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LncRNA NEAT1 facilitates the progression of sepsis through up-regulating TSP-1 *via* sponging miR-370-3p

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Abstract. – OBJECTIVE: Sepsis is a systemic inflammatory disease. LncRNA NEAT1 has been reported to be up-regulated in sepsis patients. Nevertheless, the modulatory network of NEAT1 in sepsis remains to be revealed.

PATIENTS AND METHODS: The abundance of long noncoding RNA nuclear enriched abundant transcript 1 (lncRNA NEAT1), miR-370-3p, and thrombospondin-1 (TSP-1) were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) in sepsis patients and lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Enzyme-linked immunosorbent assay (ELISA) was performed to examine the concentration of cytokines in RAW 264.7 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry assay, and Western blot assay were conducted to detect the proliferation and apoptosis of RAW 264.7 cells. Dual-Luciferase reporter assay, RNA immunoprecipitation (RIP) assay, and RNA-pull down assay were conducted to confirm the combination between miR-370-3p and NEAT1 or TSP-1 in RAW 264.7 cells.

RESULTS: The enrichment of NEAT1 was enhanced in sepsis patients and LPS-stimulated RAW 264.7 cells. NEAT1 contributed to LPS-induced inflammation and apoptosis of RAW 264.7 cells. MiR-370-3p bound to NEAT1, and it was negatively regulated by NEAT1 in RAW 264.7 cells. LPS promoted the inflammation and apoptosis while restrained the proliferation of RAW 264.7 cells *via* NEAT1/miR-370-3p axis. TSP-1 was a target of miR-370-3p in RAW 264.7 cells, and miR-370-3p suppressed the inflammation and apoptosis while it facilitated the proliferation of LPS-induced RAW 264.7 cells *via* TSP-1.

CONCLUSIONS: LncRNA NEAT1 promoted the inflammation and apoptosis while restrained the proliferation of LPS-stimulated RAW 264.7 cells through the miR-370-3p/TSP-1 axis.

Key Words:
Sepsis, LncRNA NEAT1, MiR-370-3p, TSP-1, Inflammation, Proliferation, Apoptosis.

Introduction

Sepsis is a deadly inflammatory disease caused by a disordered host response to bacterial infection[1]. The prognosis of sepsis patients remains poor and finding sensitive markers for early diagnosis is crucial for sepsis treatment[2,3]. Lipopolysaccharide (LPS) could activate inflammatory cells, and then pro-inflammatory factors releasing from inflammatory cells, including tumor necrosis factor α (TNF-α), exacerbate to sepsis[4].

Long noncoding RNAs (lncRNAs) are long-chain RNAs with at least 200 nucleotides in length. LncRNAs could modulate the expression of target genes serving as competing endogenous RNAs (ceRNAs) of microRNAs (miRNAs)[5-9]. LncRNA nuclear enriched abundant transcript 1 (NEAT1) was abnormally up-regulated in multiple cancers, including esophageal squamous cell carcinoma and gastric adenocarcinomas, and its high expression was related to poor prognosis[10,11]. Herein, we concentrated on the biological role of

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NEAT1 in sepsis. He et al[12] claimed that the enhanced enrichment of lncRNA NEAT1 was correlated with the decreased survival rate of sepsis patients, and miR-124 was a functional target of NEAT1. Zhang et al[13] reported that NEAT1 accelerated the inflammatory response by enhancing the level of TLR4 *via* Let-7a in sepsis-induced liver injury. Nevertheless, the underlying mechanism of NEAT1 in sepsis is not fully addressed.

MiRNAs are involved in cell growth, metastasis, and apoptosis by down-regulating their target messenger RNAs (mRNAs) or restraining their translation[14-17]. MiR-370-3p has been reported to be down-regulated in many cancers. Chen et al[18] found that the enrichment of miR-370-3p was declined, and circular RNA NEK6 (circRNA NEK6) facilitated the progression of thyroid cancer *via* down-regulating miR-370-3p. Shen et al[19] demonstrated that the enrichment of miR-370-3p was declined in colon cancer, and miR-370-3p suppressed growth and facilitated the apoptosis of colon cancer cells through MDM4. However, there were few studies concentrating on the biological significance of miR-370-3p in sepsis. Herein, we aimed to investigate the biological significance of miR-370-3p in sepsis.

Thrombospondin-1 (TSP-1) was first found in human blood platelets[20], and it functions through the interaction with cell surface receptors and proteins. Gawaz et al[21] found that TSP-1 was up-regulated on the platelet surface of sepsis patients, suggesting the pivotal role of TSP-1 in sepsis. However, the potential signal regulatory network behind TSP-1 in sepsis remains poorly understood.

In this study, we assessed the role of NEAT1 in sepsis and explored the underlying mechanism by which NEAT1 accelerates the inflammatory response and apoptosis while inhibits the growth of LPS-treated Raw 264.7 cells.

Patients and Methods

Patients

Sepsis patients (n=25) and normal patients (n=25) were recruited from the Danzhou People's Hospital. The serum of the above samples was used for the detection of the abundance of NEAT1, miR-370-3p, and TSP-1. This research was approved by the Ethics Committee of Danzhou People's Hospital. Patients who participated in this study had provided written informed consents.

Cell Culture and LPS Treatment

Mouse macrophage cell line Raw 264.7 was obtained from BeNa Culture Collection (Beijing, China) and was cultivated with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) added with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 10% penicillin (100 U/mL)-streptomycin (100 μg/mL) mixed solution in a 37° C, 5% CO_2 humidified incubator. RAW 264.7 cells were incubated with 1 μg/mL LPS for 24 h to establish sepsis cell model.

Quantitative Real Time-Polymerase Chain Reaction (QRT-PCR)

QRT-PCR was performed using SYBR green and special primers (GeneCopoeia; Rockville, MD, USA). The levels of NEAT1, miR-370-3p, and TSP-1 were normalized to U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the $2^{-\Delta\Delta Ct}$ method[22]. The qRT-PCR procedure was as follows: denaturation at 94°C for 2 min, amplification for 30 cycles at 94°C for 0.5 min, annealing at 58°C for 0.5 min and extension at 72°C for 1 min, terminal elongation at 72°C for 10 min. The qRT-PCR assay was carried out on a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The primers were listed in Table I.

Cell Transfection

Transfection was executed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Small interfering RNA negative control (si-NC), small interfering RNA against NEAT1 (si-NEAT1), small interfering RNA against TSP-1 (si-TSP-1), pcDNA, NEAT1 overexpression plasmid (NEAT1) and TSP-1 overexpression plasmid (TSP-1) were purchased from GenePharma (Shanghai, China). MiRNA-NC (miR-NC), miR-370-3p, inhibitor NC and miR-370-3p inhibitor were synthesized from Ribobio (Guangzhou, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was applied as described previously[23]. The concentrations of cytokines (TNF-α, interleukin 6 (IL-6), interleukin 8 (IL-8) and interleukin 1β (IL-1β)) in the cell suspension were determined by their matching ELISA kits (R&D Systems, Minneapolis, MN, USA). RAW 264.7

Table I. Primer sequences.

Target ID		Sequence
Human NEAT1	Forward	TGTCCCTCGGCTATGTCAGA
	Reverse	GAGGGGACGTGTTTCCTGAG
mouse NEAT1	Forward	TGCTGCCTTTTCTGTTCCTT
	Reverse	CTAGCTAGCTTTGGGTAGGGAAGT
human miR-370-3p	Forward	ACACTCCAGCTGGGGCCTGCTGGGGTGGAACCT
	Reverse	universal primer
mouse miR-370-3p	Forward	TTACACAGCTCATGAGTGCC
	Reverse	AGACAGACAAACCAGGTTC
human TSP-1	Forward	ATGGAATTGGTGATGCCTGTG
	Reverse	ACTGAGCTGGGTTGTAATGGAATG
mouse TSP-1	Forward	GACAAAGGCTTCATCTTCCT
	Reverse	CCGGTCCTCTTGAACAAACA
human U6	Forward	CTCGCTTCGGCAGCACATATACTA
	Reverse	ACGAATTTGCGTGTCATCCTTGC
mouse U6	Forward	TGCGGGTGCTCGCTTCGGCAGC
	Reverse	CCAGTGCAGGGTCCGAGGT
human GAPDH	Forward	TGACCACAGTCCATGCCATCAC
	Reverse	GCCTGCTTCACCACCTTCTTGA
mouse GAPDH	Forward	GCCGTATTGGGCGCCTGGTC
	Reverse	TCCCGTTGATGACAAGCTTC

cells were seeded into 96-well plates at 5000 cells per well overnight. RAW 264.7 cells were treated with different conditions and the supernatants were collected, and the concentrations of cytokines were determined according to the user manual.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

RAW 264.7 cells were seeded into 96-well plates overnight. 10 μL MTT (Invitrogen, Carlsbad, CA, USA) was pipetted into the wells of 96-well plates after transfection for 0 h, 24 h, 48 h, and 72 h, and the cells were mixed with MTT for 4 h. After removing the cell supernatant, 200 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to the wells of 96-well plates, the optical density was determined at 490 nm using a Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell Apoptosis Analysis

RAW 264.7 cells were collected using cold phosphate-buffered saline (PBS) buffer. The cells were mixed with Annexin V combined fluorescein isothiocyanate (FITC) and propidine iodide (PI; Solarbio, Beijing, China) for 10 min. Then, the apoptotic cells were analyzed by the flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot Assay

RAW 264.7 cells were lysed using Radio Immunoprecipitation Assay (RIPA) lysis solution (Beyotime, Shanghai, China). The proteins were quantified, and then the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked for 1 h and was incubated with the following antibodies overnight. The antibodies against B cell leukemia/lymphoma 2 (Bcl-2; ab185002), Bcl-2 associated X, apoptosis regulator (Bax; ab32503), Caspase-3 (ab13847), Cyclin D1 (ab16663), GAPDH (ab181602), and TSP-1 (ab88529) were obtained from Abcam (Cambridge, MA, USA). The PVDF membrane was then incubated with secondary antibody (ab205718, Abcam). The protein signal was determined *via* enhanced chemiluminescence (ECL) system (Beyotime, Shanghai, China).

Dual-Luciferase Reporter Assay

StarBase online software was used for predicting the targets of NEAT1 and miR-370-3p. The combination between miR-370-3p and NEAT1 or TSP-1 was verified by Dual-Luciferase reporter assay. We constructed reporter vector, including wild-type or mutant type binding sites of NEAT1 sequences, named as WT-NEAT1 or MUT-NEAT1. Luciferase activity was determined in RAW 264.7 cells co-transfected with miR-NC or miR-370-3p and WT-NEAT1 or MUT-NEAT1.

For the validation of the combination between miR-370-3p and TSP-1 in RAW 264.7 cells, luciferase reporter vector was constructed using the 3' UTR of TSP-1, including wild-type or mutant type binding sites. The other steps are the same as above.

RNA Immunoprecipitation (RIP) Assay

RIP buffer (Millipore, Billerica, MA, USA) was used to lyse RAW 264.7 cells, and proteins were incubated with beads conjugated with anti-Argonaute-2 (Anti-Ago2; Millipore, Billerica, MA, USA). Anti-Immunoglobulin G (Anti-IgG; Millipore) was used as a control in this study. The expression of NEAT1, miR-370-3p, and TSP-1 was quantified by qRT-PCR.

RNA-Pull Down Assay

Biotin was attached to miR-370-3p (Bio-miR-370-3p) or negative control (Bio-NC). Bio-miR-370-3p or Bio-NC was transfected into RAW 264.7 cells. Cells were collected and lysed after transfection for 48 h. RNA-pull down assay was conducted according to the previous report[24]. The level of NEAT1 or miR-370-3p was determined by qRT-PCR.

Statistical Analysis

All experiments were repeated three times, and data were presented as mean±standard deviation (SD). The comparisons between the two groups were assessed by Student's *t*-test. Spearman's correlation coefficient was performed to analyze the liner relationship between levels of miR-370-3p and NEAT1 or TSP1 in the serum of sepsis patients. $p<0.05$ was considered as statistically significant.

Results

LncRNA NEAT1 Is Up-Regulated in Sepsis Patients and LPS-Stimulated RAW 264.7 Cells

To elucidate the biological role of NEAT1 in sepsis, we detected the abundance of NEAT1 in sepsis patients and healthy patients. As showed in Figure 1A, the expression of NEAT1 was higher in sepsis patients than that in healthy controls, suggesting its crucial role in sepsis. Subsequently, we established sepsis cell model using RAW 264.7 cells treated with 1 ug/mL LPS for 24 h. The level of NEAT1 was enhanced in LPS stimulated RAW 264.7 cells compared with that in control RAW 264.7 cells (Figure 1B).

The Knockdown of NEAT1 Alleviates the Promoting Effects of LPS Treatment on the Inflammatory Response and Apoptosis and the Suppressive Impact on the Proliferation of RAW 264.7 Cells

We conducted ELISA assay, MTT assay, flow cytometry, and Western blot assay to clarify the influence of NEAT1 depletion on the inflammatory, proliferation, and apoptosis of RAW 264.7 cells stimulated by LPS. As showed in Figure 2A, the expression of NEAT1 was notably decreased in si-NEAT1 group compared with that in si-NC group in RAW 264.7 cells. The concentration of cytokines was enhanced with LPS treatment in RAW 264.7 cells, and the addition of si-NEAT1 abolished the pro-inflammatory effect of LPS treatment on RAW 264.7 cells (Figure 2B). MTT assay revealed that the proliferation of RAW 264.7 cells was restrained with

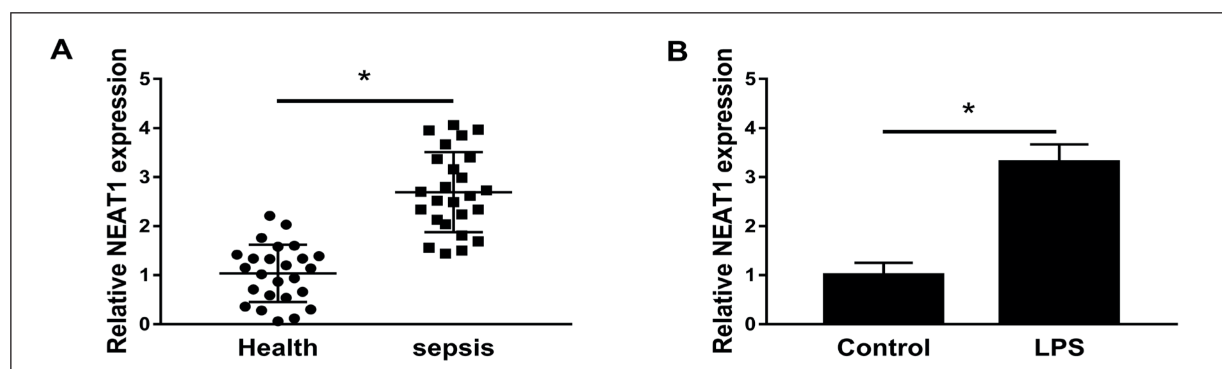

Figure 1. LncRNA NEAT1 is up-regulated in sepsis patients and LPS-stimulated RAW 264.7 cells. **A**, Abundance of lncRNA NEAT1 was measured in the serum of sepsis patients (n=25) and healthy samples (n=25) by qRT-PCR. **B**, Level of NEAT1 was determined in LPS-stimulated RAW 264.7 cells and control RAW 264.7 cells by qRT-PCR. *$p<0.05$.

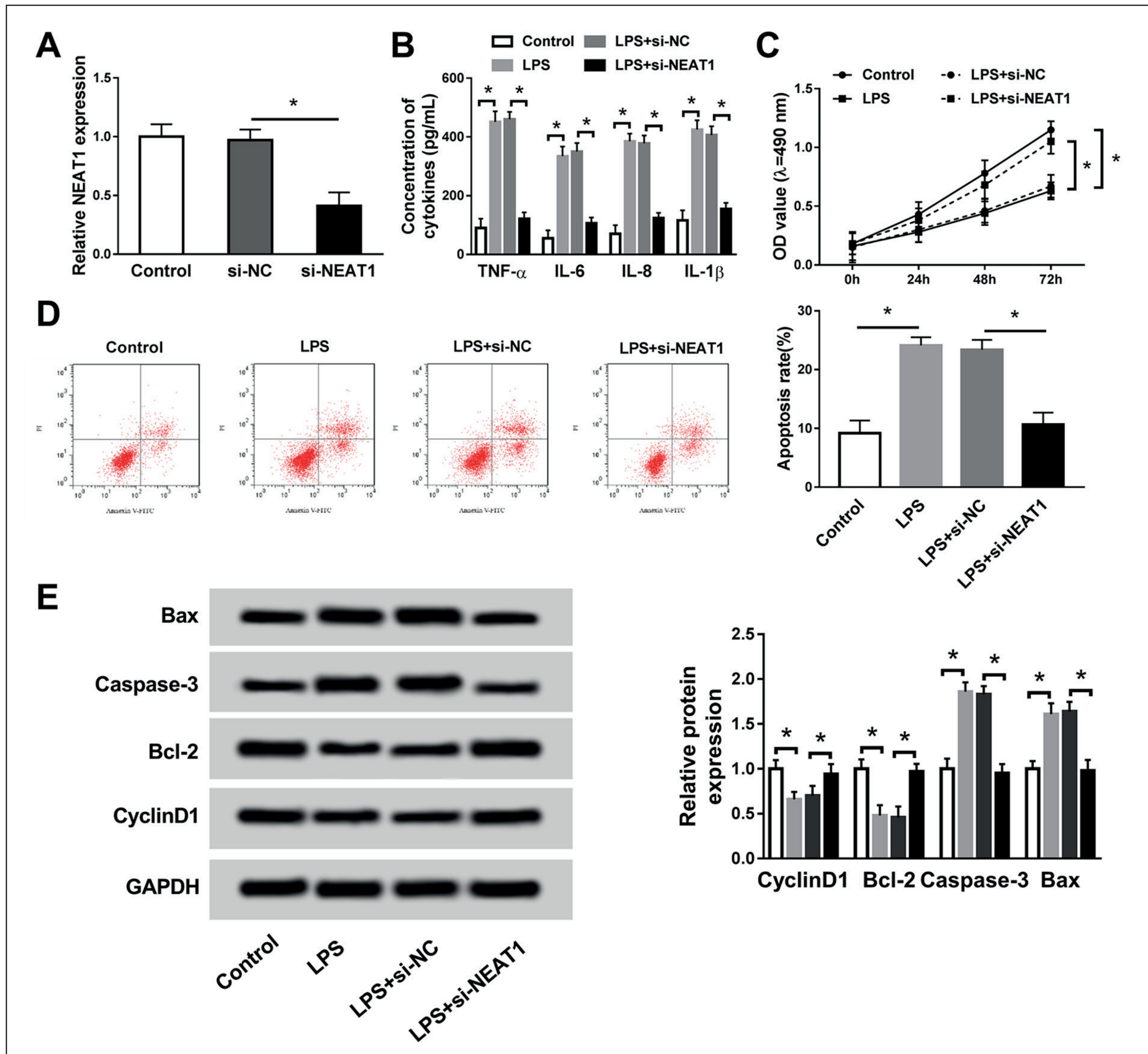

Figure 2. The knockdown of NEAT1 alleviates the promoting effects of LPS treatment on the inflammatory response and apoptosis and the suppressive impact on the proliferation of RAW 264.7 cells. **A**, Level of NEAT1 was detected in RAW 264.7 cells transfected with si-NC or si-NEAT1 by qRT-PCR. RAW 264.7 cells treated with Control, LPS, LPS + si-NC or LPS + si-NEAT1 were used for the following experiments. **B**, Concentration of cytokines (TNF-α, IL-6, IL-8, and IL-1β) was determined in RAW 264.7 cells by ELISA assay. **C**, MTT assay was performed to detect the proliferation of RAW 264.7 cells. **D**, Flow cytometry was conducted to measure the apoptosis of the above RAW 264.7 cells. **E,** Western blot was carried out to examine the abundance of apoptosis-related proteins (Bax, Caspase-3, and Bcl-2) and proliferation-associated protein (Cyclin D1) in RAW 264.7 cells. *p<0.05.

LPS treatment in RAW 264.7 cells, and it was recovered in LPS + si-NEAT1 group (Figure 2C). Meanwhile, the apoptosis of RAW 264.7 cells was elevated with LPS stimulation in RAW 264.7 cells, and the intervention of NEAT1 counteracted the promoting effect of LPS treatment on the apoptosis of RAW 264.7 cells (Figure 2D). Apart from this, the enrichment of anti-apoptotic protein Bcl-2 was down-regulated with LPS stimulation in RAW 264.7 cells, and it was recovered with the addition of si-NEAT1 (Figure 2E). The expression of pro-apoptotic proteins, including Bax and Caspase-3, exhibited an opposite trend to Bcl-2 in RAW 264.7 cells. Besides, the level of proliferation-related protein Cyclin D1 was reduced in LPS group, and the silencing of NEAT1 recovered the level of Cyclin D1. Taken together, LPS exerted its

pro-inflammatory, pro-apoptotic, and anti-proliferative functions by up-regulating NEAT1 in RAW 264.7 cells.

MiR-370-3p Is a Direct Target of NEAT1 in RAW 264.7 Cells

LncRNAs could serve as ceRNAs of target miRNAs to exert their function. Herein, we concentrated on the role of miR-370-3p in sepsis. Figure 3A, indicated that miR-370-3p was down-regulated in sepsis patients compared with that in healthy samples. Additionally, we found that the level of miR-370-3p was lower in LPS-stimulated RAW 264.7 cells than that in control group (Figure 3B). As mentioned above, the expression of NEAT1 was higher in sepsis patients and LPS-stimulated RAW 264.7 cells, and we suggested that there was an inverse correlation between the expression of miR-370-3p and NEAT1. The correlation analysis confirmed this hypothesis (Figure 3C).

MiR-370-3p was predicted as a target of NEAT1 by starBase software (Figure 3D), and this combination was validated by Dual-Luciferase reporter assay, RIP assay, and RNA-pull

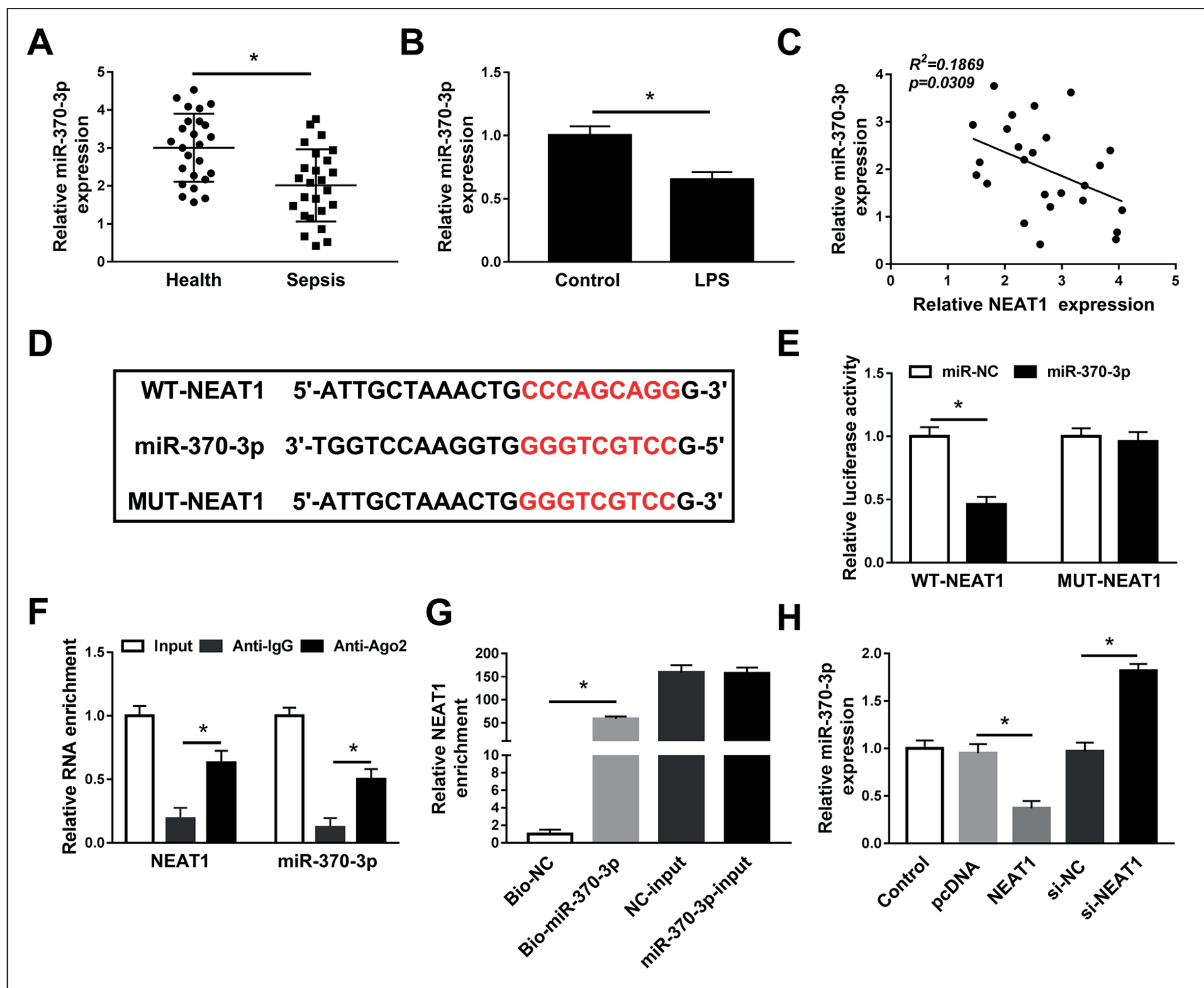

Figure 3. MiR-370-3p is a direct target of NEAT1 in RAW 264.7 cells. **A**, QRT-PCR was conducted to examine the enrichment of miR-370-3p in sepsis patients (n=25) and healthy patients (n=25). **B**, Expression of miR-370-3p was determined in LPS-stimulated RAW 264.7 cells and control RAW 264.7 cells by qRT-PCR. **C**, Correlation between the expression of miR-370-3p and the enrichment of NEAT1 in the serum of sepsis patients was analyzed. **D**, Binding sites between miR-370-3p and NEAT1 were predicted by starBase online software. **E**, Dual-Luciferase reporter assay was conducted in RAW 264.7 cells co-transfected with miR-NC or miR-370-3p and WT-NETA1 or MUT-NEAT1. **F**, **G**, RIP assay and RNA-pull down assay were performed to confirm the combination between miR-370-3p and NEAT1 in RAW 264.7 cells. **H**, Level of miR-370-3p was examined in pcDNA, NEAT1, si-NC or si-NEAT1 transfected RAW 264.7 cells. *p<0.05.

down assay. The luciferase activity was significantly reduced in miR-370-3p and WT-NEAT1 co-transfected group compared with that in miR-370-3p and MUT-NEAT1 co-transfected group, proposing that NEAT1 bound to miR-370-3p in RAW 264.7 cells (Figure 3E). RIP assay showed that NEAT1 bound to RNA-induced silencing complex (RISC) in RAW 264.7 cells, likely through the combination with miR-370-3p (Figure 3F). Furthermore, RNA-pull down assay also demonstrated that miR-370-3p was a target of NEAT1 in RAW 264.7 cells (Figure 3G). We wondered whether lncRNA NEAT1 could regulate the level of miR-370-3p in RAW 264.7 cells, and overexpression and knockdown experiments revealed that miR-370-3p was negatively modulated by NEAT1 in RAW 264.7 cells (Figure 3H). Collectively, miR-370-3p bound to NEAT1, and miR-370-3p was negatively regulated by NEAT1 in RAW 264.7 cells.

MiR-370-3p Depletion Reverses the Inhibitory Effects of NEAT1 Interference on the Inflammation and Apoptosis and the Promoting Impact on the Proliferation of LPS-Stimulated RAW 264.7 Cells

We wondered whether miR-370-3p was involved in NEAT1-mediated injury of LPS-stimulated RAW 264.7 cells. We first measured the enrichment of miR-370-3p in RAW 264.7 cells transfected with inhibitor NC or miR-370-3p inhibitor. As referred in Figure 4A, the level of miR-370-3p was conspicuously reduced in miR-370-3p inhibitor group. RAW 264.7 cells treated with Control, LPS, LPS + si-NC, LPS + si-NEAT1, LPS + si-NEAT1 + inhibitor NC or LPS + si-NEAT1 + miR-370-3p inhibitor were used for the detection of inflammation, growth, and apoptosis by ELISA assay, MTT assay, flow cytometry, and Western blot assay. As reported in Figure 4B and 4D, miR-370-3p silencing

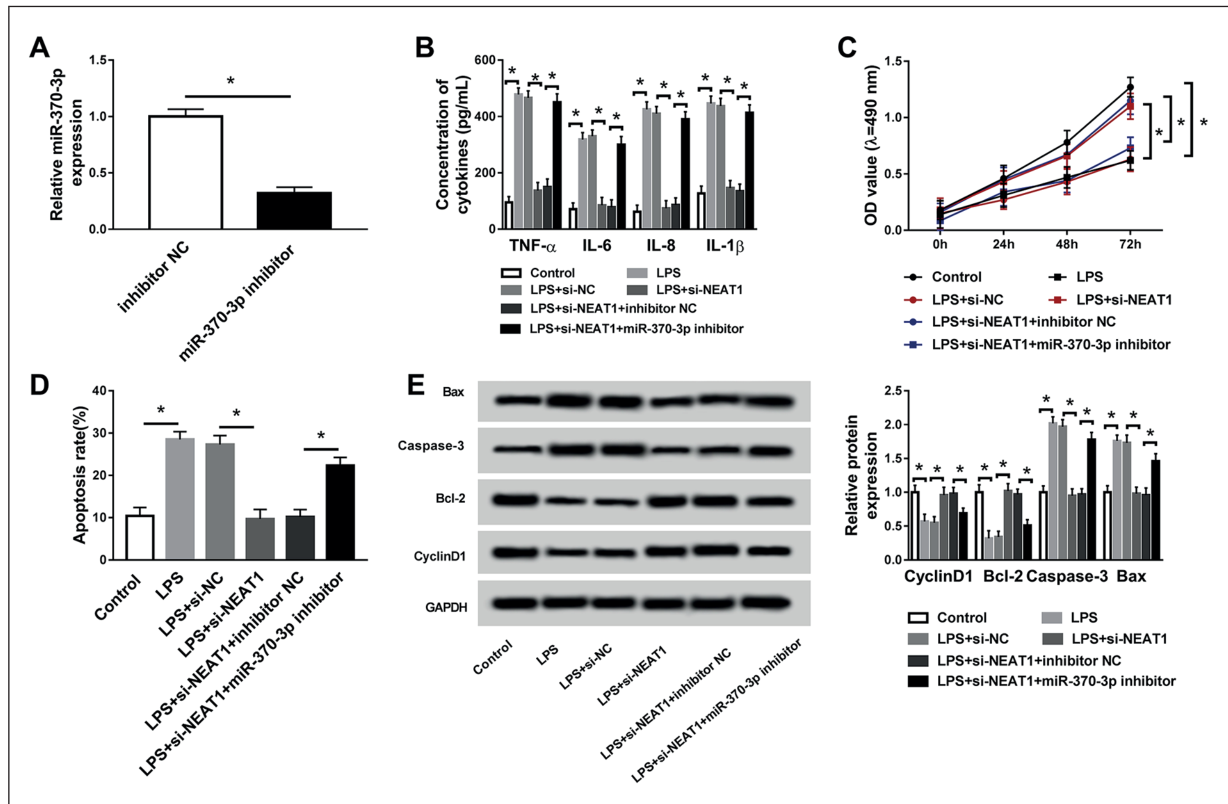

Figure 4. MiR-370-3p depletion reverses the inhibitory effects of NEAT1 interference on the inflammation and apoptosis and the promoting impact on the proliferation of LPS-stimulated RAW 264.7 cells. **A**, Expression of miR-370-3p was measured in RAW 264.7 cells transfected with inhibitor NC or miR-370-3p inhibitor by qRT-PCR. RAW 264.7 cells were treated with Control, LPS, LPS + si-NC, LPS + si-NEAT1, LPS + si-NEAT1 + inhibitor NC or LPS + si-NEAT1 + miR-370-3p inhibitor. **B**, ELISA assay was performed to detect the concentration of cytokines in RAW 264.7 cells. **C**, Proliferation of RAW 264.7 cells was measured by MTT assay. **D**, Apoptosis of RAW 264.7 cells was examined by flow cytometry. **E**, Western blot was conducted to measure the levels of Bax, Caspase-3, Bcl-2, and Cyclin D1 in RAW 264.7 cells. *p<0.05.

abolished the suppressive effects of NEAT1 depletion on the inflammation and apoptosis of RAW 264.7 cells. Besides, the proliferation was promoted by si-NEAT1 transfection in LPS-stimulated RAW 264.7 cells, and it was suppressed by the co-transfection of si-NEAT1 and miR-370-3p inhibitor in RAW 264.7 cells stimulated by LPS (Figure 4C). Furthermore, Western blot assay also validated the above results that miR-370-3p silencing reversed the inhibitory impact of NEAT1 interference on the apoptosis and the promoting effect on the proliferation of RAW 264.7 cells stimulated by LPS (Figure 4E). These findings suggested that LPS promoted the inflammation and apoptosis while inhibited the proliferation of RAW 264.7 cells through the NEAT1/miR-370-3p axis.

TSP-1 Binds to MiR-370-3p in RAW 264.7 Cells

Accruing documents reported that miRNAs functioned by modulating their target mRNAs. The abundance of TSP-1 mRNA and protein was up-regulated in sepsis patients compared with that in healthy patients (Figure 5A and 5B). As indicated in Figure 5C and 5D, the mRNA and protein expression of TSP-1 was also enhanced in LPS-treated RAW 264.7 cells compared with that

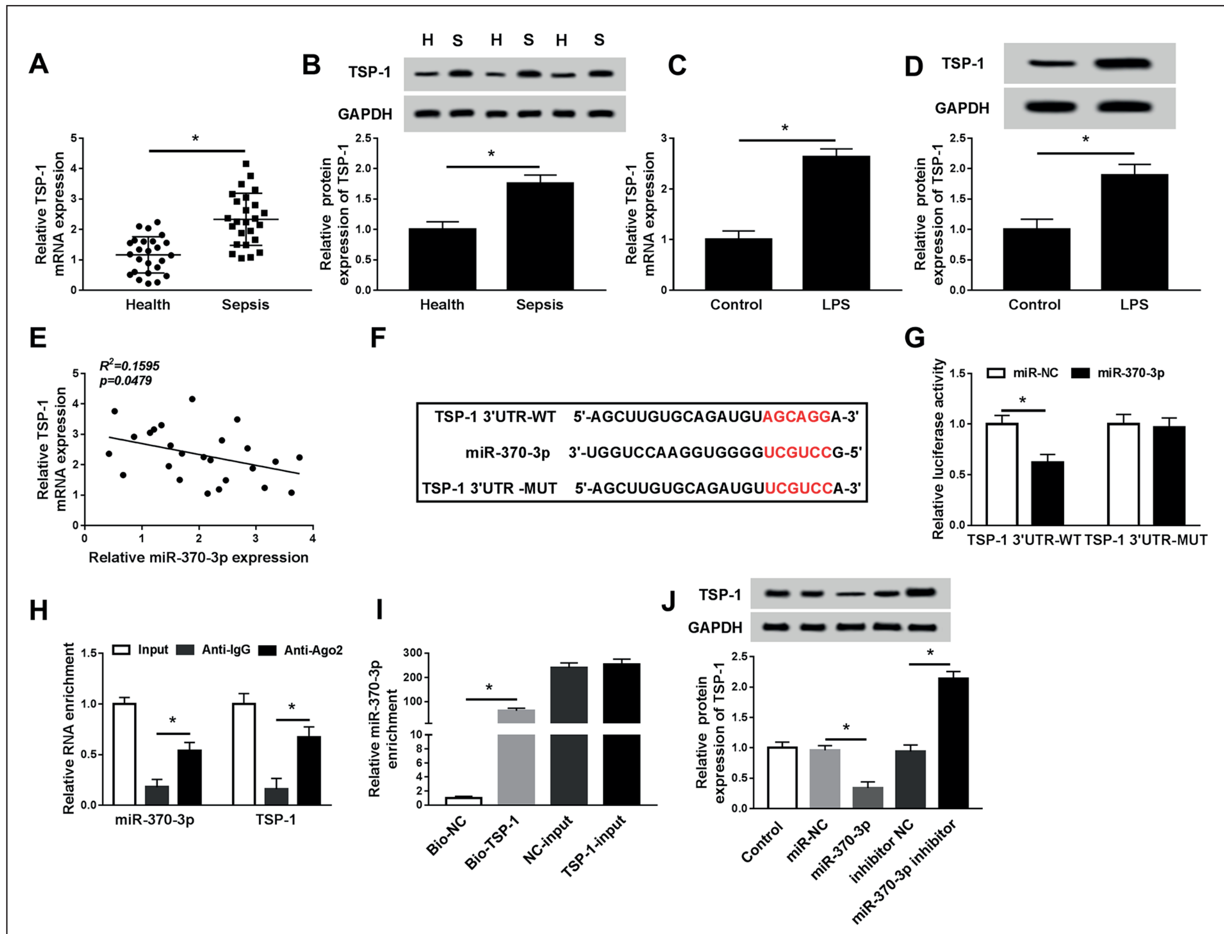

Figure 5. TSP-1 binds to miR-370-3p in RAW 264.7 cells. **A**, **B**, Enrichment of TSP-1 mRNA and protein was examined in sepsis patients (n=25) and healthy controls (n=25) by qRT-PCR and Western blot assay. **C**, **D**, QRT-PCR and Western blot assay were conducted to determine the abundance of TSP-1 mRNA and protein in control RAW 264.7 cells and LPS-stimulated RAW 264.7 cells. **E**, Correlation analysis was conducted between the mRNA expression of TSP-1 and the level of miR-370-3p in the serum of sepsis patients. **F**, Binding sites between TSP-1 and miR-370-3p were predicted by starBase online software. **G**, Luciferase activity was measured in RAW 264.7 cells co-transfected with TSP-1 3' UTR-WT or TSP-1 3' UTR-MUT and miR-NC or miR-370-3p. **H**, **I**, Combination between TSP-1 and miR-370-3p was verified by RIP experiment and RNA-pull down assay in RAW 264.7 cells. **J**, MiR-NC, miR-370-3p, inhibitor NC or miR-370-3p inhibitor transfected RAW 264.7 cells were used for the detection of the protein expression of TSP-1 by Western blot assay. *p<0.05.

in control cells. The correlation analysis revealed that the expression of TSP-1 mRNA was negatively correlated with the enrichment of miR-370-3p in the serum of sepsis patients (Figure 5E). Besides, we predicted the target of miR-370-3p by starBase (Figure 5F). To verify the combination between TSP-1 and miR-370-3p in RAW 264.7 cells, we constructed luciferase reporter vector, including the wild-type or mutant type binding sites of TSP-1 3' UTR (TSP-1 3' UTR-WT or TSP-1 3' UTR-MUT), and co-transfected with miR-370-3p or miR-NC into RAW 264.7 cells. The accumulation of miR-370-3p conspicuously reduced the luciferase activity in TSP-1 3' UTR-WT group, whereas it had little effect on TSP-1 3' UTR-MUT group, suggesting TSP-1 was a target of miR-370-3p in RAW 264.7 cells (Figure 5G). Meanwhile, this combination between miR-370-3p and TSP-1 was also confirmed by RIP experiment and RNA-pull down assay (Figure 5H and 5I). Subsequently, we found that TSP-1 was inversely regulated by miR-370-3p in RAW 264.7 cells (Figure 5J). Taken together, TSP-1 was a direct target of miR-370-3p in RAW 264.7 cells and was negatively modulated by miR-370-3p.

MiR-370-3p Inhibits the Inflammatory Response and Apoptosis While Promotes the Proliferation of RAW 264.7 Cells Through TSP-1

The abundance of TSP-1 mRNA and protein was declined in RAW 264.7 cells transfected with si-TSP-1 compared with that in si-NC group (Figure 6A and 6B). To elucidate whether the effect of miR-370-3p on LPS-induced damage of RAW 264.7 cells was achieved *via* TSP-1, RAW 264.7 cells were treated with Control, LPS, LPS + inhibitor NC, LPS + miR-

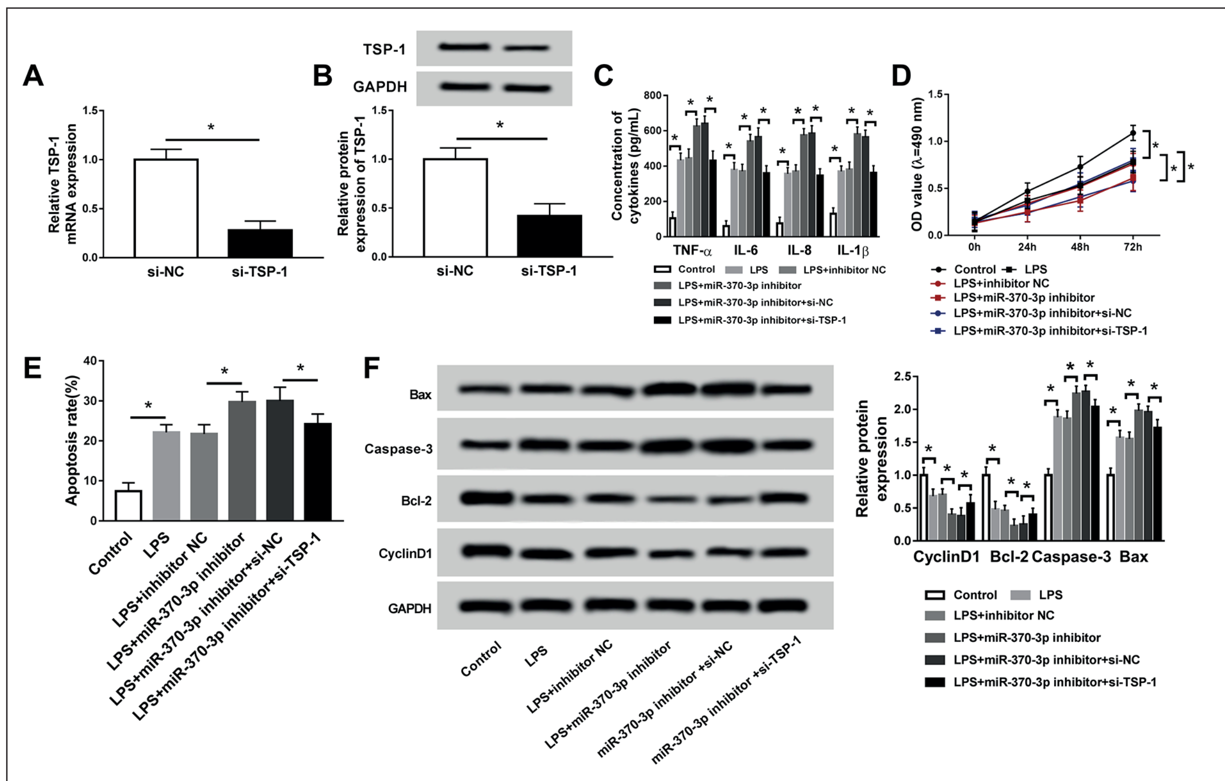

Figure 6. MiR-370-3p inhibits the inflammatory response and apoptosis while promotes the proliferation of RAW 264.7 cells through TSP-1. **A**, **B**, Abundance of TSP-1 mRNA and protein was determined in RAW 264.7 cells transfected with si-NC or si-TSP-1 by qRT-PCR and Western blot. RAW 264.7 cells were treated with Control, LPS, LPS + inhibitor NC, LPS + miR-370-3p inhibitor, LPS + miR-370-3p inhibitor + si-NC or LPS + miR-370-3p inhibitor + si-TSP-1. **C**, Concentration of cytokines was examined in RAW 264.7 cells by ELISA assay. **D**, MTT assay was carried out to detect the proliferation of RAW 264.7 cells. **E**, Flow cytometry was performed to examine the apoptosis of the above RAW 264.7 cells. **F**, Western blot assay was conducted to detect the abundance of apoptosis-related proteins (Bax, Caspase-3 and Bcl-2) and proliferation-associated protein (Cyclin D1) in RAW 264.7 cells. *$p<0.05$.

370-3p inhibitor, LPS + miR-370-3p inhibitor + si-NC or LPS + miR-370-3p inhibitor + si-TSP-1. Figure 6C-6F, showed that the silencing of TSP-1 reversed the promoting effects of miR-370-3p depletion on the inflammation and apoptosis and the inhibitory impact on the proliferation of RAW 264.7 cells stimulated by LPS. Taken together, miR-370-3p inhibited the inflammation and apoptosis while accelerated the proliferation of LPS-induced RAW 264.7 cells through TSP-1.

NEAT1 Contributes to LPS-Mediated Inflammation and Apoptosis and Proliferation Inhibition Through Elevating the Level of TSP-1 Via Sponging MiR-370-3p

To clarify the modulatory relationship among NEAT1, miR-370-3p and TSP-1 in RAW 264.7 cells, RAW 264.7 cells were treated with Control, LPS, LPS + si-NC, LPS + si-NEAT1, LPS + si-NEAT1 + inhibitor NC, LPS + si-NEAT1 + miR-370-3p inhibitor. Figure 7A and 7B, indicated that NEAT1 knockdown declined the mRNA and pro-

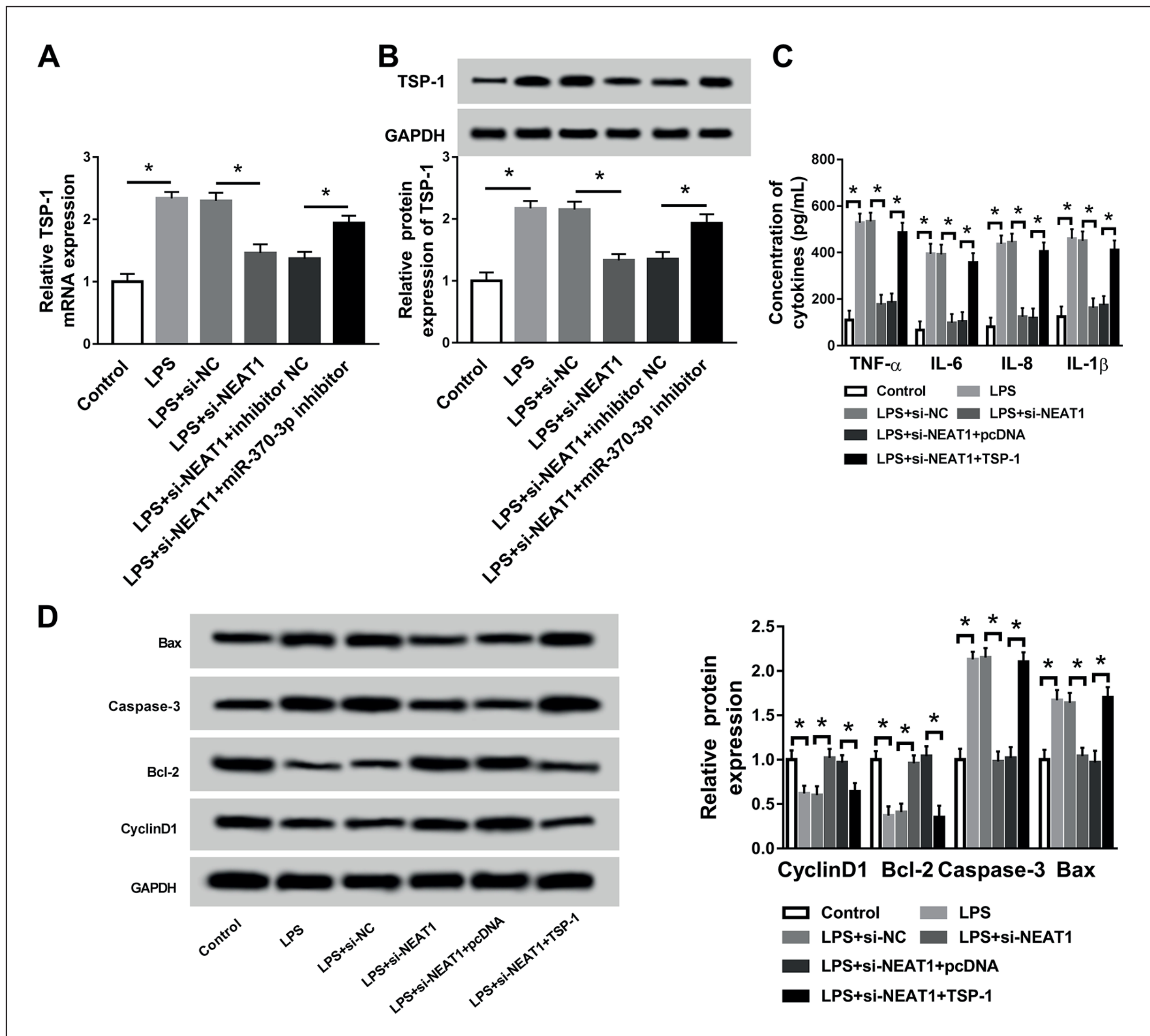

Figure 7. NEAT1 contributes to LPS-mediated inflammation and apoptosis and proliferation inhibition through elevating the level of TSP-1 *via* sponging miR-370-3p. **A**, **B**, MRNA and protein expression of TSP-1 was determined in RAW 264.7 cells treated with Control, LPS, LPS + si-NC, LPS + si-NEAT1, LPS + si-NEAT1 + inhibitor NC, LPS + si-NEAT1 + miR-370-3p inhibitor by qRT-PCR and Western blot assay. **C**, **D**, RAW 264.7 cells were treated with Control, LPS, LPS + si-NC, LPS + si-NEAT1, LPS + si-NEAT1 + pcDNA or LPS + si-NEAT1 + TSP-1. C, ELISA assay was conducted to detect the concentration of cytokines in the above RAW 264.7 cells. **D**, Western blot assay was performed to measure the abundance of Bax, Caspase-3, Bcl-2 and Cyclin D1 in RAW 264.7 cells. *$p<0.05$.

tein expression of TSP-1 in RAW 264.7 cells stimulated by LPS, and the depletion of miR-370-3p abolished the inhibitory effect of ENAT1 silencing on the expression of TSP-1 in LPS-stimulated RAW 264.7 cells. These results suggested that LPS enhanced the abundance of TSP-1 through NEAT1/miR-370-3p axis in RAW 264.7 cells.

The accumulation of TSP-1 abated the promoting effect of NEAT1 intervention on the proliferation and the inhibitory impacts on the apoptosis and inflammation of RAW 264.7 cells stimulated by LPS (Figure 7C and 7D). Collectively, LPS promoted the progression of sepsis through the NEAT1/miR-370-3p/TSP-1 axis *in vitro*.

Discussion

LncRNA NEAT1 played a crucial role in multiple inflammation-associated diseases. For example, Wang et al[25] claimed that NEAT1 depletion suppressed the inflammatory response *via* miR-342-3p in THP-1 cells. We found that the abundance of NEAT1 was higher in sepsis patients and LPS-stimulated RAW 264.7 cells than that in healthy controls and control RAW 264.7 cells. Subsequently, loss-of-function experiments revealed that LPS promoted the inflammatory response and apoptosis while impeded the proliferation of RAW 264.7 cells by up-regulating the level of NEAT1. MiR-370-3p was predicted as a target of NEAT1, and the combination between NEAT1 and miR-370-3p in RAW 264.7 cells was verified by Dual-Luciferase reporter assay, RIP, and RNA-pull down experiments. MiR-370-3p has been reported to play a suppressive role in inflammation. Zhang et al[23] reported that XIST accelerated the apoptosis and inflammatory response *via* inversely modulating miR-370-3p in WI-38 cells. Tian et al[26] found that miR-370-3p repressed the inflammation *via* TLR4 in THP-1 cells. Consistent with the above findings, we found that miR-370-3p played a suppressive role in cell inflammation and apoptosis. The knockdown of miR-370-3p reversed the inhibitory effects of NEAT1 depletion on the inflammation and apoptosis and the promoting impact on the proliferation of LPS-induced RAW 264.7 cells, suggesting that LPS facilitated the inflammation and apoptosis while repressed the proliferation of RAW 264.7 cells through the NEAT1/miR-370-3p axis.

TSP-1 has been reported to be an inflammation-related protein. Vallejo et al[27] claimed TSP-1 was overexpressed in rheumatoid synovial inflammatory tissues. Consistent with the above findings, we found that the enrichment of TSP-1 was elevated in the serum of sepsis patients and LPS-stimulated RAW 264.7 cells compared with that in healthy samples and normal RAW 264.7 cells. StarBase software predicted that TSP-1 was a target of miR-370-3p, and we confirmed the combination between TSP-1 and miR-370-3p by Dual-Luciferase reporter assay, RIP assay and RNA-pull down assay. Besides, TSP-1 silencing abolished the promoting impacts of miR-370-3p depletion on the inflammation and apoptosis and the suppressive effect on the proliferation of LPS-stimulated RAW 264.7 cells.

To further illustrate the modulatory relationship between NEAT1, miR-370-3p, and TSP-1 in RAW 264.7 cells, we conducted the following experiments. The mRNA and protein levels of TSP-1 were declined in LPS-stimulated RAW 264.7 cells transfected with si-NETA1. The co-transfection of miR-370-3p inhibitor and si-NEAT1 recovered the abundance of TSP-1 mRNA and protein, suggesting that TSP-1 was positively regulated by NEAT1, and was negatively modulated by miR-370-3p in RAW 264.7 cells. Apart from this, TSP-1 accumulation reversed the inhibitory effects of NEAT1 depletion on the inflammatory response and apoptosis and the promoting impact on the proliferation of LPS-induced RAW 264.7 cells.

Conclusions

In summary, NEAT1 promoted the inflammation and apoptosis while repressed the proliferation in LPS-induced RAW 264.7 cells *via* the miR-370-3p/TSP-1 axis. The NEAT1/miR-370-3p/TSP-1 axis might be an underlying target for sepsis treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The present study was approved by the Ethical Review Committee of Danzhou People's Hospital.

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