

Triptolide improves Alzheimer's disease by regulating the NF- κ B signaling pathway through the lncRNA NEAT1/microRNA 361-3p/TRAF2 axis

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Abstract. Alzheimer's disease (AD) is the most common type of dementia and is a serious social and medical problem threatening human health. The present study investigated the effect and underlying action mechanism of triptolide (Tri) on AD progression. Reverse transcription-quantitative PCR and western blotting analysis were used to determine the changes in RNA expression and levels of NF- κ B signaling pathway proteins before and after lipopolysaccharide (LPS) induction. Nucleocytoplasmic separation experiments determined the intracellular localization of long non-coding RNA (lncRNA) nuclear paraspeckle assembly transcript 1 (NEAT1). A dual-luciferase assay was used to analyze the binding between NEAT1 and microRNA (miRNA/miR)-361 or tumor necrosis factor receptor-associated factor 2 (TRAF2) and miR-361-3p and RNA pull-down was used to analyze the binding between NEAT1 and miR-361-3p. Cell Counting Kit-8, flow cytometry and ELISA were used to detect the effects of interaction between Tri and NEAT1/miR-361-3p/TRAF2 on cell viability, apoptosis and inflammatory factor levels, respectively. The results showed that LPS-mediated human

microglial clone 3 cell line (HMC3) viability decreased and apoptosis and inflammatory factors (IL-1 β , IL-6, IL-18 and TNF- α) increased. Tri inhibited LPS-mediated effects in a dose-dependent manner by downregulating NEAT1 expression. NEAT1 is highly expressed in the cytoplasm and reduces the transcription and translation of downstream TRAF2 by acting as a competitive endogenous RNA that adsorbs miR-361-3p. LPS-mediated HMC3 cell injury, inflammation and activation of NF- κ B signaling were partially reversed in presence of Tri. The miR-361-3p mimic promoted the Tri effect and overexpression of (ov)-NEAT1 partially reversed the Tri-miR-361-3p combined effect. The effects of ov-NEAT1 were partially attenuated by small interfering (si)-TRAF2. Overall, Tri inhibited the LPS-induced decrease in viability, increase in apoptosis and inflammation and activation of NF- κ B signaling in HMC3 cells. Tri regulation affected the NEAT1/miR-361-3p/TRAF2 axis. These findings suggested a potential therapeutic role for Tri in the clinical management of AD by modulating this molecular axis.

Introduction

Alzheimer's disease (AD) is common in older adults. Its pathogenesis is attributed to the extracellular accumulation of amyloid beta (A β) plaques in the cerebral cortex and limbic regions (1) and it is an irreversible neurodegenerative disease associated with neuroinflammation that can lead to memory loss and dementia (2,3). Although several drugs (e.g., bismarckine and ladostigil) have been approved for treating AD with therapeutic efficacy, the AD pathogenesis is unknown and no effective treatment is available (4-6). Therefore, the study of new drugs and diagnostic biomarkers is important for treating AD.

Most traditional Chinese medicine (TCM) interventions have multiple targets, fewer side effects compared with conventional medicine, good therapeutic effects and are widely used in China to treat a variety of diseases, including cardiovascular diseases (7), chronic heart failure (8) and AD (9). TCM preparations, such as triptolide (Tri), have various biological activities and pharmacological effects, including anticancer and anti-inflammatory effects. Elevated

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Abbreviations: AD, Alzheimer's disease; LPS, lipopolysaccharide; lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; miR/miRNA, microRNA; ceRNA, competitive endogenous RNA; TRAF2, tumor necrosis factor receptor associated factor 2; NC, negative control; Bio, biotinylated; FCM, flow cytometry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: Alzheimer's disease, nuclear paraspeckle assembly transcript 1, microRNA 361-3p, tumor necrosis factor receptor associated factor 2, NF- κ B

levels of inflammatory cytokines (such as IL-1 β , IL-6, IL18 and TNF- α) and the accumulation of activated microglia cells in the damaged area are markers of AD brain inflammation (10). Tri has been reported to protect cognitive function (11), reduce neuroinflammation (12) and an active treatment for neurodegenerative diseases (13). Currently, only a few studies on Tri in treating AD are available and its mechanism of action remains unclear.

Dysregulated long non-coding RNA (lncRNAs) may be key AD factors and it has been reported that the upregulation of growth arrest-specific 5 lncRNA is closely associated with AD progression (14). X-inactive specific transcript lncRNA mediates neuronal inflammation and injury and promotes AD (15). In addition, the regulation of cellular functions by long intergenic non-coding RNAs (lincRNAs) expressed in the cytoplasm may involve a competitive endogenous RNA (ceRNA) mechanism; that is, lincRNAs act as ceRNAs to sponge microRNAs (miRNA/miR) that can target messenger RNA (mRNA) and inhibit its transcription and translation (16). lncRNA-miRNA-mRNA networks are critical for developing therapeutic strategies for AD (17). Studies have shown that small nucleolar RNA host gene 14 acts as a ceRNA to adsorb miR-223-3p, regulate nucleotide-binding domain leucine-rich repeat-containing family pyrin domain containing 3 (NLRP3) expression, promote inflammation and mediate AD development (18). lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) promotes AD development through the miR-27a-3p/beta-secretase 1 axis (19).

Microglial cells are involved in a range of neurodegenerative diseases, such as AD and Parkinson's disease and can exert neuroprotective or neurotoxic effects that depend on the type and degree of stimulation produced when neurons appear damaged (20,21). NF- κ B exists widely in mammals and is a key factor in the cellular inflammatory response and neuroprotection (10). Inhibition of NF- κ B activation plays a neuroprotective role in lipopolysaccharide (LPS)-induced microglia in neurodegenerative diseases (22). Fu *et al* (23) demonstrated that blocking the activation of NF- κ B can inhibit the activation of NLRP3 inflammation in human microglial clone 3 cell line (HMC3) microglia and reduce cell damage and apoptosis.

The purpose of the present study was to investigate the therapeutic effect of Tri on AD and its mechanism of action and to provide a theoretical basis for its potential clinical application in AD treatment and future drug development.

Materials and methods

Cell culture and transfection. HMC3 cells were purchased from Procell Life Science & Technology Co., Ltd. and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Tri was dissolved in dimethyl sulfoxide (DMSO) and an equal volume of DMSO was added to HMC3 cells as a control. HMC3 cells were pre-treated with Tri (5, 10, 20, 50, and 100 nM) for 24 h and then stimulated with LPS (100 ng/ml) for 24 h. HMC3 cells were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) with an overexpression (ov) NEAT1 plasmid, a miR-361-3p mimic/inhibitor and a small interfering (si)-tumor necrosis factor receptor-associated factor 2 (TRAF2)

plasmid. All ribonucleic acids (RNAs) were synthesized by GenePharma Biotechnology Co., Ltd. and the sequences are shown in Table SI.

Cell viability assay. According to the manufacturer's instructions, HMC3 cells were cultured for 72 h at 37°C and incubated for 60 min with 10 ml Cell Counting Kit-8 (CCK8; Solarbio, Beijing, China) reagent every 24 h at 37°C. The optical density values were determined using a microplate reader (Thermo Fisher Scientific, Inc.).

Flow cytometry (FCM) analysis. Annexin V-fluorescein isothiocyanate (FITC; BD Biosciences) and propidium iodide (PI; BD Biosciences) were incubated with HMC3 cells in the dark for 15 min at 25°C. A flow cytometer (FC500; Beckman Coulter, Inc.) was used to detect the staining. FlowJo software (version 10.0.6; FlowJo LLC) was used to analyze the apoptosis rate (percentage of early + late apoptotic cells).

ELISA. The HMC3 supernatant of each subgroup was collected to analyze the concentrations of IL-1 β (cat. no. SEKH-0002), IL-6 (cat. no. SEKH-0013), IL-18 (cat. no. SEKH-0028) and TNF- α (cat. no. SEKH-0047) using ELISA kits (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. The absorbance was recorded using a multifunctional microplate reader (Thermo Fisher Scientific, Inc.).

Nucleocytoplasmic separation and reverse transcription-quantitative (RT-q) PCR. According to the manufacturer's protocols, a cytoplasmic and nuclear RNA purification kit (Norgen Biotek Corp.) was used to isolate cytoplasmic and nuclear RNA from HMC3 (5x10⁶ cells) for RT-qPCR analysis. According to the manufacturer's protocols, total RNA from HMC3 (1x10⁶ cells) was extracted using the TRIzol[®] reagent (Thermo Fisher Scientific, Inc.). copy deoxyribonucleic acid (cDNA) was reverse transcribed using the PrimeScript RT reagent kit (Takara Bio, Inc.) and then using the SYBR[®] Premix Ex Taq[®] II kit (Takara Bio, Inc.) and the Applied Biosystems 7500 Real-Time PCR System (CXF96; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The PCR conditions were as follows: 95°C for 32 sec, followed by 40 cycles of 95°C for six sec and 62°C for 32 sec. Primers (GenePharma Biotechnology Co., Ltd.) used are listed in Table SII. *NEAT1* and *TRAF2* RNA levels were normalized to GAPDH and miRNAs were normalized with U6 and calculated using the 2^{- $\Delta\Delta$ C_q} method (24). The assay was repeated three times for each subgroup.

Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) assays. The raw gene expression data (GSE150696) were divided into two groups: a control group (elderly patients without neurological or psychiatric diseases) and an AD group (patients with AD). The Affymetrix Transcriptome Analysis Console (version 4.0.1; Affymetrix; Thermo Fisher Scientific, Inc.) was used to analyze the data and generate a volcano plot of differentially expressed lncRNAs. Differentially expressed lncRNAs were considered significant at P \leq 0.01 and a log₂ (fold change, FC) >1.5.

Screening of miRNA and mRNA. Screening of miRNAs: miRNAs that may bind to lncRNA NEAT1 were jointly analyzed using StarBase (<https://starbase.sysu.edu.cn/>) and Lncbase (<https://diana.e-ce.uth.gr/lncbasev3>) databases and subsequent prediction of putative binding sites was performed using miRanda software (version 1.9; <http://www.microrna.org/microrna/home.do/>). Finally, RT-qPCR was performed as aforementioned to verify miRNA expression in LPS-induced HMC3 cells. Screening of mRNA: downstream targets that may potentially bind to the screened miRNAs were analyzed jointly by Tarbase (version 8; <https://bio.tools/tarbase#!>), Targetscan (https://www.targetscan.org/vert_72/) and miRDB (<https://mirdb.org/mirdb/index.html>) databases. The Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/>) database was used to analyze the possibility of these mRNAs being regulators of growth, apoptosis and inflammation-related pathways.

Dual luciferase assays. The amplified 3'-UTR fragments of NEAT1 and TRAF2 containing miR-361-3p binding sites were cloned into the psiCHECK-2 dual-luciferase reporter vector (Promega Corporation). Next, HMC3 cells were cotransfected with reporter vector and miR-361-3p mimics for 4 h at 37°C using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, the luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation). The firefly to *Renilla* luciferase activity ratio was used to normalize the firefly luciferase values.

RNA pull-down assay. Biotinylated NEAT1 (Bio-NEAT1, 100 ng/ μ l) and miR-361-3p (Bio-miR-361-3p, 100 ng/ μ l) were prepared using the Biotin RNA Labeling Mix (Beyotime Institute of Biotechnology) and were cloned into the pCMV6-AC plasmid (OriGene Technologies, Inc.) using pCMV6-Entry plasmid, and transfected into HMC3 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cell lysates (500 μ l per reaction) were incubated (30 min; 25°C) with 50 μ l of Dynabeads M-280 streptavidin (Invitrogen; Thermo Fisher Scientific, Inc.) and slowly shaken. The beads were then washed thrice with 1X Phosphate Buffered Saline (Gibco; Thermo Fisher Scientific, Inc.) and subsequently isolated by magnetic separation. The supernatant was subsequently removed by centrifugation at 2,000 \times g for 5 min at 4°C. RT-qPCR was performed as aforementioned for quantitative analysis.

Western blotting. After HMC3 cells were lysed with lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), protein concentrations in the extracts were determined using a bicinchoninic acid protein assay kit (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) was used to separate the denatured proteins (20 μ g), which were then transferred onto polyvinylidene fluoride membranes (MilliporeSigma). The membranes were rinsed with 10% tris-buffered saline (TBS)-Tween-20 (0.1%, v/v; Beijing Solarbio Science & Technology Co., Ltd.), blocked with 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) for 60 min at 25°C, and

incubated overnight at 4°C with antibodies against TRAF2 (1:1,000; cat. no. ab126758; Abcam), NF- κ B (p65; 1:10,000; cat. no. ab32536; Abcam) and p-NF- κ B (p65; 1:1,000; cat. no. ab76302; Abcam). Membranes were then incubated at 25°C for 2 h with a secondary antibody (goat anti-rabbit, 1:10,000; cat. no. ab205718; Abcam) and GAPDH (1:10,000; cat. no. ab181602; Abcam) was used as the loading control. Finally, protein bands were visualized by addition of ELC enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) in conjunction with an imaging system (DNR Bio-Imaging Systems Ltd.). All blots in each experiment were performed simultaneously under the same experimental conditions.

Statistical analysis. All experiments were performed in triplicate. The data were expressed as the mean \pm standard deviation. Statistical analysis was performed using a one-way analysis of variance with the Bonferroni post-hoc test. The means of the two groups were analyzed using the Student's t-test (unpaired). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Tri inhibits LPS-mediated inflammation and promoted HMC3 cell growth. Following Tri pre-treatment, Tri at 50 and 100 nM slightly decreased the viability of normal HMC3 cells (Fig. 1A); therefore, 5, 10 and 20 nM Tri were chosen for subsequent experiments to exclude the interference of Tri on HMC3 cells. It was subsequently observed that LPS mediation decreased HMC3 cell viability (Fig. 1B) and increased apoptosis (Fig. 1C) and inflammatory factor levels (Fig. 1D-G). Tri inhibited LPS-mediated effects in a dose-dependent manner. Different doses of Tri (5, 10 and 20 nM) had no significant effect on normal HMC3 cell viability, apoptosis, or inflammatory cytokine levels.

Tri inhibits LPS-mediated upregulation of NEAT1 expression. To explore the mechanism of lncRNAs in AD, the present study screened for differences in lncRNA expression between patients with AD and those without neurological or psychiatric diseases using the GEO dataset GSE150696. NEAT1 may be critical for Tri to alleviate LPS induction, as it had the highest FC value in the AD group in the GSE150696 dataset (Fig. 2A) and has been confirmed by previous research to be a key lncRNA affecting AD progression (19). In HMC3 cells, LPS mediated the upregulation of NEAT1 expression. Tri inhibited LPS-mediated high NEAT1 expression in a dose-dependent manner and had no significant effect on NEAT1 expression in normal HMC3 cells (Fig. 2B). An ov-NEAT1 plasmid was then constructed and transfected into HMC3 cells. Results showed that NEAT1 expression was upregulated in the ov-NEAT1 group compared with the ov-NC group (Fig. 2C). In addition, ov-NEAT1 promoted LPS-mediated expression of NEAT1. After Tri (20 nM) treatment inhibited NEAT1 expression, ov-NEAT1 reversed this effect (Fig. 2D). In addition, this dose was used in subsequent mechanistic investigations. Thus, it was confirmed that Tri inhibited LPS-mediated cell function through the NEAT1 pathway.

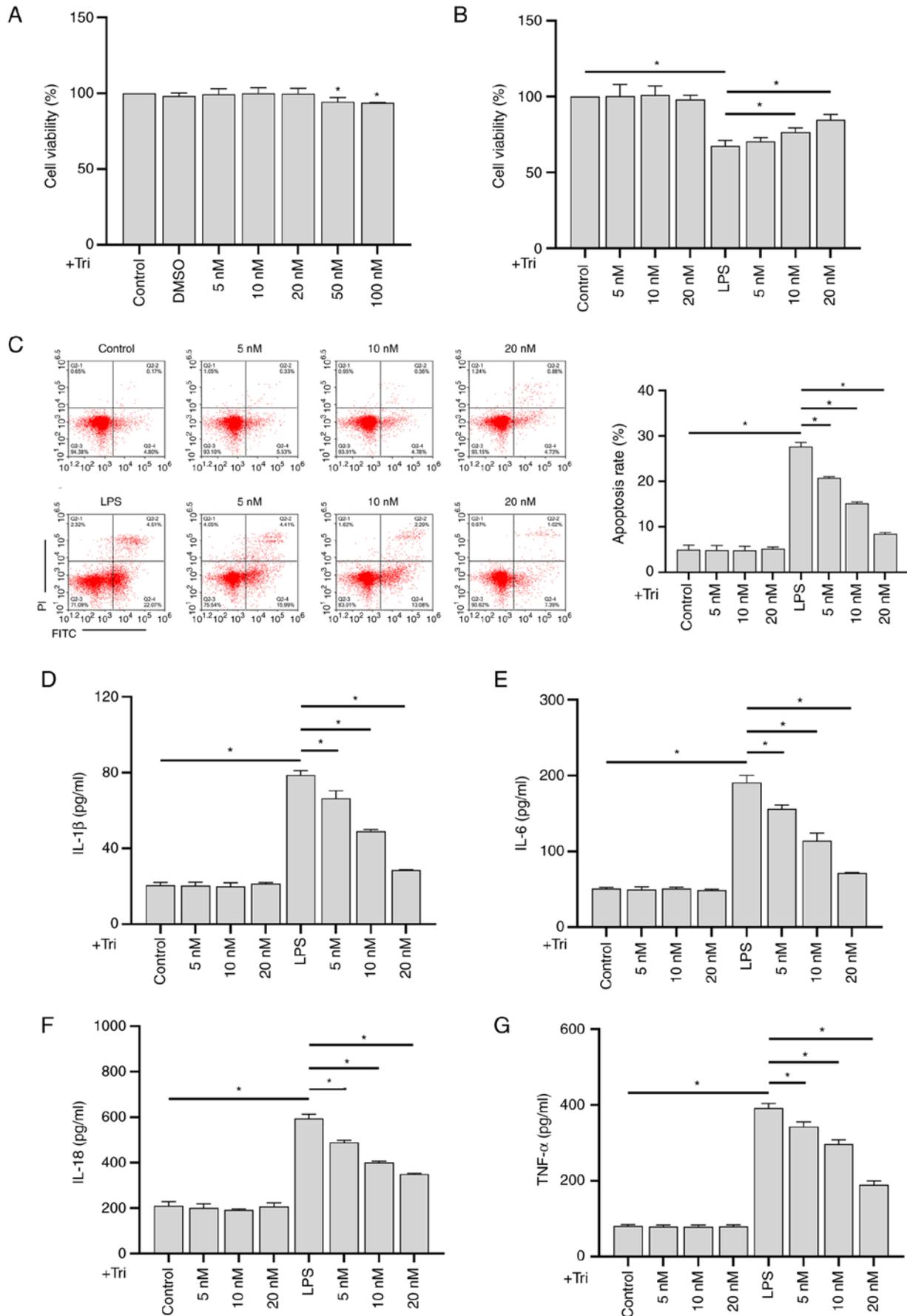


Figure 1. Tri inhibited LPS (100 ng/ml)-mediated inflammation and promoted HMC3 cell growth. (A) CCK8 analysis of the impact of different doses of Tri on the viability of normal HMC3 cells. (B) CCK8 analysis of the Tri impact on the viability of LPS-induced HMC3 cells. (C) Flow cytometry analysis of the Tri impact on the apoptosis of LPS-induced HMC3 cells. (D-G) ELISA analysis of the Tri impact on the contents of inflammatory factors IL-1β (D), IL-6 (E), IL-18 (F) and TNF-α (G) in LPS-induced HMC3 cells. *P<0.05. Tri, triptolide; LPS, lipopolysaccharide.

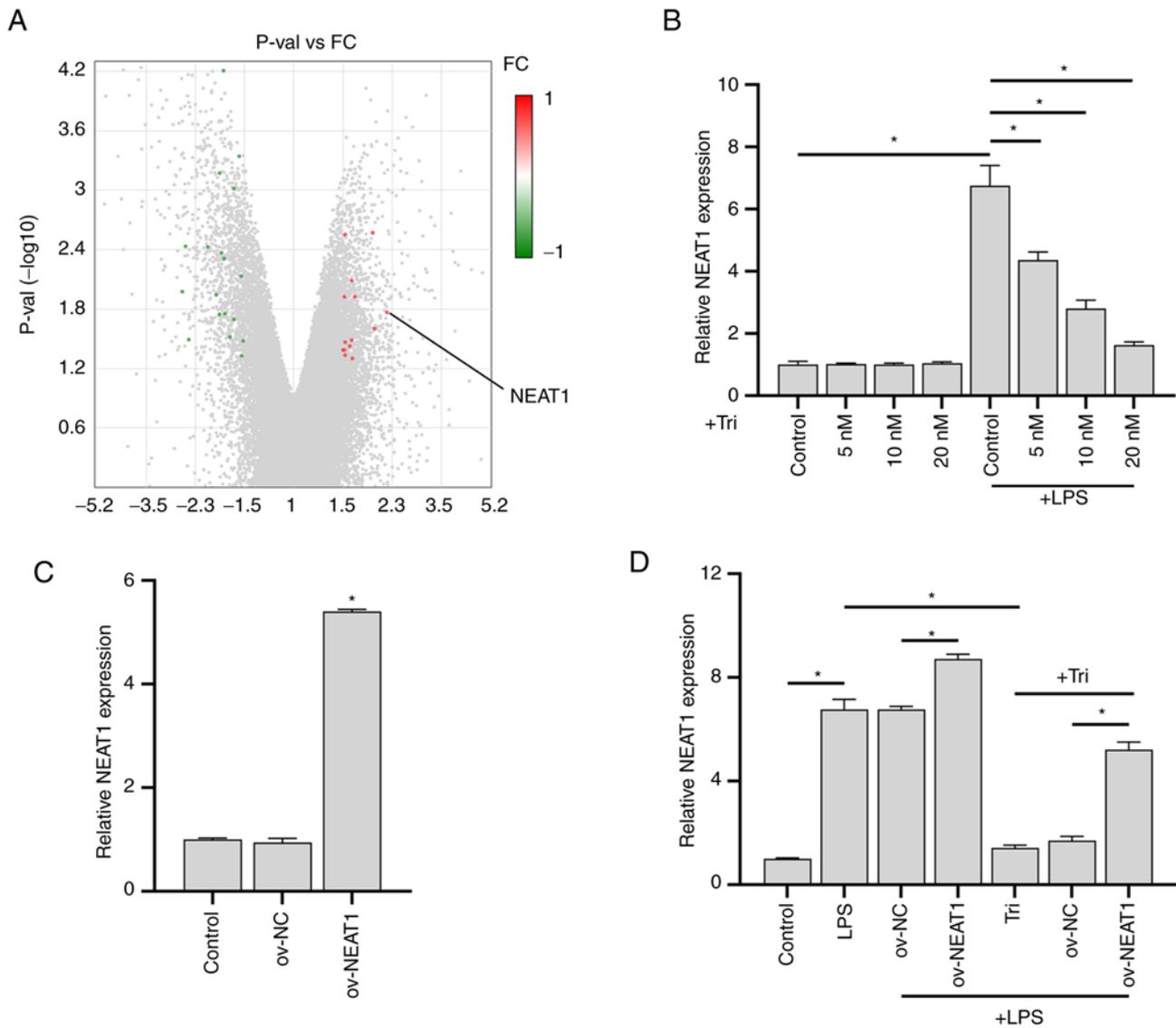


Figure 2. Tri inhibits LPS (100 ng/ml)-mediated up-regulation of NEAT1 expression. (A) Volcano plot showing differential expression of lncRNAs between patients with AD and older adults without AD in the GSE150696 dataset. Green dots represent low expression lncRNAs, red dots represent high expression lncRNAs and gray dots represent lncRNAs that do not meet the criteria of $P \leq 0.01$ and $\log_2 FC > 1.5$. (B) RT-qPCR analysis of the Tri impact on LPS-induced NEAT1 expression in HMC3 cells. (C) RT-qPCR verification of the validity of the ov-NEAT1 plasmid. (D) RT-qPCR analysis of ov-NEAT1 effects on LPS-induced and Tri (20 nM) pre-treated on NEAT1 expression. $P < 0.05$. Tri, triptolide; LPS, lipopolysaccharide; NEAT1, nuclear paraspeckle assembly transcript 1; lncRNA, long non-coding RNA; AD, Alzheimer's disease; RT-qPCR, reverse transcription-quantitative PCR; ov, overexpression; NC, negative control; P-val, P-value; FC, fold change.

miRNA screening. Nucleoplasmic separation determined the high cytoplasmic expression of NEAT1 (Fig. 3A). According to the joint analysis of the StarBase and LncBase databases, there were 27 assumed downstream miRNAs in NEAT1 (Fig. 3B), which are listed in Table SIII. Of these miRNAs (labeled with # in Table SIII) 13 are upregulated in animal models of AD or cognitive deficits (25-36). Furthermore, an analytical targeting analysis of the remaining miRNAs was performed using the miRanda database. The seed regions of miR-361-3p, miR-493-5p, miR-542-3p, miR-129-3p and miR-942-5p had potential binding sites for lncRNA NEAT1 (Fig. 3C). Subsequent RT-qPCR assays showed that the expression of miR-361-3p was lowest following LPS induction (Fig. 3D) and it is a key miRNA in alleviating the AD process (37,38). Compared with the ov-NC group, miR-361-3p expression was downregulated in the ov-NEAT1 group

(Fig. 3E). It was observed that LPS mediation decreased miR-361-3p expression and Tri promoted miR-361-3p expression in a dose-dependent manner (Fig. 3F). ov-NEAT1 further inhibited the LPS-mediated decrease in miR-361-3p expression and reversed the recovery of miR-361-3p expression induced by Tri (Fig. 3G) under LPS-mediated conditions.

NEAT1 acts as a ceRNA to adsorb miR-361-3p. It was observed that the expression of miR-361-3p increased in the miR-361-3p mimic group and decreased in the miR-361-3p inhibitor group (Fig. 4A), suggesting that the synthesis of the miR-361-3p mimic/inhibitor was effective. Bioinformatics analysis showed that NEAT1 has a potential binding site for miR-361-3p (Fig. 4B). A dual-luciferase assay confirmed that the fluorescence activity of the wild-type (WT) NEAT1+miR-361-3p mimic group was significantly lower than that of the WT

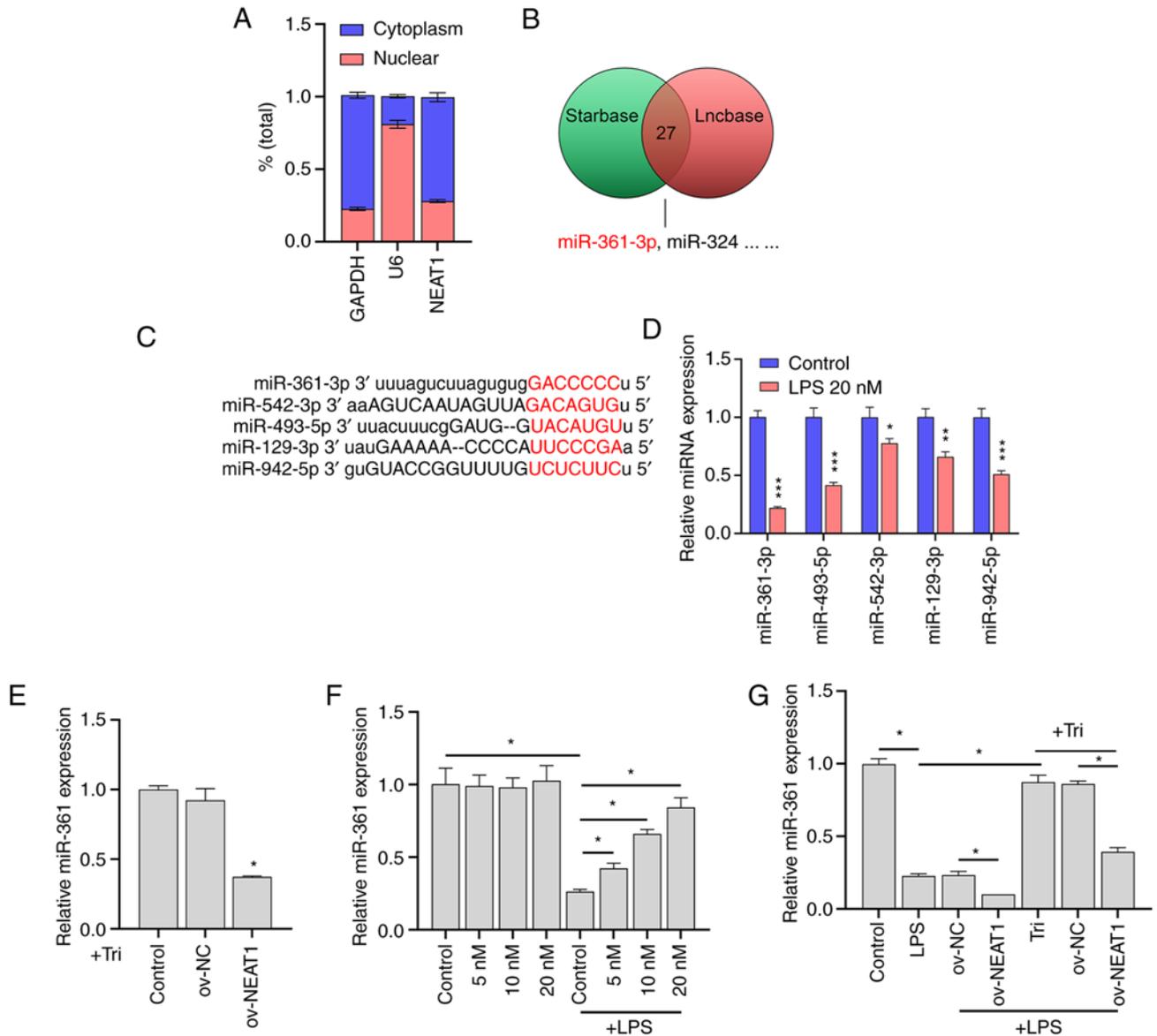


Figure 3. miRNA screening. (A) Nucleocytoplasmic separation determines the localization of NEAT1 in cells. (B) The joint screening of the StarBase and Lncbase databases for potential miRNAs that can bind to NEAT1. (C) The miRanda database was used to analyze the potential binding sites of miR-361-3p, miR-493-5p, miR-542-3p, miR-129-3p and miR-942-5p to lncRNA NEAT1. MiR-129-3p represents miR-129-1-3p and miR-129-2-3p. (D) RT-qPCR analysis of the effect of LPS induction on the expression of miRNAs. (E) RT-qPCR analysis of the impact of ov-NEAT1 on miR-361-3p expression. (F) RT-qPCR analysis of the impact of Tri on the expression of miR-361-3p in LPS-induced HMC3 cells. (G) RT-qPCR analysis of ov-NEAT1 effects on LPS-induced and Tri (20 nM) pre-treatment on miR-361-3p expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. miR/miRNA, microRNA; NEAT1, nuclear paraspeckle assembly transcript 1; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative PCR; ov, overexpression; NC, negative control.

NEAT1+miR-361-3p mimic NC group. However, the fluorescence activity of the NEAT1+miR-361-3p mimic group was not significantly different from that of the NEAT1+miR-361-3p mimic NC group (Fig. 4C). Thus, NEAT1 directly targeted miR-361-3p. RNA pull-down results revealed that, compared with the Bio-NC and Bio-mut groups, the expression of miR-361-3p and NEAT1 was upregulated in the Bio-NEAT1 and Bio-miR-361-3p groups, respectively (Fig. 4D and E). The results further confirmed the binding efficiency of NEAT1 to miR-361-3p. Moreover, miR-361-3p expression did not change significantly during the expression of NEAT1 (Fig. 4F).

TRAF2 is directly targeted by miR-361-3p. Through joint analysis of the Tarbase, TargetScan and miRDB databases, it

was observed that TRAF2 is a potential target of miR-361-3p (Fig. 5A). In addition, the KEGG database showed that TRAF2 is an upstream effector of the NF- κ B signaling pathway and it was reported that NF- κ B is a key factor in the development of AD and that its activation is not conducive to the improvement of memory and cognitive impairment (39,40). The results showed no significant difference in TRAF2 expression in Tri-pretreated HMC3 cells; however, Tri had a dose-dependent inhibitory effect on the LPS-mediated high expression and protein levels of TRAF2 (Fig. 5B and C). Bioinformatics analysis revealed a potential binding site between TRAF2 and miR-361-3p (Fig. 5D). Dual-luciferase assays confirmed that the fluorescence activity of the WT TRAF2+miR-361-3p mimic group was significantly lower than that of the WT TRAF2+miR-361-3p

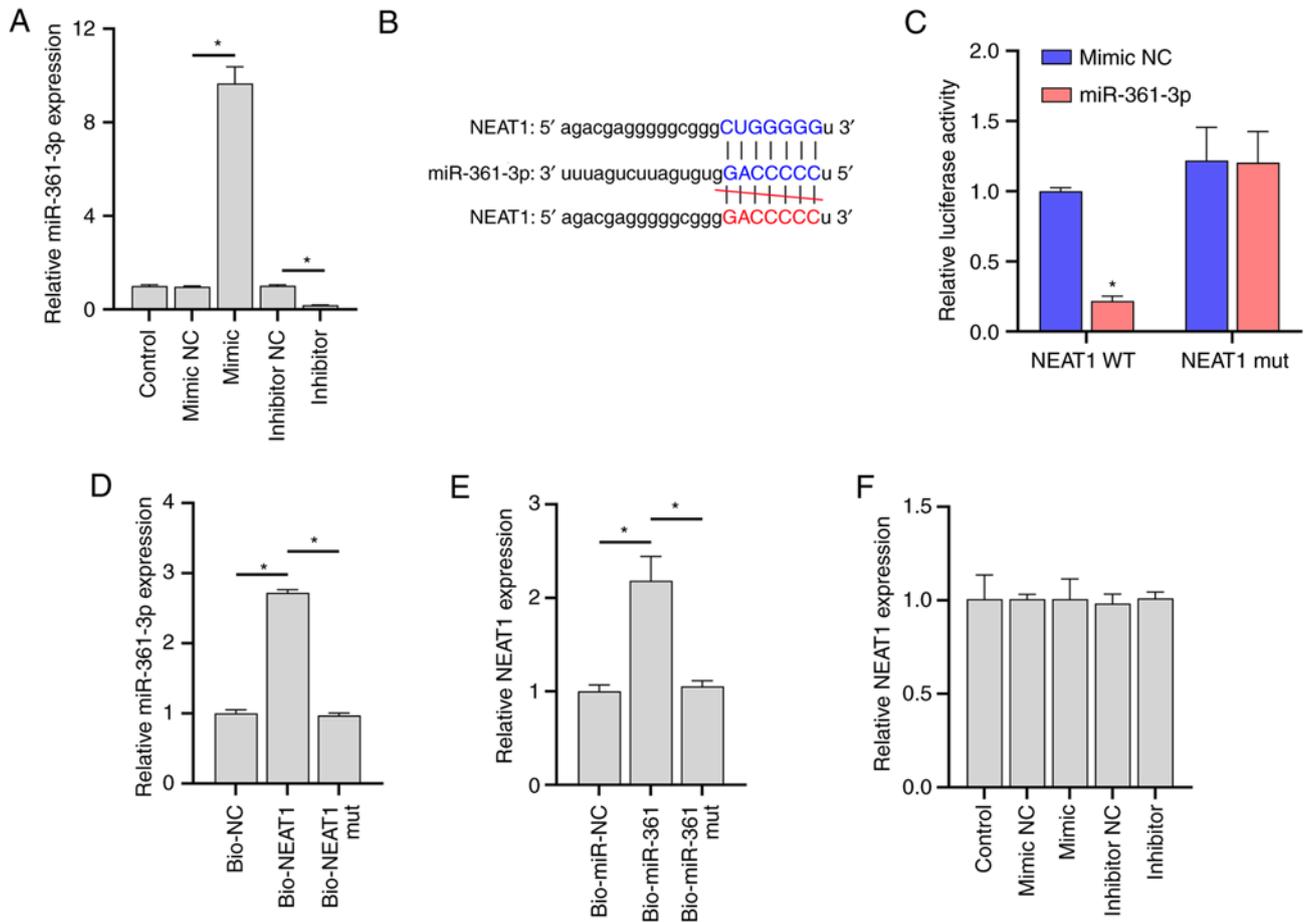


Figure 4. NEAT1 acts as a ceRNA to adsorb miR-361-3p-3p. (A) RT-qPCR was used to verify the effectiveness of the miR-361-3p mimic/inhibitor. (B) Binding sites and mutated sequences of NEAT1 and miR-361-3p. (C) Dual-luciferase analysis of the binding of NEAT1 to miR-361-3p. RNA pull-down analysis of the binding efficiency of (D) miR-361-3p and (E) NEAT1. (F) RT-qPCR analysis of the effect of changes in miR-361-3p expression on NEAT1 expression. * $P < 0.05$. NEAT1, nuclear paraspeckle assembly transcript 1; ceRNA, competitive endogenous RNA; RT-qPCR, reverse transcription-quantitative PCR; miR/miRNA, microRNA; WT wild-type; mut, mutant; NC, negative control.

mimic NC group. The fluorescence activity of the mutant TRAF2+miR-361-3p mimic group was not significantly different from that of the mutant TRAF2+miR-361-3p mimic NC group (Fig. 5E). Thus, TRAF2 is directly targeted by miR-361-3p. Moreover, TRAF2 expression decreased in the miR-361-3p mimic group and increased in the miR-361-3p inhibitor group (Fig. 5F). Compared with si-NC, the expression and protein levels of TRAF2 were downregulated in the si-TRAF2 transfection group (Fig. 5G and H), suggesting that the synthesis of si-TRAF2 was effective. The si-TRAF2-3 showed the highest inhibitory efficiency and was used in subsequent experiments. Decreased TRAF2 expression had no significant effect on the expression of miR-361-3p (Fig. 5I).

Tri inhibits AD development through the NEAT1/miR-361-3p/TRAF2 axis. To investigate the interaction between Tri and NEAT1/miR-361-3p/TRAF2, ov-NEAT1 was co-transfected with miR-361-3p mimic/si-TRAF2. The results showed that LPS-mediated decreased HMC3 cell viability (Fig. 6A), increased apoptosis (Fig. 6B), increased inflammatory cytokine content (Fig. 6C-F) and upregulated TRAF2 expression (Fig. 6G) and that Tri partially alleviated the LPS-mediated effect. Intervention with the miR-361-3p mimic promoted the effect of Tri, while ov-NEAT1 partially reversed the combined effect

of Tri-miR-361-3p. These effects of ov-NEAT1 were partially mitigated by si-TRAF2 treatment. In addition, Tri reduced LPS-mediated TRAF2 protein level and NF- κ B phosphorylation and the addition of miR-361-3p mimic facilitated Tri's effects. si-TRAF2 mitigated the reverse effects of ov-NEAT1 on the miR-361-3p mimic (Fig. 6H). Thus, Tri promotes the expression of miR-361-3p by inhibiting NEAT1, which inhibits the transcription and translation of TRAF2 and suppresses the NF- κ B signaling pathway to achieve a protective effect on HMC3 cells.

Discussion

A number of recent studies have shown that the low side effects and good and high therapeutic effects of TCM are beneficial for treating AD (9,41). For example, garlic extract can reduce brain A β levels and inflammation (42) and ginsenosides can reduce A β peptide production (43) and inhibit NF- κ B-induced neuroinflammation in microglia (44). As a TCM, Tri has promising neuroprotective characteristics and the ability to cross the blood-brain barrier and it has attracted extensive attention from the medical community (45). Because of the key role played by microglia in AD, the present study attempted to elucidate the mechanism of Tri through its effect on the viability, apoptosis and inflammatory factor content of LPS-induced HMC3

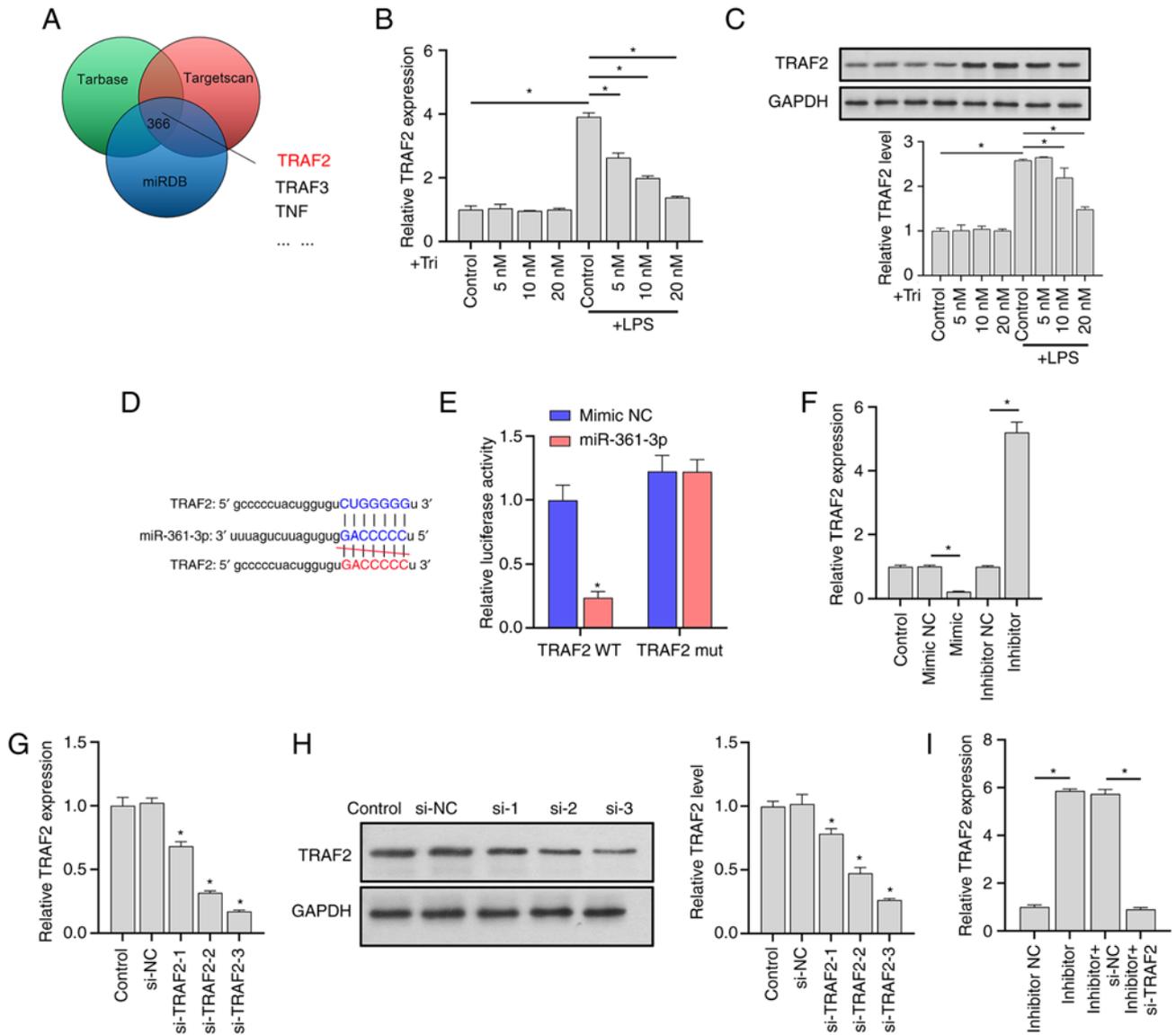


Figure 5. TRAF2 is directly targeted by miR-361-3p. (A) Venn diagram showing the joint screening of the downstream targets of miR-361-3p by the Tarbase, TargetScan and miRDB databases. (B) RT-qPCR analysis of the effect of Tri on LPS-induced TRAF2 expression in HMC3 cells. (C) Western blotting analysis of the effect of Tri on LPS-induced TRAF2 protein level in HMC3 cells. (D) Bioinformatics analysis of the binding site between miR-361-3p and TRAF2. (E) Dual-luciferase analysis of the binding of miR-361-3p to TRAF2. (F) RT-qPCR analysis of the effect of changes in miR-361-3p expression on TRAF2 expression. (G) RT-qPCR verification of the effectiveness of the si-TRAF2 plasmid. (H) Western blotting to verify the effectiveness of the si-TRAF2 plasmid. (I) RT-qPCR analysis of the effect of changes in TRAF2 expression on miR-361-3p expression. * $P < 0.05$. TRAF, tumor necrosis factor receptor associated factor; miR/miRNA, microRNA; RT-qPCR, reverse transcription-quantitative PCR; Tri, triptolide; LPS, lipopolysaccharide; WT wild-type; mut, mutant; NC, negative control; si, small interfering.

microglial cells. In accordance with previous studies (46-48), LPS was used to induce HMC3 microglia to construct an AD model *in vitro* and used gradient doses (5, 10, 20, 50 and 100 nM) of Tri (49,50) to determine the safe dose of Tri for normal HMC3 cells to avoid the interference of Tri in subsequent experiments. Subsequent experiments determined a safe Tri dose of 20 nM. LPS-mediated decreases in HMC3 cell viability, increased apoptosis and increased inflammatory factor content confirmed the successful construction of an *in vitro* AD model. Under the action of gradient concentrations of Tri, the effects of LPS on HMC3 cells were partially alleviated. This is the first study, to the best of the authors' knowledge, to show that Tri effectively treats an AD model (HMC3 cells) *in vitro*. Therefore, it is necessary to understand

further the intrinsic principles and molecular mechanisms involved.

Abnormally expressed RNAs, such as lncRNAs, miRNAs and mRNAs, are key to degenerative diseases (17,51,52). Additionally, lncRNAs act as ceRNAs that regulate the transduction of intercellular signals through the miR/mRNA axis, thereby affecting microglial viability and inflammation (53). The present study revealed that Tri altered LPS-mediated cellular functions by reducing the expression of the lncRNA NEAT1. It first confirmed high expression of NEAT1 in the cytoplasm, which is a prerequisite for NEAT1 to function as a ceRNA (17). A joint analysis of the StarBase and Lncbase databases observed that miR-361-3p might be a potential target of NEAT1 and a previous study confirmed that miR-361-3p

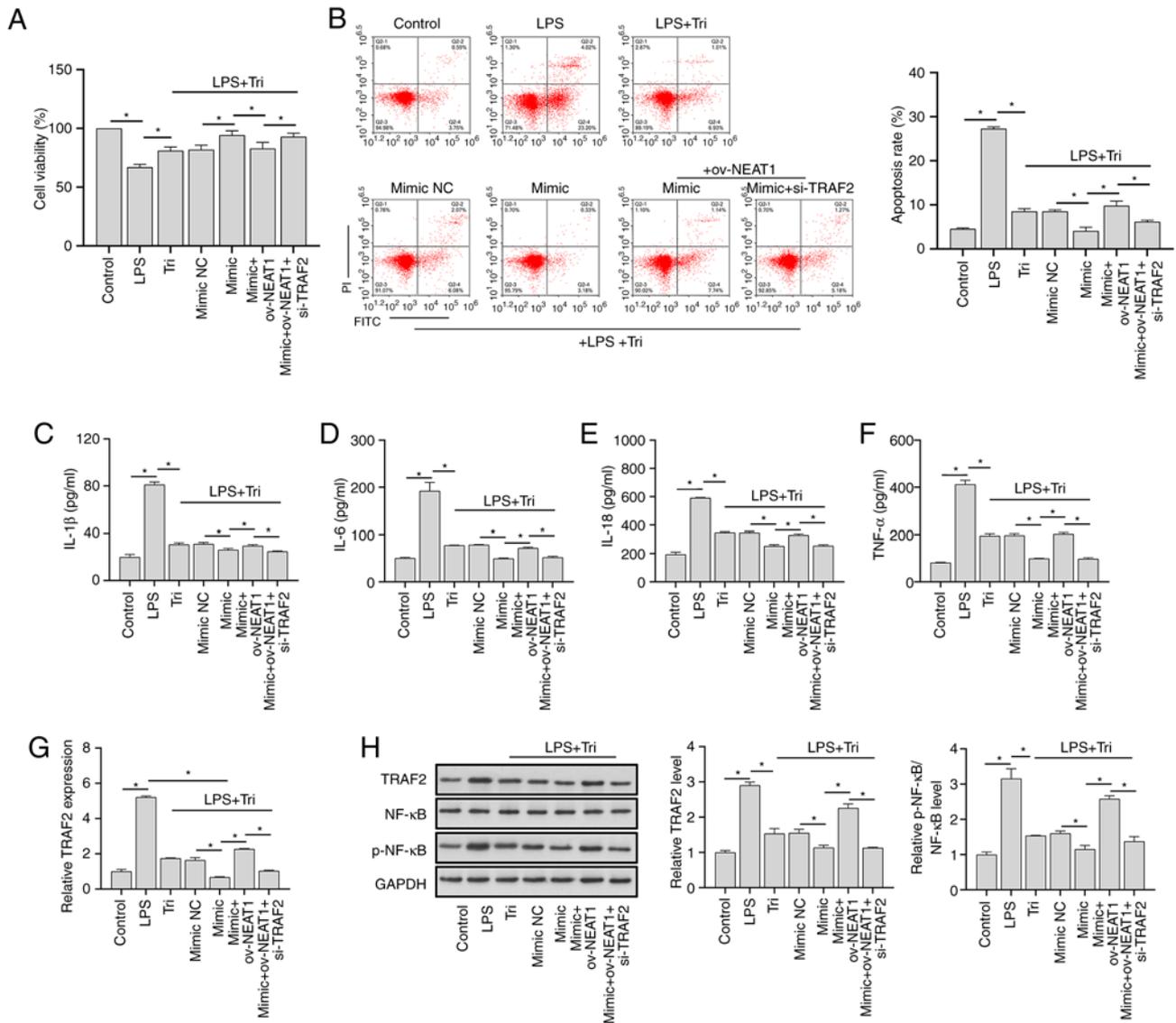


Figure 6. Tri inhibits the AD process through the NEAT1/miR-361-3p/TRAF2 axis. (A) CCK8 analysis of the role of the NEAT1/miR-361-3p/TRAF2 axis in Tri on the LPS-induced viability of HMC3 cells. (B) Flow cytometry analysis of the role of the NEAT1/miR-361-3p/TRAF2 axis in Tri on LPS-induced HMC3 cell apoptosis. (C-F) ELISA analysis of the role of the NEAT1/miR-361-3p/TRAF2 axis in Tri on LPS-induced HMC3 cell inflammatory cytokine content of (C) IL-1 β , (D) IL-6, (E) IL-18 and (F) TNF- α . (G) RT-qPCR analysis of the role of the NEAT1/miR-361-3p/TRAF2 axis in Tri on LPS-induced TRAF2 expression in HMC3 cells. (H) Western blotting analysis of the role of the NEAT1/miR-361-3p/TRAF2 axis in Tri on LPS-induced protein levels of TRAF2, NF- κ B and p-NF- κ B in HMC3 cells. *P<0.05. Tri, triptolide; AD, Alzheimer's disease; NEAT1, nuclear paraspeckle assembly transcript 1; TRAF2, tumor necrosis factor receptor associated factor 2; LPS, lipopolysaccharide; NC, negative control; p-, phosphorylated; ov, overexpression; si, small interfering.

is poorly expressed in AD (37). Dual-luciferase and RNA pull-down analyses revealed that NEAT1 is a sponge of miR-361-3p and adsorbs miR-361-3p by acting as a ceRNA, which is consistent with a previous study (54).

miRNA can inhibit the transcription and translation of mRNA by binding to the 3' untranslated region of mRNA (55). Through the joint analysis of the Tarbase, TargetScan and miRDB databases, a number of mRNAs with potential binding sites for miR-361-3p were observed, a few of which were involved in the NF- κ B signaling pathway (such as TRAF2). High levels of TRAF2 are strongly associated with poor prognosis in patients with AD (56) and normalization of its levels helps alleviate memory impairment and cognitive deficits (57). Subsequent results confirmed that miR-361-3p targeted TRAF2 and was negatively associated with it. Therefore, Tri may regulate injury

and inflammation in HMC3 microglia by regulating NF- κ B signaling via the NEAT1/miR-361-3p/TRAF2 axis. The present study showed that LPS-mediated injury and elevated inflammation in HMC3 cells were attenuated by Tri treatment. The effect of miR-361-3p on Tri was significantly promoted but was reversed by ov-NEAT1. Furthermore, reduced TRAF2 expression inhibited ov-NEAT1 function. These results demonstrated that Tri treatment of LPS-mediated microglial injury and inflammation is achieved by regulating NF- κ B signaling through the NEAT1/miR-361-3p/TRAF2 axis.

The present study had certain limitations. First, no clinical data supported the role of Tri in patients with AD. Second, a number of lncRNAs from GEO data mining have not been analyzed and verified. Third, although LPS can induce oxidative stress and neuroinflammation, which was also exploited in

previous studies to induce LPS in HMC3 cells mimicking the *in vitro* AD environment (46-48), this may not fully reflect the complexity of the inflammatory response in AD. Finally, the present study did not explore other signaling pathways between cells. However, these are the directions and goals of future research, as the effective treatment of HMC3 cells *in vitro* with Tri gives reason to consider its prospect for *in vivo* and even clinical treatment of AD.

In conclusion, Tri attenuated LPS-mediated decreased microglial viability and increased apoptosis and inflammation, involving activation of the NEAT1/miR-361-3p/TRAF2 axis and inhibiting NF- κ B. Tri is a promising therapeutic drug for AD and NEAT1 is a potential diagnostic biomarker. This provides an excellent theoretical basis for future drug development and clinical treatment of AD.

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Availability of data and materials

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

Authors' contributions

LZ designed the study and developed the methodology. XH and HL performed the experiments. JW and ZL collected and interpreted data. LZ drafted the manuscript. LZ and XH confirm the authenticity of all the raw data. All the authors have read and approved the final version of the 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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