addition to miRNAs, another type of non-coding RNA, which have lengths of >200 nucleotides, termed long non-coding RNAs (lncRNAs), have been reported to regulate gene or protein expression levels in the course of fibrogenesis. The lncRNA nuclear enriched abundant transcript 1 (NEAT1) was highly expressed in murine fibrotic livers and promoted liver fibrosis by regulating miRNA-122 and Kruppel-like factor 6 (12). Huang *et al* (13) revealed that the downregulation of NEAT1 repressed the proliferation of mesangial cells and fibrosis in diabetic nephropathy by inactivating the Akt/mTOR signaling pathway. However, the expression of NEAT1 in lung epithelial cells and the regulatory mechanism involved remains largely unknown.

Numerous studies have described that lncRNA function, as a competing endogenous RNA, could mediate miRNAs and the targets of miRNAs (14,15). Binding sites between the sequences of NEAT1 and miR-455-3p were predicted using bioinformatics analysis. The present study aimed to determine the function of NEAT1 in TGF-β1-treated human alveolar epithelial and bronchial epithelial cell lines, and investigate its potential association with miR-455-3p.

Materials and methods

Cell culture and transfection. The human HPAEpiC alveolar epithelial cells (ScienCell Research Laboratories, Inc.) were cultured in DMEM/F12 (Hyclone; Cytiva) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO_2. The BEAS-2B human bronchial epithelial cells (American Type Culture Collection) were cultured in complete bronchial epithelial growth medium (Lonza Group, Ltd.) at 37˚C in a humidified incubator with 5% CO_2.

TGF-β1 (R&D Systems China Co., Ltd.) is a potent pro-fibrotic factor, and 10 ng/ml was used to stimulate the two types of epithelial cells for 48 h. Two interfering sequences of short hairpin NEAT1 (shRNA-NEAT1-1, 5'-GTGAGAAGT TGCTTAGAAA-3'; and shRNA-NEAT1-2, 5'-TGGTAATGG TGGAGGAAGA-3') were ligated into the pGPH6/Neo vector (50 nM; Shanghai GenePharma Co., Ltd.). miR-455-3p mimic (50 nM; 5'-GCAGUCCAUGGGCAUAUACAC-3') and inhibitor (50 nM; 5'-GUGUAUAUGCCCAUGGACUGC-3'), as well as their corresponding negative controls [50 nM; NC; mimic-NC; 5'-UUCUCCGAACGUGUCACGUTT-3') and inhibitor-NC (50 nM; 5'-CAGUACUUUUGUGUAGUA CAA-3')] were also purchased from Shanghai GenePharma Co., Ltd. The empty vector plasmid (50 nM; pcDNA3.1) and pcDNA3.1-SMAD3 (50 nM; 5'-GAATCGCCACCATGTCGTCCATCCTGCCC TTC-3' and 5'-CTCGAGCCTGGGGTTTTCTTCTGTG GTC-3') were constructed by Sangon Biotech Co., Ltd. Briefly, cells were seeded ($5x10^5$) into 12-well plates and grown to 80% confluence. Subsequently, cells were transfected with shRNA or mimic using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) for 48 h, according to the manufacturer's instructions. At 48 h post-transfection, transfection efficacy was evaluated using reverse transcription-quantitative PCR (RT-qPCR).

Prediction of target genes. The Encyclopedia of RNA Interactomes (ENCORI, http://starbase.sysu.edu.cn). ENCORI is an open-source platform for studying the miRNA-ncRNA, miRNA-mRNA, ncRNA-RNA, RNA-RNA, RBP-ncRNA and RBP-mRNA interactions from CLIP-seq, degradome-seq and RNA-RNA interactome data.

RT-qPCR. Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), while the PrimeScript™ RT reagent kit (Takara Bio, Inc.; 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min) and SYBR Green qPCR kit (Thermo Fisher Scientific, Inc.) were used for reverse transcription and qPCR, respectively. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 5 min; followed by 40 cycles of denaturation at 95˚C for 20 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 20 sec. β-actin was used as the endogenous control of lncRNA and mRNA. U6 was used to normalize the relative expression levels of miRNA. Relative expression levels of miRNA and mRNA were determined using the $2^{-\Delta\Delta Cq}$ method (16). The primer sequences used are stated in Table I.

Wound healing assay. Cells were seeded ($3x10^5$ cells/well) into a 6-well plate and incubated at 37˚C in 5% CO_2. At 80% confluence, the scratches were created using a sterile 200 μl pipette tip. Then, cells were washed gently to remove the floating cells and the medium was replaced with serum-free medium for 24 h. Images of the cells that had migrated into the wound were captured under a light microscope (magnification, x100; Zeiss AG). Quantitative analysis of the wound healing area was performed using ImageJ software (version 1.52r; National Institutes of Health).

Transwell invasion assay. A Transwell invasion assay was used to analyze the invasive rate of cells. Briefly, AMC-HN-8 cells in 100 μl ($2x10^5$) serum-free medium (Thermo Fisher Scientific, Inc.) were plated into the upper chambers of an 8-μm Transwell plate (Corning, Inc.) precoated with Matrigel (BD Biosciences) at 24 h (37˚C) after transfection. DMEM/F12 (Hyclone; Cytiva) containing 20% FBS was plated in the lower chamber to serve as a chemoattractant. Following the incubation (37˚C, 24 h), the invading cells on the bottom surface of the filter were fixed with methanol (100%, 4˚C) for 30 min and stained with hematoxylin at room temperature for 20 min. Cell invasion was analyzed in three randomly selected fields under a fluorescent microscope (magnification, x20).

Western blot analysis. Total protein in the cells was extracted using a RIPA lysis buffer (Beyotime Institute of Biotechnology). After quantification using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology), protein (40 μg/lane) in the different experimental groups were separated via 10% SDS-PAGE, and then transferred onto PVDF membranes using electrophoresis. Following blocking with 5% skimmed milk for 1.5 h at room temperature, the PVDF membranes were subsequently incubated with primary antibodies overnight at 4˚C. Next, the primary antibodies were removed and the membrane was incubated with the secondary antibodies for 1 h at room temperature. The primary antibodies against α smooth muscle actin (α-SMA; cat. no. ab7817; 1:1,000), collagen I (cat. no. ab34710; 1:1,000), collagen III (cat. no. ab184993; 1:1,000) and E-cadherin

Table I. Primers used in the present study.

Primer	Sequence (5'→3')
U6 snRNA	F: GCGCGTCGTGAAGCGTTC R: GTGCAGGGTCCGAGGT
miR-455-3p	F: ACACTCCAGCTGGGGCAGTCCACGGGCATATACAC R: GTGCAGGGTCCGAGGT
β-actin	F: CAGAGCAAGAGAGGCATCC R: CTGGGGTGTTGAAGGTCTC
NEAT1	F: CAGGGTGTCCTCCACCTTTA R: AAACCAGCAGACCCCTTTTT
SMAD3	F: AAACTAGTGTCACAGTCCAACCAGAAAC R: GGAAGCTTTTGTACCAAGCCTGCAATT

F, forward; R, reverse; miR, microRNA; NEAT1, nuclear enriched abundant transcript 1.

(cat. no. ab1416; 1:1,000) were obtained from Abcam, while the antibodies against SMAD3 (cat. no. 9523T; 1:1,000), fibronectin1 (cat. no. 26836S; 1:1,000) and β-actin (cat. no. 4970T; 1:1,000) were obtained from Cell Signaling Technology, Inc. The secondary antibodies (cat. nos. ab7090 and ab97040; 1:5,000) were purchased from Abcam.

Luciferase reporter gene. The 3'untranslated region (UTR) of miR-455-3p, containing NEAT1 wild-type (WT) or mutant (MUT) sites which were amplified by Shanghai GenePharma Co., Ltd., were subcloned into the pmirGLO vector (Promega Corporation). The cells were co-transfected with miR-455-3p mimic or NC-mimic, and with a vector containing NEAT1 WT or MUT sites using Lipofectamine 3000. The 3'UTR of miR-455-3p containing SMAD3 WT or MUT sites were subcloned into the pmirGLO vector (Promega Corporation). The cells were co-transfected with miR-455-3p mimic or NC-mimic and with a vector containing SMAD3 WT or MUT sites using Lipofectamine 3000. Relative luciferase activity was measured by normalizing firefly luciferase activity to *Renilla* luciferase activity at 48-h post-transfection using a Dual-Luciferase Reporter assay (Promega Corporation).

Statistical analysis. The data are presented as the mean ± standard deviation, and were analyzed using GraphPad Prism v6.0 statistical software (GraphPad Software, Inc.). A Student's t-test was used for comparisons between two groups, while ANOVA followed by the Tukey's post hoc test was used for comparisons among multiple groups. All experiments were performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Knockdown of NEAT1 inhibits migration, EMT and collagen generation of epithelial cells. The mRNA expression level of NEAT1 was significantly increased in TGF-β1-treated HPAEpiC and BEAS-2B cells compared with that in the control group (Fig. 1A and B). The HPAEpiC and BEAS-2B cell lines were transfected with shRNA-NEAT1 (Fig. 1C and D), and shRNA-NEAT1-1 was selected for the further experiments due to lower NEAT1 expression levels induced by shRNA-NEAT1-1 compared with shRNA-NEAT1-2. The migratory and invasive abilities of the TGF-β1-induced HPAEpiC cells were weakened by silencing NEAT1 (Fig. 1E-H), and similar results were also found in the BEAS-2B cell line (Fig. 1I-L). The epithelial cell marker, E-cadherin, was decreased in the HPAEpiC and BEAS-2B cell lines treated with TGF-β1, whereas transfection with shRNA-NEAT1-1 reversed the effect of TGF-β1 (Fig. 1M). Fibronectin1 and α-SMA act as markers of mesenchymal cells and were upregulated in TGF-β1-treated HPAEpiC and BEAS-2B cell lines, while knockdown of NEAT1 reduced the protein expression level of fibronectin1 and α-SMA. Similarly, shRNA-NEAT1-1 partially abrogated the promotional effects of TGF-β1 on the protein expression levels of collagen I and III (Fig. 1M). Collectively, these findings illustrated the important roles of NEAT1 in cell migration, EMT and collagen production of epithelial cells.

NEAT1 modulates the expression of miR-455-3p. NEAT1 was predicted to be a sponge of miR-455-3p using the StarBase software (Fig. 2A). miR-455-3p expression in TGF-β1-treated HPAEpiC and BEAS-2B cell lines were decreased (Fig. 2B and C). The transfection efficiency of the miR-455-3p mimic is shown in Fig. 2D and E. A luciferase reporter assay was performed to validate the interaction between miR-455-3p and NEAT1, and the luciferase activity was found to be downregulated in cells co-transfected with NEAT1-WT and miR-455-3p mimic, suggesting that miR-455-3p could bind to NEAT1 (Fig. 2F and G). Furthermore, the expression of miR-455-3p was significantly higher in shRNA-NEAT1-1-transfected cells compared with that in the shRNA-NC group (Fig. 2H and I). These data suggested that NEAT1 may bind to miR-455-3p and regulate the expression levels of miR-455-3p.

Regulation of NEAT1 in migration, collagen production and EMT depends on miR-455-3p. Subsequently, a miR-455-3p inhibitor was generated and the transfection efficiency was determined (Fig. 3A and B). As shown in Fig. 3C-J, the results of the wound healing and Transwell invasion assays

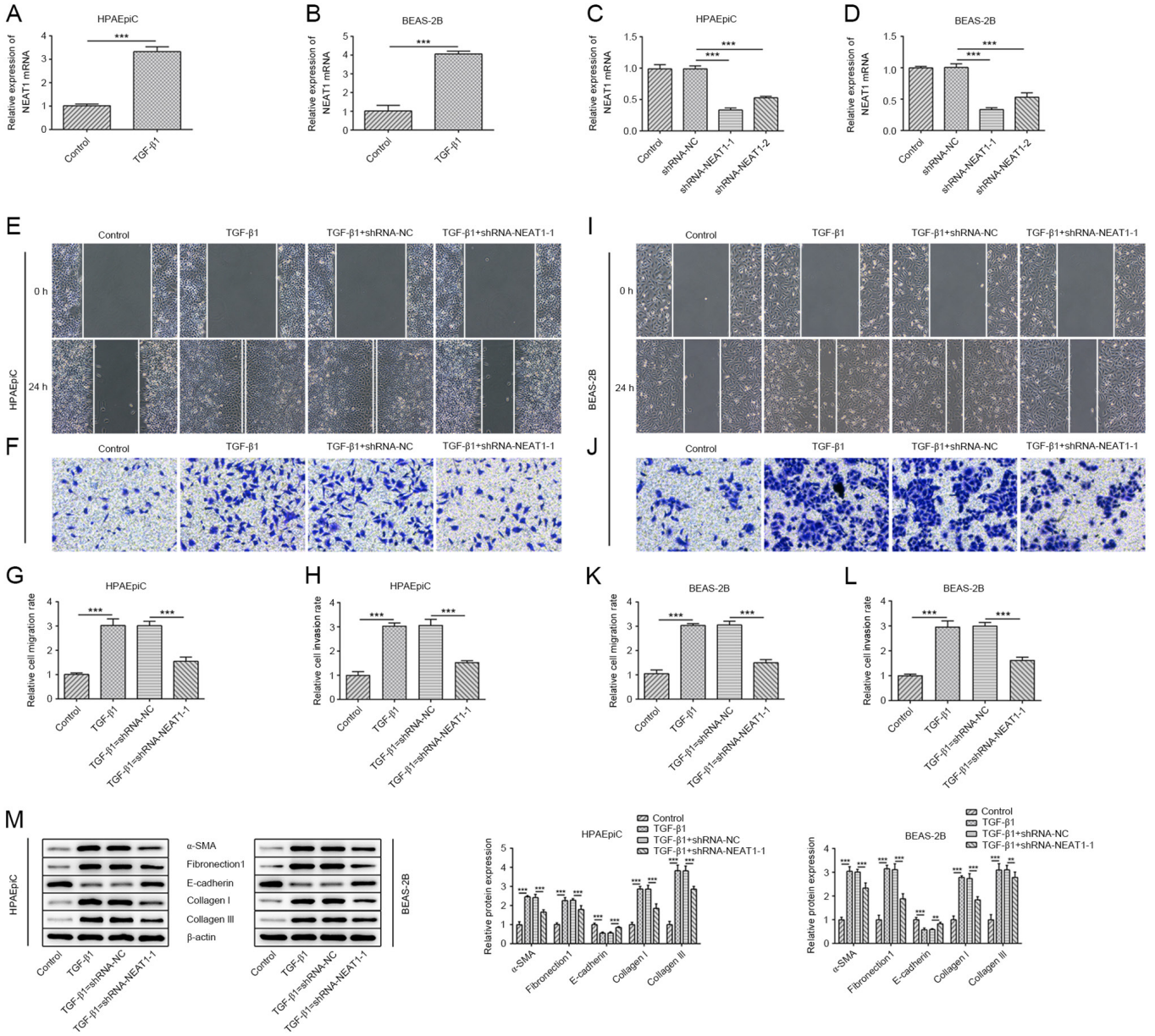

Figure 1. NEAT1 knockdown inhibits epithelial cell migration, EMT and collagen generation. (A) HPAEpiC and (B) BEAS-2B cells were treated with 10 ng/ml TGF-β1 for 48 h then, the mRNA expression of NEAT1 was determined using RT-qPCR. Transfection efficiency of shRNA-NEAT1 was validated using RT-qPCR in (C) HPAEpiC and (D) BEAS-2B cells. A wound healing assay was performed to assess the migratory ability of the (E) HPAEpiC and (I) BEAS-2B cell lines (magnification, x100). The migration rate of (G) HPAEpiC and (K) BEAS-2B cells. A Transwell assay was performed to assess the invasive ability of the (F) HPAEpiC and (J) BEAS-2B cell lines (magnification, x100). The invasion rate of (H) HPAEpiC and (L) BEAS-2B cell lines. (M) The protein expression levels of E-cadherin, α-SMA, collagen I, collagen III and fibronectin1 in the HPAEpiC and BEAS-2B cell lines were evaluated using western blot analysis. **P<0.01, ***P<0.001. sh, short hairpin; RT-qPCR, reverse transcription-quantitative PCR; α-SMA, α smooth muscle actin; NEAT1, nuclear enriched abundant transcript 1; NC, negative control.

indicated that the miR-455-3p inhibitor promoted the migratory and invasive abilities of the HPAEpiC and BEAS-2B cell lines. Furthermore, knockdown of NEAT1 counteracted the effects of TGF-β1 on the expression levels of E-cadherin, whereas the miR-455-3p inhibitor further abolished the function of shRNA-NEAT1-1. The protein expression levels of fibronectin1, α-SMA, collagen I and collagen III showed the opposite trend to E-cadherin (Fig. 3K).

SMAD3 serves as a target of miR-455-3p. SMAD3 has been identified to be an important mediator in the progression of pulmonary fibrosis (17,18). In the TGF-β1-treated HPAEpiC and BEAS-2B cell lines, the mRNA expression levels of SMAD3 were increased compared with that in the control group (Fig. 4A and B), while SMAD3 was also elevated at the protein level (Fig. 4C and D). Notably, SMAD3 was predicted as a target gene of miR-455-3p using the StarBase software (Fig. 4E), and the results from a luciferase reporter assay revealed decreased luciferase activity in the group co-transfected with SMAD3 WT and miR-455-3p mimic, demonstrating that there was an interaction between miR-455-3p and SMAD3 (Fig. 4F and G). Furthermore, the mRNA and protein expression levels of SMAD3 were downregulated in the HPAEpiC or BEAS-2B cell lines transfected with miR-455-3p mimic (Fig. 4H-K).

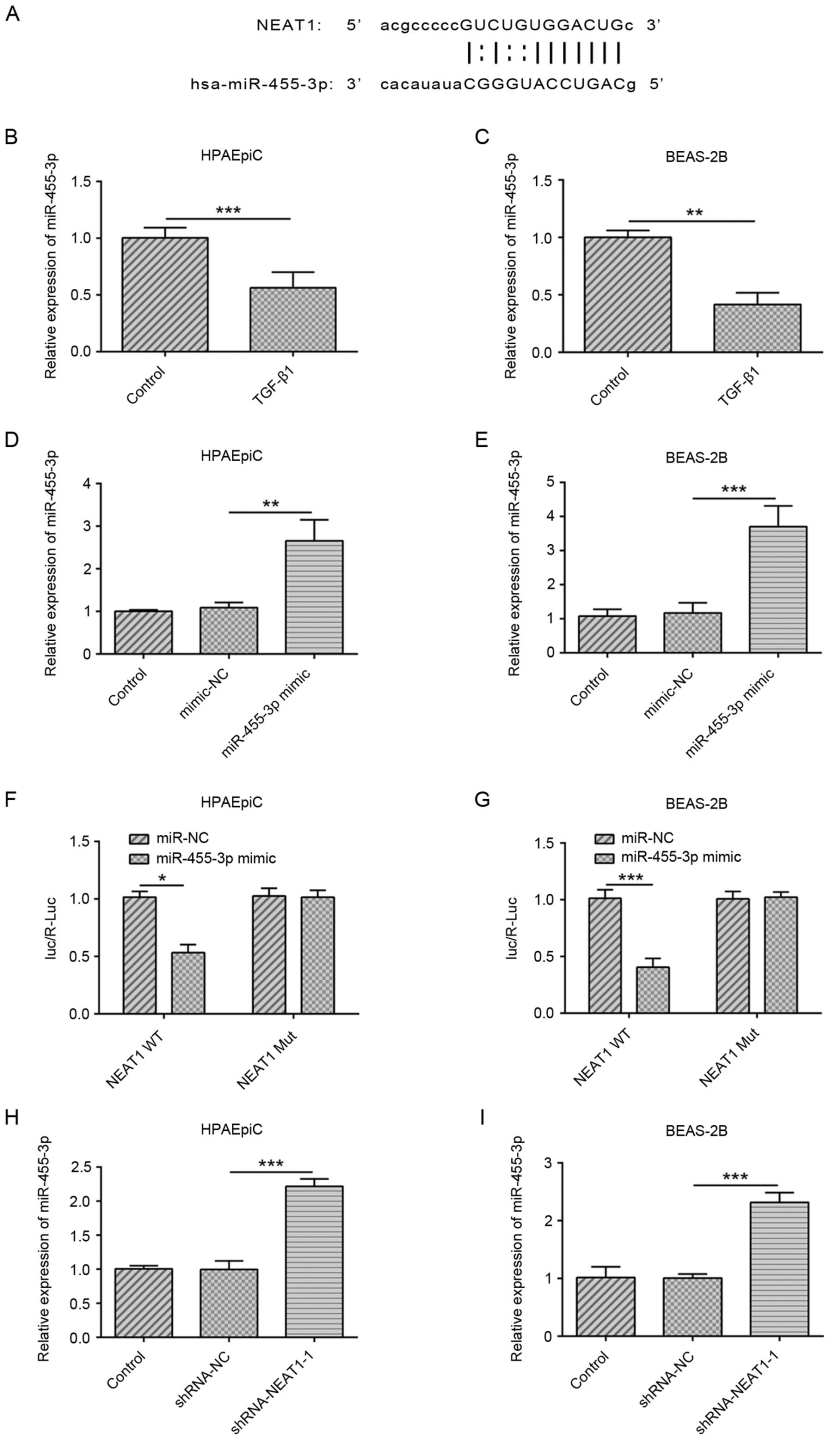

Figure 2. NEAT1 modulates miR-455-3p expression. (A) The binding sites between NEAT1 and miR-455-3p were predicted using the StarBase software. The mRNA expression levels of miR-455-3p in the (B) HPAEpiC and (C) BEAS-2B cell lines treated with TGF-β1 were determined using RT-qPCR. The mRNA expression levels of miR-455-3p in the (D) HPAEpiC and (E) BEAS-2B cell lines transfected with miR-455-3p mimic were determined using RT-qPCR. (F and G) The luciferase activity in each experimental group. The mRNA expression levels of miR-455-3p in the (H) HPAEpiC and (I) BEAS-2B cell lines transfected with shRNA-NEAT1-1 were determined using RT-qPCR. *P<0.05, **P<0.01, ***P<0.001. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; NEAT1, nuclear enriched abundant transcript 1; NC, negative control.

NEAT1/miR-455-3p mediates migration, EMT and collagen generation via SMAD3. Compared with the control group, knockdown of NEAT1 was found to significantly inhibit the expression of SMAD3, which was rescued by the miR-455-3p inhibitor (Fig. 5A). As shown in Fig. 5B, the transfection efficiency of pcDNA3.1-SMAD3 was validated using western blot analysis. It was found that the effects of co-transfection with shRNA-NEAT1-1 and miR-455-3p mimic could further inhibit the migratory and invasive abilities of the epithelial cells compared with that in cells transfected with shRNA-NEAT1-1 alone, following treatment with TGF-β1; however, overexpression of SMAD3 weakened the synergistic effect of shRNA-NEAT1-1 and miR-455-3p mimic (Fig. 5C-J). Similar results were observed in EMT; miR-455-3p mimic enhanced

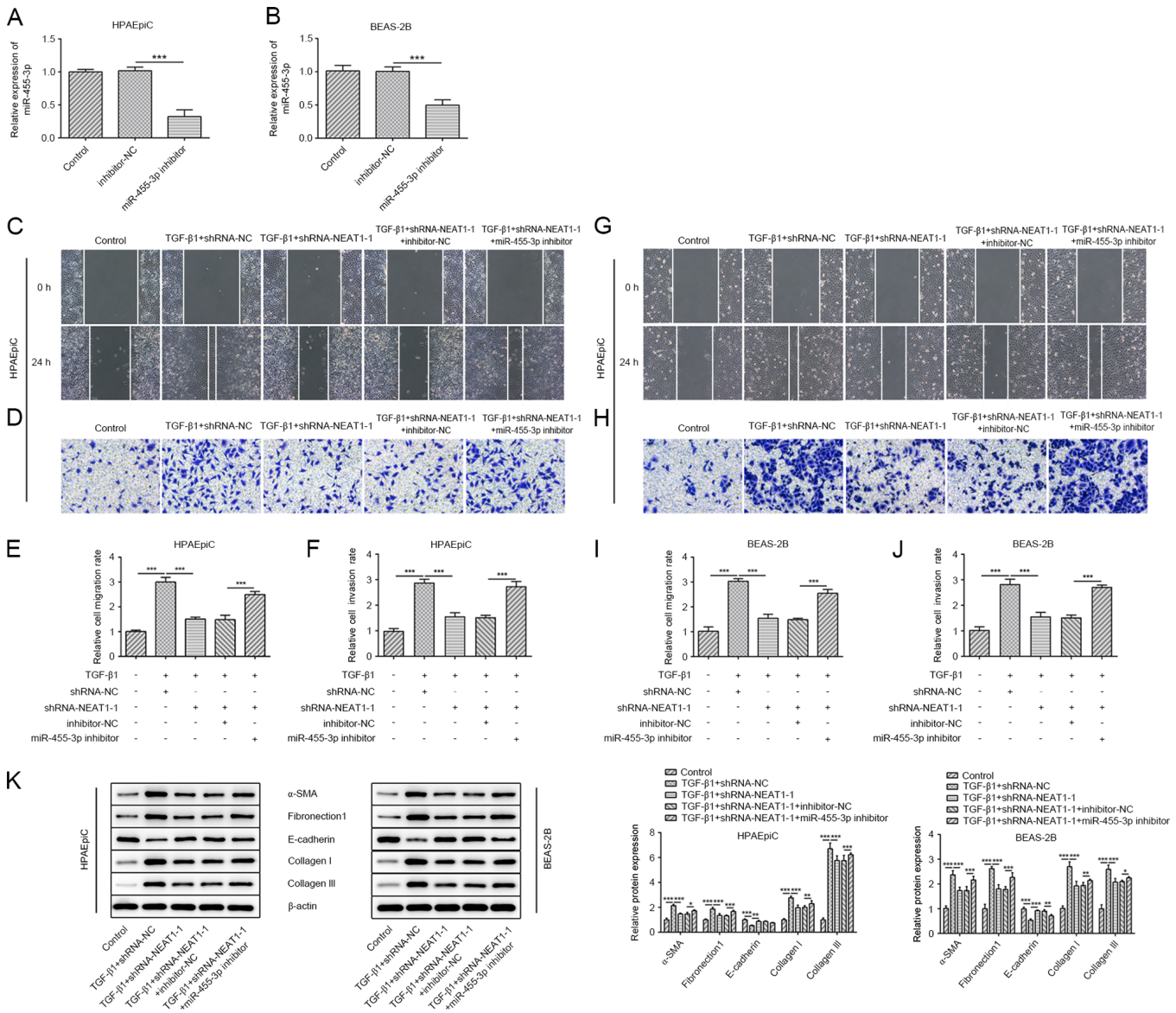

Figure 3. Regulation of NEAT1 in migration, collagen production and EMT depends on miR-455-3p. The mRNA expression levels of miR-455-3p in the (A) HPAEpiC and (B) BEAS-2B cell lines transfected with miR-455-3p inhibitor were determined using RT-qPCR. A wound healing assay was performed to assess the migratory ability of the (C) HPAEpiC and (G) BEAS-2B cell lines (magnification, x100). The migration rate of (E) HPAEpiC and (I) BEAS-2B cells. A Transwell assay was performed to assess the invasion ability of the (D) HPAEpiC and (H) BEAS-2B cell lines (magnification, x100). The invasion rate of (F) HPAEpiC and (J) BEAS-2B cells. (K) The protein expression levels of E-cadherin, α-SMA, collagen I, collagen III and fibronectin1 in the HPAEpiC and BEAS-2B cell lines were evaluated using western blot analysis. *P<0.05, **P<0.01, ***P<0.001. RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; α-SMA, α smooth muscle actin; NC, negative control; NEAT1, nuclear enriched abundant transcript 1; sh, short hairpin.

the inhibition of shRNA-NEAT1-1 on the protein expression levels of α-SMA, collagen I, collagen III and fibronectin1, but upregulated the expression of E-cadherin. SMAD3 overexpression reversed the effect of shRNA-NEAT1-1 and miR-455-3p mimic (Fig. 5K). Taken together, these data demonstrated that the inhibitory effects of shRNA-NEAT1-1 and miR-455-3p on migration, EMT and collagen generation were abrogated by the overexpression of SMAD3.

Discussion

Numerous studies have reported the emerging role of lncRNAs in the pathogenesis of pulmonary fibrosis (19-21). A previous study found that NEAT1 was increased in human fibrotic liver samples and murine fibrotic livers (12), while Huang *et al* (13) reported that NEAT1 accelerated fibrosis in diabetic nephropathy. Non-coding RNA-NEAT1 has been demonstrated to play a role in the differentiation and regulation of the development of various diseases (22,23). For example, NEAT1 was reported to be highly expressed in Parkinson's disease, and NEAT1 knockdown inhibited PD progression via regulating the miR-212-3p/axin 1 signaling pathway (24). NEAT1 also accelerated apoptosis and inflammation in lipopolysaccharide-induced sepsis models by targeting miR-590-3p (25). In the present study, NEAT1 was found to be upregulated in the TGF-β1-treated HPAEpiC and BEAS-2B cell lines, suggesting that the high expression level of NEAT1 could be associated with pulmonary fibrosis. TGF-β1 is commonly considered to be a potent pro-fibrotic factor, which could stimulate epithelial-derived fibroblasts to produce fibronectin and collagen, the main components of the ECM (26,27).

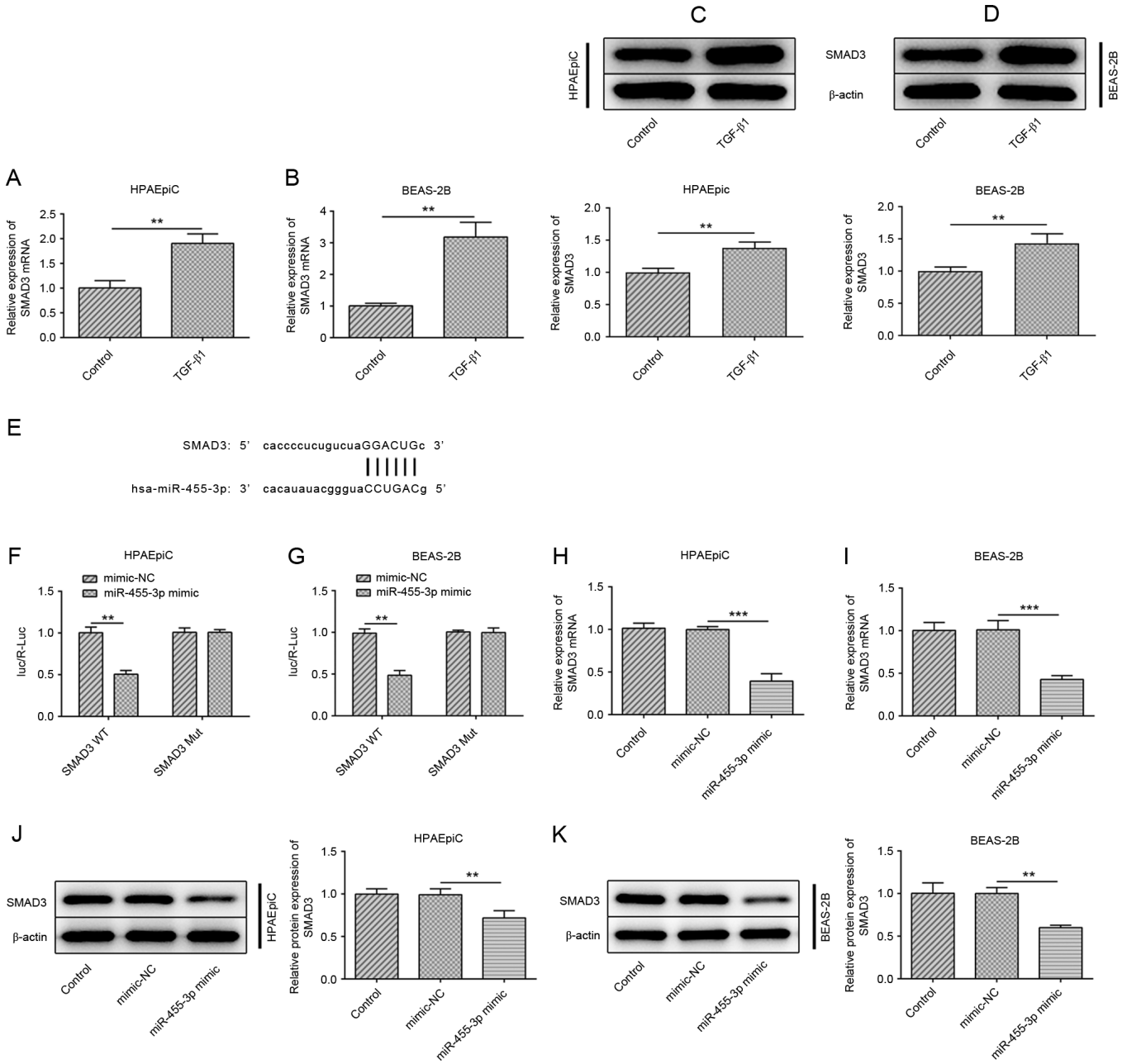

Figure 4. SMAD3 is a target of miR-455-3p. The mRNA expression levels of SMAD3 in the (A) HPAEpiC and (B) BEAS-2B cell lines treated with TGF-β1 were determined using RT-qPCR. The protein expression levels of SMAD3 in the (C) HPAEpiC and (D) BEAS-2B cell lines treated with TGF-β1 were determined using western blot analysis. (E) The binding sites between miR-455-3p and SMAD3 were predicted using the StarBase software. (F and G) The luciferase activity in each experimental group. The mRNA expression levels of SMAD3 in the (H) HPAEpiC and (I) BEAS-2B cell lines transfected with miR-455-3p mimic were determined using RT-qPCR. The protein expression levels of SMAD3 in the (J) HPAEpiC and (K) BEAS-2B cell lines transfected with miR-455-3p mimic were determined using western blot analysis. **P<0.01, ***P<0.001. RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; NC, negative control.

Knockdown of NEAT1 weakened the abilities of EMT and collagen production in epithelial cells in the present study. A previous study demonstrated that downregulation of NEAT1 reduced the expression levels of collagen I and fibronectin in mouse mesangial cells (28).

Jin *et al* (29) found that NEAT1 promoted liver fibrosis by mediating miR-506 and transcriptional activator GLI3. In addition, NEAT1 has been found to impair lung function through the interaction with miR-124 (30). Understanding the crosstalk between lncRNA, miRNA and mRNA, and their regulatory pattern could provide a novel perspective for the therapy of pulmonary fibrosis. In the present study, NEAT1 was identified to be a sponge of miR-455-3p. miR-455-3p is lowly expressed in various tumors, including prostate (31), colorectal (32), pancreatic (33) and breast cancer (34). miR-455-3p serves as an important regulator in organ fibrosis, including pulmonary fibrosis (10,35,36). The present study found that the miR-455-3p inhibitor partially reversed the regulatory effects of shRNA-NEAT1-1 on EMT and collagen generation. SMAD3 is commonly known as a downstream intracellular effector of TGF-β1 (37); in addition, SMAD3 was identified as a target mRNA of miR-455-3p and overexpression

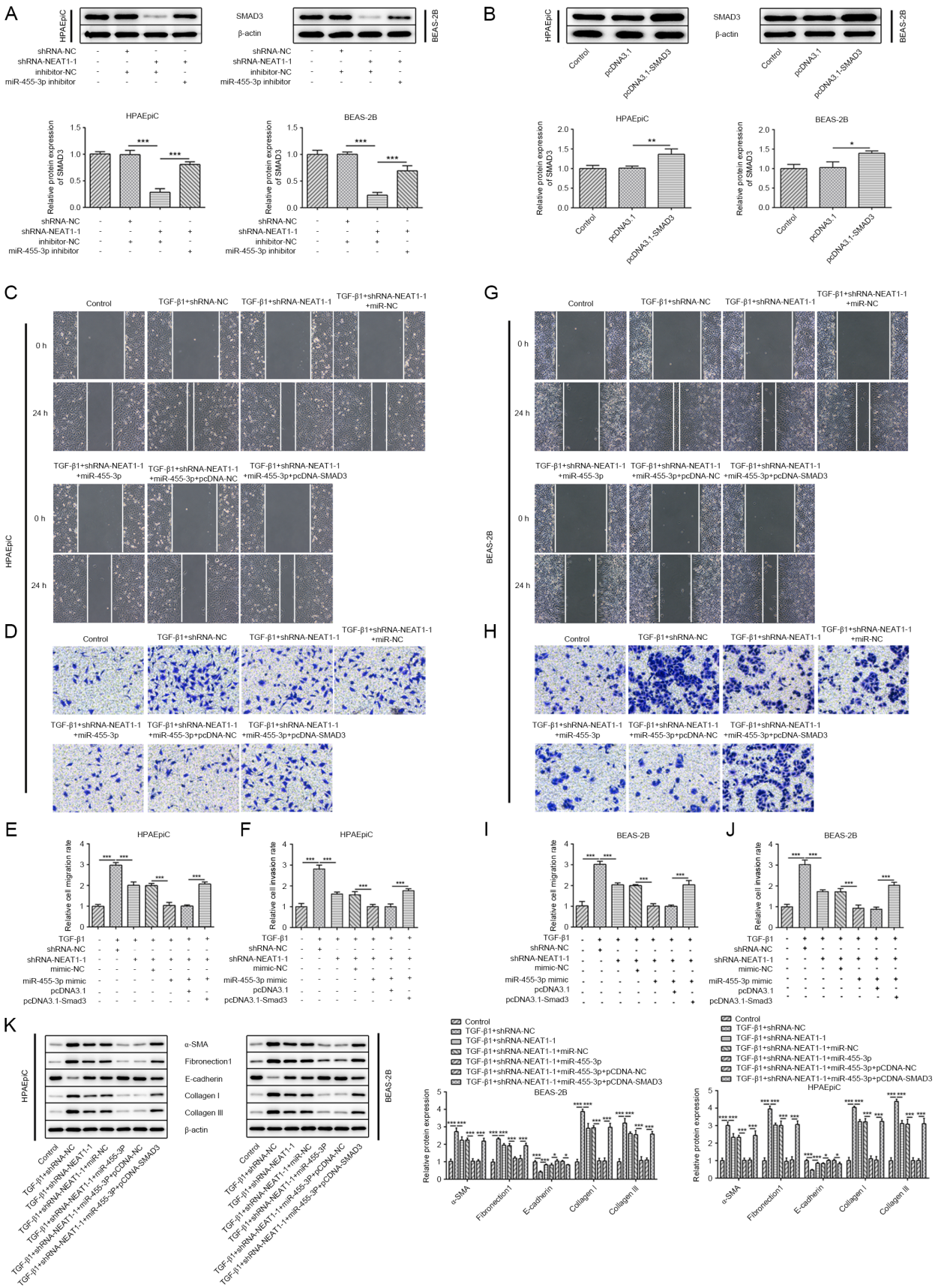

Figure 5. NEAT1/miR-455-3p mediates migration, EMT and collagen generation via SMAD3. (A) The protein expression levels of SMAD3 in the HPAEpiC and BEAS-2B cell lines transfected with shRNA-NEAT1-1 and/or miR-455-3p inhibitor were determined using western blot analysis. (B) The protein expression levels of SMAD3 in the HPAEpiC and BEAS-2B cell lines transfected with pcDNA3.1-SMAD3. A wound healing assay was performed to assess the migratory ability of (C) HPAEpiC and (G) BEAS-2B cell lines (magnification, x100). Migration rate of (E) HPAEpiC and (I) BEAS-2B cells. A Transwell assay was performed to assess the invasive ability of the (D) HPAEpiC and (H) BEAS-2B cell lines (magnification, x100). Invasion rate of (F) HPAEpiC and (J) BEAS-2B cells. (K) The protein expression levels of E-cadherin, α-SMA, collagen I, collagen III and fibronectin1 in the HPAEpiC and BEAS-2B cell lines were evaluated using western blot analysis. *P<0.05, **P<0.01, ***P<0.001. NEAT1, nuclear enriched abundant transcript 1; sh, short hairpin; miR, microRNA; NC, negative control; α-SMA, α smooth muscle actin.

of SMAD3 abolished the effects of NEAT1/miR-455-3p on cell fibrosis in the present study.

In summary, the present study illustrated that NEAT1 knockdown alleviated TGF-β1-induced epithelial cell migration, EMT and collagen production by regulating the miR-455-3p/SMAD3 axis. These findings suggested that NEAT1 and miR-455-3p may be potential targets for treatment of pulmonary fibrosis. However, the use of only *in vitro* methods was a limitation of the present study. Therefore, in subsequent research, an *in vivo* pulmonary fibrosis model will be established to determine the expression levels of NEAT1, miR-455-3p or SMAD3 in fibrotic lung tissues, furthermore the role of NEAT1 in pulmonary fibrosis requires validation by interfering with the expression of NEAT in lung tissue.

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Availability of data and materials

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

Authors' contributions

YL, FAL, LW and CFW searched the literature, designed the experiments and performed the experiments. FAL and YFW analyzed and interpreted the data. YFW and CFW wrote the manuscript. CFW revised the manuscript. YL and CFW confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ley B, Collard HR and King TE Jr: Clinical course and prediction of survival in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 183: 431-440, 2011.
2. Povedano JM, Martinez P, Flores JM, Mulero F and Blasco MA: Mice with Pulmonary Fibrosis Driven by Telomere Dysfunction. Cell Rep 12: 286-299, 2015.
3. Selman M and Pardo A: Role of epithelial cells in idiopathic pulmonary fibrosis: From innocent targets to serial killers. Proc Am Thorac Soc 3: 364-372, 2006.
4. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM and Borok Z: Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: Potential role in idiopathic pulmonary fibrosis. Am J Pathol 166: 1321-1332, 2005.
5. Hou J, Shi J, Chen L, Lv Z, Chen X, Cao H, Xiang Z and Han X: M2 macrophages promote myofibroblast differentiation of LR-MSCs and are associated with pulmonary fibrogenesis. Cell Commun Signal 16: 89, 2018.
6. Saito S, Zhuang Y, Shan B, Danchuk S, Luo F, Korfei M, Guenther A and Lasky JA: Tubastatin ameliorates pulmonary fibrosis by targeting the TGFβ-PI3K-Akt pathway. PLoS One 12: e0186615, 2017.
7. Esteller M: Non-coding RNAs in human disease. Nat Rev Genet 12: 861-874, 2011.
8. Fabian MR, Sonenberg N and Filipowicz W: Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79: 351-379, 2010.
9. Li S, Geng J, Xu X, Huang X, Leng D, Jiang D, Liang J, Wang C, Jiang D and Dai H: miR-130b-3p Modulates Epithelial-Mesenchymal Crosstalk in Lung Fibrosis by Targeting IGF-1. PLoS One 11: e0150418, 2016.
10. Wei S, Wang Q, Zhou H, Qiu J, Li C, Shi C, Zhou S, Liu R and Lu L: miR-455-3p Alleviates Hepatic Stellate Cell Activation and Liver Fibrosis by Suppressing HSF1 Expression. Mol Ther Nucleic Acids 16: 758-769, 2019.
11. Zhu XJ, Gong Z, Li SJ, Jia HP and Li DL: Long non-coding RNA Hottip modulates high-glucose-induced inflammation and ECM accumulation through miR-455-3p/WNT2B in mouse mesangial cells. Int J Clin Exp Pathol 12: 2435-2445, 2019.
12. Yu F, Jiang Z, Chen B, Dong P and Zheng J: NEAT1 accelerates the progression of liver fibrosis via regulation of microRNA-122 and Kruppel-like factor 6. J Mol Med (Berl) 95: 1191-1202, 2017.
13. Huang S, Xu Y, Ge X, Xu B, Peng W, Jiang X, Shen L and Xia L: Long noncoding RNA NEAT1 accelerates the proliferation and fibrosis in diabetic nephropathy through activating Akt/mTOR signaling pathway. J Cell Physiol 234: 11200-11207, 2019.
14. Yao W, Li Y, Han L, Ji X, Pan H, Liu Y, Yuan J, Yan W and Ni C: The CDR1as/miR-7/TGFBR2 Axis Modulates EMT in Silica-Induced Pulmonary Fibrosis. Toxicol Sci 166: 465-478, 2018.
15. Zhao X, Sun J, Chen Y, Su W, Shan H, Li Y, Wang Y, Zheng N, Shan H and Liang H: lncRNA PFAR Promotes Lung Fibroblast Activation and Fibrosis by Targeting miR-138 to Regulate the YAP1-Twist Axis. Mol Ther 26: 2206-2217, 2018.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
17. Wang X, Cheng Z, Dai L, Jiang T, Jia L, Jing X, An L, Wang H and Liu M: Knockdown of Long Noncoding RNA H19 Represses the Progress of Pulmonary Fibrosis through the Transforming Growth Factor β/Smad3 Pathway by Regulating MicroRNA 140. Mol Cell Biol 39: e00143-19, 2019.
18. Wu Q, Han L, Yan W, Ji X, Han R, Yang J, Yuan J and Ni C: miR-489 inhibits silica-induced pulmonary fibrosis by targeting MyD88 and Smad3 and is negatively regulated by lncRNA CHRF. Sci Rep 6: 30921, 2016.
19. Sun J, Su W, Zhao X, Shan T, Jin T, Guo Y, Li C, Li R, Zhou Y, Shan H, *et al*: lncRNA PFAR contributes to fibrogenesis in lung fibroblasts through competitively binding to miR-15a. Biosci Rep 39: BSR20190280, 2019.
20. Zhao X, Sun J, Chen Y, Su W, Shan H, Li Y, Wang Y, Zheng N, Shan H and Liang H: lncRNA PFAR Promotes Lung Fibroblast Activation and Fibrosis by Targeting miR-138 to Regulate the YAP1-Twist Axis. Mol Ther 26: 2206-2217, 2018.
21. Hao X, Du Y, Qian L, Li D and Liu X: Upregulation of long noncoding RNA AP003419.16 predicts high risk of aging associated idiopathic pulmonary fibrosis. Mol Med Rep 16: 8085-8091, 2017.
22. Li JH, Zhang SQ, Qiu XG, Zhang SJ, Zheng SH and Zhang DH: Long non-coding RNA NEAT1 promotes malignant progression of thyroid carcinoma by regulating miRNA-214. Int J Oncol 50: 708-716, 2017.
23. Li X, Wang S, Li Z, Long X, Guo Z, Zhang G, Zu J, Chen Y and Wen L: Retracted: NEAT1 induces epithelial-mesenchymal transition and 5-FU resistance through the miR-129/ZEB2 axis in breast cancer. FEBS Lett: Nov 1, 2016 (Epub ahead of print). doi: 10.1002/1873-3468.12474.

24. Liu T, Zhang Y, Liu W and Zhao J: lncRNA NEAT1 Regulates the Development of Parkinson's Disease by Targeting AXIN1 Via Sponging miR-212-3p. Neurochem Res: Nov 26, 2020 (Epub ahead of print). doi: 10.1007/s11064-020-03157-1.
25. Liu L, Liu F, Sun Z, Peng Z, You T and Yu Z: lncRNA NEAT1 promotes apoptosis and inflammation in LPS-induced sepsis models by targeting miR-590-3p. Exp Ther Med 20: 3290-3300, 2020.
26. Dong C, Gongora R, Sosulski ML, Luo F and Sanchez CG: Regulation of transforming growth factor-beta1 (TGF-β1)-induced pro-fibrotic activities by circadian clock gene BMAL1. Respir Res 17: 4, 2016.
27. Yoshida M, Romberger DJ, Illig MG, Takizawa H, Sacco O, Spurzem JR, Sisson JH, Rennard SI and Beckmann JD: Transforming growth factor-beta stimulates the expression of desmosomal proteins in bronchial epithelial cells. Am J Respir Cell Mol Biol 6: 439-445, 1992.
28. Ma J, Zhao N, Du L and Wang Y: Downregulation of lncRNA NEAT1 inhibits mouse mesangial cell proliferation, fibrosis, and inflammation but promotes apoptosis in diabetic nephropathy. Int J Clin Exp Pathol 12: 1174-1183, 2019.
29. Jin SS, Lin XF, Zheng JZ, Wang Q and Guan HQ: lncRNA NEAT1 regulates fibrosis and inflammatory response induced by nonalcoholic fatty liver by regulating miR-506/GLI3. Eur Cytokine Netw 30: 98-106, 2019.
30. Li X, Ye S and Lu Y: Long non-coding RNA NEAT1 overexpression associates with increased exacerbation risk, severity, and inflammation, as well as decreased lung function through the interaction with microRNA-124 in asthma. J Clin Lab Anal 34: e23023, 2020.
31. Zhao Y, Yan M, Yun Y, Zhang J, Zhang R, Li Y, Wu X, Liu Q, Miao W and Jiang H: MicroRNA-455-3p functions as a tumor suppressor by targeting eIF4E in prostate cancer. Oncol Rep 37: 2449-2458, 2017.
32. Zheng J, Lin Z, Zhang L and Chen H: MicroRNA-455-3p Inhibits Tumor Cell Proliferation and Induces Apoptosis in HCT116 Human Colon Cancer Cells. Med Sci Monit 22: 4431-4437, 2016.
33. Zhan T, Huang X, Tian X, Chen X, Ding Y, Luo H and Zhang Y: Downregulation of MicroRNA-455-3p Links to Proliferation and Drug Resistance of Pancreatic Cancer Cells via Targeting TAZ. Mol Ther Nucleic Acids 10: 215-226, 2018.
34. Guo J, Liu C, Wang W, Liu Y, He H, Chen C, Xiang R and Luo Y: Identification of serum miR-1915-3p and miR-455-3p as biomarkers for breast cancer. PLoS One 13: e0200716, 2018.
35. Zhou Y and Chai X: Protective effect of bicyclol against pulmonary fibrosis via regulation of microRNA-455-3p in rats. J Cell Biochem 121: 651-660, 2020.
36. Wu J, Liu J, Ding Y, Zhu M, Lu K, Zhou J, Xie X, Xu Y, Shen X, Chen Y, *et al*: MiR-455-3p suppresses renal fibrosis through repression of ROCK2 expression in diabetic nephropathy. Biochem Biophys Res Commun 503: 977-983, 2018.
37. Derynck R and Zhang YE: Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425: 577-584, 2003.