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Original Article

A novel NUTM2A-AS1/miR-769–5p axis regulates LPS-evoked damage in human dental pulp cells via the TLR4/MYD88/NF-κB signaling

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Abstract *Background/purpose:* Long non-coding RNAs (lncRNAs) can function as competing endogenous RNAs (ceRNAs) for microRNAs (miRNAs) to be involved in the pathogenesis of multiple human diseases, including pulpitis. Here, we explored the ceRNA activity of NUTM2A-AS1 in regulating lipopolysaccharide (LPS)-evoked cytotoxicity in human dental pulp cells (HDPCs).
Materials and methods: NUTM2A-AS1, miR-769–5p and toll-like receptor 4 (TLR4) were quantified by qRT-PCR and Western blot. Cell viability, proliferation, and apoptosis were detected by XTT, EdU, and flow cytometry assays, respectively. The direct relationship between miR-769–5p and NUTM2A-AS1 or TLR4 was verified by dual-luciferase reporter and RNA immunoprecipitation (RIP) assays.
Results: NUTM2A-AS1 was upregulated in pulpitis tissues and LPS-exposed HDPCs. NUTM2A-AS1 depletion relieved LPS-evoked cell damage in HDPCs. Mechanistically, NUTM2A-AS1 had a binding site for miR-769–5p, and reduced expression of miR-769–5p reversed NUTM2A-AS1 depletion-mediated alleviative effect on LPS-evoked HDPC damage. TLR4 was a direct miR-769–5p target, and miR-769-5p-mediated inhibition of TLR4 relieved LPS-evoked HDPC damage. Furthermore, NUTM2A-AS1 regulated TLR4 expression by acting as a ceRNA for miR-769–5p, and the NUTM2A-AS1/miR-769–5p axis modulated the TLR4/MYD88/NF-κB pathway in LPS-exposed HDPCs.
Conclusion: Our findings establish that NUTM2A-AS1 regulates LPS-evoked damage in HDPCs at least partially through the miR-769–5p/TLR4/MYD88/NF-κB pathway.

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Introduction

Pulpitis, which is known as a typical inflammation of dental pulp, is one of the most frequent dental disorders.[1] Microbial infection in dental pulp tissues initiates immune responses and the induction of inflammatory factors contributes to the pathogenesis of pulpitis.[2,3] Pivotal modulators of inflammatory response in dental pulp cells (DPCs), including proteins and non-coding RNAs (ncRNAs), are under research at the moment.[4–6] Identifying the functional actions of these regulatory molecules will provide an opportunity to develop mechanism-based therapies for pulpitis.

Long ncRNAs (lncRNAs) are RNAs of at least 200 nucleotides in length that have emerged in recent years as essential players in normal development and human diseases.[7] Moreover, deregulation of lncRNAs has been demonstrated to be implicated in the pathogenesis of pulpitis.[8] For instance, MEG3, an overexpressed lncRNA in inflamed pulp and lipopolysaccharide (LPS)-exposed human DPCs (HDPCs), can control LPS-evoked cell inflammation via the p38/MAPK pathway.[9] The competing endogenous RNA (ceRNA) hypothesis suggests that lncRNAs can work as microRNA (miRNA) decoys to involve the post-transcriptional modulation of RNA transcripts, highlighting the implication of the ceRNA networks in pulpitis.[10,11] NUTM2A-AS1 is upregulated in LPS-stimulated HDPCs and its deficiency can protect the cells against LPS-evoked cytotoxicity through the let-7c-5p/HMGB1 axis.[12] Nonetheless, the ceRNA activity of NUTM2A-AS1 in regulating LPS-evoked HDPC damage largely remains to be identified.

Aberrant miRNA expression is suggested to be associated with the pathogenesis of pulpitis.[13,14] For example, miR-410−3p is underexpressed in human inflamed dental pulp;[15] miR-21 exerts an anti-inflammation role in LPS-exposed HDPCs.[16] A recent report unveils that dysregulation of miR-769−5p is closely associated with the progression of chronic periodontitis.[17] Toll-like receptor 4 (TLR4), a crucial player in the inflammatory response, can activate LPS-evoked pro-inflammatory signaling.[18,19] Enhanced expression of TLR4 is known to be involved in pulpitis pathogenesis.[20,21] In the preliminary research, we observed the putative binding relationship between miR-769−5p and NUTM2A-AS1 or TLR4 3'untranslated region (3'UTR) by bioinformatics analysis. Here, we demonstrate the novel NUTM2A-AS1/miR-769−5p/TLR4 ceRNA network in the regulation of LPS-evoked damage in HDPCs.

Materials and methods

Human tissue specimens

We obtained the dental pulp tissue specimens from 23 pulpitis patients (mean years: 9.8 ± 3.2) and 21 healthy donors (mean years: 10.2 ± 2.4) who gave written informed consent at Chongqing Youyoubaobei Women's and Children's Hospital. Tissues specimens were processed in compliance with a protocol approved by the Ethics Committee of Chongqing Youyoubaobei Women's and Children's Hospital. In this study, partial specimens were transferred to a −80 °C freezer immediately for storage for expression analysis, and partial specimens were used to isolate the HDPCs as described[22] using enzymatic method (2 mg/mL collagenase and 0.25% trypsin, all from Sigma−Aldrich, Milan, Italy).

Cell culture and treatment

The HDPCs were maintained at 37 °C, 5% CO_2 and 85% humidity. For cellular growth, we used the culture medium consisting of Minimum Essential Medium α (α-MEM, Life Technologies, Paisley, UK), 10% foetal bovine serum (SAFC Biosciences, Lenexa, KS, USA), and 1% streptomycin/penicillin (Life Technologies). For LPS exposure, HDPCs were seeded in 24-well dishes at 5×10^5 cells per well and then stimulated with the indicated concentrations (0, 2.5, 5, and 10 μg/mL) or 5 μg/mL of LPS (*Escherichia coli* 055:B5, ≥99% purity; Solarbio, Beijing, China) for 24 h.

Transient transfection of cells with oligonucleotides and plasmids

We obtained Silencer® NUTM2A-AS1 siRNA (si-NUTM2A-AS1) and the corresponding Negative Control#2 siRNA (si-NC), and Silencer® TLR4 siRNA (si-TLR4) and Silencer® Negative Control#1 (si-con) from Life Technologies. We purchased micrON hsa_miR-769−5p mimic, mimic mock (miR-NC mimic), hsa_miR-769−5p inhibitor (anti-miR-769−5p) and inhibitor mock (anti-miR-NC) from Ribobio (Guangzhou, China). We obtained a pcDNA3.1-TLR4-coding sequence plasmid (without 3'UTR) and a pcDNA3.1-NUTM2A-AS1 expression plasmid from VectorBuilder (Guangzhou, China), and a negative pcDNA3.1 vector served as a control. For transient transfection, HDPCs cultured in 24-well dishes at 5×10^5 cells per well were submitted to lipofection using Lipofectamine 3000 reagent (Life Technologies) and siRNA (100 nM/well), miRNA mimic or inhibitor (50 nM/well), or plasmid (200 ng/well). Eight hours later, medium was changed and HDPCs were subjected to further culture for 24 h. Then, transfected HDPCs were stimulated with LPS (5 μg/mL) for 24 h for further analyses.

RNA preparation and expression analysis by quantitative real-time PCR (qRT-PCR)

We extracted total RNA from tissue specimens and cultured HDPCs (5×10^6) using peqGOLD total RNA Kit (PeqLab,

Erlangen, Germany) and the accompanying protocol. We prepared nuclear and cytoplasmic RNA from HDPCs with Cytoplasmic & Nuclear RNA Purification Kit as described by the manufacturers (NORGEN, Thorold, ON, Canada) For NUTM2A-AS1 and TLR4 analysis, we used a Bio-Rad IScript Kit and iTaq SYBR Green Supermix (all from Bio-Rad, Sundbyberg, Sweden) for cDNA synthesis and amplification, respectively. For miR-769–5p analysis, we used miRCURY LNA Universal RT miRNA PCR setup for miRNA cDNA synthesis and amplification as recommended by the manufacturers (Exiqon, Copenhagen, Denmark). The GAPDH or U6 was used as a reference gene to correct for differences in the amount of RNA in each sample. All primers were supplied by Sangon Biotech (Shanghai, China; Supplement Table 1). Using the $2^{-\Delta\Delta Ct}$ formula, we calculated the fold change.

XTT cell viability assay

The effects of LPS exposure, the indicated transfections, and their combinations on the viability of HDPCs were examined by the XTT (2,3,-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay. In brief, 4×10^3 treated HDPCs were grown in 100 μL standard medium in 96-well dishes at 37 °C. After 24 h, 10 μL of XTT reagent (Abcam, Cambridge, UK) was added into each well. Following a 3-h incubation at 37 °C, we read the results by measuring absorption at 450 nm with a plate reader (Tecan, Grödig, Austria).

EdU cell proliferation assay

We used the Cell-Light EdU Apollo567 Kit (Ribobio) to evaluate cell proliferation. Briefly, 4×10^3 treated HDPCs were maintained in 100 μL standard medium in 96-well dishes for 24 h. Subsequently, cells were incubated with EdU reagent and stained with Apollo567, followed by the nucleus staining with DAPI (Sigma–Aldrich). We scored the EdU positive cells (red) using a fluorescence microscope (Olympus, Markham, Ontario, Canada).

Flow cytometry for cell apoptosis

Flow cytometry assay was carried out using Annexin V/PI double staining method (BD Biosciences, Milan, Italy). Briefly, 4×10^3 treated HDPCs were grown in 100 μL standard medium in 96-well dishes for 48 h. After that, cells were stained with Annexin V-FITC and PI as per the manufacturing guideline and then analyzed on a flow cytometer (Epics XL-MCL, Beckman Coulter, Brea, CA, USA).

Western blot

We performed Western blot under standard method,[23] using primary antibodies for PCNA (ab29, Abcam), Cleaved-caspase-3 (ab32042, Abcam), TLR4 (ab13556, Abcam), MYD88 (ab107585, Abcam), p-P65 (ab76302, Abcam), P65 (ab76311, Abcam), IκBα (#4812, Cell Signaling Technology, Danvers, MA, USA), p-IκBα (#2859, Cell Signaling Technology), and GAPDH (ab9485, Abcam). Lysates were electrophoresed on 5–20% Tris–HCl-ready gels (Bio-Rad), transferred to nitrocellulose membranes (Bio-Rad), and then probed with the indicated primary antibodies. After protein bands were developed with Immun-Star™ HRP Enhancer (Bio-Rad), we analyzed the results using BIOMAX XAR films (Kodak, Stuttgart, Germany).

Enzyme-linked immunosorbent assay (ELISA)

We determined the levels of IL-6, IL-1β, and TNF-α in cell culture medium by ELISA using Human IL-6 ELISA Kit (Life Technologies), Human IL-1β ELISA Kit (Life Technologies), and Human TNF-α ELISA Kit (Abcam), respectively, based on the manufacturer's recommendations.

Computational prediction

To search the miRNA-binding sites to NUTM2A-AS1, we downloaded the output results of the computational algorithm starBase. To identify miR-769–5p target genes, we analyzed the output results of the prediction program TargetScan.

Dual-luciferase reporter assay

NUTM2A-AS1 fragments harboring the predicted miR-769–5p binding sequence or mutant seed sequence, TLR4 3′UTR and TLR4 3′UTR carrying a mutant miR-769–5p binding sequence were each ligated in psiCHECK-2 vector (Promega, Barcelona, Spain). HDPCs (5×10^4) were cotransfected with the indicated reporter construct (200 ng) and miRNA mimic (50 nM). Lysates were harvested at 24 h posttransfection, and the dual-luciferase reporter assay was carried out (Promega).

RNA immunoprecipitation (RIP) and RNA pull-down assays

In RIP assays, lysates of 5×10^6 HDPCs were incubated with Protein A/G Agarose-coupled an antibody for Ago2 (ab233727) or IgG (ab172730, all from Abcam) at 4 °C for 6 h. In RNA pull-down assays, lysates of 5×10^6 HDPCs were incubated with streptomycin magnetic beads-coupled biotinylated miR-769–5p mimic (bio-miR-769–5p, Ribobio) or control probe (bio-miR-NC, Ribobio) overnight at 4 °C. We collected the beads and extracted the RNA to gauge the enrichment levels of NUTM2A-AS1, miR-769–5p and TLR4 mRNA by qRT-PCR.

Statistical analysis

Data were expressed as mean ± SEM of separate experiments ($n \geq 3$). For statistical comparison, we used a Student's *t*-test (two-tailed) or ANOVA with a post hoc Tukey's test, where appropriate, with a *P* value less than 0.05 considered significant. We analyzed the RNA expression correlations in 23 pulpitis tissue specimens using Pearson's correlation coefficients.

Results

NUTM2A-AS1 depletion attenuates HDPC damage evoked by LPS

To establish the function of NUTM2A-AS1 in human pulpitis, we firstly analyzed its expression by qRT-PCR in 23 human pulpitis tissue specimens. Relative to normal pulp tissues, NUTM2A-AS1 levels were markedly augmented in pulpitis tissues (Fig. 1A). In HDPCs, subcellular fractionation assays showed that NUTM2A-AS1 was mainly present in the cytoplasm (Fig. 1B). Moreover, LPS exposure resulted in increased expression of NUTM2A-AS1 in a dose-dependent manner in HDPCs (Fig. 1C).

Having demonstrated upregulation of NUTM2A-AS1 in LPS-exposed HDPCs, we then examined its detailed role. A

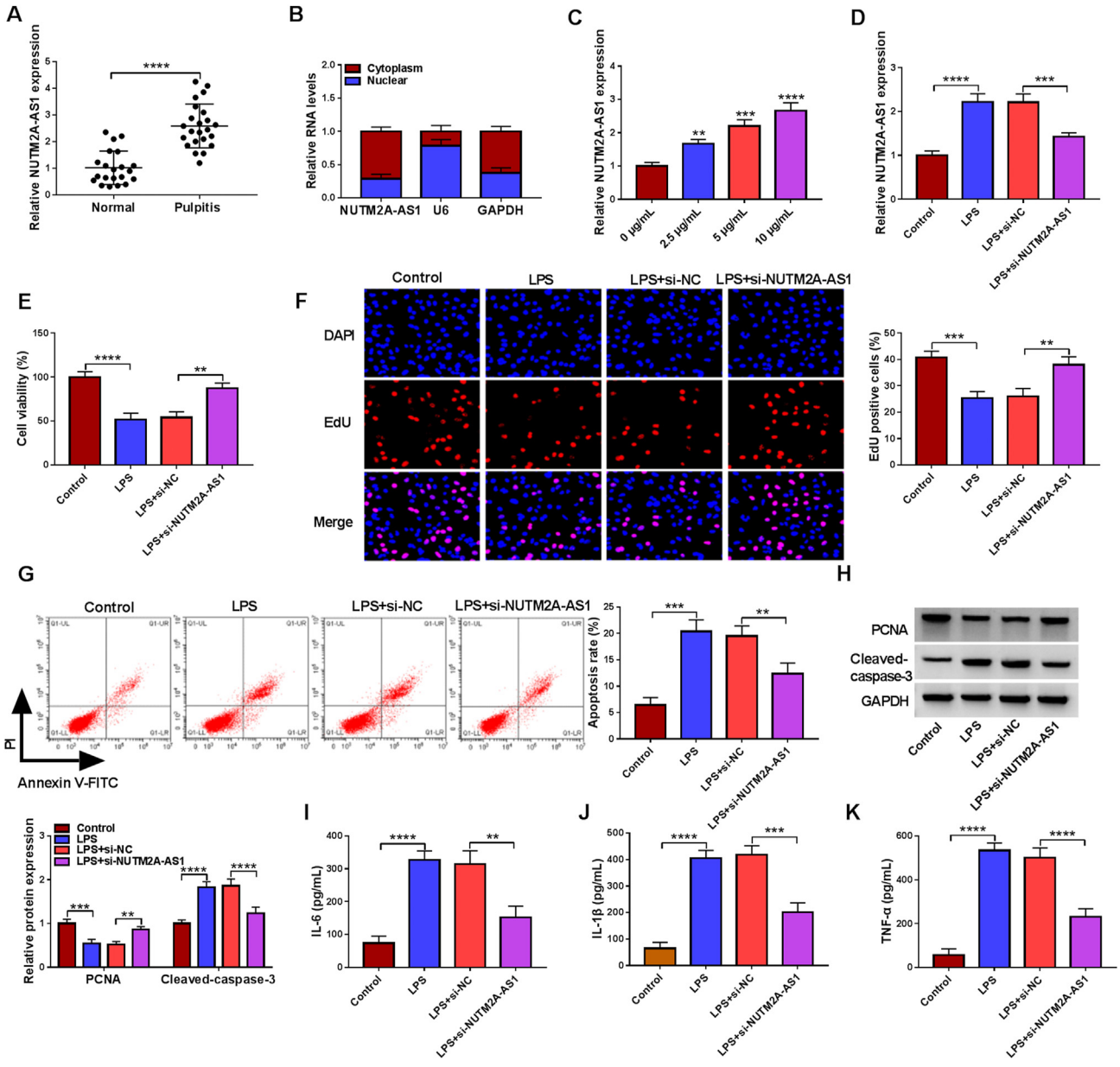

Figure 1 NUTM2A-AS1 is upregulated in human pulpitis and its depletion relieves LPS-evoked HDPC damage. (A) qRT-PCR showing NUTM2A-AS1 expression in 23 human pulpitis tissue specimens and 21 healthy pulp tissues. (B) Subcellular fractionation assay showing the localization of NUTM2A-AS1 in HDPCs. (C) qRT-PCR showing NUTM2A-AS1 expression in HDPCs exposed to the indicated concentrations of LPS for 24 h. (D–K) HDPCs were transfected with or without si-NUTM2A-AS1 or si-NC and then exposed to 5 μg/mL of LPS for 24 h. (D) qRT-PCR of NUTM2A-AS1 expression in the HDPCs treated as indicated. (E) Cell viability by XTT assay with the HDPCs treated as indicated. (F) Cell proliferation by EdU assay with the HDPCs treated as indicated. (G) Cell apoptosis by flow cytometry with the HDPCs treated as indicated. (H) Representative Western blot showing the levels of PCNA and Cleaved-caspase-3 in the HDPCs treated as indicated. (I–K) The production of IL-6, IL-1β, and TNF-α by ELISA in the HDPCs treated as indicated. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

siRNA specific for NUTM2A-AS1 (si-NUTM2A-AS1) was used to silence the expression of NUTM2A-AS1 in LPS-exposed HDPCs, and its transfection efficiency was confirmed by qRT-PCR (Fig. 1D). Knockdown of NUTM2A-AS1 remarkably rescued LPS-imposed suppression on cell viability and proliferation (Fig. 1E and F). NUTM2A-AS1 knockdown also reduced cell apoptosis induced by LPS in HDPCs (Fig. 1G). Our Western blot results revealed that LPS led to reduced expression of proliferating marker PCNA and elevated level of apoptosis-related protein Cleaved-caspase-3 in HDPCs, and the depletion of NUTM2A-AS1 significantly abolished these effects (Fig. 1H). Furthermore, in HDPCs, LPS induced the secretion of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α), which was reversed by NUTM2A-AS1 depletion (Fig. 1I–K). Conversely, overexpression of NUTM2A-AS1 by the expression plasmid transfection aggravated cell apoptosis and the production of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) in LPS-treated HDPCs (Supplement Fig. 1). All these results validate that NUTM2A-AS1 depletion mitigates cell damage induced by LPS.

NUTM2A-AS1 contains a binding site for miR-769–5p

To study the molecular mechanism underlying the function of NUTM2A-AS1, we attempted to identify the miRNAs that potentially bind to NUTM2A-AS1. Using the computational algorithm starBase, a putative complementary sequence for miR-769–5p was found in NUTM2A-AS1 (Fig. 2A). We confirmed that the binding site is functional, using a luciferase reporter linked to NUTM2A-AS1 fragment encompassing the seed region. Overexpression of miR-769–5p, gauged by qRT-PCR (Fig. 2B), resulted in a marked reduction in the luciferase activity of wild-type reporter (WT-NUTM2A-AS1) (Fig. 2C). Mutant of the miR-769–5p binding site in the reporter (MUT-NUTM2A-AS1) completely abolished its effect (Fig. 2C). To test whether NUTM2A-AS1 associates with the RNA-induced silencing complexes (RISCs), RIP experiments were carried out using an antibody against Ago2, which is the core component of the RISC.[24] Consistent with miR-769–5p enrichment level, NUTM2A-AS1 was substantially enriched in Ago2-containing RISCs relative to control IgG immunoprecipitates (Fig. 2D). Furthermore, RNA pull-down assays revealed that incubation of cell lysates with biotinylated miR-769–5p mimic (bio-miR-769–5p) led to a clear elevation in NUTM2A-AS1 enrichment level (Fig. 2E), suggesting the direct relationship between NUTM2A-AS1 and miR-769–5p. Analysis of pulpitis tissue specimens revealed that miR-769–5p levels were markedly reduced in pulpitis tissues compared with healthy controls (Fig. 2F). Importantly, there existed a strong inverse association between the levels of miR-769–5p and NUTM2A-AS1 in 23 pulpitis tissue specimens (Fig. 2G). Additionally, in HDPCs, LPS exposure led to suppressed expression of miR-769–5p in a dose-dependent manner (Fig. 2H). These data

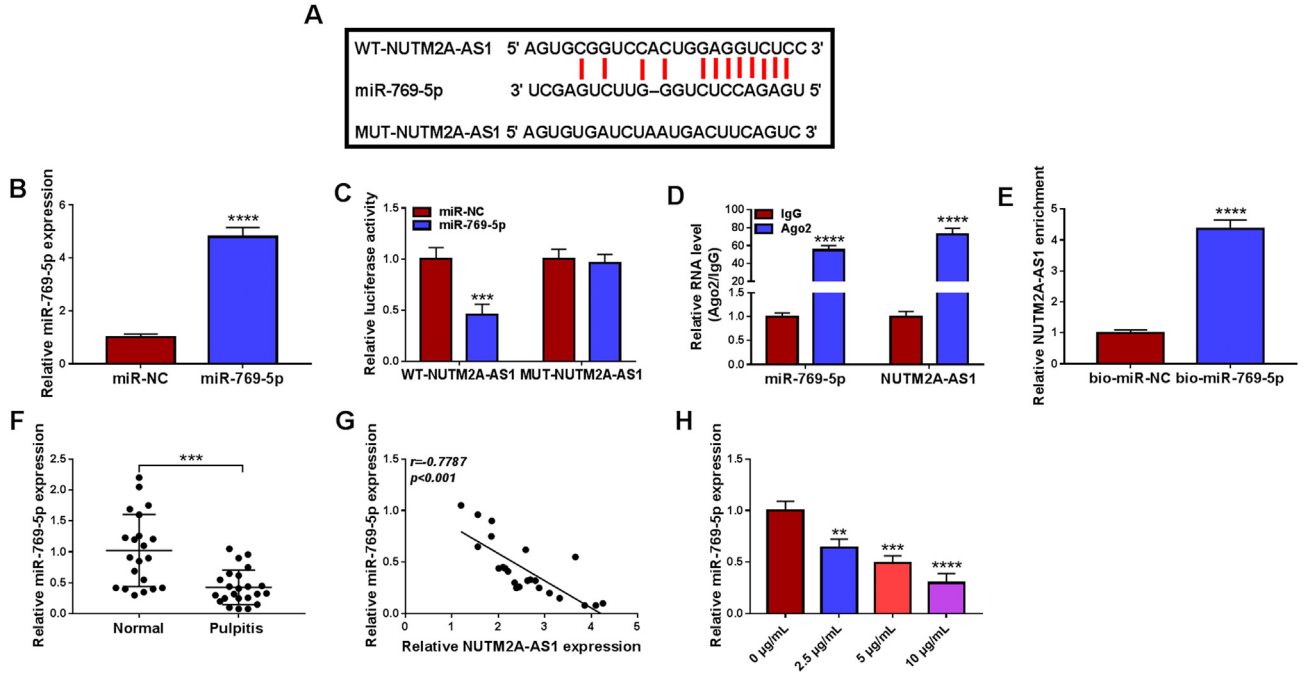

Figure 2 NUTM2A-AS1 contains a miR-769-5p binding site. (A) Sequence of miR-769–5p and partial sequences of NUTM2A-AS1 containing a wild-type or mutant-type miR-769–5p binding site. (B) qRT-PCR of miR-769–5p in miR-769–5p mimic- or miR-NC mimic-transfected HDPCs. (C) The indicated constructs were each transfected into HDPCs together with mimic of miR-769–5p or miR-NC, followed by the measurement of luciferase activity. (D) RIP assay with an antibody against Ago2 or IgG from extracts of HDPCs. (E) RNA pull-down assay with total extractions of HDPCs using bio-miR-769–5p or bio-miR-NC. (F) MiR-769–5p qRT-PCR in 23 human pulpitis tissue specimens and 21 healthy pulp tissues. (G) Scatter plots of miR-769–5p level versus NUTM2A-AS1 expression in 23 human pulpitis tissue specimens. Pearson's correlation coefficient (r) and *P* value were shown. (H) MiR-769–5p qRT-PCR in HDPCs exposed to the indicated concentrations of LPS for 24 h $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

together establish that NUTM2A-AS1 contains a functional binding site for miR-769—5p.

Reduced expression of miR-769—5p reverses NUTM2A-AS1 depletion-mediated alleviative effect on LPS-evoked HDPC damage

In LPS-exposed HDPCs, we observed a striking augmentation in the level of the endogenous miR-769—5p following NUTM2A-AS1 depletion (Fig. 3A), reinforcing that NUTM2A-AS1 can affect miR-769—5p expression. To determine whether miR-769—5p is a functional mediator of NUTM2A-AS1 regulation, we transfected NUTM2A-AS1-silenced HDPCs with a miR-769—5p inhibitor (anti-miR-769—5p) before LPS exposure. The transfection efficacy of anti-miR-769—5p in reducing miR-769—5p expression was verified by qRT-PCR (Fig. 3A). In NUTM2A-AS1-silenced HDPCs under LPS, reduced expression of miR-769—5p reversed, at least partially, NUTM2A-AS1 depletion-driven viability and proliferation enhancement and apoptosis defect (Fig. 3B—F), as well as production increase of IL-6, IL-1β, and TNF-α (Fig. 3G—I) compared with controls. Taken together, these findings suggest that the effects of NUTM2A-AS1 depletion are at least in part due to the augmentation of miR-769—5p expression.

MiR-769—5p directly targeted TLR4 and miR-769-5p-mediated repression of TLR4 attenuates HDPC damage evoked by LPS

To further understand the role of miR-769—5p, we decided to identify the target genes of miR-769—5p using TargetScan algorithm. Among these candidates, TLR4 was of particular interest because TLR4 is known to be involved in the pathogenesis of pulpitis.[21,25] The TLR4 mRNA was predicted to contain a 3′UTR element that is partially complementary to miR-769—5p (Fig. 4A). To demonstrate the targeting of TLR4 by miR-769—5p, we cloned TLR4 3′UTR into a luciferase

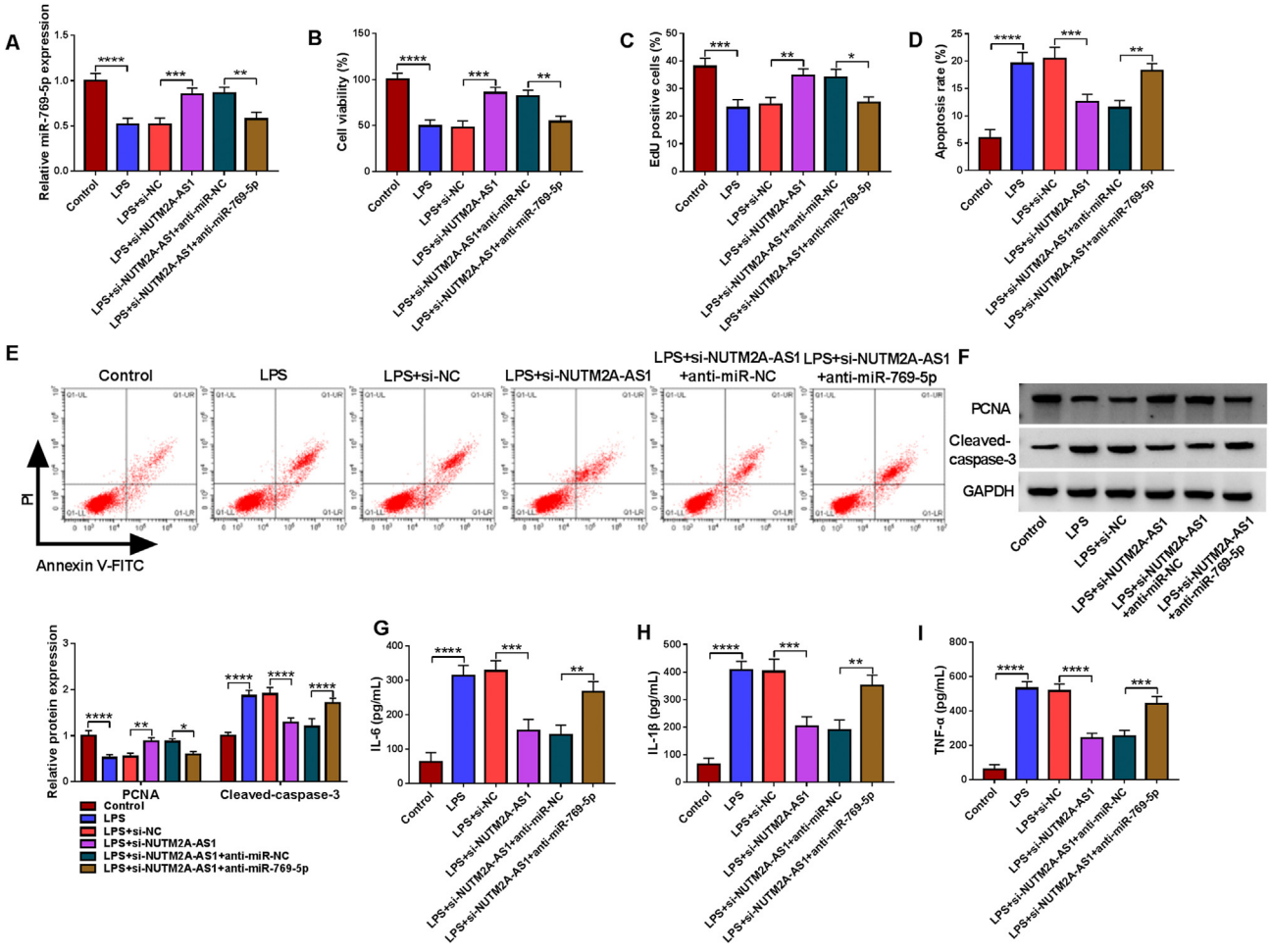

Figure 3 Reduction of miR-769-5p reverses the effects of NUTM2A-AS1 depletion. (A—I) HDPCs were transfected with si-NUTM2A-AS1+anti-miR-769—5p, si-NUTM2A-AS1+anti-miR-NC, si-NUTM2A-AS1, or si-NC and then exposed to 5 μg/mL of LPS for 24 h. (A) MiR-769—5p qRT-PCR in the HDPCs treated as indicated. (B) XTT assay for cell viability of the HDPCs treated as indicated. (C) EdU assay for cell proliferation of the HDPCs treated as indicated. (D and E) Cell apoptosis by flow cytometry with the HDPCs treated as indicated. (F) Representative Western blot showing PCNA and Cleaved-caspase-3 levels in the HDPCs treated as indicated. (G—I) IL-6, IL-1β, and TNF-α levels by ELISA in the HDPCs treated as indicated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

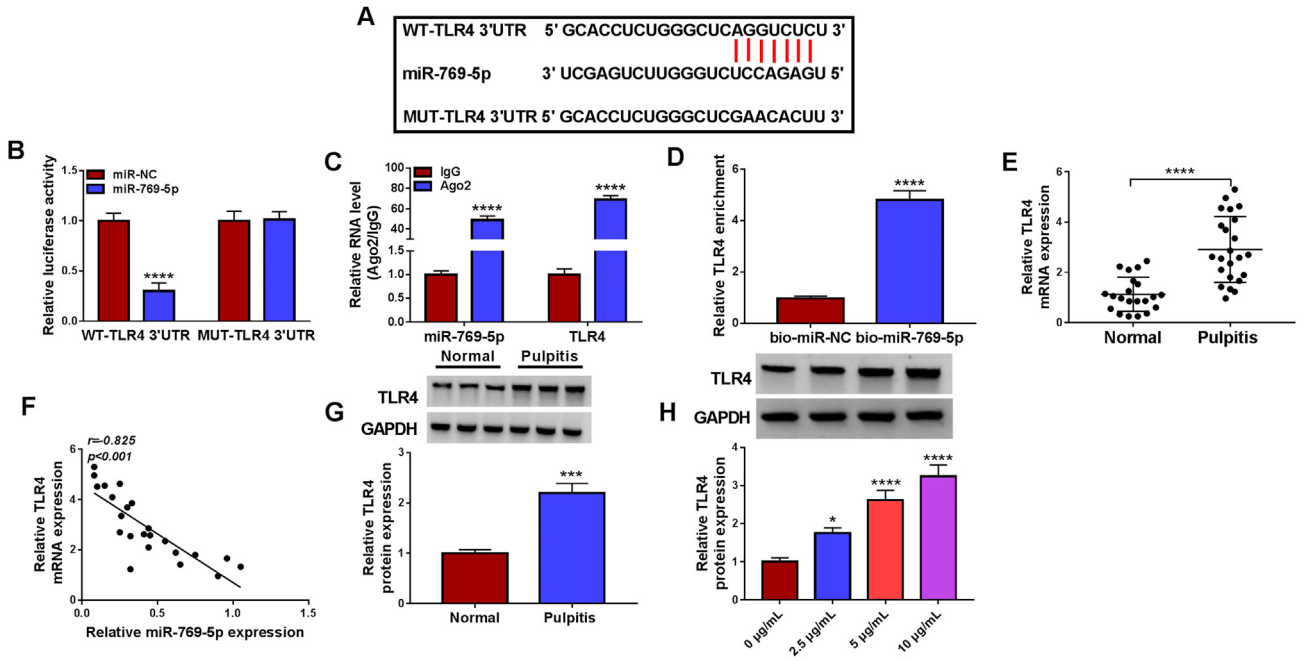

Figure 4 TLR4 is directly targeted by miR-769-5p. (A) Sequence of miR-769–5p and partial sequences of TLR4 3′UTR containing a wild-type or mutant-type miR-769–5p binding site. (B) The indicated constructs were each transfected into HDPCs together with mimic of miR-769–5p or miR-NC, followed by the measurement of luciferase activity. (C) RIP assay with an antibody against Ago2 or IgG from extracts of HDPCs. (D) RNA pull-down assay with total extractions of HDPCs using bio-miR-769–5p or bio-miR-NC. (E) qRT-PCR of TLR4 mRNA in 23 human pulpitis tissue specimens and 21 healthy pulp tissues. (F) Scatter plots of TLR4 mRNA level versus miR-769–5p expression in 23 human pulpitis tissue specimens. Pearson's correlation coefficient (r) and *P* value were shown. (G) Representative Western blot showing TLR4 protein level in 3 human pulpitis tissue specimens and 3 healthy pulp tissues. (H) Representative Western blot showing the level of TLR4 protein in HDPCs exposed to the indicated concentrations of LPS for 24 h $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

construct. Reporter assays with miR-769-5p-expressing HDPCs showed that miR-769–5p suppressed the expression of TLR4 3′UTR; mutation of the predicted miR-769–5p binding sequence abolished responsiveness to miR-769–5p (Fig. 4B), suggesting that TLR4 is a direct miR-769–5p target. RIP experiments revealed that TLR4 mRNA was significantly enriched in Ago2-containing RISCs compared with control IgG immunoprecipitates (Fig. 4C), indicating that TLR4 associates with the RISCs. Moreover, RNA pull-down assays validated the direct relationship between miR-769–5p and TLR4 (Fig. 4D). In pulpitis tissues, TLR4 mRNA and protein levels were strikingly elevated relative to normal controls, and TLR4 mRNA expression inversely correlated with the level of miR-769–5p (Fig. 4E–G). Furthermore, LPS exposure resulted in a remarkable increase in the expression of TLR4 protein in HDPCs (Fig. 4H).

In support of the targeting of TLR4 by miR-769–5p, we found that overexpression of miR-769–5p significantly repressed the level of TLR4 protein in LPS-exposed HDPCs (Fig. 5A). To elucidate whether TLR4 is a downstream effector of miR-769–5p, we transfected HDPCs before LPS exposure with miR-769–5p mimic alone or together with a TLR4 expression construct rendered miR-769–5p insensitive by deletion of its 3′UTR. In LPS-exposed HDPCs, enforced expression of miR-769–5p led to enhanced viability and proliferation, suppressed apoptosis (Fig. 5B–F), as well as decreased secretion of IL-6, IL-1β, and TNF-α (Fig. 5G–I) compared with miR-NC controls. Our Western blot data showed that the TLR4 expression construct significantly elevated TLR4 protein level in miR-769-5p-expressing HDPCs under LPS (Fig. 5A). Notably, restoration of TLR4 abrogated, at least in part, these effects of miR-769–5p overexpression in LPS-exposed HDPCs (Fig. 5B–I). Additionally, silencing of TLR4 upon si-TLR4 transfection, confirmed by Western blot, strongly promoted viability and proliferation, and hindered apoptosis, as well as decreased the production of IL-6, IL-1β, and TNF-α in LPS-exposed HDPCs (Supplement Fig. 2). These results together establish that TLR4 is a direct and functional target of miR-769–5p.

NUTM2A-AS1 regulates the TLR4/MYD88/NF-κB pathway via miR-769–5p

Based on the shared binding sequence for NUTM2A-AS1 and TLR4 3′UTR in miR-769–5p, we attempted to evaluate whether NUTM2A-AS1 can impact TLR4 expression by miR-769–5p. Indeed, in LPS-exposed HDPCs, NUTM2A-AS1 depletion led to the striking downregulation in the levels of TLR4 mRNA and protein; reduced expression of miR-769–5p abrogated these effects (Fig. 6A and B), indicating that NUTM2A-AS1 post-transcriptionally modulates TLR4 expression via miR-769–5p.

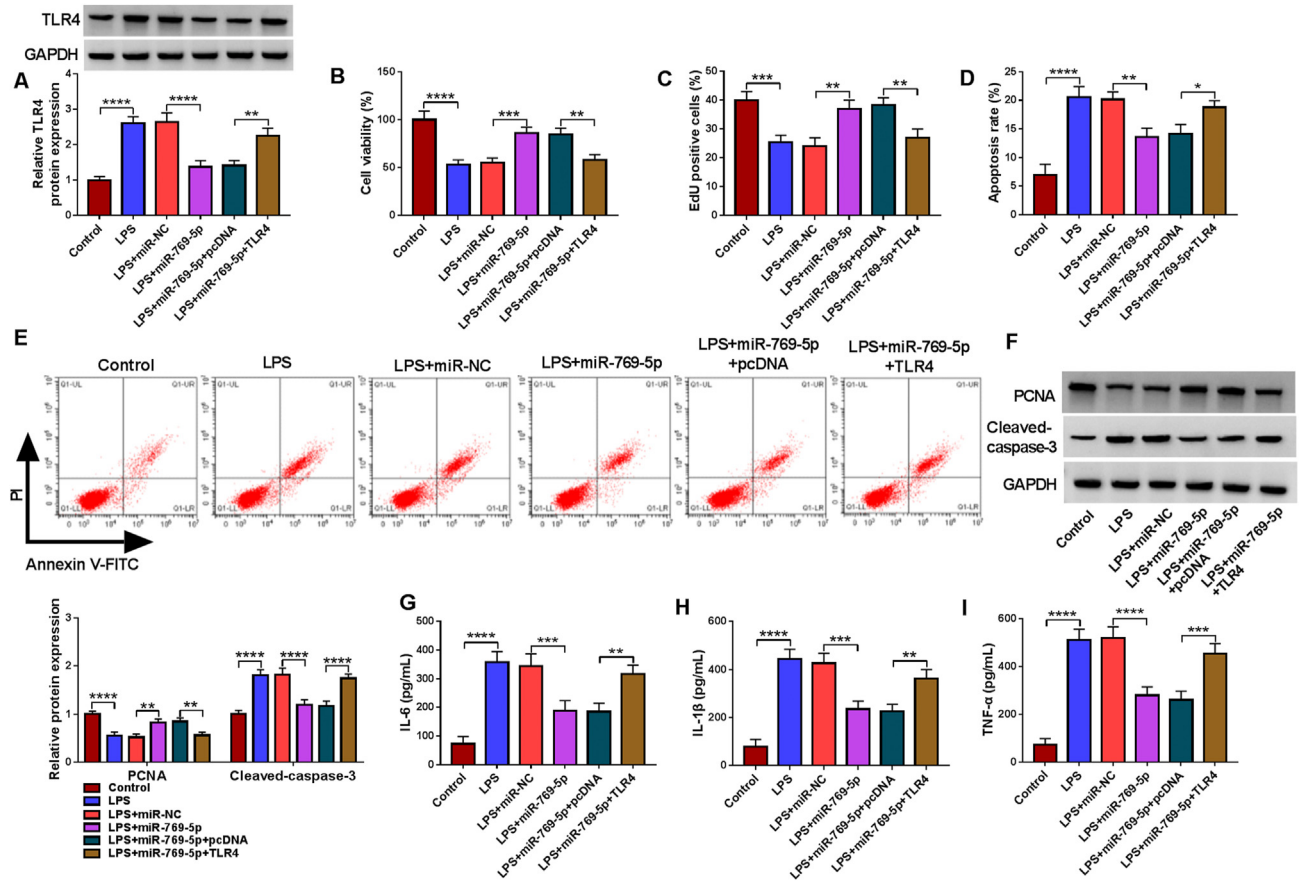

Figure 5 TLR4 is a downstream effector of miR-769-5p. (A–I) HDPCs were transfected with or without miR-769–5p mimic+a TLR4 expression plasmid, miR-769–5p mimic + pcDNA, miR-769–5p mimic, or miR-NC mimic and then exposed to LPS (5 μg/mL) for 24 h. (A) Representative Western blot showing the level of TLR4 protein in the HDPCs treated as indicated. (B) XTT assay for cell viability of the HDPCs treated as indicated. (C) Cell proliferation by EdU assay with the HDPCs treated as indicated. (D and E) Cell apoptosis by flow cytometry with the HDPCs treated as indicated. (F) Representative Western blot showing the levels of PCNA and Cleaved-caspase-3 in the HDPCs treated as indicated. (G–I) The production of IL-6, IL-1β, and TNF-α by ELISA in the HDPCs treated as indicated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

The TLR4/MYD88/NF-κB pathway possesses a key role in LPS-evoked cell injury and the pathogenesis of pulpitis.[26–28] Having established the regulation of TLR4 by the NUTM2A-AS1/miR-769–5p axis, we also asked whether the NUTM2A-AS1/miR-769–5p axis can regulate this pathway. In HDPCs, LPS led to increased levels of MYD88, p-P65 and p-IκBα (Fig. 6C), demonstrating that LPS can activate the MYD88/NF-κB pathway. Intriguingly, NUTM2A-AS1 depletion blocked the MYD88/NF-κB pathway, as presented by the reduction of MYD88, p-P65 and p-IκBα levels in LPS-exposed HDPCs (Fig. 6C). Furthermore, reduced expression of miR-769–5p or restoration of TLR4 markedly abolished the blockage by NUTM2A-AS1 depletion (Fig. 6C). Taken together, these findings suggest that the NUTM2A-AS1/miR-769–5p axis can modulate the TLR4/MYD88/NF-κB pathway in LPS-exposed HDPCs.

Discussion

Recent advances have led to a better understanding of the implication of lncRNAs and their ceRNA activity in human diseases, including pulpitis.[8,10,11] Consistent with a recent document,[12] our results demonstrated that NUTM2A-AS1 is overexpressed in LPS-exposed HDPCs and its depletion can relieve HDPC damage evoked by LPS. Moreover, we show the upregulation of NUTM2A-AS1 in pulpitis tissues, implying its relevance to the pathogenesis of pulpitis. More importantly, we unveil a novel ceRNA crosstalk mediated by NUTM2A-AS1 in the regulation of LPS-evoked damage in HDPCs.

NUTM2A-AS1 has established an oncogenic role in lung adenocarcinoma and gastric cancer.[29,30] Functional enrichment analysis also shows that NUTM2A-AS1 may be involved in the ceRNA network in lymphoma and hepatocellular carcinoma.[31,32] Our findings confirm that NUTM2A-AS1 is mainly present in the cytoplasm of HDPCs, which provides the possibility for the direct relationship between NUTM2A-AS1 and miRNAs, because miRNAs are known to be present in the cytoplasm in the RISCs.[24] Wang et al. ascertain that NUTM2A-AS1 can modulate LPS-evoked inflammation in HDPCs by sponging let-7c-5p.[12]

Numerous reports have uncovered the conflicting roles of miR-769–5p in human tumorigenesis.[33–36] The

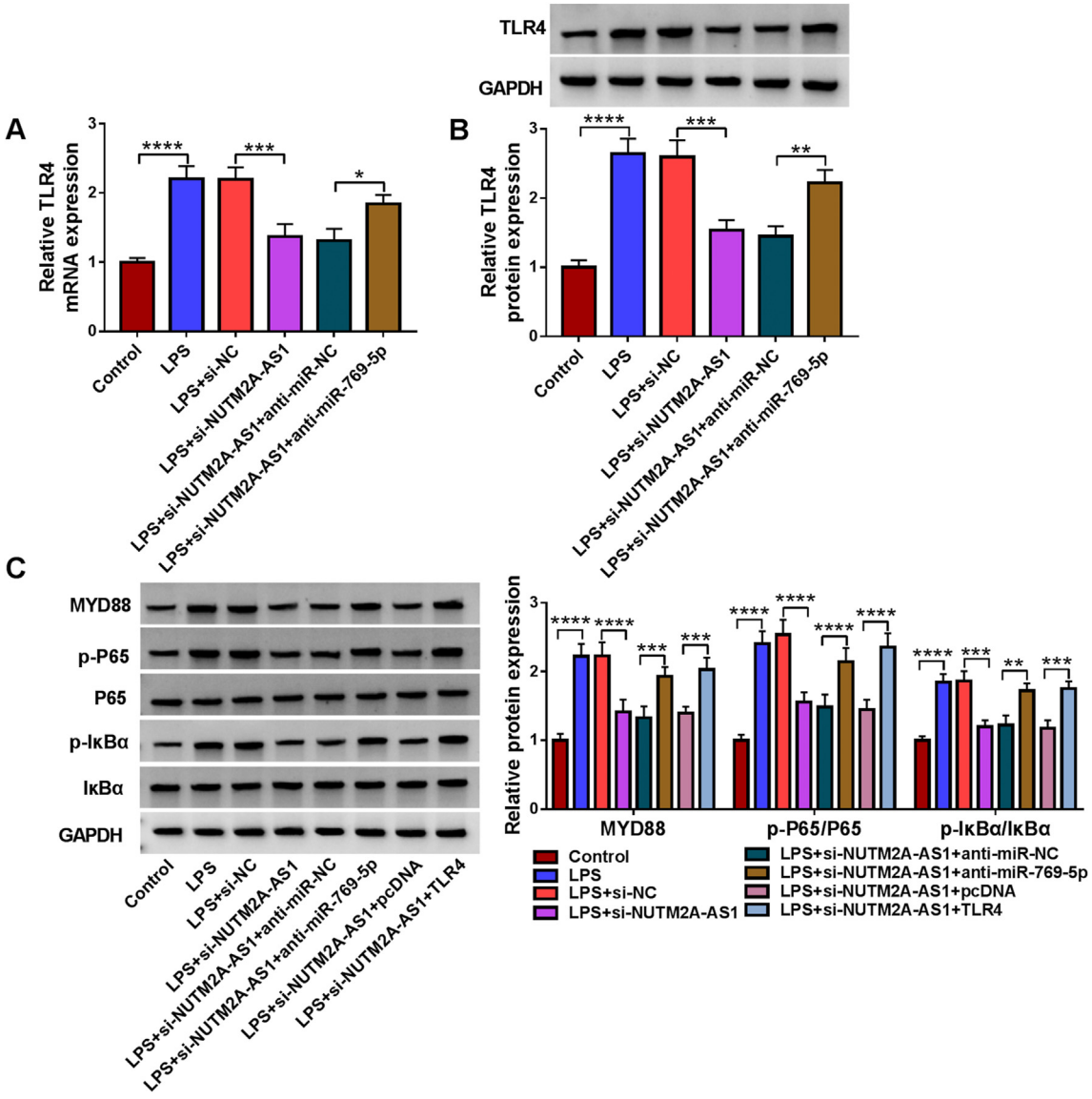

Figure 6 NUTM2A-AS1 regulates the TLR4/MYD88/NF-κB pathway via miR-769-5p. (A and B) HDPCs were transfected with or without si-NUTM2A-AS1+anti-miR-761–5p, si-NUTM2A-AS1+anti-miR-NC, si-NC, or si-NUTM2A-AS1 before LPS or Control exposure and checked for TLR4 mRNA level by qRT-PCR and TLR4 protein level by Western blot. (C) HDPCs were transfected with or without si-NUTM2A-AS1+anti-miR-761–5p, si-NUTM2A-AS1+anti-miR-NC, si-NUTM2A-AS1+a TLR4 expression plasmid, si-NUTM2A-AS1+pcDNA, si-NC, or si-NUTM2A-AS1 before LPS or Control exposure and checked for MYD88, P65, p-P65, IκBα, p-IκBα and GAPDH levels by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

incompatible conclusions may dependent, at least partially, on the different types of tumor in these reports, where miR-769–5p exerts an anti-tumor function in bladder cancer[35] and oral squamous cell carcinoma[36] and enhance the development of gastric cancer[33] and glioma.[34] Moreover, miR-769–5p is present at low levels in gingival tissues of patients with chronic periodontitis and its upregulation can protect human periodontal ligament cells from LPS-triggered cytotoxicity.[17] Here, we first point to the underexpression of miR-769–5p in pulpitis tissues. Furthermore, we first demonstrate that NUTM2A-AS1 depletion attenuates LPS-evoked HDPC damage at least in part by directly binding to miR-769–5p.

TLR4, activated by Gram-negative bacteria and LPS, has been unveiled to possess crucial activity in pulpitis pathogenesis and trigeminal ganglion neurons for dental pain.[21,37,38] Melatonin is reported to mitigate inflammation of acute pulpitis in the DPCs by suppressing TLR4.[39] The TLR4/MYD88/NF-κB pathway, an important inflammation-related signaling pathway,[40,41] has been implicated in LPS-evoked cytotoxicity in HDPCs and pulpitis pathogenesis.[28] Here we demonstrate, for the first time, that TLR4 is a direct target and a functionally downstream effector of miR-769–5p in modulating LPS-evoked HDPC damage. Furthermore, we first establish that NUTM2A-AS1 regulates the TLR4/MYD88/NF-κB pathway through functioning as a

ceRNA for miR-769—5p. Wang and colleagues ascertain that NUTM2A-AS1 operates as a ceRNA for let-7c-5p to modulate LPS-triggered damage in HDPCs by post-transcriptionally regulating HMGB1 expression.[12] The NUTM2A-AS1/miR-769—5p/TLR4 and NUTM2A-AS1/let-7c-5p/HMGB1 axes may be two paralleled or interactional ceRNA networks in LPS-exposed HDPCs. Previous work indicates that the TLR4/MYD88/NF-κB pathway participates in the inflammatory response process via the NLRP3 inflammasome.[42,43] The mechanisms underlying the regulation of the NUTM2A-AS1/miR-769—5p/TLR4/MYD88/NF-κB pathway in HDPC damage evoked by LPS will be further explored in future work. The p38/STAT1 signaling pathway is associated with cell inflammatory response under some conditions.[44—46] In macrophages, the LPS-p38-STAT1 pathway is reported to be indispensable for the LPS-induced upregulation of lncRNA Mirt2.[47] However, no studies showed the mechanism by which LPS upregulates NUTM2A-AS1. Future work will explore whether the p38/STAT1 signaling is involved in LPS-induced upregulation of NUTM2A-AS1 in HDPCs.

Collectively, we show that NUTM2A-AS1 regulates LPS-evoked damage in HDPCs at least in part through the miR-769—5p/TLR4/MYD88/NF-κB pathway. This provides evidence for the idea that NUTM2A-AS1 is an important player in pulpitis.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2022.05.010.

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