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Original Article

# Knockdown of LncRNA NEAT1 inhibits myofibroblast activity in oral submucous fibrosis through miR-760/TPM1 axis

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## KEYWORDS

Oral submucous fibrosis;  
LncRNA NEAT1;  
MiR-760;  
TPM1;  
Fibrous buccal mucosa fibroblasts

**Abstract** *Background/purpose:* Oral submucous fibrosis (OSF) is a fibrotic disease with high transformation of malignant disorders. Aberrant expression of lncRNA nuclear enriched abundant transcript 1 (NEAT1) was engaged with various fibrosis models, but its mechanism in OSF remained elusive.

*Materials and methods:* Fibrous buccal mucosa fibroblasts (fBMFs) were from OSF specimens. Myofibroblast activities including the alpha smooth muscle actin ( $\alpha$ -SMA) distribution and invasion capacities were determined by Immunocytochemistry and Transwell assays. Gene and protein were identified by quantitative real time polymerase chain reaction or western blotting. Binding relationship was analyzed via Starbase and dual-luciferase reporter or RNA immunoprecipitation assays.

*Results:* NEAT1 and Tropomyosin-1 (TPM1) were significantly increased in OSF specimens, but miR-760 was decreased. NEAT1 knockdown repressed myofibroblast activities and reduced the fibrosis and Wnt/ $\beta$ -catenin pathway via miR-760/TPM1 axis. MiR-760 inhibition could reverse the regulation of NEAT1 knockdown via TPM1 in fBMFs.

*Conclusion:* NEAT1 knockdown inhibited myofibroblast activities and Wnt/ $\beta$ -catenin pathway via miR-760/TPM1 axis in fBMFs. NEAT1 could be the target for inhibiting myofibroblast activities in fBMFs for OSF treatment.

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## Introduction

Oral submucous fibrosis (OSF) is amply defined as a chronic disorder of the oral cavity.<sup>1</sup> It can exert adverse effects on the function and cell metabolic homeostasis of oral cavity or pharynx, and have a compelling risk of malignant transformation.<sup>2</sup> And preponderant epidemiological research evidence indicates that areca nut chewing is the main etiological factor for OSF, especially common in southeast Asian countries.<sup>3</sup> In addition, the progression of OSF is closely accompanied with atrophic oral epithelium and extraordinarily extracellular matrix deposition (such as collagen), which involving the dysplasia level of buccal mucosa fibroblasts.<sup>4</sup> Apparently, one of the primary fibrosis cellular regulators have been myofibroblasts identified by alpha smooth muscle actin (alpha-SMA) and phosphorylated or drosophila mothers against decapentaplegic protein (p-/Smad) maker, and their activation can be induced to fibrous buccal mucosa fibroblasts (fBMFs) in OSF patients.<sup>4–6</sup> Myofibroblasts activation and fBMFs formation have been crucially implicated in the progression and presentation stage of OSF disease.<sup>7</sup> But the detailed molecular regulatory mechanism of myofibroblasts' activities including the fBMFs' collagen contraction, migration and invasion capacities is still remaining relatively elusive. As one of pre-cancerous diseases, the pathogenesis and progression mechanism of OSF is remain indistinct and requisite to be explored to supplement the current limited clinical treatment of OSF.

Long noncoding RNAs (LncRNAs) have been reported that they are engaged in the regulation of aberrant cell proliferation and differentiation process. And aberrant expression of LncRNAs also has been involved the pathomechanism of fibrotic diseases including heart, liver and oral cavity.<sup>8–10</sup> Recent relevant research has shown that knockdown of LncRNA nuclear enriched abundant transcript 1 (NEAT1) could repress the renal fibrosis and inflammation response in mice model by modulating epithelial–mesenchymal transition (EMT) process, another crucial cause of myofibroblasts' activation.<sup>11</sup> In addition, NEAT1 could promote the progression of liver fibrosis in primary mouse hepatic stellate cells.<sup>12</sup> Also, the expression of NEAT1 has been also enhanced in diabetic nephropathy model, another fibrosis related disease, and NEAT1 overexpression promoted the fibrosis of mouse mesangial cells.<sup>13</sup> All the mentioned research indicates the vital regulatory role of NEAT1 in various fibrosis model and diseases. Notably, the extreme high expression of NEAT1 has been reported to closely link with the progression of clinical stage and shorter survival time in the patients with oral squamous cell carcinoma, and the oral squamous cell carcinoma can be induced and developed by the OSF.<sup>14,15</sup> It has indicated the importance of the NEAT1 in the patients with OSF. But based on current research results, the role of NEAT1 in the activation myofibroblasts and formation of fBMFs is still unknown, which is the crucial process of OSF.

Meanwhile, microRNAs (miRNAs) also pose vital regulatory effects on the development of OSF.<sup>16</sup> Related study results have manifested that the level of miR-760 is decreased in glaucoma patients and associated with glaucoma tissue fibrosis.<sup>17</sup> Also, compared the control group,

the expression levels of miR-760 in buccal mucosa has also declined in OSF patients.<sup>18</sup> And our bioinformation analysis results predicted that the NEAT1 and miR-760 could be target binding. But their individual and combined regulatory mechanism of OSF pathogenesis is still rare in relevant researches.

Tropomyosin-1 (TPM1) is one member of the tropomyosin family and aberrant expression of it is found to be in miscellaneous diseases including tumor, cardiomyopathy and fibrosis.<sup>19–21</sup> Based on recent study results, TPM1 could significantly repress the metastasis of various tumor cells such as renal cancer cells, which suggesting its potential regulatory role in the EMT process.<sup>22</sup> Previous study results indicated that TPM1 exhibited a high level in the TGF- $\beta$ 1-induced renal fibrosis model of NRK-49F cells, and knock-down of it could repress myofibroblast formation by inactivation of Wnt/ $\beta$ -catenin signaling pathway.<sup>23</sup>

Wnt/ $\beta$ -catenin signaling pathway has been reported as the vital regulatory element that promotes EMT and increases collagen secretion by mesenchymal cells, induce the extracellular matrix (ECM) deposition in related tissues or organs.<sup>24</sup> Based on previous research, the abnormal activation of Wnt/ $\beta$ -catenin signaling pathway and its downstream proteins C-myc and CyclinD1 has been observed in OSF tissues, suggesting its potential modulatory role in OSF.<sup>25</sup> In addition, we found that TPM1 could also have potential binding target with miR-760, based on the bioinformation prediction. But there has been no related research involved the interaction mechanism among the miR-760, TPM1 and Wnt/ $\beta$ -catenin signaling in the myofibroblasts' activities including fBMFs' collagen contraction, migration and invasion capacities of the OSF.

Our research objective was to explore the mechanism of NEAT1, miR-760 and TPM1 in the fBMFs for myofibroblast activities and the regulation of Wnt/ $\beta$ -catenin signaling pathway in fBMFs. It clarified the NEAT1's molecular mechanism in fBMFs and provided a potential target for OSF treatment.

## Materials and methods

### Tissues acquisition and cell culture

In our study, all procedures were conducted in conformity to the approved guidelines from the Institutional Review Committee of The First People's Hospital of Jingzhou in Hubei. Oral mucosa tissues (n = 30) were collected from normal or OSF patients from The First People's Hospital of Jingzhou in Hubei. For the pathological analysis, Patient's age, gender, chewing duration, OSF disease duration and OSF histopathological grade were applied as parameters as shown in Table 1. And the other excised mucosa samples were shredded and washed respectively in PBS (which containing antibiotics with 100 Unit/ml penicillin, streptomycin and 0.25 mg/mL amphotericin). Then 3 mg/mL dispase II (Roche, Mannheim, Germany, #04942078001) was continuously shaken to get fibrotic BMFs at 37 °C for 1 h<sup>26</sup> fBMFs which were migrated from margin of tissue were maintained in Dulbecco's-modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA, #11965092) with 10% fetal bovine serum (FBS, Gibco, #10091155) and antibiotics.

**Table 1** Associations between miR-411-3p/KIA0087/SOCS1 expression and clinicopathological characteristics in osteosarcoma patients.

Clinical parameters	Cases (n)	Expression level		P value (*p < 0.05)	Expression level		P value (*p < 0.05)	Expression level		P value (*p < 0.05)
		miR-760 <sup>high</sup>	miR-760 <sup>low</sup>		TPM1 <sup>high</sup>	TPM1 <sup>low</sup>		LncRNA NEAT1 <sup>high</sup>	LncRNA NEAT1 <sup>low</sup>	
<b>Age (years)</b>										
<30 years	18	10	8	0.710	8	10	0.710	7	11	0.264
≥30 years	12	5	7		7	5		8	4	
<b>Gender</b>										
Male	16	6	10	0.272	5	11	0.066	6	10	0.272
Female	14	9	5		10	4		9	5	
<b>Chewing duration (years)</b>										
<4	12	7	5	0.710	4	8	0.264	9	3	0.0600
≥4	18	8	10		11	7		6	12	
<b>Disease duration (years)</b>										
<1	14	12	2	0.001	3	11	0.009	2	12	0.001
≥1	16	3	13		12	4		13	3	
<b>Histological grade</b>										
E stage	12	10	2	0.009	1	11	0.001	2	10	0.011
M stage	8	3	5		6	2		6	2	
A stage	10	2	8		8	2		7	3	

E stage: early stage; M stage: moderately advanced stage; A stage: advanced stage.

## Cell transfection

Synthesized short hairpin RNA and overexpression vector for NEAT1 (sh-NEAT1 or oe-NEAT1), shRNA targeting TPM1 (sh-TPM1), and their negative control (NC) were synthesized and obtained from Genepharma (Shanghai, China). The miR-760 mimics, miR-760 inhibitor, mimics NC and inhibitor NC were also synthesized and bought from Genepharma. For the overexpressing of TPM1, the full-length human TPM1 cDNA was synthesized and sub-cloned into the pcDNA3.1 vector (Genepharma). Transient transfection of fBMFs (single or cotreated transfection) were performed by the Lipofectamine 2000 which obtained from Invitrogen (Carlsbad, CA, USA, #11668-027) in according with the manufacturer's protocol.

## Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated by the Total RNA-extraction kit (Yeasen Biotech, Shanghai, China, #10606ES60) and converted to cDNA by High-Capacity Prime Script RT reagent (Yeasen Biotech, # 11121ES60) according to the appendant instruction. The sequent qPCR analysis was performed by the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green Supermix (Takara, Dalian, China, #RR420L). And for detecting the level of miR-760, qRT-PCR was conducted with the using of TaqMan miRNA assays accompanied with specific primer sets (Applied Biosystems).<sup>27</sup> The  $2^{-\Delta\Delta C_t}$  calculative method was selected for detection the relative change level of each specific gene. The chosen primer sequences (5'-3') in the qPCR analysis were listed below:

NEAT1-F: 5'-GTTCCGTGCTTCTCTTCTG-3',  
 NEAT1-R: 5'-GTGTCTCCGACTTTACCAG-3',  
 miR-760-F: 5'-TCAATCCACCAGAGCATGGATAT-3',

miR-760-R: 5'-CTCTACAGCTATATTGCCAGCCA-3',  
 TPM1-F: 5'-GGCACCGAAGATGAACTGGACA-3',  
 TPM1-R: 5'-GCGTCTGTTCAGAGAAGCTACG-3',  
 GAPDH-F: 5'-GTCTCGTCTGACTTCAACAGCG-3',  
 GAPDH-R: 5'-ACCACACTGTTGCTGTAGCCAA-3',  
 U6-F: 5'-CTCGCTTCGGCAGCACT-3',  
 U6-R: 5'-AACGCTTCACTAATTTGCGT-3'.

## Western blot analysis

Cells and tissues were harvested and lysed in RIPA buffer with protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China, #P0013B) to obtain total protein. The protein concentration was determined by the Bicinchoninic Acid Protein Assay Kit (Beyotime Institute of Biotechnology, #P0012). For detecting the protein abundance of  $\alpha$ -SMA (CST, Danvers, MA, USA, #19245, 1:1000), p-Smad2 (CST, #18338, 1:1000), Smad2 (CST, #18338, 1:1000), CyclinD1 (CST, #55506, 1:1000), C-myc (Abcam, Cambridge, UK, #ab32072, 1:1000),  $\beta$ -catenin (Abcam, #ab184919, 1:1000), and GAPDH (CST, #8884, 1:1000), equal protein samples were separated by 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Abcam, #ab133411). Then, PVDF membranes were conducted in 5% Bovine Serum Albumin (BSA, Sangon Biotech, Shanghai, China, #E661003-0200) by 1 h to blockade at room temperature and incubated with specific primary antibodies overnight at 4 °C temperature condition. And the peculiar horseradish peroxidase (HRP)-conjugated secondary antibodies were administered to incubate the PVDF membranes by 1 h. The specific protein bands' emergence was observed with the Enhanced Chemiluminescence Kit (ECL, Thermo Fisher Scientific, Waltham, MA, USA, #WP20005) and captured by the ImageQuant LAS system (GE Healthcare, Piscataway, NJ, USA).

## Immunocytochemistry assays (ICC)

For immunocytochemical analysis, fBMFs cultured on coverslips with the appropriate density were incubated with sodium citrate buffer (pH 6.0). Cells were incubated overnight at 4 °C with  $\alpha$ -SMA (CST, #19245, 1:2000) antibodies before blocked with 3% H<sub>2</sub>O<sub>2</sub> and bovine serum albumin (BSA). After that, the cell samples were covered by secondary antibodies and visualized with 0.03% 3, 3'-diaminobenzidine. The expression level was quantified using Image Pro Plus software (Media Cybernetics, Rockville, MD, USA).

## Transwell assays

Transwell Assays (Corning Incorporated, Corning, NY, USA, #3422) was used to determine the invasion and migration capacities of fBMFs. For the migration assays, DMEM with serum was added in the lower chamber for chemoattraction. And the membrane was coated with Matrigel (BD Pharmingen, Franklin Lakes, NJ, USA, #356234) for the invasion capacity detection.  $1 \times 10^5$  Cells were cultured in the upper chamber filled with the serum-free DMEM for 48 h of incubation. Cells on the upper surface were stained by 0.5% crystal violet (Sigma–Aldrich, Burlington, MA, USA, #V5265) for 15 min and followed fixation. Stained fBMFs were counted in five random visual areas of by a microscope (Olympus, Tokyo, Japan) with 100-fold magnification.

## Luciferase reporter assay

The predicted binding sites of miR-760 on NEAT1 or TPM1 (WT-NEAT1 or WT-TPM1) and their mutated site (MUT-NEAT1 or MUT-TPM1) were constructed and cloned into the firefly luciferase gene in PGL3 vector (Promega, Madison, WI, USA, #E1751). fBMFs were seeded into 24-well plates and cultured by 12 h, co-transfected with the WT-NEAT1/MUT-NEAT1 or WT-TPM1/MUT-TPM1 reporter gene plasmid and miR-760 mimics or mimics NC. After the incubation by 48 h, Dual-Luciferase Reporter Assay System (Promega, #E1960) was utilized to determine luciferase activities.

## RNA immunoprecipitation (RIP) assays

RIP analysis was conducted with Magna RNA-binding protein immunoprecipitation kit (Millipore, Burlington, MA, USA), according to the previously research protocols.<sup>28</sup> fBMFs were administrated in RIP buffer and cell extraction was interacted with magnetic beads bound with antibodies immunoglobulin G (IgG, as the control; Abcam, #ab32381) or Argonaute-2 (Ago2; Abcam, #ab6702). And the protease K (Sangon Biotech, #A004220) was applied for the protein digestion and immunoprecipitated RNA isolation. Thereafter, the enrichment of NEAT1, TPM1 and miR-760 in the immunoprecipitant complexes were determined by RT-qPCR analysis.

## Statistical analysis

Data were statistically analyzed by SPSS 22.0. Data was presented as the mean  $\pm$  standard deviation (SD). Comparison of differences between groups was assessed using one-way

ANOVA and Student's *t*-test. Pearson's correlation was conducted to analyze the relationship among the expression levels of NEAT1, miR-760 and TPM1. Results were reported as statistic significant when  $P < 0.05$ .

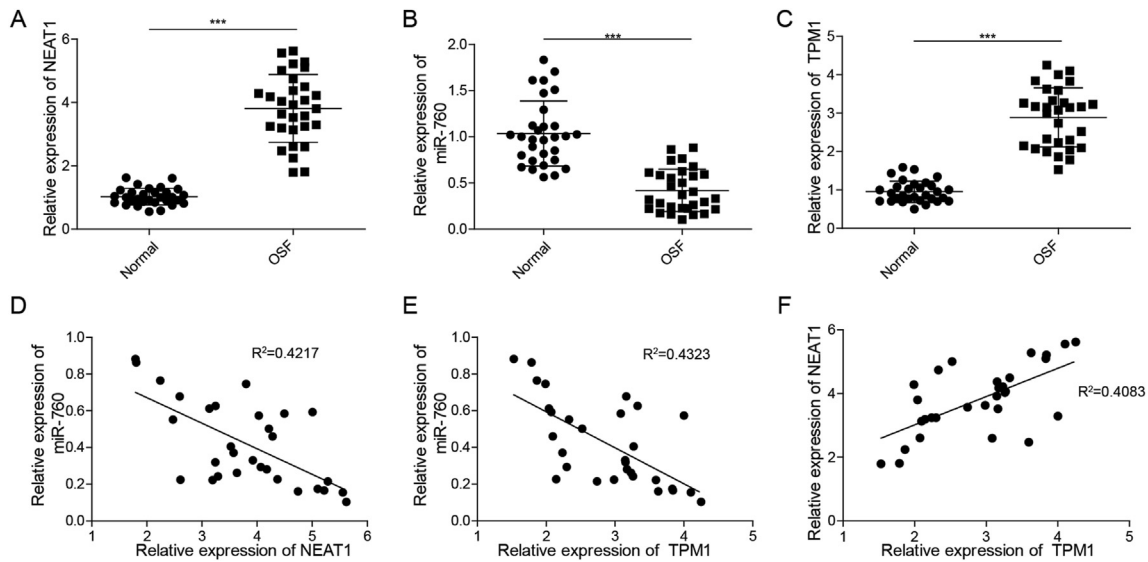
## Results

### NEAT1 and TPM1 were significantly up-regulated but miR-760 was down-regulated in OSF specimens

To explore the regulatory role of NEAT1 in the development of OSF, we determined the gene expression level of it in OSF tissue samples. As indicated in Fig. 1A, the expression of NEAT1 ( $p < 0.05$ ) was significantly increased in OSF tissues, compared with the normal tissues (Fig. 1A). And, for identifying the interaction effect of NEAT1 with its potential target TPM1 and miR-760 in OSF pathogenesis, we also determined the gene expression levels of them in OSF specimens. RT-PCR analysis results revealed that the TPM1 ( $p < 0.05$ ) was significantly up-regulated, but that of miR-760 ( $p < 0.05$ ) was down-regulated in OSF tissues (Fig. 1B and C). Subsequently, we carried the linear correlation analysis among the expression levels of NEAT1 and miR-760 in OSF patients. Spearman's correlation coefficient results showed that the negative correlation relation was exhibited between the NEAT1 and miR-760 ( $p < 0.05$ ), and between the miR-760 and TPM1 ( $p < 0.05$ ). But the NEAT1 posed a positive correlation with the level of TPM1 in OSF patients. More importantly, the up-regulated expression of NEAT1 and TPM1 and the down-regulated expression of miR-760 were significantly correlated with the OSF disease duration and OSF histological grade (Table 1). So, the aberrant expressions of NEAT1, TPM1 and miR-760 in OSF specimens and their correlation relation of OSF disease duration and histological grade indicated their potential regulatory role in OSF development.

### NEAT1 knockdown repressed the myofibroblast activities and the activation of Wnt/ $\beta$ -catenin signaling pathway via targeting miR-760 in fBMFs

To identify the precise regulatory mechanism of NEAT1 in OSF, the constructed shRNAs (sh-NEAT1 or sh-NC) and overexpression transfection (oe-NEAT1 or oe-NC) were engaged in fBMFs to verify related regulatory effects. Compared with the negative control group, the gene expression of NEAT1 ( $p < 0.05$ ) was dramatically decreased in sh-NEAT1 group, but significantly increased in overexpression group, suggesting the high interference efficiency of the designed vectors (Fig. 2A). In addition, we used the StarBase 3.0 to predict the target miRNA of NEAT1, and the results showed that NEAT1 had putative binding points of miR-760 (Fig. 2B). And, the predicted results were validated by dual-Luciferase reporter assay, the overexpression of miR-760 ( $p < 0.05$ ) significantly reduced the luciferase of NEAT1-WT group, not NEAT1-MUT group (Fig. 2C). Then, we conducted the RIP assays to further validate the interaction between NEAT1 and miR-760. As shown in Fig. 2D, the enrichment of NEAT1 and miR-760 ( $p < 0.05$ ) were significantly boosted in Ago2-containing miRNA ribonucleoprotein complex of fBMFs.



**Figure 1** Expression of NEAT1, miR-760 and TPM1 in OSF specimens. (A) Expression of NEAT1 was increased ( $P < 0.05$ ) whereas (B) miR-760 expression was decreased ( $P < 0.05$ ), and (C) TPM1 expression was increased ( $P < 0.05$ ) in OSF specimens. (D) NEAT1 posed ( $P < 0.05$ ) the negative correlation with miR-760 and (E) miR-760 posed ( $P < 0.05$ ) the negative correlation with TPM1, whereas (F) NEAT1 posed ( $P < 0.05$ ) the positive correlation with TPM1 in OSF specimens (\* $P < 0.05$ ; \*\* $P < 0.01$ ). The data were expressed as the mean  $\pm$  SD and statistically analyzed using the Student's t-test and Spearman's correlation coefficient analysis.

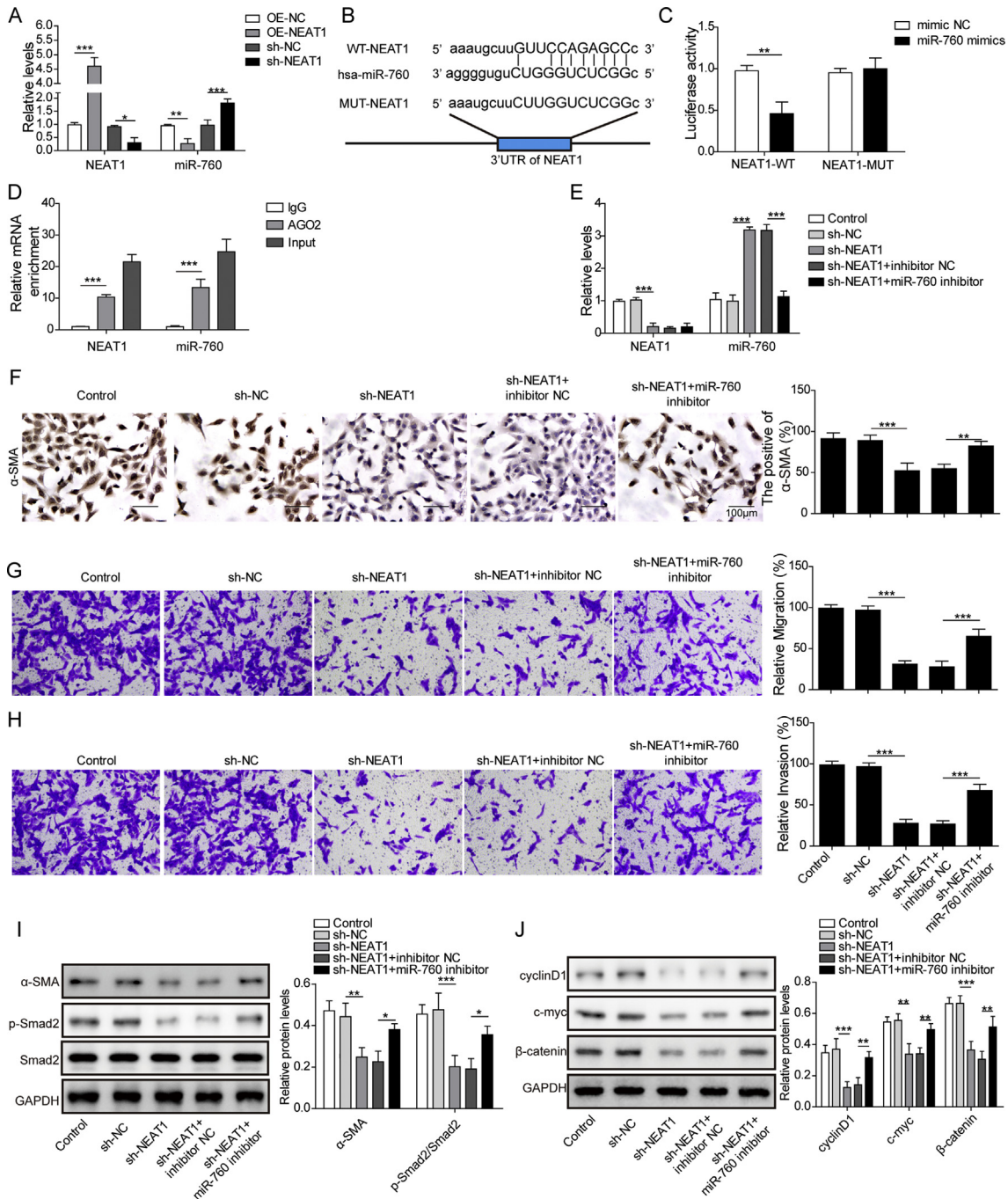
Also, the expression level of miR-760 ( $p < 0.05$ ) was increased of sh-NEAT1 group, and miR-760 inhibitor reversed the increasing effect of NEAT1 knockdown in miR-760 expression in fBMFs (Fig. 2E). These findings indicated that NEAT1 targeted miR-760 and negatively regulated its expression in fBMFs. To further explore the effects of NEAT1 and miR-760 on the activity of myofibroblasts, we detected the fibrotic marker  $\alpha$ -SMA's immunocytochemistry distribution, migration and invasion capacities of fBMFs. As showed in (Fig. 2F, G and 2H), NEAT1 knockdown ( $p < 0.05$ ) repressed the protein distribution of  $\alpha$ -SMA, migration and invasion capacities of fBMFs, but miR-760 inhibitor ( $p < 0.05$ ) counteracted these regulatory effects. And, knockdown of NEAT1 ( $p < 0.05$ ) reduced the fibrotic marker protein levels of  $\alpha$ -SMA and p-Smad in fBMFs, suggesting anti-fibrotic function of NEAT1 knockdown. But inhibition of miR-760 ( $p < 0.05$ ) also reversed these effects (Fig. 2H). As the crucial signaling pathway involved tissue fibrosis, Wnt/ $\beta$ -catenin signaling pathway's proteins of fBMFs were determined by western blotting analysis. We found that NEAT1 silencing ( $p < 0.05$ ) reduced the protein abundance of  $\beta$ -catenin and its downstream targets including CyclinD1 and C-myc, and miR-760 inhibitor ( $p < 0.05$ ) abolished the decrease of them in fBMFs. These findings showed that NEAT1 silencing could restrain the myofibroblast activities and inactivate of Wnt/ $\beta$ -catenin signaling pathway in fBMFs to exert anti-fibrotic effects via directly targeting miR-760.

### Overexpression of miR-760 repressed the myofibroblast activities via targeting TPM1 involved the activation of Wnt/ $\beta$ -catenin signaling pathway in fBMFs

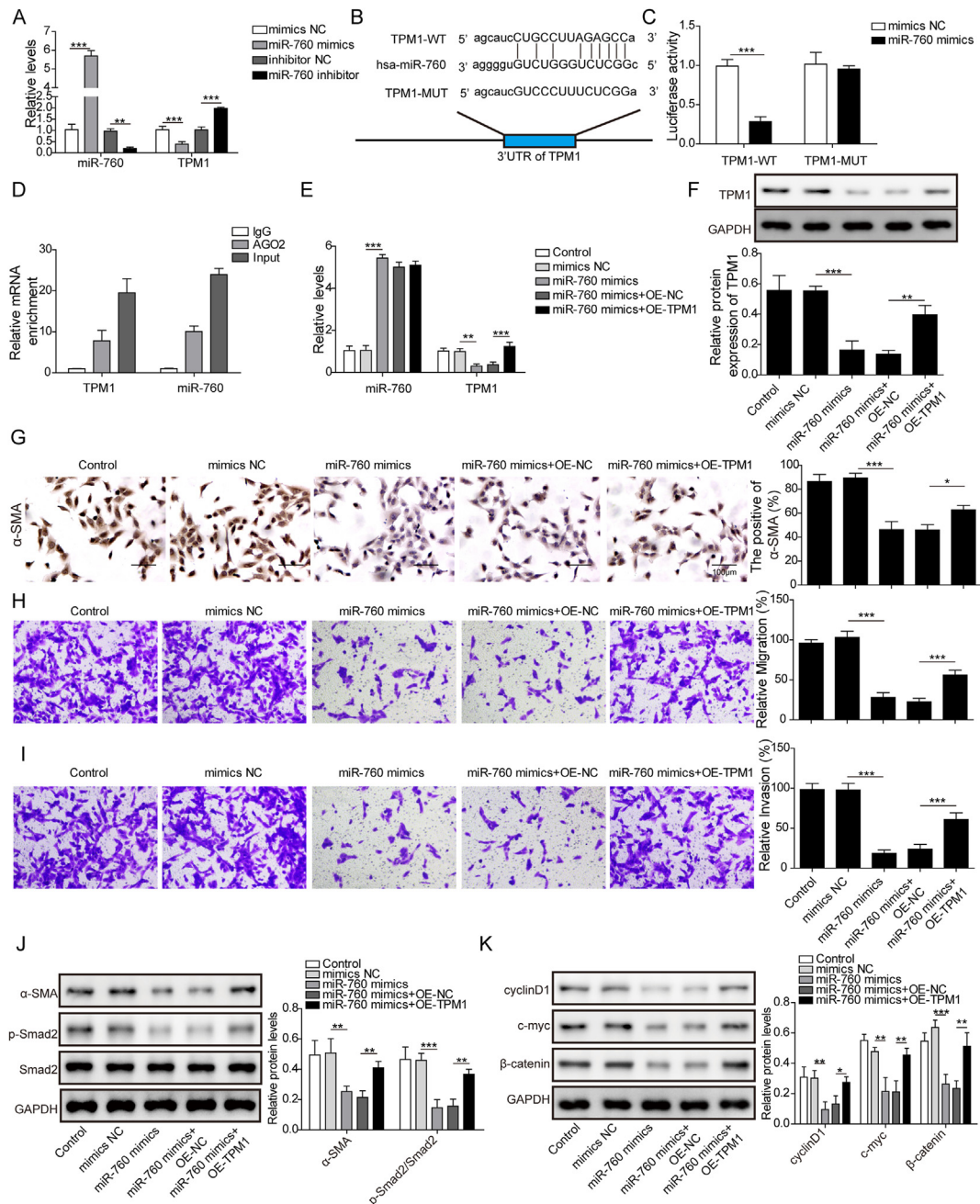
To further investigate the functional roles of miR-760 in fBMFs, we determined the gene expression levels of it in

fBMFs after relevant transfection treatment. Results showed that the miR-760 overexpression treatment ( $p < 0.05$ ) significantly boosted its expression level in mimic group, and the inhibition transfection ( $p < 0.05$ ) exerted the distinct reverse effects, showing the high efficiency of designed transfection vector (Fig. 3A). And, the prediction results of miR-760's interaction showed that it had a putative binding site with TPM1, and the prediction results were verified by dual-Luciferase reporter assay of them (Fig. 3B and C). Also, based on the results of RIP assays, both of TPM1 and miR-760 ( $p < 0.05$ ) were significantly enriched in Ago2-containing miRNA ribonucleoprotein complex of fBMFs (Fig. 3D). In addition, the gene and protein expression levels of TPM1 ( $p < 0.05$ ) were markedly repressed by miR-760 overexpression (Fig. 3E and F). These effects were partially reversed by TPM1 overexpression ( $p < 0.05$ ) in fBMFs cotreated overexpression of miR-760 and TPM1 (Fig. 3E and F). Above results revealed the negative interaction relation between miR-760 and TPM1 in fBMFs.

Then, we also determined the protein distribution of  $\alpha$ -SMA, migration and invasion capacities to identify modulatory effects of miR-760 and TPM1 in fBMFs. As shown, the protein distribution of  $\alpha$ -SMA, migration and invasion capacities of fBMFs posed a visibly decrease in miR-760 mimic group ( $p < 0.05$ ). Interestingly, the repressive effects of miR-760 overexpression on fBMFs' myofibroblast activities were reversed by TPM1 overexpression ( $p < 0.05$ ) (Fig. 3G, H and 3I). And overexpression of miR-760 ( $p < 0.05$ ) reduced protein levels of  $\alpha$ -SMA and p-Smad in fBMFs, whereas overexpression of TPM1 partially abolished these effects (Fig. 3J). Moreover, the levels of CyclinD1, C-myc and  $\beta$ -catenin ( $p < 0.05$ ) were declined in miR-760 mimic group, and these inhibited effects of miR-760 in fBMFs were markedly reversed by TPM1 overexpression ( $p < 0.05$ ). Altogether, these findings indicated that knockdown of



**Figure 2** Effects of NEAT1 on the miR-760 expression, myfibroblast activities and Wnt/ $\beta$ -catenin signaling pathway in fBMFs. (A) Transfection of Oe-NEAT1 vector enhanced ( $P < 0.05$ ) the expression of NEAT1 and inhibited ( $P < 0.05$ ) the expression of miR-760, whereas transfection of sh-NEAT1 vector inhibited ( $P < 0.05$ ) NEAT1 expression and enhanced the expression of miR-760. (B) Bioinformatics predicted the interaction binding sites between NEAT1 and miR-760, and their interaction was validated ( $P < 0.05$ ) by (C) Dual luciferase assays and (D) RIP assays. (E) NEAT1 knockdown enhanced ( $P < 0.05$ ) the expression of miR-760. (F) NEAT1 knockdown repressed ( $P < 0.05$ ) the protein level of  $\alpha$ -SMA, (G) collagen contractility (H) migration and (I) invasion capacities of fBMFs, whereas miR-760 inhibition reversed ( $P < 0.05$ ) the trend. (J) NEAT1 knockdown repressed ( $P < 0.05$ ) the fibrotic marker  $\alpha$ -SMA and p-Smad expression, whereas inhibition of miR-760 reversed ( $P < 0.05$ ) the trend. (I) NEAT1 knockdown repressed ( $P < 0.05$ ) the Wnt/ $\beta$ -catenin pathway expression, whereas inhibition of miR-760 also reversed ( $P < 0.05$ ) the trend (\* $P < 0.05$ ; \*\* $P < 0.01$ ). The data were expressed as the mean  $\pm$  SD and statistically analyzed using the Student's t-test.



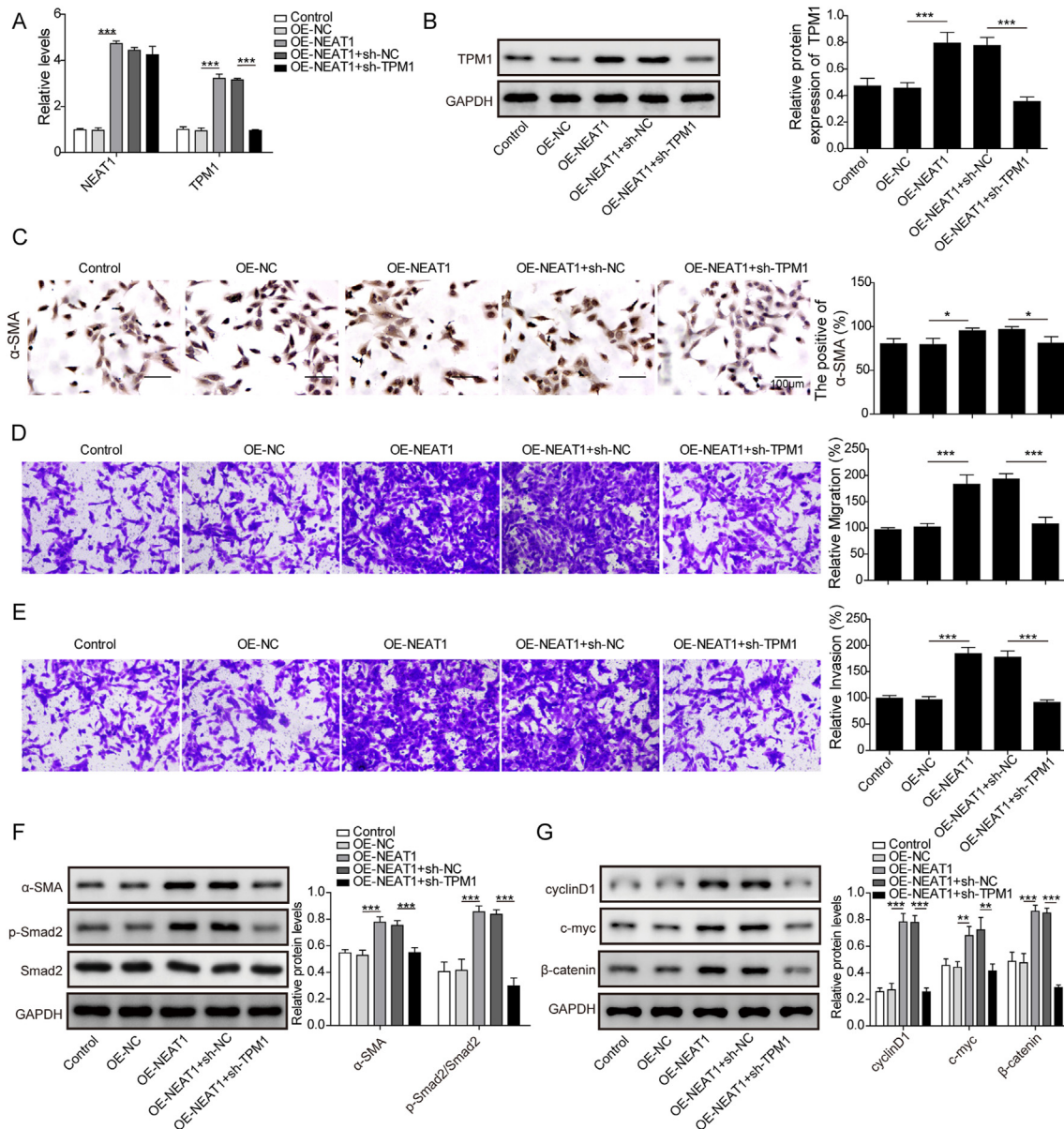
**Figure 3** Effects of miR-760 on the TPM1 expression, myfibroblast activities and Wnt/ $\beta$ -catenin signaling pathway in fBMFs. (A) Transfection of miR-760 mimic enhanced ( $P < 0.05$ ) its expression and the inhibitor inhibited ( $P < 0.05$ ) its expression, whereas transfection of miR-760 inhibitor enhanced ( $P < 0.05$ ) TPM1 expression. (B) Bioinformatics predicted the interaction binding sites between miR-760 and TPM1, and their interaction was validated ( $P < 0.05$ ) by (C) Dual luciferase assays and (D) RIP assays. (E, F) MiR-760 overexpression repressed ( $P < 0.05$ ) the gene and protein expression of TPM1. (G) MiR-760 overexpression repressed ( $P < 0.05$ ) the protein level of  $\alpha$ -SMA, (H) collagen contractility (I) migration and (J) invasion capacities of fBMFs, whereas TPM1 overexpression reversed ( $P < 0.05$ ) the trend. (K) MiR-760 overexpression repressed ( $P < 0.05$ ) the fibrotic marker  $\alpha$ -SMA and p-Smad expression, whereas overexpression of TPM1 reversed ( $P < 0.05$ ) the trend. (L) Overexpression of miR-760 repressed ( $P < 0.05$ ) the Wnt/ $\beta$ -catenin pathway expression, whereas overexpression of miR-760 also reversed ( $P < 0.05$ ) the trend (\* $P < 0.05$ ; \*\* $P < 0.01$ ). The data were expressed as the mean  $\pm$  SD and statistically analyzed using the Student's t-test.

miR-760 could exert a negative effect on OSF in modulating the myfibroblast activities and Wnt/ $\beta$ -catenin signaling pathway in fBMFs through TPM1.

### NEAT1 enhanced the myfibroblast activities and activated Wnt/ $\beta$ -catenin signaling pathway through TPM1 in fBMFs

To investigate the interaction mechanism between NEAT1 and TPM1 in OSF pathogenesis, the genes and protein densities of them in fBMFs after respective transfection

treatment were detected. Detection results indicated that the overexpression of NEAT1 ( $p < 0.05$ ) significantly enhanced its gene expression level and TPM1's gene and protein abundance in fBMFs than the control group (Fig. 4A and B). And NEAT1 overexpression ( $p < 0.05$ ) exhibited the enhanced the protein distribution of  $\alpha$ -SMA and myfibroblast activities of fBMFs, but TPM1 knockdown ( $p < 0.05$ ) reversed these pro-fibrogenic phenotype effects (Fig. 4C, D and 4E). Moreover, overexpression of NEAT1 ( $p < 0.05$ ) boosted the protein levels of  $\alpha$ -SMA and p-Smad in fBMFs, whereas inhibition of TPM1 ( $p < 0.05$ ) could suppress these



**Figure 4** Effects of NEAT1 on the TPM1 expression, myfibroblast activities and Wnt/ $\beta$ -catenin signaling pathway in fBMFs. (A, B) Transfection of Oe-NEAT1 vector enhanced ( $P < 0.05$ ) the gene and protein expression of TPM1. (C) NEAT1 overexpression repressed ( $P < 0.05$ ) the protein level of  $\alpha$ -SMA, (D) collagen contractility (E) migration and (F) invasion capacities of fBMFs, whereas TPM1 knockdown reversed ( $P < 0.05$ ) the trend. (G) MiR-760 overexpression repressed ( $P < 0.05$ ) the fibrotic marker  $\alpha$ -SMA and p-Smad expression, whereas overexpression of TPM1 reversed ( $P < 0.05$ ) the trend. (H) Overexpression of miR-760 repressed ( $P < 0.05$ ) the Wnt/ $\beta$ -catenin pathway expression, whereas overexpression of miR-760 also reversed ( $P < 0.05$ ) the trend (\* $P < 0.05$ ; \*\* $P < 0.01$ ). The data were expressed as the mean  $\pm$  SD and statistically analyzed using the Student's t-test.



negative effects for OSF progression (Fig. 4F). In addition, NEAT1 overexpression ( $p < 0.05$ ) enhanced the protein expression levels of  $\beta$ -catenin, CyclinD1 and C-myc and TPM1 inhibitor also abolished these relevant implications in fBMFs (Fig. 4G). Overall, these results revealed that NEAT1 overexpression could enhance the expression of  $\alpha$ -SMA and p-Smad, myofibroblast activities, and promote the activation of Wnt/ $\beta$ -catenin signaling pathway through regulation of TPM1 in fBMFs with the manifestation of deterioration for OSF.

## Discussion

OSF is defined as an emblematic chronic fibrotic disease and its transformation rate ranks the third highest among the subtypes for oral potentially malignant disorders (OPMD).<sup>29</sup> Based on the epidemiological research results, the habit of areca nut chewing is regard as the main factor with OSF occurrence and dramatically contribute to the pathogenesis of OSF. As the vital constitution of areca nut, the arecoline can induce the myofibroblast transformation from the BMFs, myofibroblast' migration and collagen formation with the enhancement of the protein abundance of fibrotic marker  $\alpha$ -SMA and p-Smad in OSF specimens.<sup>30</sup> Activated myofibroblast is the vital element engaged in the accumulation of ECM<sup>31</sup> and development of fibrosis process.<sup>32</sup> So, in consideration of the boundedness for current OSF's treatment and detection of transformation extent, we need the brand-new molecular biomarker and therapeutic strategy for OSF. In our study, we found that the aberrant high expression of NEAT1 in OSF tissues and knockdown of it could repress the myofibroblast activities and inactivate of Wnt/ $\beta$ -catenin signaling pathway via targeting miR-760/TPM1 axis in fBMFs. It provided a potential biomarker and therapeutic target of OSF and hopeful to strain the OSF development.

Based on the previous study results, aberrant high expression of NEAT1 was involved the fibrosis processes of various tissues including liver, lung, and kidney, engaged in the enhancement of myofibroblasts' activity of the mentioned models.<sup>11,33,34</sup> NEAT1 silencing could repress the inflammation reaction, hepatocyte apoptosis and liver fibrosis in the liver.<sup>34</sup> And, NEAT1 knockdown could decrease the fibrotic marker protein levels of Collagen I, Collagen III and  $\alpha$ -SMA in TGF- $\beta$ 1-induced pulmonary cells.<sup>33</sup> In our study, we also found the expression of NEAT1 posed a high level in OSF specimens than the normal group. And NEAT1 knockdown could significantly repressed the migration, invasion capacities and the fibrotic marker  $\alpha$ -SMA and p-Smad expression in fBMFs, suggesting the anti-fibrotic effect of it. And our found anti-fibrotic effect of NEAT1 knockdown was similar with the other organs' fibrosis models, which implying that it may be engaged with the extensive or systemic fibrosis needing more researches to validate its role. Our results demonstrated the accelerative effect of NEAT1 on myofibroblast activity and fibrosis process in OSF.

lncRNAs' interaction with miRNAs always participated in the regulation of disease development. For example, NEAT1 could regulate fibrosis and inflammation via targeting miR-506 in the nonalcoholic fatty liver disease.<sup>35</sup> And, there were abundant miRNAs exerting abnormal expression in the

OSF tissues such as miR-1246 and miR-155-5p by bio-information screening and analysis.<sup>5,36</sup> In others and our research, the results indicate that the expression of miR-760 is declined in OSF patients.<sup>18</sup> But the interaction relation between NEAT1 and miR-760 is still unclear. In the present study, the results showed that miR-760 was also decreased and overexpression of it significantly repressed the myofibroblast activities in fBMFs. And overexpression of miR-760 inhibited the expression of  $\alpha$ -SMA and p-Smad, the marker proteins involved the tissues fibrosis. And we offered evidence that NEAT1 knockdown could regulate the myofibroblast activities and via directly targeting miR-760 in fBMFs to restrain OSF, which also similar with the published researches about the regulation mechanism of lncRNAs and miRNAs in the disease's models.

TPM1 is one member of the tropomyosins (TPMs) and has been defined as an anti-tumor gene acting the regulator of cancer progression.<sup>37</sup> For example, TPM1 could inhibit the renal cancer cells' proliferation and metastasis.<sup>19</sup> And TPM1 significantly repressed the migration and invasion in esophageal squamous cell carcinoma cell line.<sup>38</sup> Recent study indicated that the TPM1 knockdown could reduce the expression level of  $\alpha$ -SMA in rat renal tubular epithelial cells treated with TGF- $\beta$  to simulate kidney fibrosis.<sup>23</sup> Also, the relevant research showed that arecoline treatment markedly enhanced the expression of TPM1 and activated the TGF- $\beta$ /Smad pathway in the oral keratinocytes cell model HaCaT cell line, and inhibition of TGF- $\beta$  receptor could repressed the increase of TPM1 induced by arecoline treatment.<sup>39</sup> And in our study, we found that the expression of TPM1 was enhanced in the fBMFs, in accordance with the arecoline treated HaCaT cell line. In addition, as above mentioned, overexpression of miR-760 could pose the anti-fibrotic effects in fBMFs, but TPM1 overexpression abolished these anti-fibrotic effects of overexpression of miR-760 in fBMFs. Our bioinformation prediction and dual-Luciferase reporter assay results also identified that miR-760 had directly binding site with TPM1. Further results about NEAT1 and TPM1 showed that NEAT1 could enhanced the myofibroblast activities through TPM1 in fBMFs. So, our research provided the evidence that NEAT1 could regulate the myofibroblast activities in fBMFs through miR-760/TPM1 axis. And, Wnt/ $\beta$ -catenin signaling pathway and its downstream proteins including C-myc and CyclinD1 also have been reported that they are the vital modulators for collagen secretion and can inhibit the degradation of extracellular matrix to promote tissues fibrosis.<sup>40,41</sup> Of noted, we also found that NEAT1 activated Wnt/ $\beta$ -catenin signaling pathway through miR-760/TPM1 axis in fBMFs.

In conclusion, our study showed that NEAT1 knockdown could repress the myofibroblast activities and inactivate of Wnt/ $\beta$ -catenin signaling pathway via miR-760/TPM1 axis in fBMFs. Whereas we need to go on exploring the effects of silencing or overexpressing of NEAT1 and the miR-760/TPM1 axis on the atrophic oral epithelium and extraordinarily extracellular matrix deposition condition, and the key element of OSF development, and the relevant research in vivo OSF model to further explore the deeper mechanism of NEAT1 in the future study. In view of the negative impacts of OSF on the patients' health and life quality, and the current treatment of OSF, our finding offers a molecular mechanism of NEAT1 knockdown in OSF and provide a

potential target for inhibiting myofibroblast activities in fbMFs for OSF treatment.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

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