Long non-coding RNA NEAT1 contributes to lipopolysaccharide-induced inflammation and apoptosis of human middle ear epithelial cells via regulating the miR-301b-3p/TLR4 axis

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Abstract. Acute otitis media (AOM) is a common infectious disease in children that is accompanied by signs and symptoms of middle ear inflammation and infection. Previous studies have shown that the long non-coding (lnc)RNA nuclear-enriched abundant transcript 1(NEAT1) participates in various inflammatory conditions and plays an important regulatory role. The focus of the present study was the biological function of NEAT1 and underlying molecular mechanism in lipopolysaccharide (LPS)-induced human middle ear epithelial cells (HMEECs). The expression of NEAT1, miR-301b-3p and toll-like receptor 4 (TLR4) protein were determined by reverse transcription-quantitative PCR and western blot assays, respectively. Dual-luciferase reporter assay was performed to investigate the combination of miR-301b-3p and NEAT1 or *TLR4*. In addition, cell viability, apoptosis and the levels of pro-inflammatory factors (IL-1β, TNF-α and IL-6) were measured by Cell Counting Kit-8 assay, flow cytometry and ELISA, respectively. Cell viability was significantly decreased, whereas apoptosis and inflammation were increased in LPS-stimulated HMEECs. Functional analyses demonstrated that NEAT1 was upregulated following LPS treatment, whereas knockdown of NEAT1 significantly increased cell viability and alleviated apoptosis and inflammation. Mechanistically, NEAT1 directly bound to and negatively regulated miR-301b-3p expression, whereas miR-301b-3p inhibitors abolished the inhibitory effect of NEAT1 knockdown on cell apoptosis and inflammation. As a target of miR-301b-3p, TLR4 was regulated by NEAT1 and miR-301b-3p. TLR4 overexpression alleviated NEAT1 silencing-induced inflammatory suppression. Rescue experiments demonstrated that NEAT1 promoted TLR4 expression by inhibiting miR-301b-3p. Collectively, the results of the present study suggested that NEAT1 may attenuate LPS-induced inflammation and apoptosis in HMEECs by modulating the miR-301b-3p/TLR4 axis, and may provide a new therapeutic target for the clinical treatment of AOM.

Introduction

Acute otitis media (AOM) is one of the most common infectious diseases in childhood caused by bacteria (1), such as *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* (*H. influenzae*) and *Moraxella catarrhalis* (2). AOM is characterized by the presence of middle ear effusion together with an acute onset of signs and symptoms caused by middle ear inflammation. The pathophysiology, pathogenesis and progression of AOM are associated with multiple factors, such as pathogenic bacteria, reactive oxygen species and inflammatory cytokines (3). Currently, due to the extensive use of antibiotics, which may cause resistance of AOM to treatment, 10-20% of children may experience continuous recurrences of otitis media and long-term deafness (4). Therefore, identifying effective therapies is crucial for the prevention and treatment of AOM.

Long non-coding (lnc)RNAs are a group of heterogeneous non-coding RNAs with a length of >200 nucleotides, which are widely distributed in the genome (5). Previous research has shown that lncRNAs play an important role in transcription regulation, maintenance of genomic integrity, alternative splicing, gene chromatin rearrangement (6,7) and various pathophysiological processes (8,9). Several studies have revealed that lncRNAs are implicated in immune defense against bacterial

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infections, involving *Salmonella*, *Mycobacterium bovis* and *Mycobacterium tuberculosis* (10-12). The lncRNA nuclear-enriched abundant transcript 1 (NEAT1) has two isoforms (short isoform NEAT1-1 and long isoform NEAT1-2), which are associated with the prognosis of human tumors (13). In addition, NEAT1 has been reported to be involved in a number of inflammatory diseases. It has been reported that overexpression of NEAT1 protected cardiomyo-cytes from As_2O_3-induced injury (14), whereas other studies re-ported that downregulating NEAT1 inhibited inflamma-tion, fibrosis and apoptosis (15-17). In addition, NEAT1 was found to be upregulated by lipopolysaccharide (LPS) (15,18). However, the regulatory mechanism of NEAT1 in AOM remains unclear and must be further investigated. Therefore, the present study focused on the biological function of NEAT1 in AOM.

MicroRNAs (miRNAs/miRs) are non-coding, endog-enous single-stranded small RNAs. Studies have shown that miRNAs may serve as nodes of signaling networks, partici-pating in the regulation of basic cell activities, such as cell inflammation (19). miR-301b-3p was found to be significantly reduced in LPS-induced human middle ear epithelial cells (HMEECs) (20), but the biological function of miR-301b-3p in AOM has not been reported to date. Furthermore, miRNAs can negatively regulate protein expression by targeting the 3'-untranslated region (UTR) of their target mRNAs (21). Based on bioinformatics prediction, the toll-like receptor 4 gene (*TLR4*) was shown to be a target of miR-301b-3p in the present study. TLR4 has been proven to play an essential role in triggering the inflammatory response, and TLR4 signal activation promotes an increase in the production and upregulation of pro-inflammatory factors (22,23). Furthermore, the TLR4 protein was found to be upregulated by non-typeable *H. influenzae* infection and LPS induction in animal models and patients with AOM (24-27). Hence, it was hypothesized that miR-301b-3p and TLR4 may be involved in NEAT1-mediated inflammation in AOM. The aim of the present study was to investigate the effect of NEAT1 on inflammatory response in LPS-treated HMEECs and explore the potential interactions among NEAT1, miR-301b-3p and TLR4.

Materials and methods

Cell culture and LPS treatment. HMEECs were purchased from American Type Culture Collection; human renal epithe-lial cells (HK2) and human liver epithelial cells (THLE-3) were purchased from Beijing Beina Chuanglian Institute of Biotechnology. HMEECs and THLE-3 cells were routinely cultured in RPMI-1640 medium supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. HK2 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml strepto-mycin. The cells were kept in a humidified atmosphere with 5% CO_2 and 95% humidity at 37°C in an incubator. HMEECs were stimulated with LPS (Sigma-Aldrich; Merck KGaA) at different concentrations (0, 0.1, 1, 3, 5 and 10 μg/ml) for 24 h, and then cultured for 12, 24 and 48 h to determine cell viability. The cells were incubated with 5 μg/ml LPS for 6, 12,

24 and 48 h to determine the optimal action time. Finally, 5 μg/ml LPS was used for 24 h to establish an inflammatory cell model.

Cell transfection. Transfections were performed using 1.0 μg small interfering (si)RNA of NEAT1 (si-NEAT1; 5'-GUGAGA AGUUGCUUAGAAACUUUCC-3'), 1.0 μg si-*TLR4* (5'-TTGC TAAATGCTGCCGTTTTATC-3'), 1.0 μg si-RNA-negative control (si-NC; 5'-UACUGUCUAGUCGCCGUAC-3'), 100 nM miR-301b-3p mimic (5'-CAGUGCAAUGAUAUUGUCAA AGC-3'), 100 nM NC mimic (5'-UUGUACUACACAAAAGU ACUG-3'), 100 nM miR-301b-3p inhibitor (5'-GCUUUGACAA UAUCAUUGCACUG-3') and 100 nM NC inhibitor (5'-CAG UACUUUUGUGUAGUACAA-3'), which were obtained from Shanghai GenePharma Co., Ltd.. To overexpress NEAT1 or *TLR4*, the full-length sequence was inserted into the pcDNA3.1 vector (2 μg; Shanghai GenePharma Co., Ltd.), and an empty vector (2 μg) was used as a control. HMEECs cells ($1x10^5$) were transfected with si-RNA, miR-mimic, miR-inhbitor, overexpression vectors and corresponding NCs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C. After transfection for 48 h, the efficiency of transfection was detected by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis.

RNA extraction and RT-qPCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The first cDNA of total RNA was synthesized according to the instructions of PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.). qPCR was subsequently performed using the SYBR Green qPCR kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The following primer sequences were used for RT-qPCR: Taurine upregu-lated gene 1, forward 5'-AAATCTGGCTCTGCTGACCC-3' and reverse 5'-CAGAAAGGCCAGCCATACCA-3'; zinc finger antisense 1, forward 5'-AACCATTAGCTAGCTGG GGC-3' and reverse 5'-CAAGTTAACCCCGGAGGGAC-3'; plasmacytoma variant translocation 1, forward 5'-CCTGTGA CCTGTGGAGACAC-3' and reverse 5'-GTCCGTCCAGAGT GCTGAAA-3'; zinc finger E-box binding homeobox 1 anti-sense 1, forward 5'-CCGTGGGCAC-TGCTGAAT-3' and reverse 5'-CTGCTGGCAAGCGGAACT-3'; X-inactive-specific transcript, forward 5'-CTTGGATGGGTTGCCAGCTA-3' and reverse 5'-TCATGCCCCATCTCCACCTA-3'; NEAT1, forward 5'-CAGTTAGTTTATCAG-TTCTCCCATCCA-3' and reverse 5'-GTTGTTGTCGTCACCTTTCAACTCT-3'; miR-301b-3p, forward 5'-TGCTGCTAACGAATGCTCTGA-3' and reverse 5'-CTCTGCTTTCAGATGCTTTGAC-3'; GAPDH, forward 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse 5'-AGGG GCCATCCACAGTCTTC-3'; and U6, forward 5'-CTCGCTTC GGCAGCACATA-3' and reverse 5'-AACGATTCACGAATT TGCGT-3'. RT-qPCR experiments were performed with the 7900HT Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocy-cling conditions were used for qPCR: Initial denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and elongation at 72°C for 20 sec, with final extension at 72°C for 10 min. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (28) and

normalized to the internal reference genes GAPDH (mRNA) and U6 (miRNA).

Cell viability assay. Cell viability was assessed using the Cell Counting Kit-8 proliferation de-tection kit (Beyotime Institute of Biotechnology). Briefly, cells were seeded in 96-well plates at a density of 10^4 cells/well; 10 μl CCK-8 solution was added to each well and cultured for 4 h. The OD value at 450 nm was measured with a microplate reader (BioTek Instruments, Inc.).

Detection of cell apoptosis. Apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis detection kit (Beijing Solarbio Science & Technology Co., Ltd.). In brief, HMEECs were collected using cold PBS and then cultured with 5 μl Annexin V-FITC reagent and 5 μl PI in the dark for 15 min. The cell apoptosis rate was analyzed by flow cytometry (BD Biosciences) and FlowJo software (version 10; FlowJo LLC).

ELISA. Cell-free culture supernatant was collected after treatment. The secretory levels of IL-6 (cat. no. 900-T16), TNF-α (cat. no. 900-M25) and IL-1β (cat. no. 900-M95) were analyzed using their corresponding ELISA kits (Neobioscience Technology Co., Ltd.) according to the manufacturers' instructions. The concentration of inflammatory cytokines was measured at 450 nm using a microplate spectrophotometer (BioTek Instruments, Inc.).

Western blot analysis. Cells were lysed with RIPA buffer [50 mM Tris-HCl (pH 8.0), 50 mM NaCl and 0.5% NP-40]. The total protein (20 μg/well) in the supernatant was separated by 10% SDS-PAGE and then transferred to PVDF membranes. After blocking with 5% skimmed milk at room temperature for 2 h, the membrane was incubated with primary antibodies against TLR4 (dilution 1:1,000; cat. no. ab13556; Abcam) or GAPDH (dilution 1:1,000; cat. no. ab8245; Abcam) at 4˚C overnight, and then incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:4,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The immunoreactive bands were visualized using an enhanced chemilumi-nescence reagent (Beyotime Institute of Biotechnology). The blots were semiquantified by ImageJ software, version 1.47 (National Institutes of Health).

Bioinformatics and luciferase reporter analysis. The starBase (version 2.0: star-base.sysu.edu.cn) online software was used to predict targeting binding sites of lncRNA to miRNA and miRNA to mRNA. Therefore, the targeting associations between miR-301b-3p and NEAT1 or *TLR4* were predicted. Reporter vectors were constructed using pmirGLO dual-lucif-erase expression vector (Promega Corporation), including wild-type (WT) and mutant (MUT) binding sequences of NEAT1 and *TLR4*, named as WT-NEAT1, MUT-NEAT1, WT-*TLR4* and MUT-*TLR4*, and were co-transfected with miR-301b-3p mimics or negative control (sequences as afore-mentioned) into 293 cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C. After 48 h of transfection, luciferase activities were measured with a dual-luciferase reporter assay

system (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity and all experiments were performed in triplicate.

Statistical analysis. All experiments were repeated three times. GraphPad Prism 8 (GraphPad Software, Inc.) was used for statistical analysis, and data are presented as mean ± standard deviation. Differences between the two groups were analyzed using unpaired Student's t-tests and differences between multiple groups data were analyzed by one-way ANOVA followed by Tukey's post hoc tests. P<0.05 was considered to indicate statistically significant differences.

Results

LPS induces apoptosis and inflammatory response in HMEECs and inhibits cell viability. To investigate whether LPS causes HMEEC injury, different concentrations (0, 0.1, 1, 3, 5 and 10 μg/ml) of LPS were used to treat HMEECs for 12, 24 and 48 h. The CCK-8 assay demonstrated that the viability of HMEECs was inhibited after LPS induction in a time- and concentration-dependent manner (Fig. 1A). Following treatment with 3 and 5 μg/ml for 48 h, the cell viability decreased by ~50% (P<0.05), and it was significantly decreased in the 5 μg/ml group compared with that in the 0 μg/ml (control) group (P<0.01; Fig. 1B). Therefore, HMEECs were treated with 5 μg/ml for 24 h in the subsequent experiments. LPS-induced HMEEC apoptosis was significantly increased (P<0.01; Fig. 1C), and the concentration of inflammatory factors (IL-6, IL-1β and TNF-α) was also significantly increased (P<0.001; Fig. 1D), indicating that LPS induced an inflammatory response in HMEECs.

NEAT1 knockdown alleviates cell apoptosis and inflammatory response in LPS-induced HMEECs. RT-qPCR was conducted to explore the possible involvement of lncRNAs in the regulation of AOM-related mechanisms. As shown in Fig. 2A, all six inflammation-related lncRNAs were found to be upregulated in LPS-stimulated HMEECs, among which NEAT1 exhibited the most prominent increase (P<0.001). Subsequently, si-NEAT1 was transfected into HMEECs to inhibit the expression of NEAT1. The results demonstrated that NEAT1 was successfully silenced (Fig. 2B; P<0.01). CCK-8 assay, flow cytometry and ELISA were conducted to elucidate the effect of NEAT1 knockdown on the proliferation, apoptosis and inflammation of LPS-stimulated HMEECs (Fig. 2C-E). The results revealed that NEAT1 knockdown relieved the proliferation inhibition of LPS-induced HMEECs (P<0.01), and markedly alleviated cell apoptosis and the levels of the inflammatory factors IL-6, IL-1β and TNF-α (P<0.05 and P<0.001, respectively). Taken together, these results suggested that the downregulation of NEAT1 inhibited LPS-induced HMEEC inflammatory injury.

miR-301b-3p targets NEAT1 and TLR4 in HMEECs. Epithelial cells are protected from injury due to different stressors through inflammation response regulation. To investigate the abnor-mal expression of miR-301b-3p and TLR4 in LPS-induced epithe-lial cells, as described by Ma *et al* (29) in HMEECs, HK-2 and THLE-3 cells were selected, and then exposed to LPS
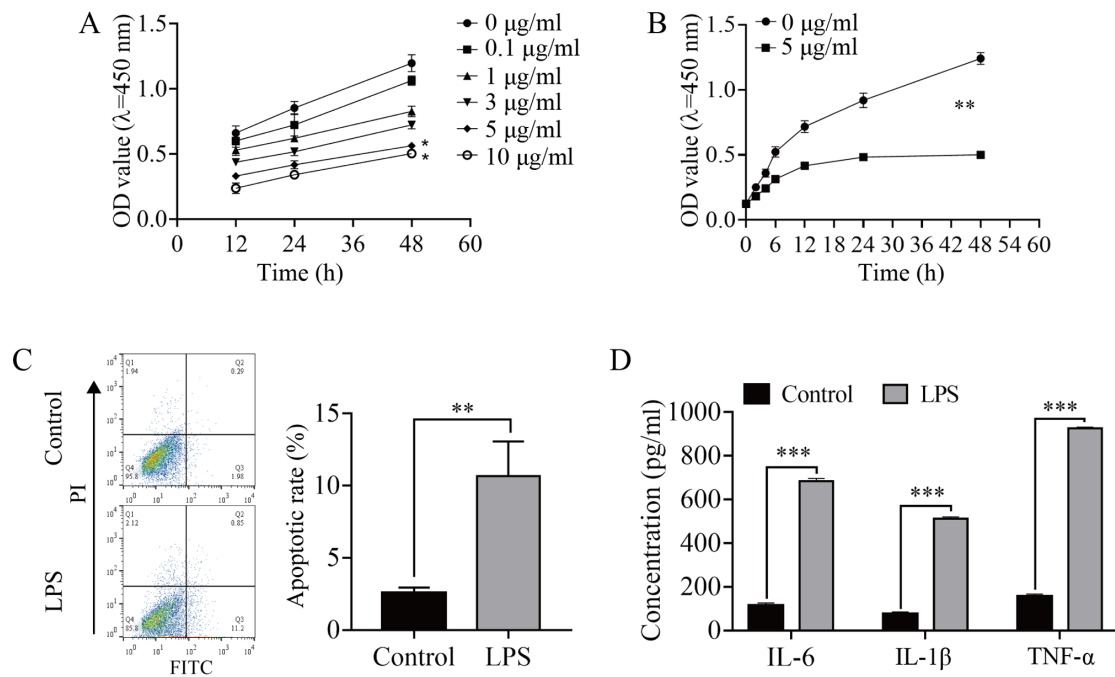
Figure 1. LPS reduces cell viability, and increases apoptosis and inflammatory response in human middle ear epithelial cells. (A and B) Cell viability was estimated by Cell Counting Kit-8 assay. *P<0.05, **P<0.01 vs. 0 μg. (C) Cell apoptosis was evaluated by flow cytometry. **P<0.01 vs. control. (D) IL-6, IL-1β and TNF-α expression was evaluated by ELISA. ***P<0.001 vs. control. LPS, lipopolysaccharide; OD, optical density; FITC, fluorescein isothi-ocyanate; PI, propidium iodide.

for 48 h. miR-301b-3p expression in HMEECs was markedly downregulated following LPS treat-ment (Fig. 3A; P<0.01), while TLR4 expression was significantly upregulated (Fig. 3B; P<0.05). Moreover, miR-301b-3p and TLR4 levels in HK-2 and THLE-3 cells, as well as in HMEECs, were detected by RT-qPCR and western blot assays. miR-301b-3p and TLR4 levels were down- and upregulated, respectively, following LPS induction (Fig. 3A and B; P<0.05). These results suggested that LPS down- and upregulated miR-301b-3p and TLR4 levels, re-spectively, in HMEECs. A search through the bioinformatics database starBase revealed a putative interac-tion between NEAT1 and miR-301b-3p (the target binding sequence is shown in Fig. 3C). Dual-luciferase reporter assay demonstrated that NEAT1-WT and miR-301b-3p mimics cotransfection significantly decreased luciferase activity in HMEECs, while NEAT1-MUT and miR-301b-3p mimics cotransfection failed to affect luciferase activity in HMEECs (Fig. 3D; P<0.01). Furthermore, NEAT1 silencing significantly increased miR-301b-3p expression (Fig. 3E; P<0.01). These results indicated that miR-301b-3p was targeted by NEAT1. Subsequently, based on the biological information database starBase, the target gene of miR-301b-3p was predicted, and it was found that TLR4, which is involved in inflammation, and miR-301b-3p had a targeted binding sequence (Fig. 3F). Dual-luciferase reporter assay demonstrated that the luciferase activity of TLR4-WT was decreased by miR-301b-3p mimics in HMEECs, while TLR4-MUT and miR-301b-3p mimics cotransfection failed to affect luciferase activity (Fig. 3G; P<0.01). Subsequently, pc-NEAT1 vector and miR-301b-3p mimic were transfected into HMEECs to upregulate the expression of NEAT1 and miR-301b-3p, and the results revealed that NEAT1and miR-301b-3p expression was strongly

enhanced (Fig. 3H and 3I; P<0.01). As shown in Fig. 3J, over-expression of miR-301b-3p inhibited TLR4 expression, which was reversed by overexpressing TLR4 (Fig. 3J; P<0.05). Taken together, these results proved that miR-301b-3p directly targets both NEAT1 and *TLR4*.

NEAT1 regulates LPS-induced proliferation, apoptosis and inflammation of HMEECs via the miR-301-3p/TLR4 axis. To explore how NEAT1 regulates LPS-induced HMEEC proliferation, inflammation and apoptosis, miR-301b-3p inhib-itors, si-NEAT1, si-*TLR4* and pc-*TLR4* were transfected into HMEECs to detect their regulatory effects on inflammation. Silencing miR-301b-3p significantly decreased miR-301b-3p expression compared with the NC group (Fig. 4A; P<0.01), and then transfection of si-*TLR4* and pc-*TLR4* resulted in effective decrease and increase in the expression of TLR4, respectively (Fig. 4B and C; P<0.01). The CCK-8 assay revealed that LPS inhibited cell proliferation, which was recovered with si-NEAT1 and si-*TLR4*, but proliferation was finally suppressed by miR-301b-3p inhibitors or overexpression TLR4 (Fig. 4D). Similarly, the inflammation and apoptosis of HMEECs was analyzed. Flow cytometry and ELISA revealed an opposite trend than the CCK-8 assay (Fig. 4E and F). Next, western blotting was performed to verify whether NEAT1 regulated the miR-301-3p/TLR4 axis and protein expression of TLR4. As shown in Fig. 4G, miR-301b-3p inhibitors or overexpression of TLR4 abolished the inhibitory effect of NEAT1 silencing on the protein expression of TLR4. In addition, miR-301b-3p inhibitors reversed the suppression of the protein expression of TLR4 by TLR4 silencing. Taken together, these results indicated that NEAT1 attenuated LPS-induced inflammation in HMEECs via modulating the miR-301b-3p/TLR4 axis.
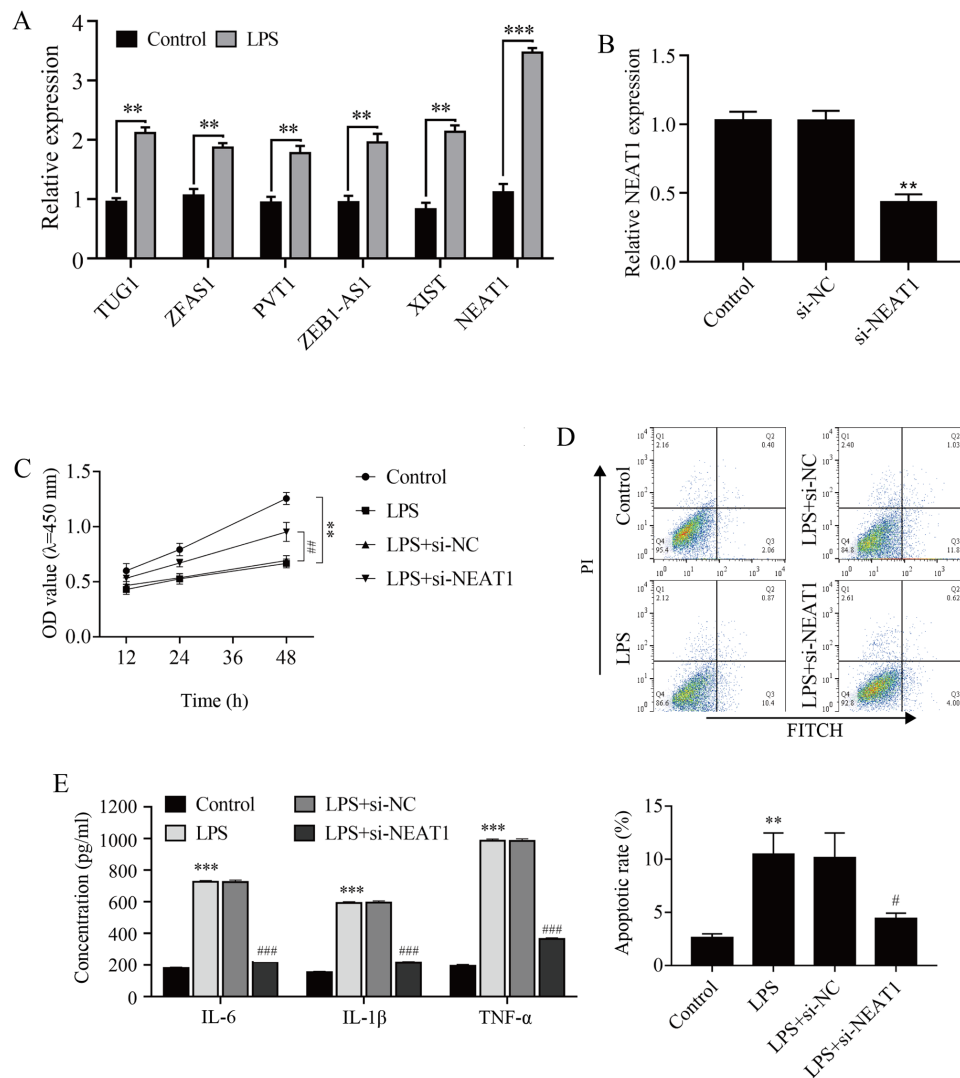
Figure 2. NEAT1 knockdown alleviates cell apoptosis and inflammatory response in LPS-induced human middle ear epithelial cells. (A) The expression of different long non-coding RNAs and (B) transfection efficiency of si-NEAT1 were analyzed by reverse transcription-quantitative PCR. (C) Cell viability was analyzed by Cell Counting Kit-8 assay. (D) Cell apoptosis was evaluated by flow cytometry. (E) IL-6, IL-1β and TNF-α expression was measured by ELISA. **P<0.01 and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. LPS+si-NC. LPS, LPS-stimulated; NEAT1, nuclear enriched abundant transcript 1; TUG1, taurine upregulated gene 1; ZFAS1, zinc finger antisense 1; PVT1, plasmacytoma variant translocation 1; ZEB1-AS1, zinc finger E-box binding homeobox 1 antisense 1; XIST, X-inactive-specific transcript; LPS, lipopolysaccharide; NC, negative control; si, small interfering; OD, optical density; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Discussion

AOM is one of the most common infectious diseases in children. Its pathological characteristic is inflammation, with induced secretion of cytokines TNF-α, IL-6 and IL-8 (30). AOM may develop into recurrent or persistent otitis media, which causes progressive hearing loss and severely affects the quality of life of the patients (1), even causing language disability and intellectual impairment (31). The specific molecular mechanism of AOM is poorly understood and finding effective therapeutic strategies is urgently required. Therefore, the present study aimed to investigate the NEAT1/miR-301b-3p/TLR4 regulatory network, in the hope of revealing a new theoretical basis for the application of NEAT1 in the treatment of AOM. In the present study, LPS was used to establish the cell model of AOM and investigate the potential mechanism (26,32). According to previous reports, NEAT1 is induced in multiple immune system diseases,

such as pneumonia (15), hepatitis (17) and osteoarthritis (33). Thus, it was hypothesized that NEAT1 may be induced in LPS-stimulated HMEECs. In the present study, NEAT1 was found to be significantly increased in LPS-induced HMEECs, and knockdown of NEAT1 significantly improved cell proliferation and inhibited cell apoptosis and inflammation. These data suggested that NEAT1 knockdown attenuated LPS-induced inflammation and apoptosis.

NEAT1 has been previously found to be induced in various tumors (13). For example, NEAT1 was shown to increase cell survival and facilitate gallbladder cancer progression by sponging miR-335 (34). Furthermore, NEAT1 is not only im-plicated in cancer, but may also affect the inflammatory response. Li et al (35) reported that NEAT1 may promote LPS-induced inflammatory injury in macrophages by regulating miR-17-5p/TLR4. In addition, downregulation of NEAT1 promoted cell proliferation and inhibited the apoptosis of rat cardiac muscle cells (36). However, to the best
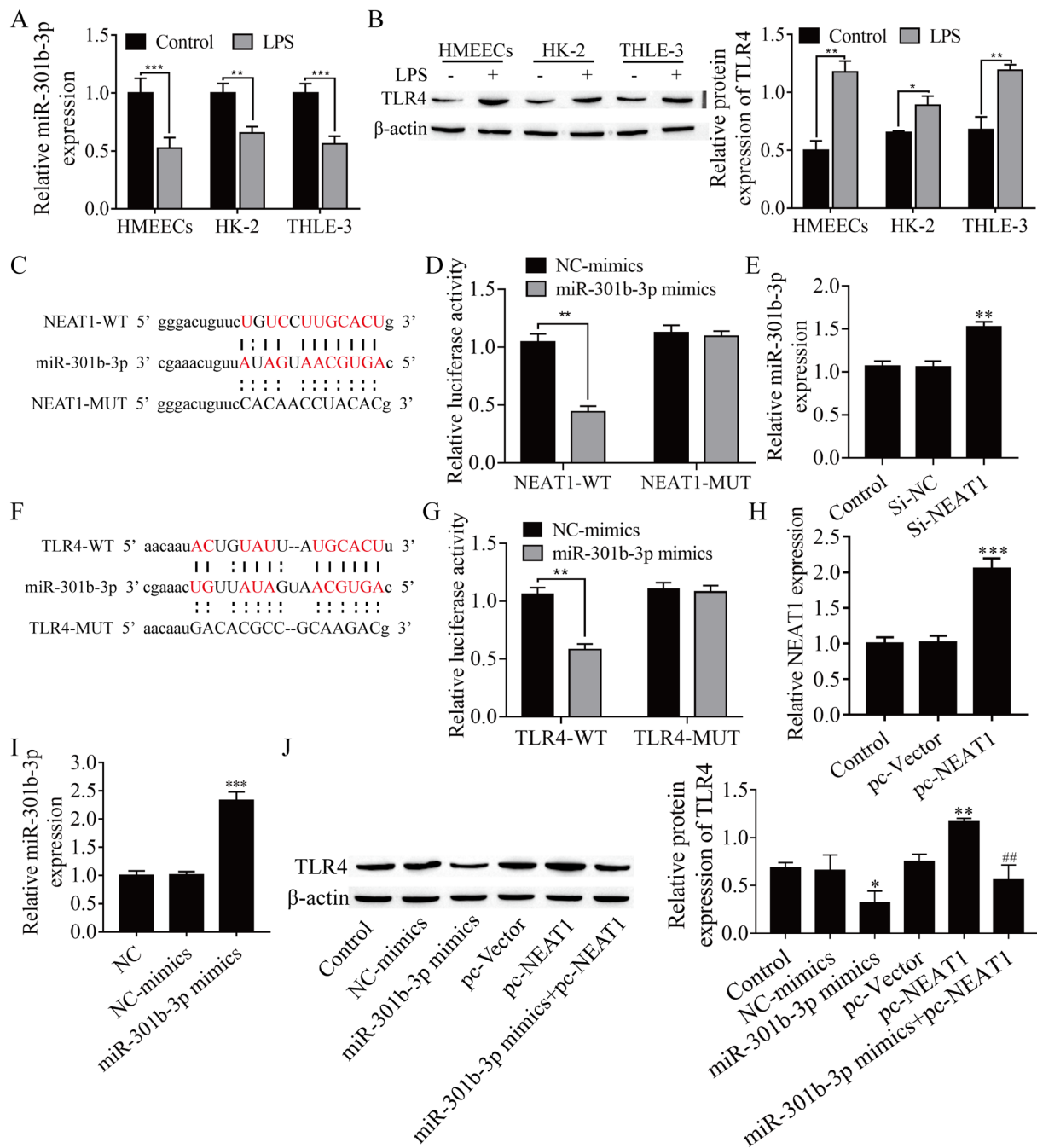
Figure 3. miR-301b-3p directly targets both NEAT1 and *TLR4*. (A) miR-301b-3p expression was detected by RT-qPCR. (B) TLR4 protein level was analyzed with western blotting. (C) Binding site of miR-301b-3p and NEAT1. (D) Dual-luciferase reporter assay was used to prove NEAT1 can target miR-301b-3p. (E) NEAT1 silencing significantly increased miR-301b-3p expression in HMEECs. (F) Binding site of miR-301b-3p and *TLR4*. (G) Dual-luciferase reporter assay was used to prove that miR-301b-3p can target *TLR4*. (H) NEAT1 expression was determined by RT-qPCR. (I) miR-301b-3p expression was detected by RT-qPCR. (J) TLR4 protein expression was measured by western blotting. [*]P<0.05 vs. control or NC-mimics; [**]P<0.01 vs. control, NC-mimics or si-NC; and [***]P<0.001 vs. control or NC-mimics; [##]P<0.01 compared with pc-vector. HMEECs, human middle ear epithelial cells; NEAT1, nuclear enriched abundant transcript 1; TLR4, toll-like receptor 4; LPS, lipopolysaccharide; miR, microRNA; NC, negative control; si, small interfering; WT, wild-type; MUT, mutant; RT-qPCR, reverse transcription-quantitative PCR.

of our knowledge, the function and mechanism of action of NEAT1 in AOM have not been reported in the literature to date. The present study demonstrated that NEAT1 expression was higher in LPS-stimulated HMEECs compared with other pro-inflammatory lncRNAs. Subsequently, loss-of-function experiments revealed that NEAT1 silencing inhibited LPS-stimulated HMEEC inflammation and apoptosis, while it promoted cell proliferation. Thus, these data suggested

that NEAT1 may regulate LPS-induced inflammation of HMEECs.

It has been previously demonstrated that lncRNAs may serve as miRNA competitive endogenous RNAs (ceRNAs) in several diseases (37). For example, NEAT1 may serve as a ceRNA, regulating LPS-induced apoptosis and inflammatory injury of WI-38 cells by spong-ing miR-193a-3p to target *TLR4* (15). In the present study, miR-301b-3p was
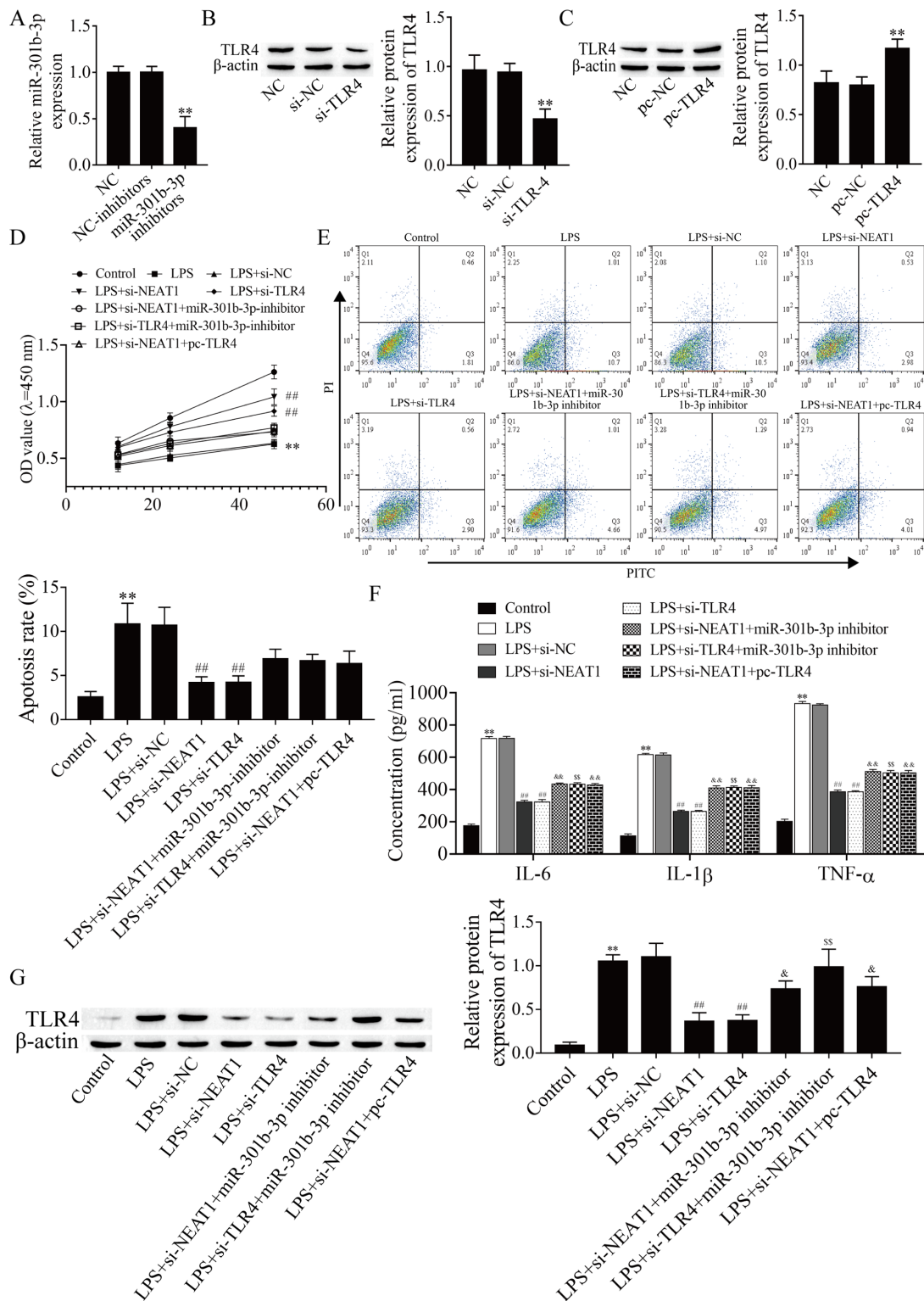
Figure 4. NEAT1 regulates LPS-induced cell viability, apoptosis and inflammation of human middle ear epithelial cells via the miR-370-3p/TLR4 axis. (A) miR-301b-3p expression was determined by reverse transcription-quantitative PCR. TLR4 protein expression was detected by western blot assay after transfection with (B) si-*TLR4* and (C) pc-*TLR4*. (D) Cell viability was analyzed by Cell Counting Kit-8 assay. (E) Cell apoptosis was evaluated by flow cytometry. (F) IL-6, IL-1β and TNF-α expression levels were detected by ELISA. (G) TLR4 protein expression in different groups was detected by western blot assay. **P<0.01 vs. control, si-NC or pc-NC; ##P<0.01 vs. control and LPS+si-NC; &P<0.05 vs. LPS+si-NEAT1; &&P<0.01 vs. LPS+si-NEAT1; $$P<0.01 vs. LPS+*si-TLR4*. LPS, lipopolysaccharide; miR, microRNA; NC, negative control; si, small interfering; NEAT1, nuclear enriched abundant transcript 1; TLR4, toll-like receptor 4; OD, optical density; FITC, fluorescein isothiocyanate; PI, propidium iodide.

downregulated and determined to be a target of and negatively regulated by NEAT1, thereby inhibiting LPS-induced apoptosis and inflammation of HMEECs. According to previous reports, miR-301b-3p was shown to contribute to tumor growth in human hepatocellular carcinoma and gastric cancer (38,39). In addition, the expression of miR-301b-3p was significantly

upregulated by *Pseudomonas aeruginosa* and *Candida albicans* (22,40,41), but miR-301b-3p was downregulated in LPS-induced HMEECs (20). miR-301b-3p and TLR4 expression was also investigated in the HK2 and THLE-3 cells. The results demonstrated that the expression of miR-301b-3p was reduced in LPS-induced HK2 and THLE-3 cells, whereas TLR4 expression was increased. The present study found that miR-301b-3p was a target of NEAT1, and miR-301b-3p inhibitors reversed the inhibitory effect of NEAT1 silencing on the inflammatory response of HMEECs. In this context, our data suggested that NEAT1 may regulate LPS-induced inflammation of HMEECs by sponging miR-301b-3p.

Previous studies have reported that TLR4 is highly expressed in necrotizing enterocolitis (42), oral cancer and brain injury (43). Of note, TLR4 is also highly expressed in AOM (31,44). Consistent with the aforementioned findings, it was observed herein that the enrichment of TLR4 increased in LPS-stimulated HMEECs compared with normal HMEECs. In addition, miRNAs can regulate gene expression through binding to the 3'-UTR of their target mRNAs (45). For example, miR-301b-3p targets the 3'-UTR of PTEN and promotes cell migration and invasion in bladder cancer (46). Previous studies reported that miR-301b-3p was transcriptionally upregulated by the TLR4/NF-κB axis in human bladder cancer and *Candida albicans*-induced lung injury (40,47). Notably, in patients with AOM, the expression of TLR4 was upregulated, and *H. influenza*e inoculation and LPS-induced inflammation also upregulated the TLR4 level in animal models with AOM (24-27), but LPS induction significantly reduced miR-301b-3p expression in HMEECs (20). It was also observed that LPS induction significantly increased TLR4 and decreased miR-301b-3p expression in HMEECs. Notably, *TLR4* was found to be the target gene of miR-301b-3p, and miR-301b-3p inhibited TLR4 expression in LPS-induced HMEECs. Li *et al* (35) reported that NEAT1 may promote LPS-induced inflammatory injury in macrophages by regulating miR-17-5p/TLR4. Consistently, the present study found that miR-301b-3p inhibitors and TLR4 overexpression upregulated the inhibitory effect of NEAT1 silencing on TLR4, indicating that NEAT1 affects TLR4 expression by modulating miR-301b-3p, thereby promoting LPS-induced apoptosis and inflammation of HMEECs. However, the present study only investigated the potential role and mechanism of the NEAT1/miR-301b-3p/TLR4 axis in LPS-induced HMEECs, and several limitations should also be considered. Preclinical data are expected to improve our understanding of the potential mechanism by using mouse models *in vivo* or primary cells *in vitro* in future studies.

In summary, it was herein demonstrated that interference with lncRNA NEAT1 expression may protect HMEECs from LPS-induced inflammation and apoptosis via the miR-301b-3p/TLR4 axis, which contributes to improving the pathological status of AOM. The findings of the present study also indicated that the NEAT1/miR-301b-3p/TLR4 axis may constitute a potential therapeutic target for AOM.

Availability of data and materials

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

Authors' contributions

ZL and RL conceived and designed the research. TL, SL, FZ, JY, SD and BR performed the experiments and analyzed the data. ZL, TL and SL wrote the manuscript. ZL and RL interpreted the data. RL, BR and RL confirm the authenticity of all the raw data. All the authors have 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.

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