ORIGINAL RESEARCH

4cRNA NEAT1 Sponge Adsorption of miR-378 Modulates Activity of Lipopolysaccharide-treated Articular Chondrocytes and Influences the Pathological Development of Osteoarthritis

Xinting Wang, MD; Yue Hu, MD; Chenglong Fang, MD; Chongtian Zhu

ABSTRACT

Context • Osteoarthritis (OA) is a chronic joint disease that can eventually lead to degeneration, fibrosis, fractures, and defects of the articular cartilage. Long non-coding RNA (lncRNA) is a key substance in many processes, such as epigenetic regulation and cell-cycle and cell-differentiation modulation, and its relationship with OA has been repeatedly verified.

Objective • The study intended to clarify the influence of lncRNA nuclear enriched abundant transcript 1 (NEAT1), lncRNA NEAT1, on lipopolysaccharide (LPS)-induced OA chondrocytes through sponge adsorption of microRNA-378 (miR-378) and to provide novel insights into future diagnosis and treatment of OA.

Design • The research team performed an animal study.

Setting • The study took place in the Department of Rehabilitation Medicine at Linyi People's Hospital in Linyi, Shangdong, China.

Animals • The study's animals were 10 Sprague Dawley (SD) rats, 3-5 days old and 10-15 g in weight, of the specific-pathogen-free (SPF) grade.

Intervention • The rat chondrocytes for the positive control group (the model group) were treated with 500 ng/mL of LPS to induce OA. Chondrocytes treated with the same amount of normal saline were used as the negative control group. The chondrocytes of the LPS-induced rats were into six groups: (1) a positive control group transfected with NEAT1-interfering RNA, the sh-NEAT1 group; (2) a negative control group not transfected with NEAT1-interfering RNA, the NEAT1 empty vector (NC-NEAT1) group; (3) an intervention group co-transfected with NEAT1 interfering RNA and the miR-378 inhibitor sequence (Inh-miR-378 the sh-NEAT1+ Inh-miR-378 group; (4) a negative control group transfected with NEAT1 interfering RNA but not transfected with the miR-378 inhibitor sequence, the sh-NEAT1+ miR-378 negative control (NC-miR-378) group; (5) a negative control group transfected with the miR-378 inhibitor sequence but not transfected with NEAT1 interfering RNA, the NEAT1 empty vector (NC-NEAT1) + Inh-miR-378 group; (6) a negative control group not transfected with either NEAT1 interfering RNA or the miR-378 inhibitor sequence, the NC-NEAT1 + NC-miR-378 group.

Outcome Measures • An OA-chondrocyte model was induced by LPS and measurements of NEAT1 and miR-378 expression were made by real-time quantitative reverse transcription (qRT)- polymerase chain reaction (PCR). Then, small NEAT1-interfering RNA (sh-NEAT1), empty vector NEAT1 (NC-NEAT1), inhibitor-sequence-miR-378 (Inh-miR-378), and negative-control-miR-378 (NC-miR-378) were transfected into cells, and cell viability and apoptosis rate were measured. Finally, the study verified the relationship between NEAT1 and miR-378.

Results • Compared to the control group, NEAT1 was significantly elevated in the model group, and its miR-378 was significantly decreased. Silencing NEAT1 can enhance OA-chondrocyte activity and decrease apoptosis. When NEAT1 and miR-378 were inhibited together, as shown fort the NC-NEAT1 + NC-miR-378 group, NEAT1 expression, as well as the multiplication and apoptosis ability of the OA-model cells, were the same as those of cells transfected with an empty vector, the NC-NEAT1 group. Also, the NEAT1 + NC-miR-378 group's cell activity was lower than that of the sh-NEAT1+NC-miR-378 group but higher than that of the NC-NEAT1 + Inh-miR-378 group. Finally, higher fluorescence activity occurred for NEAT1-mutant type (MUT) transfected with Inh-miR-378.

Conclusions • NEAT1, which is highly expressed in OA, mediates LPS-induced OA-chondrocyte activity through sponge adsorption of miR-378. (*Altern Ther Health Med.* 2022;28(6):103-111)

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Osteoarthritis (OA) is a chronic joint disease with articular-cartilage damage as its main characteristic. It's also one of the most common types of joint disease and can eventually lead to degeneration, fibrosis, fractures, and defects of the articular cartilage.[1] The most typical clinical presentations of OA are joint pain and stiffness, accompanied by varying degrees of limitation of motion.[2]

One survey in 2021 indicated that nearly 50% of people over the age of 65 worldwide suffer from OA, and its incidence is on the rise year by year because of global aging.[3] In addition, Wang et al found that OA shows a trend toward prevalence in younger people, the harm from which deserves more attention from clinics and patients.[4] Currently, the specific pathogenesis of OA hasn't been fully clarified.

Treatments vary, depending on the specific type of OA as well as on the degree of disease development.[5] For end-stage OA, only surgery, such as an osteotomy or joint replacement, can achieve a therapeutic purpose.[6]

In OA's pathogenic process, oxidative stress caused by overexpression of inflammatory mediators can lead to an increase in catabolic enzymes, degradation of the extracellular matrix, and chondrocyte apoptosis, thus promoting the overall progression of OA.[7] Therefore, future breakthroughs in the prevention and treatment of OA may lie in ensuring the functional integrity of cartilage and chondrocytes.

With the improvements in molecular research in recent years, the attempts to achieve a breakthrough in clinical diagnosis and treatment of OA has focused on human DNA inheritance.[8] At present, long non-coding RNA (lncRNA), which currently is a leading topic for research in cell biology, has been well documented to exert obvious effects on chondrocytes.[9-11]

LncRNA is a key substance in many processes, such as epigenetic regulation and cell-cycle and cell-differentiation modulation, and its relationship with OA has been repeatedly verified. LncRNA nuclear enriched abundant transcript 1 (NEAT1), lncRNA NEAT1, is a kind of lncRNA transcribed from multiple endocrine-tumor loci and was first discovered in 2015 to have abnormal expression in multiple sclerosis and some tumor diseases.[12]

NEAT1 can alleviate myocardial-cell damage in a state of peroxidation,[13] showing that it may have an influence on the functional stability of normal cells. One study has confirmed that NEAT1 ameliorates LPS-induced inflammation in MG63 cells by activating autophagy and suppressing the NLRP3 inflammasome,[14] and another study has shown that it can regulate the release of inflammatory factors during hepatitis progression,[15] which suggests that NEAT1 may be strongly associated with the progression of an inflammatory response. The inflammatory response is one of the most important pathogenic factors in OA and has been repeatedly confirmed to have abnormal expression in OA in previous studies.[16,17]

In recent years, a number of studies have found that NEAT1 plays a regulatory role in gene expression via adsorbing microRNA (miR). For example, Sun et al found that NEAT1 could block the development of Parkinson's disease through sponge adsorption of miR-1301-3p.[18] Two recent studies have shown that NEAT1 can significantly suppress the functional development of chondrocytes and is closely related to the remodeling of the skeletal system.[19,20]

Based on the information above, the current research team preliminarily speculated that NEAT1 is closely related to the occurrence and development of OA. Zhao et al found that NEAT1 can cause cardiomyocyte injury through miR-378,[21] which is considered to be a typical miR in early-stage OA.[22]

Zhao et al. found that the expression of miR-378 increased after transfection of a lentivirus that interfered with NEAT1 expression into cardiomyocytes, indicating a negative regulatory relationship between the two.[23] In addition, two studies have found that both NEAT1 and miR-378 can significantly regulate inflammatory responses,[24,25] and two other studies have confirmed the close connection of the two with OA.[26,27]

All preceding studies have revealed the potential impact of NEAT1 on OA, but no relevant research has confirmed the research team's views at present. Therefore, the current study intended to clarify the influence of lncRNA NEAT1 on lipopolysaccharide (LPS)-induced OA chondrocytes through sponge adsorption of miR-378 and to provide novel insights into future diagnosis and treatment of OA.

METHODS

Animals

The study's animals were 10 Sprague Dawley (SD) rats, 3-5 days old and 10-15 g in weight, of the specific-pathogen-free (SPF) grade that were supplied by Guangzhou Ruige Biotechnology (Guangdong Guangzhou, China) SYXK 2021-0259. They were caged at three rats per cage. All animals were adaptively reared in an environment with free light, food, water, and 12h day/night alternation.

Procedures

The research team built an LPS-induced OA cell model to study the impacts of NEAT1 and miR-378 on OA.

Chondrocyte extraction. The rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium and then killed. Their bilateral femoral heads and knee joints, retaining the surrounding muscle tissue, were obtained under aseptic operation and then immersed in phosphate-buffered saline (PBS) comprising 1% penicillin/streptomycin.

Following removal of the pericartilage tissue, the specimens were cut into small pieces of about one mm^3 and digested with trypsin for 30 min. Subsequently, they were cultivated with 2 mg/mL of type II collagenase for 4 hours at 37°C, centrifuged and resuspended, and then immersed in Dulbecco's modified eagle medium (DMEM) for a constant-temperature culture. The fluid was changed once every other day, and a subculture was conducted when the cell growth reached 80% to 90%. The third-generation chondrocytes were used for follow-up experiments.

OA-cell model. Induction of an OA-cell model using LPS is a mature technology that has been repeatedly verified.[28,29] Referring to Guo et al's research,[30] the rat

Table 1. Primer Sequences

	F (5'-3')	R (5'-3')
NEAT1	TGGCTAGCTCAGGGCTTCAG	TCTCCTTGCCAAGCTTCCTTC
Co II	AGAACAGCATTGCCTACCT	ATGGTCTTGCCCCACTTAC
GAPDH	AAGGCTGTGGGCAAGGTCATC	GCGTCAAAGGTGGAGGAGTGG
MMP-9	CGGTTTGGTAACGCAGATCG	GCGTGATGAGTTCCTCCCT
β-actin	CTCGATCGGGCCTCGCTGTT	GCAGTCACGTACACCGTG
miR-378	GGGACTGGACTTGGAGTCA	GTGCGTGTCGTGGAGTCG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Abbreviations: NEAT1, nuclear enriched abundant transcript 1; Co II, collagen type II; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP-9, matrix metalloproteinase-9; β-actin, beta actin; miR-378, microRNA-378; U6, PCR primer U6 gene.

chondrocytes for the positive control group (the model group) were seeded onto 24-well plates (1×10^5/well) and treated with 500 ng/mL of LPS after the growth reached 80% to induce OA. Chondrocytes treated with the same amount of normal saline were used as the negative control group.

After building the OA-cell model, the research team performed a real-time quantitative reverse transcription (qRT)-polymerase chain reaction (PCR), qRT-PCR, to detect the mRNA expressions of collagen type II (Co II) and matrix metalloproteinase-9 (MMP-9) in the cells to verify the modeling results of the cell model, two of the outcome measures for the study. These measurements were performed to determine if the OA cell model, induced by LPS, was successfully established.

QRT-PCR experiment. The treated cells were subjected to inoculation into a 96-well plate and then cultured for 24 h at 37°C, with subsequent total RNA isolation with Trizol (ThermoFisher, Waltham, Massachusetts, USA). After verifying the purity, a reverse transcription into complementary DNA (cDNA) was performed, followed by an amplification reaction using the PCR kit.

Primer sequences were constructed by Beijing Tsingke Biotechnology (Table). NEAT1 expression relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and miR-378 expression relative to U6 were calculated using the $2^{-\Delta\Delta CT}$ formula.

Cell-transfection groups. Using a Lipofectamine 2000 transfection kit (ThermoFisher, Waltham, Massachusetts, USA), the following sequences were transfected to create groups from the model group's LPS-treated chondrocytes: (1) to create a positive control group transfected with NEAT1-interfering RNA, the sh-NEAT1 group; (2) to create a negative control group not transfected with NEAT1-interfering RNA, the NEAT1 empty vector (NC-NEAT1) group; (3) to create an intervention group co-transfected with NEAT1 interfering RNA and the miR-378 inhibitor sequence (Inh-miR-378), the sh-NEAT1+ Inh-miR-378 group; (4) to create a negative control group transfected with NEAT1 interfering RNA but not transfected with the miR-378 inhibitor sequence, the sh-NEAT1+ miR-378 negative control (NC-miR-378) group; (5) to create a negative control group transfected with the miR-378 inhibitor sequence but not transfected with NEAT1 interfering RNA, the NEAT1 empty vector (NC-NEAT1) + Inh-miR-378 group; (6) to create a negative control group not transfected with either NEAT1 interfering RNA or the miR-378 inhibitor sequence, the NC-NEAT1 + NC-miR-378 group.

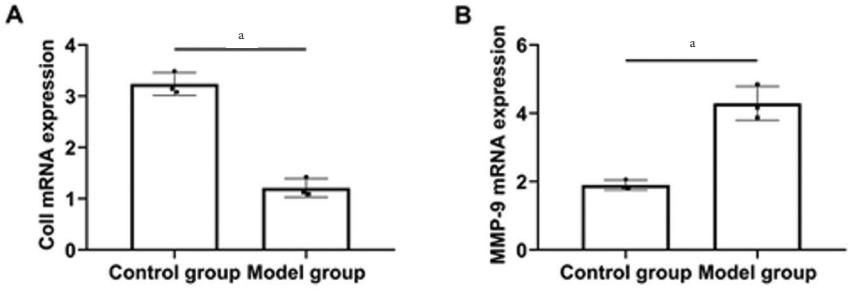
The expression levels of NEAT1 in the sh-NEAT1 and NC-NEAT1 groups were used to verify the success rate of transfection.

Cell-multiplication-ability test. The treated cells were inoculated into 96-well plates, with five multiple wells set in each group. At 0, 24, 48, and 72 h of culture, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed (20 μL) was added to one well for 4h of incubation. Then, the supernatant was discarded, and 200 μLs of dimethyl sulfoxide were added. The absorbance value was detected by microplate reader at a 490-nm wavelength, and the cell-growth curve was drawn. In addition, the treated cells were inoculated into six-well plates at 300/well and immersed in a medium containing 50 μmol/L of chlorogenic acid (CGA) for two weeks of culture and stained with crystal violet after methanol immobilization, to microscopically count the numbers of cell formed.

Apoptosis-rate detection. With the use of a PI-Annexin V apoptosis detection kit (ThermoFisher, Waltham, Massachusetts, USA), the cell-apoptosis rate was detected. After trypsin digestion, cells were suspended with 300 μL of binding buffer and 5 μL of Annexin V-FITC (ThermoFisher, Waltham, Massachusetts, USA was added for 15 min of light-tight incubation at ambient temperature. Following the addition of 5 μL of propidium iodide (PI), the apoptosis rate was detected using flow cytometry (FCM).

Apoptosis-related protein detection. The total proteins of radioimmunoprecipitation assay (RIPA)-lysed cells were collected for quantification. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were shifted to a polyvinylidene difluoride (PVDF) membrane and then closed with 5% defatted milk and I antibodies. After incubation at 4°C overnight, the membrane subjected to three washes with (hydroxymethyl) aminomethane (Tris)-buffered saline with 0.1% Tween 20 detergent (TBST), the addition of the Co II antibody, and enhanced chemiluminescence (ECL) development, for gray-value analysis of protein bands.

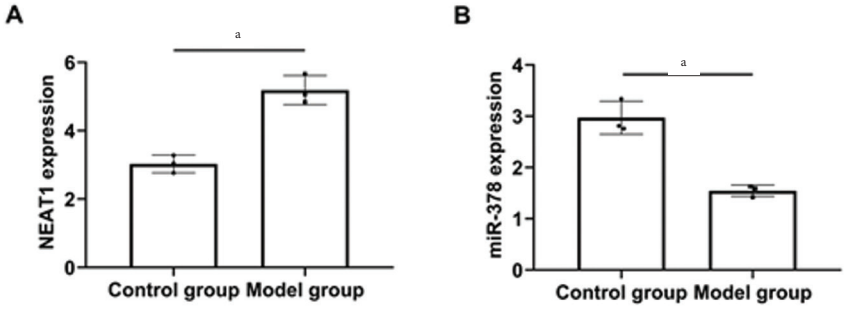
Figure 1. Modeling Results for the Model Group With LPS-induced OA and the Control Group. Figure 1A compares the Co II mRNA expression, and Figure 1B compares the MMP-9 mRNA expression.



[a]$P < .05$, indicating significantly lower Co II mRNA expression and significantly higher MMP-9 mRNA expression for the model group compared to the expressions for the control group

Abbreviations: Co II, collagen type II; MMP-9, matrix metalloproteinase-9.

Figure 2. Expression Levels of NEAT1 and miR-378 in OA Cells for the Model Group With LPS-induced OA and the Control Group. Figure 2A compares the NEAT1 expression, and Figure 2B compares the miR-378 expression.



[a]$P < .05$, indicating a significantly higher NEAT1 and a significantly lower miR-378 for the model group compared to the NEAT1 and miR-378 for the control group

Abbreviations: miR-378, microRNA-378; NEAT1, nuclear enriched abundant transcript 1; OA, osteoarthritis.

Double luciferase reporter (DLR) gene assay. An online target-gene database was used to predict the targeting relationship between NEAT1 and miR-378. Then NEAT1 wild-type (NEAT1-WT) and mutant (NEAT1-MUT) luciferase reporter vectors were constructed and were then co-transfected with Inh-miR-378 and NC-miR-378 into chondrocytes treated with LPS, respectively. The enzyme activity was detected using the DLR gene assay kit (MedChemExpress, New Jersey, North America, USA).

Outcome Measures

mRNA expressions of Co II. Co II is one of the markers of chondrocytes, with low expression in chondrocytes and high expression in normal cells. Therefore, we detected Co II mRNA to judge the success of chondrocyte isolation. The CoII mRNA in the model group was lower than that in the control group, indicating that CoII was lowly expressed.

MMP-9. Similarly, MMP-9 is one of the markers of chondrocytes, with low expression in chondrocytes and high expression in normal cells. Therefore, we detected MMP-9 mRNA to judge the success of chondrocyte isolation. The MMP-9 mRNA in the model group was lower than that in the control group, indicating that MMP-9 was lowly expressed.

NEAT1 expression. The role of NEAT1 in osteoarthritis was determined by detecting the expression of NEAT1.

miR-378 expression. The role of miR-378 in osteoarthritis was determined by detecting the expression of NEAT1.

Cell viability. The effects of NEAT1 and miR-378 on the proliferation of osteoarthritis cells were determined by MTT assay.

Apoptosis rate and apoptosis-related proteins. The effects of NEAT1 and miR-378 on the apoptosis of osteoarthritis cells were determined by MTT assay.

Statistical Analysis

All experiments in this study were run in triplicate. The results, recorded as means ± standard deviations (SDs), were statistically analyzed using SPSS22.0 software (IBM, Armonk, New York, USA), compared by independent samples *t* test between groups, and verified by one-way analysis of variance (ANOVA) and Bonferroni post-hoc test among the groups, with $P < .05$ as the significance level.

RESULTS

Modeling Results

The qRT-PCR showed that the level of Co II mRNA in the model group's cells was 1.21 ± 0.18, which was significantly lower than that of the control group, 3.24 ± 0.22, at $P < .05$ (Figure 1A), while the level of MMP-9 mRNA was 4.29 ± 0.50, which was significantly higher than that of the control group, 1.90 ± 0.14, with $P < .05$ (Figure 1B). These findings indicate that the OA cell model, induced by LPS, was successfully established.

NEAT1 and miR-378

NEAT1 was 5.19 ± 0.42 in the model group and 3.03 ± 0.26 in the control group, with $P < .001$ indicating a statistically significant difference (Figure 2A). The miR-378 was significantly lower in the model group, at 1.55 ± 0.11, compared to the control group at 2.97 ± 0.32, with $P < .001$ (Figure 2B).

LPS-induced OA-cell Multiplication

The transfection success rate was first verified by detecting the NEATI1 expression in the sh-NEAT1 and NC-NEAT1 groups. NEAT1 was lower in the sh-NEAT1 group compared with that of the NC-NEAT1 group, at 1.55 ± 0.11 and 2.97 ± 0.32, respectively, with $P < .05$ (Figure 3A), confirming a successful transfection.

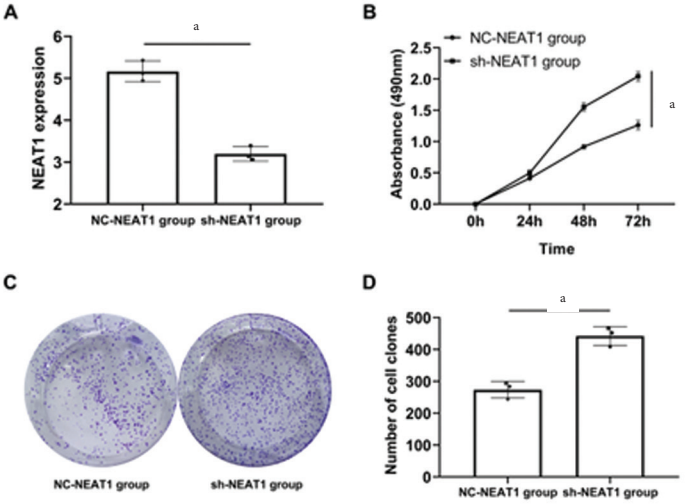
Subsequently, the MTT assay found that the cell-optical densities of the sh-NEAT1 group at 0, 24, 48, and 72 h, were 0, 0.50 ± 0.05, 1.55 ± 0.07, and 2.04 ± 0.08, respectively, while those of the NC-NEAT1 group were 0, 0.41 ± 0.04, 0.92 ± 0.03, and 1.26 ± 0.08, respectively. These findings suggest a higher cell-multiplication ability for the sh-NEAT1 group than for the NC-NEAT1 group, with $P < .05$ (Figure 3B). This indicated that the proliferation ability of cells increased after inhibiting the expression of NEAT1.

In the clone formation experiment (Figure 3C), the number of cell clones in the sh-NEAT1 group was found to be higher than that of the NC-NEAT1 group, at 442.33 ± 29.70 and 442.33 ± 29.70, respectively, with $P < .05$ (Figure 3D). This indicated that the cloning ability of cells increased after inhibiting the expression of NEAT1.

LPS-induced OA-cell Apoptosis

The FCM (Figure 4A) determined that the sh-NEAT1 group had a lower apoptosis rate than that of the NC-NEAT1 group, at 4.26 ± 1.17 % and 10.67 ± 0.90 %, respectively, with $P < .05$ (Figure 4B). The Western blotting (Figure 4C) showed that the B-cell lymphoma-2 (Bcl-2)-associated X apoptosis regulator (Bax) and the Bcl-2 protein levels were 0.23 ± 0.03 and 1.07 ± 0.09, respectively, in the sh-NEAT1 group, while they were 0.67 ± 0.06 and 0.62 ± 0.06, respectively, in the NC-NEAT1 group (Figure 4D). The Bax was lower while Bcl-2 was higher in the sh-NEAT1 group than the Bax and Bcl-2 in the NC-NEAT1 group, with $P < .05$.

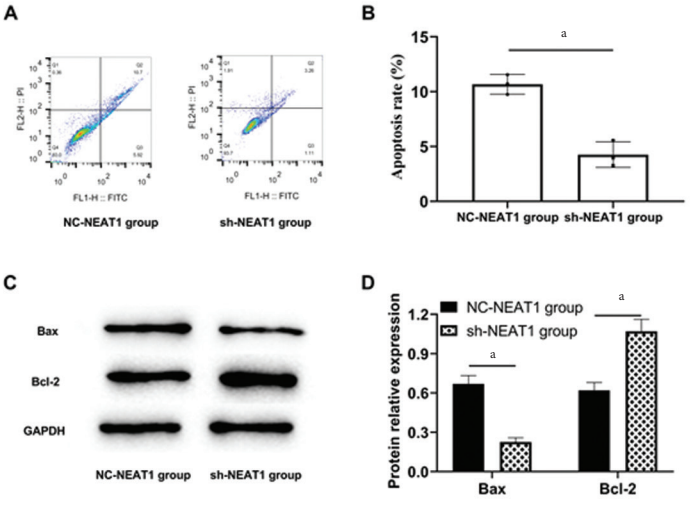
Figure 3. Impacts of NEAT1 on LPS-induced, OA-cell Multiplication. Figure 3A shows the results of the detection of the expression level of NEAT1 in the sh-NEAT1 and NC-NEAT1 groups used to verify the success rate of transfection; Figure 3B shows the results of the MTT assay that was used to detect cell multiplication; Figure 3C shows the experimental results of cell-clone formation; and Figure 3D shows the number of cell clones.



[a]$P < .05$, indicating a statistical difference between the NC-NEAT1 and sh-NEAT1 groups

Abbreviations: LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NC-NEAT1, empty vector NEAT1; NEAT1, nuclear enriched abundant transcript 1; OA, osteoarthritis; sh-NEAT1, small NEAT1-interfering RNA.

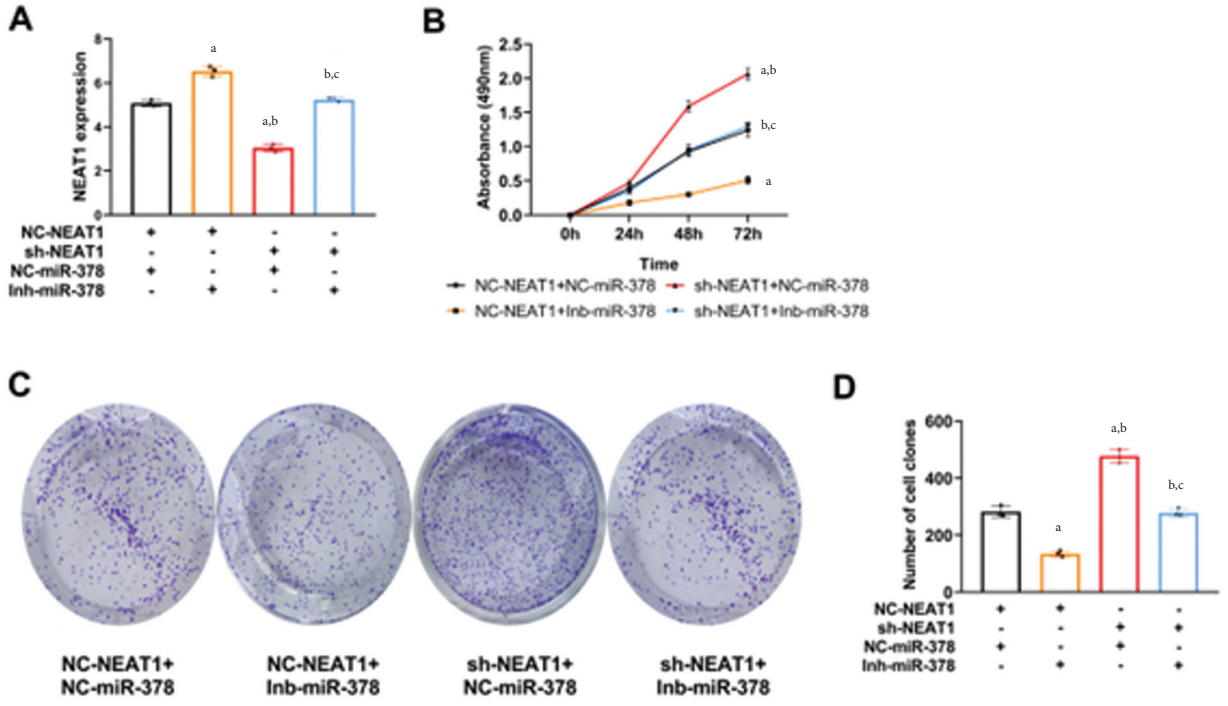
Figure 4. Impacts of NEAT1 on LPS-induced OA-cell Apoptosis. Figure 4A shows the experimental results for the flow cytometry (FCM); Figure 4B shows the cell-apoptosis rate; Figure 4C shows the results for detection of the apoptosis-related proteins; and Figure 4D shows the relative expression of the apoptosis-related proteins.



[a]$P < .05$, indicating a statistical difference between the NC-NEAT1 and sh-NEAT1 groups

Abbreviations: Bax, BCL2 associated X, apoptosis regulator; Bcl-2, B-cell lymphoma-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; NC-NEAT1, empty vector NEAT1; NEAT1, nuclear enriched abundant transcript 1; OA, osteoarthritis; sh-NEAT1, small NEAT1- interfering RNA.

Figure 5. Impacts of NEAT1 on OA-cell Multiplication Through Sponge Adsorption of miR-378. Figure 5A shows the results of the detection of the expression level of NEAT1 in the sh-NEAT1 and NC-NEAT1 groups used to verify the success rate of transfection; Figure 5B shows the results of the MTT assay that was used to detect cell multiplication; Figure 5C shows the experimental results of cell-clone formation; and Figure 5D shows the number of cell clones. The plus signs under Figures 5A and 5D identify the groups represented.



Note: The black graph is the NC-NEAT1+NC-miR-378 group, the orange graph is the NC-NEAT1+Inb-miR-378 group, the red graph is the sh-NEAT1+NC-miR-378 group, and the blue graph is the sh-NEAT1+ Inb-miR-378 group.

[a]$P < .05$, indicates comparison with NC-NEAT1+NC-miR-378 group
[b]$P < .05$, indicates comparison with NC-NEAT1+Inb-miR-378 group
[c]$P < .05$, indicates comparison with sh-NEAT1+NC-miR-378 group

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Inh-miR-378, inhibitor-sequence-miR-378; miR-378, microRNA-378; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NC-NEAT1, empty vector NEAT1; NC-miR-378, miR-378 negative-control-miR-378; NEAT1, nuclear enriched abundant transcript 1; OA, osteoarthritis; sh-NEAT1, small NEAT1- interfering RNA.

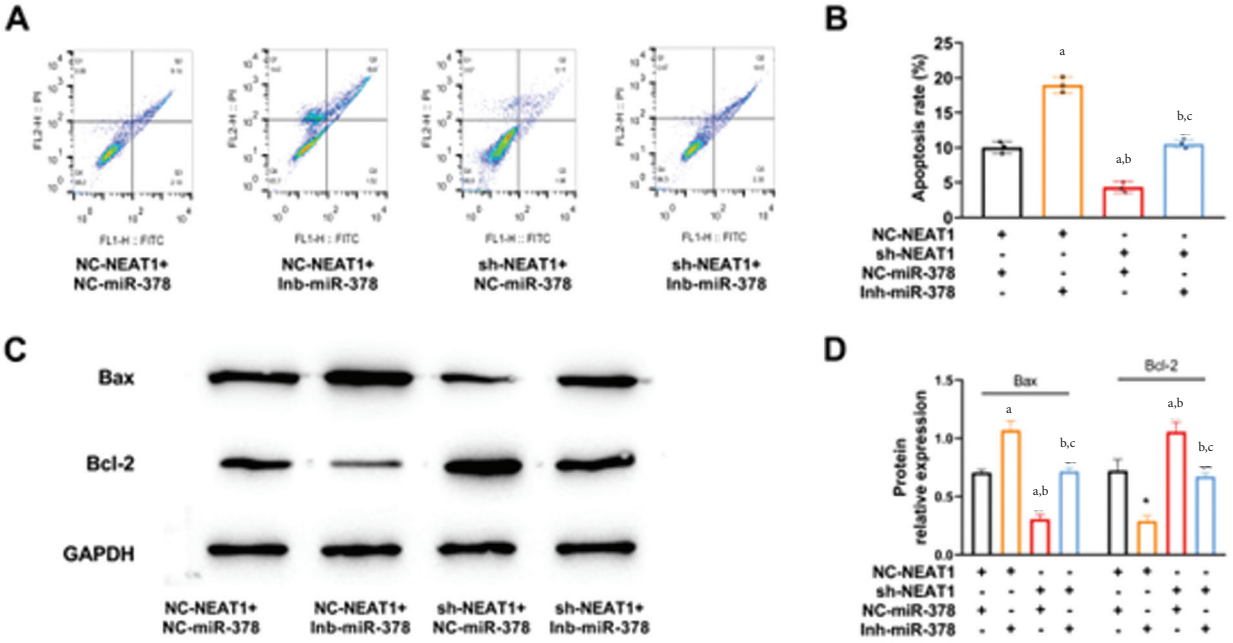
OA-cell Multiplication Through Sponge Adsorption of miR-378

PCR detection showed that the NEAT1 expression in the NC-NEAT1+ NC-miR-378 group was 5.09 ± 0.14, which wasn't significantly different than that of the sh-NEAT1+Inh-miR-378 group, at 5.24 ± 0.09 ($P > .05$). The NEAT1 expression of the sh-NEAT1+ Inh-miR-378 group was lower than that in NC-NEAT1+Inh-miR-378 group, at 6.54 ± 0.22, but higher than that in sh-NEAT1+NC-miR-378 group, at 4.26 ± 1.97, with $P < .05$ (Figure 5A).

The MTT assay revealed that the cell optical density at 72 h of the NC-NEAT1 + NC-miR-378 group was 1.24 ± 0.10, which wasn't significantly different from that of the sh-NEAT1 + Inh-miR-378 group, at 1.30 ± 0.05 ($P > .05$). The density of the sh-NEAT1 + Inh-miR-378 group was significantly lower than that of the sh-NEAT1 + NC-miR-378 group, at 2.06 ± 0.09, and significantly higher than that of the NC-NEAT1 + Inh-miR-378 group, at 0.51 ± 0.06, with $P < .05$ (Figure 5B).

The cell-cloning experiment (Figures 5C and 5D) demonstrated that the number of cell clones among the four groups discussed above was the highest in the sh-NEAT1 + NC-miR-378 group, at 477.67 ± 23.54, and the lowest in the NC-NEAT1 + Inh-miR-378 group, at 134.67 ± 10.50 ($P < .05$). The NC-NEAT1 + NC-miR-378 group and the sh-NEAT1 + Inh-miR-378 group didn't differ significantly in the number of cell clones, with $P > .05$.

Figure 6. Impacts of NEAT1 on OA-cell Apoptotic Capacity Through Sponge Adsorption of miR-378. Figure 6A shows the experimental results for the flow cytometry (FCM); Figure 6B shows the cell-apoptosis rate; Figure 6C shows the results of the detection of apoptosis-related proteins; and Figure 6D show the relative expression of apoptosis-related proteins. The plus signs under Figures 6B and 6D identify the groups represented.



Note: The black graph is the NC-NEAT1+NC-miR-378 group, the orange graph is the NC-NEAT1+Inb-miR-378 group, the red graph is the sh-NEAT1+NC-miR-378 group, and the blue graph is the sh-NEAT1+ Inb-miR-378 group.

[a]$P < .05$, indicates comparison with NC-NEAT1+NC-miR-378 group
[b]$P < .05$, indicates comparison with NC-NEAT1+Inb-miR-378 group
[c]$P < .05$, indicates comparison with sh-NEAT1+NC-miR-378 group

Abbreviations: Bax, BCL2 associated X, apoptosis regulator; Bcl-2, B-cell lymphoma-2; Inh-miR-378, inhibitor-sequence-miR-378; miR-378, microRNA-378; NC-NEAT1, empty vector NEAT1; NC-miR-378, miR-378 negative-control-miR-378; NEAT1, nuclear enriched abundant transcript 1; sh-NEAT1, small NEAT1- interfering RNA.

Apoptotic Capacity Through Sponge Adsorption of miR-378

The FCM (Figure 6A) showed that the apoptosis rate (Figure 6B) of the NC-NEAT1 + NC-miR-378 group was 10.04 ± 0.84%, which wasn't significantly different from that of the sh-NEAT1 + Inh-miR-378 group, at 10.51 ± 0.65% ($P > .05$). Among the four groups, the apoptosis rate was the highest in the NC-NEAT1 + Inh-miR-378 group, at 14.36 ± 9.28, and the lowest in the sh-NEAT1 + NC-miR-378 group, at 3.39 ± 2.02, ($P < .05$).

Similarly, the apoptosis-related protein-detection results (Figures 6C and 6D) showed that the Bax, at 0.71 ± 0.03, and the Bcl-2, at 0.72 ± 0.10, of the NC-NEAT1 + NC-miR-378 group weren't significantly different from the Bax and the Bcl-2 of the sh-NEAT1 + Inh-miR-378 group, at 0.72 ± 0.04 and 0.67 ± 0.03, respectively ($P > .05$).

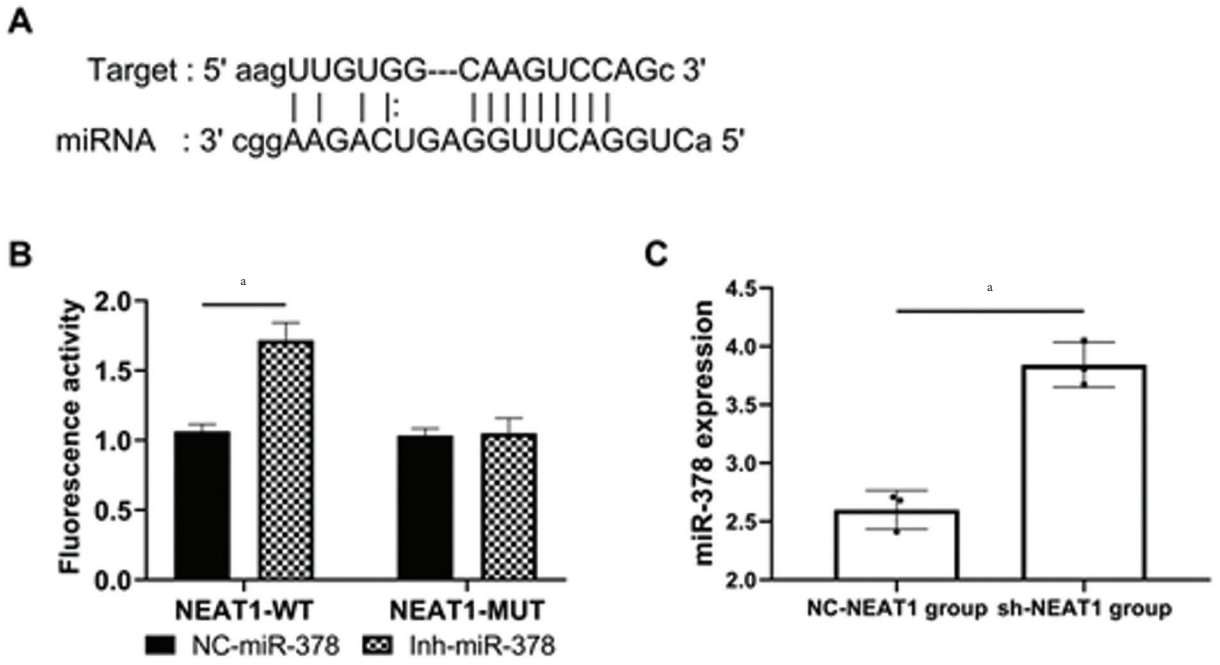
The sh-NEAT1 + Inh-miR-378 group's Bcl-2 protein at 0.67 ± 0.03, was higher than that of the NC-NEAT1 + Inh-miR-378 group (0.29 ± 0.05) and lower than that of the sh-NEAT1 + NC-miR-378 group at 1.06 ± 0.09, while its Bcl-2 protein was higher than that of the NC-NEAT1 + Inh-miR-378 group and lower than that of sh-NEAT1 + NC-miR-378 group (0.72 ±0.10), with $P < .05$.

Relationship Between NEAT1 and miR-378

The upstream target gene of miR-378 contained NEAT1, and NEAT1 and miR-378 had obvious complementary binding loci (Figure 7A). The DLR assay indicated that the intensity of the fluorescence activity of NEAT1-MUT transfected with Inh-miR-378 was 1.72 ± 0.13, which was obviously higher than that of the NEAT1-MUT transfected with NC-miR-378, at 1.05 ± 0.11, with $P < .05$ (Figure 7B), indicating the presence of a targeting relationship between the two.

In addition, by detecting the miR-378 in the sh-NEAT1 group and the NC-NEAT1 group, the study found a significantly higher miR-378 expression in the sh-NEAT1 group than in the NC-NEAT1 group, with $P < .05$ (Figure 7C).

Figure 7. Verification of the Relationship Between NEAT1 and miR-378. Figure 7A shows the upstream target genes of miR-378; Figure 7B shows the results of the double-luciferase reporter (DLR) gene assay; and Figure 7C shows the effects of NEAT1 on miR-378 expression.



[a]$P < .05$ for Figure 7C, indicating a significantly higher miR-378 expression in the sh-NEAT1 group than in the NC-NEAT1 group

Abbreviations: Inh-miR-378, inhibitor-sequence-miR-378; NC-NEAT1, empty vector NEAT1; NC-miR-378, miR-378 negative-control-miR-378; miR-378, microRNA-378; NEAT1, nuclear enriched abundant transcript 1; sh-NEAT1, small NEAT1- interfering RNA.

DISCUSSION

The current study analyzed the impact of NEAT1 sponge adsorption of miR-378 on LPS-induced OA-model cells, which is of great significance for clinical research of OA prevention and treatment. The current study found that NEAT1 expression was markedly higher in the OA-cell model than in normal rat chondrocytes, while miR-378 was lower, preliminarily confirming the close connection of the two with OA, which is consistent with the results of previous studies.[21,22]

To confirm the exact use of NEAT1 in OA, the current research team transfected NEAT1 interfering RNA expression sequences into OA cells and observed the alterations in the cells' biological behavior. The results showed that OA cell multiplication and cloning ability were significantly enhanced after inhibiting NEAT1, while the apoptosis rate and pro-apoptotic protein expression of Bax decreased, suggesting that NEAT1 inhibition can effectively alleviate the accelerated apoptosis process of OA chondrocytes.

To further confirm the mechanism of NEAT1's influence on OA, the current research team co-transfected NEAT1 and miR-378 into OA cells for further observation. The team found that NEAT1 expression in the OA cell model, after simultaneous inhibition of NEAT1 and miR-378, was the same as that of empty vector-transfected cells, and the increase in the NEAT1 level in the NC-NEAT1 + Inh-miR-378 group also preliminarily suggested that NEAT1 might be negatively modulated by miR-378.

Subsequently, results related to biological-behavior detection revealed no differences in cell multiplication, clone formation, apoptosis, or expression of apoptosis-related proteins between the sh-NEAT1 + Inh-miR-378 group and the NC-NEAT1 + NC-miR-378 group, for which the cell activity was lower than that of the sh-NEAT1 + NC-miR-378 group and higher than that of the NC-NEAT1 + Inh-miR-378 group. These results demonstrate that inhibiting miR-378 can further increase the apoptosis process of OA chondrocytes and completely reverse the effects of silencing NEAT1 on the OA cells, suggesting that NEAT1 participates in the occurrence and development of OA through sponge adsorption of miR-378. Based on the above results the current research team holds that a targeted regulatory relationship may exist between NEAT1 and miR-378.

Finally, the current research team screened the upstream target genes of miR-378 through an online website for target-gene prediction and found that NEAT1 was one for miR-378. In addition, through a DLR assay, the current research team noted obviously enhanced fluorescence activity for NEAT1-

MUT after inhibition of miR-378, which confirms the targeted regulation relationship between them. However, miR-378 expression increased after NEAT1 was silenced, indicating a negative regulatory relationship between the two, which was also consistent with the current study's results and with those of Zhao et al[26] and could prove the accuracy of the current studies experimental results.

In the current study, rat chondrocytes were induced with LPS to establish the OA-cell model. However, to determine the exact mechanism of NEAT1, researchers need to obtain human OA-chondrocyte samples for verification. In addition, animal models should be established to observe the influence of NEAT1 on the histopathological manifestations of OA in animals. Furthermore, a more in-depth analysis is warranted to investigate the downstream signal pathways of NEAT1.

Inflammatory factors in the OA cell model weren't detected in the current study. After confirming the successful establishment of the OA-cell model in the current study, the expression of inflammatory factors could be expected naturally. Therefore, these factors weren't described in the current article. Moreover, due to limited experimental conditions, the current study mainly investigated the effects of NEAT1 on the activity of OA-model cells, so its experiments focused on the detection of cell proliferation and apoptosis. The synthesis and metabolic indices of chondrocytes are also key points worthy of in-depth study. The current research team intends to address these shortcomings as soon as possible in follow-up research.

CONCLUSIONS

NEAT1, highly expressed in OA, mediates LPS-induced OA chondrocyte activity via sponge adsorption of miR-378. In the future, the targeted approach of inhibiting NEAT1 expression may become a breakthrough in the treatment of OA.

AUTHORS' DISCLOSURE STATEMENT

The authors declared there is no conflict of interest.

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