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Knocking Down lncRNA HOXA-AS2 Mitigates the Progression of Epilepsy via Regulation of the miR-372-3p/STAT3 Axis

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ABSTRACT

AIM: To explore the potential activity of HOXA cluster antisense RNA 2 (HOXA-AS2), a long non-coding RNA (lncRNA), in epilepsy progression, as well as the mechanisms behind its activity.

MATERIAL and METHODS: Kainic acid (KA) was used to treat rat astroglial CTX-TNA2 cells to establish a cellular model of epilepsy. Reverse transcription-quantitative PCR was conducted to examine the expression levels of HOXA-AS2, microRNA (miR)-372-3p and STAT3. Cell Counting Kit-8, flow cytometry and western blot assays were performed to analyze cell viability and apoptosis. The secretion levels of various inflammatory factors (IL-6, IL-1β and TNF-α) were identified by ELISA. To validate the functional interaction between HOXA-AS2/STAT3 and miR-372-3p, dual-luciferase reporter assay was performed.

RESULTS: The HOXA-AS2 and STAT3 expression levels were notably upregulated, whereas miR-372-3p was downregulated in KA-treated CTX-TNA2 cells. Silencing HOXA-AS2 or overexpressing miR-372-3p inhibited the secretion of inflammatory factors and apoptosis in KA-treated CTX-TNA2 cells. HOXA-AS2 negatively regulated miR-372-3p, and miR-372-3p targeted STAT3 mRNA. Suppression of miR-372-3p or overexpression of STAT3 abrogated the rescue effect of small interfering HOXA-AS2 in KA-treated CTX-TNA2 cells.

CONCLUSION: The current study suggested that targeting HOXA-AS2 could alleviate cellular damages in the epileptic model by regulating the miR-372-3p/STAT3 axis. Therefore, HOXA-AS2 may serve as a potential anti-epilepsy therapeutic target.

KEYWORDS: HOXA cluster antisense RNA 2, microRNA-372-3p, STAT3, Epilepsy

INTRODUCTION

Epilepsy is the second most common neurological disease after brain stroke, affecting >65 million patients worldwide (1). This neurological disorder notably undermines the life quality of patients with epilepsy, whose life span is significantly shorter than that of healthy individuals (2,3). Therefore, developing innovative therapeutic strategies based on drugs or gene therapies is necessary to improve the living conditions of patients with epilepsy. Understanding the molecular mechanisms underlying the pathophysiological progression of epilepsy is key for developing novel therapies. Previous evidence indicates that long non-coding RNA (lncRNA) dysregulation is implicated in the development of epilepsy (9). In a previous study, four dysregulated lncRNAs were identified in peripheral blood samples from healthy individuals and cases of epilepsy, among which HOXA cluster antisense RNA 2 (HOXA-AS2) showed upregulation in male patients compared with male controls (6). Moreover, HOXA-AS2 could regulate the expression of sodium voltage-gated channel α-subunit 3 (14), a recognized gene associated with pediatric epileptic encephalopathy (17). However, the mechanism by which HOXA-AS2 contributes to the progression of epilepsy needs to be clarified.

As a type of non-coding RNA, microRNAs (miRNAs or miRs) are involved in neurodegenerative disorders such as epilepsy by regulating gene expression (1). Among the most studied

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miRNAs, miR-372-3p is a well-recognized factor associated with autophagy and lipid metabolism in cancer (4). According to a recent study, miR-372-3p suppressor was capable of alleviating damages to neural stem cells and astrocytes after ischemia-reperfusion injury (16). However, whether miR-372-3p is implicated in the onset of epilepsy needs to be further investigated.

STAT3, which is a pivotal signal transducer for the JAK/STAT axis, acts as an important regulator of astrocytes following central nervous system injury (7,12). Former studies indicate that upregulation of STAT3 and the downstream effector c-Myc facilitate glial cell growth in the hippocampus (7). The function of STAT3 in the pathogenesis of epilepsy is unclear. Kainic acid (KA) has been widely used to establish *in vitro* cell models of epilepsy (8). The present study used KA to treat rat astroglial CTX-TNA2 cells to establish a cellular model of epilepsy, and the expression pattern and molecular interactions between HOXA-AS2/STAT3 with miR-372-3p were examined.

MATERIAL and METHODS

Cell Culture and Transfection

Rat CTX-TNA2 astroglial cells (cat. no. CVCL_3670) were obtained from Ningbo Mingzhou Biotechnology Co., Ltd. Cells were cultured in DMEM (Beijing Solarbio Science & Technology Co., Ltd.) containing 10% fetal bovine serum, 0.1% penicillin/streptomycin and 0.4% glutamine (Thermo Fisher Scientific, Inc.). CTX-TNA2 cells were treated with 50 μM KA (APExBIQ Technology LLC) for 8 h to establish a cellular model of epilepsy. Small interfering RNA (siRNA) against HOXA-AS2, siRNA negative control (si-NC), miR-372-3p mimic, miR-372-3p suppressor, mimic NC, NC inhibitor, pcDNA empty vector and pcDNA-STAT3 (STAT3-expressing vector, Cat#Rb2343) were purchased from Guangzhou RiboBio Co., Ltd.

Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized to transfect cells with these molecules according to the manufacturer's instructions. After reaching 80% density, the transfection of cells was accomplished using 4 μg plasmid plus 100 nM miR-372-3p mimic/suppressor or the corresponding NC into 6-well plates. Cells were collected at 48 h post-transfection to conduct subsequent analyses.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted from 1 million cells with TRIzol® reagent (Takara Bio, Inc.). After extraction, diethyl pyrocarbonate-treated water was used to dissolve total RNA, while NanoDrop (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) was employed to measure the RNA content. Total RNA (5 μg) was subjected to RT and converted into cDNA using GoScript™ Reverse Transcription System (Promega Corporation). With the aid of SYBR® Premix Ex Taq II kit (Takara Biotechnology Co., Ltd.), the gene levels were analyzed with a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR was conducted under the following thermocycling conditions: 2 min at 95˚C; followed by 30 sec at 95˚C, 30 sec at 60˚C and 60 sec at 72˚C for 40 cycles. The gene levels were analyzed by the $2^{-\Delta\Delta Cq}$ method (6,14), with U6 and GAPDH being used as the endogenous controls. The primers were synthesized by Sangon Biotech Co., Ltd., and their sequences are as follows: GAPDH forward (F), 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse (R), 5'-GGGGTCGTTGATGGCAACA-3'; U6 F, 5'-CTCGCTTCGGCAGCACA-3' and R, 5'-AACGCTTCACGAATTTGCGT-3'; HOXA-AS2 F, 5'-CTGGCTACTCTACGTCCTG-3' and R, 5'-GAGGACATGTGGGAGGTG-3'; miR-372-3p F, 5'-CTCGTGGTTGCTGTTGTGA-3' and R, 5'-GTGCAGGGTCCGAGGT-3'; and STAT3 F, 5'-CAATACCATTGACCTGCCGA-3' and R, 5'-GAGCGACTCAAACTGCCCT-3'.

Cell Counting Kit (CCK)-8 Assay

Cell proliferation was examined via the CCK-8 assay. Initially, seeding of cells was accomplished at a density of $1x10^4$ cells/well into 96-well plates, followed by overnight incubation until achieving cell adherence. After 24, 48 or 72-h cell treatment, 10 μl CCK-8 solution (Abbkine Scientific Co., Ltd.) was added to the cell culture for another 1-h incubation under humid conditions. Next, determination of the absorbance [optical density (OD)] value at 450 nm was accomplished with a Multilabel Plate Reader (EnSpire; PerkinElmer, Inc.).

Analysis of Apoptosis by Flow Cytometry

Assessment of apoptosis was accomplished following the manufacturer's protocols of the Annexin V-FITC/PI kit (Abbkine Scientific Co., Ltd.). In brief, cells were subjected to trypsin digestion, followed by resuspension in binding buffer. The cell suspension (1 ml containing $1x10^6$ cells) was then subjected to a 30-min incubation in the dark after adding 5 μl Annexin V-FITC and 5 μl PI. Upon staining, the cells were subjected to centrifugation and rinsing by adding binding buffer twice, and then resuspended in binding buffer (400 μl). Flow cytometry (Agilent Technologies, Inc.) was utilized to detect the proportion of apoptotic cells, which was calculated by combining the proportion of apoptotic cells in the lower right and upper right quadrants.

ELISA

A sandwich ELISA kit (Shanghai MEILIAN Biotechnology Co. Ltd.) was utilized to detect the levels of inflammatory factors (IL-6 (cat. ml055488), IL-1β (cat. ml028611) and TNF-α (cat. ml061137)) in line with specific protocols. Subsequently, 100 μl cell supernatants were harvested for measuring IL-6, IL-1β and TNF-α levels, and added to the capture-antibody-coated plate. The unbound material was removed by washing, and the sample was then incubated for 1 h using a biotin-labeled detection antibody, followed by streptavidin–HRP reagent. Signals were then developed with chemiluminescent reagents, and OD values were determined using a microplate reader (Guangzhou Bio-Gene Technology Co., Ltd.) at 450 nm. The cytokine levels were analyzed according by standard linear regression.

Dual-Luciferase Reporter Assay

The miR-372-3p–STAT3 mRNA and miR-372-3p–HOXA-AS2 binding sites were predicted with the aid of starBase online resources (https://starbase.sysu.edu.cn/starbase2/). Thereafter, the mutant (MUT) and wild-type (WT) binding sites

were inserted into the pGL3 vector (Promega Corporation) for generating the corresponding luciferase reporters (HOXA-AS2-MUT, HOXA-AS2-WT, STAT3-MUT and STAT3-WT). By using Lipofectamine® 3000 reagent, co-transfection of cells was accomplished using the *Renilla* luciferase (hRlucneo) control plasmid and reporter plasmid with miR-NC or miR-372-3p mimic according to the manufacturer's instructions. At 48 h post-transfection, the relative luciferase activity was examined with a luminescence microplate reader and the Dual-Luciferase® Reporter (DLR™) Assay System (Promega Corporation). As for reporter plasmid, its relative firefly luciferase activity was determined based on *Renilla* luciferase.

Western Blotting

RIPA lysis buffer (Hangzhou Fude Biotechnology Co., Ltd.) was adopted for 15-min cell lysis on ice. After 10-min centrifugation at 12,000 x g at 4°C, the protein contents within the supernatants were quantified with a BCA protein assay kit (Beyotime Institute of Biotechnology). Proteins (10 μg) were separated by SDS-PAGE, followed by transfer onto PVDF membranes (MilliporeSigma). The membranes were blocked for 1 h with skimmed milk (5%), followed by subsequent 12-h incubation at 4°C using a primary antibody. After rinsing with TBS-Tween 20, the membranes were incubated for 1 h at room temperature with a secondary antibody conjugated with HRP (1:5,000; cat. P0615, Beyotime Institute of Biotechnology). Subsequently, an ECL reagent (AmyJet Scientific, Inc.) was utilized to visualize the protein bands. The antibodies utilized in the present study were specific for cleaved caspase 3 (1:1,000; cat. no. AB2827742; Abcam), B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no. AB2783814; Abcam), STAT3 (1:1,000; cat. no. 9139; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. AB37168; Abcam). GAPDH served as a loading control for normalization.

Statistical Analysis

The results are presented as the mean ± SD of three separate replicates. SPSS 21.0 (IBM, Corp.) was employed for data analysis. Inter-group comparisons were conducted with unpaired Student's t-test, while ANOVA followed by post-hoc Tukey's test was employed for pairwise comparisons among several groups. Two-way ANOVA with Tukey's post-hoc test was adopted to compare data from different time points. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Knockdown of HOXA-AS2 Suppresses Apoptosis and Reduces the Production of Inflammatory Factors in Epileptic Cell Models

CTX-TNA2 cells (rat astroglial cells) were treated with 50 M KA for 8 h to establish a cellular model of epilepsy stimulation. To investigate the role of HOXA-AS2 in the astrocglial cells of the epileptic model, its level of expression in KA-induced CTX-TNA2 cells was assessed (KA group). The results showed that KA-treatment significantly upregulated the expression of HOXA-AS2 (Figure 1A), indicating that HOXA-AS2 upregulation may contribute to the onset of epilepsy. To explore the functional engagement of HOXA-AS2, the present study aimed to silence HOXA-AS2 using siRNA. After transfection of si-HOXA-AS2 #1, #2 and #3, it was observed that HOXA-AS2 #1 showed the strongest silencing effect, and was therefore selected for subsequent experiments (Figure 1B). CCK-8 proliferation assay showed that KA treatment could remarkably suppress CTX-TNA2 cell proliferation, whereas the knockout of HOXA-AS2 partially rescued cell proliferation upon KA treatment (Figure 1C). In addition, silencing of HOXA-AS2 also partially reduced the percentage of apoptotic events in KA-treated cells (Figure 1D). Consistently, silencing HOXA-AS2 lowered the expression of cleaved caspase 3 but elevated the level of Bcl-2 after KA treatment (Figure 1E). As depicted in Figure 1F, KA treatment also increased the secretion of inflammatory cytokines (IL-1, TNF-α and IL-6), which was significantly alleviated upon si-HOXA-AS2 transfection. These experimental results suggested that silencing HOXA-AS2 shows protective effects in an epileptic cell model.

lncRNA HOXA-AS2 Targets miR-372-3p

To investigate the downstream effector of HOXA-AS2 in KA-induced astrocyte damage, the miRNA targets of HOXA-AS2 were predicted with the starBase database. miR-372-3p exhibited complementary binding sites to HOXA-AS2 (Figure 2A). To validate their interaction, dual luciferase activity assay was carried out with the use of HOXA-AS2-MUT and HOXA-AS2-WT reporters. These findings suggested that miR-372-3p mimic could significantly reduce the WT reporter's luciferase activity, although it did not influence the MUT reporter (Figure 2B), indicating their functional interaction. Moreover, the KA group exhibited markedly declined expression of miR-372-3p (Figure 2C). To validate miR-372-3p's functionality in the cell model of epilepsy, a miR-372-3p mimic was employed, which could significantly increase the miR-372-3p level (Figure 2D). As revealed by CCK-8 assay, cell proliferation upon KA treatment was also rescued by miR-372-3p mimic transfection (Figure 2E). Moreover, miR-372-3p mimic transfection could also protect CTX-TNA2 cells from KA-induced apoptosis (Figure 2F, G) and changes in apoptotic proteins (Bcl-2 and cleaved caspase 3). As shown in Figure 2H, miR-372-3p mimic also lowered the IL-1β, TNF-α and L-6 expression levels in the KA group. Together, these findings implied that overexpression of miR-372-3p rescues the protective effect of HOXA-AS2 silencing.

HOXA-AS2/miR-372-3p Regulates STAT3 Expression

To detect the downstream target mRNA, the present study employed the online starBase software, which predicted the possible binding sites between the 3' untranslated region of STAT3 mRNA and miR-372-3p. To validate their potential interaction, the present study carried out a dual luciferase activity assay with STAT3-WT and STAT3-MUT reporters. According to the findings, miR-372-3p mimic could significantly reduce the WT reporter's luciferase activity, although was not influential to the MUT reporter (Figure 3A). miR-372-3p inhibitor was used to investigate the association between the expression of STAT3 and miR-372-3p. miR-372-3p inhibitor transfection notably decreased the level of

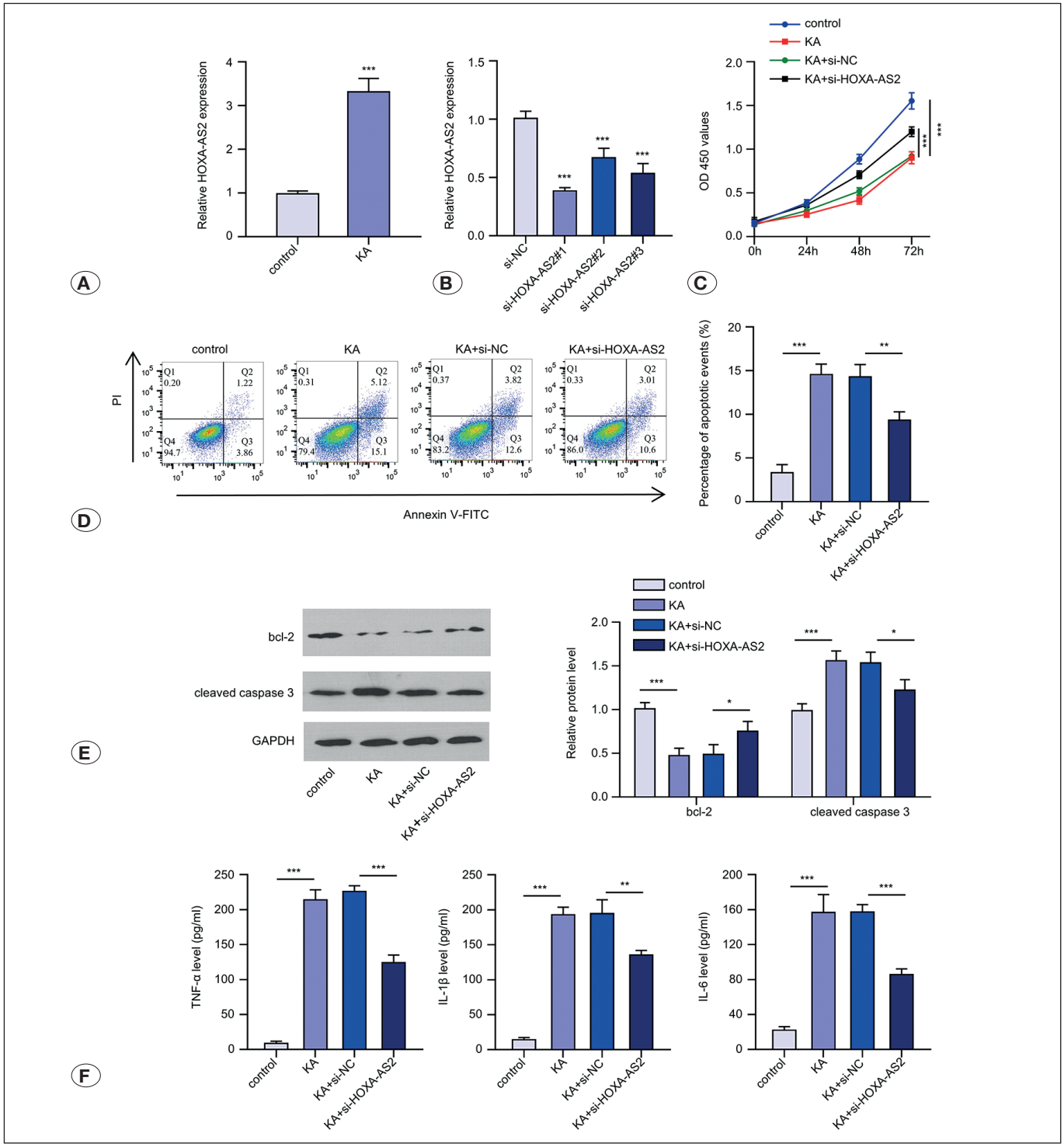

Figure 1: Knockdown of HOXA-AS2 suppresses apoptosis and inflammatory responses in an epileptic cell model. The experiments were carried out in KA-induced CTX-TNA2 cells (KA group). **A)** The RT-qPCR results showed the differences in HOXA-AS2 expression between control and KA-treated cells. **B)** Three HOXA-AS2 siRNAs were transfected into CTX-TNA2 cells, and HOXA-AS2 expression was detected by RT-qPCR. si-HOXA-AS2 #1 was selected for subsequent experiments. **C)** Cell proliferation was measured by Cell Counting Kit-8 assay following si-HOXA-AS2 transfection. **D)** Cell apoptosis was detected upon si-HOXA-AS2 transfection by flow cytometry analysis. **E)** The levels of apoptosis-related proteins were detected by western blotting after transfection of si-HOXA-AS2. **F)** The levels of inflammatory factors in cell culture supernatants were detected by ELISA following si-HOXA-AS2 transfection. *p<0.05, **p<0.01, ***p<0.001. HOXA-AS2, HOXA cluster antisense RNA 2; KA, kainic acid; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering.

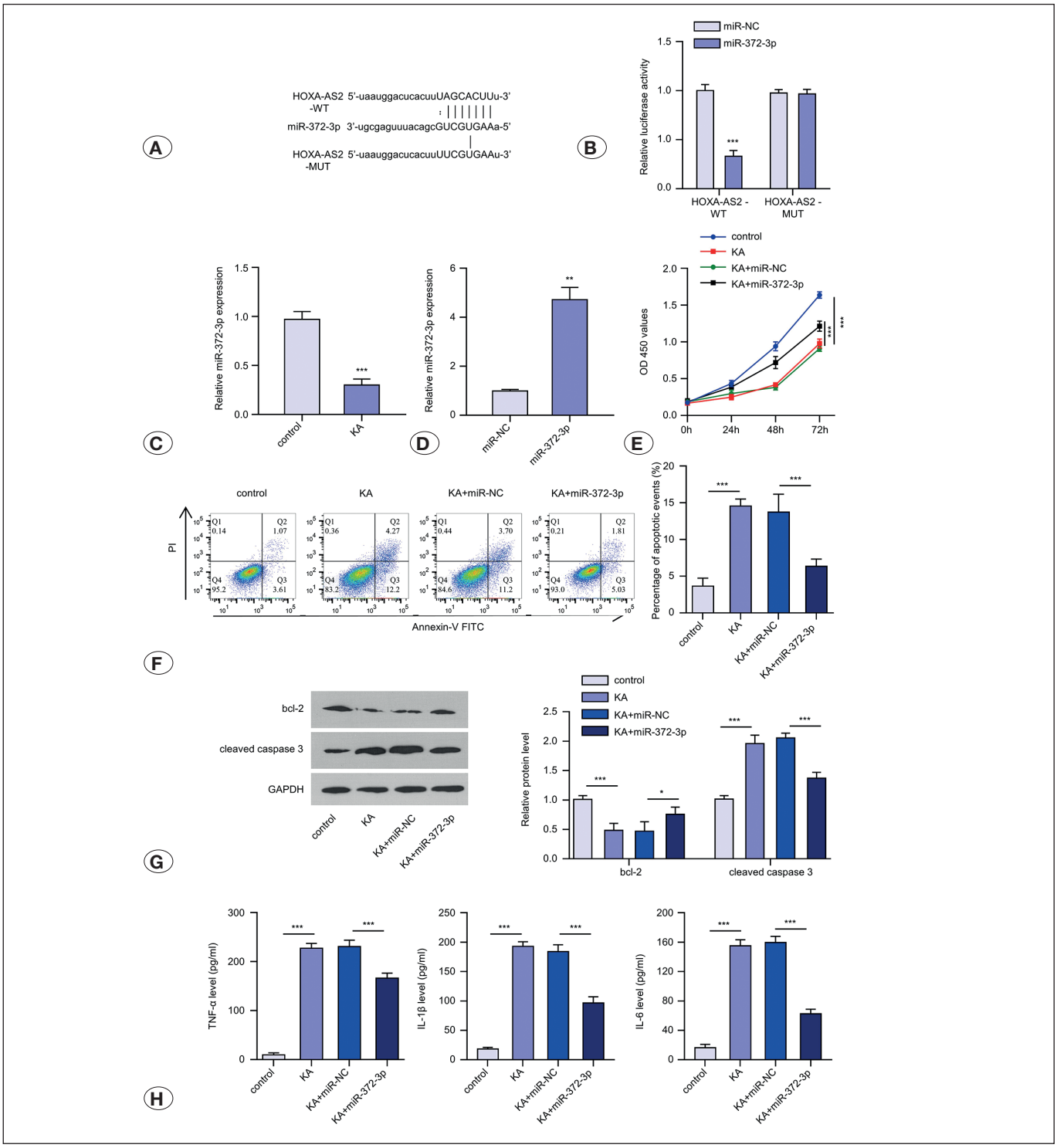

Figure 2: Long non-coding RNA HOXA-AS2 targets miR-372-3p. **A)** The complementary sites of miR-372-3p with HOXA-AS2 were predicted with the starBase database. **B)** The functional interaction between HOXA-AS2 with miR-372-3p was validated by dual luciferase reporter assay. **C)** Differences in miR-372-3p expression between the control and kainic acid groups were detected by RT-qPCR. **D)** The level of miR-372-3p after miR-372-3p mimic transfection in CTX-TNA2 cells was detected by RT-qPCR. **E)** Cell Counting Kit-8 assay was performed to detect cell proliferation in CTX-TNA2 cells subjected to different treatments. **F)** Flow cytometry analysis was conducted to determine cell apoptosis in CTX-TNA2 cells subjected to different treatments. **G)** The levels of apoptosis-related protein were detected by western blotting in CTX-TNA2 cells subjected to different treatments. **H)** The levels of inflammatory factors were measured by ELISA in cell culture supernatants of CTX-TNA2 cells subjected to different treatments. *p<0.05, **p<0.01, ***p<0.001. HOXA-AS2, HOXA cluster antisense RNA; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

miR-372-3p (Figure 3B). Western blot analysis showed that the STAT3 protein level was elevated upon transfection with miR-372-3p suppressor, but was reduced following miR-372-3p mimic transfection (Figure 3C). In addition, KA treatment promoted a higher level of STAT3 expression, while silencing of HOXA-AS2 reduced STAT3 expression (Figure 3D). However, the co-transfection of miR-372-3p inhibitor with si-HOXA-AS2 elevated STAT3 expression (Figure 3D). Together, these data indicated that the HOXA-AS2/miR-372-3p axis could regulate STAT3 expression in CTX-TNA2 cells treated with KA.

miR-372-3p/STAT3 Mediates the Effect of HOXA-AS2 Silencing in an Epileptic Cell Model

To further study whether STAT3 or miR-372-3p mediated the protective function of HOXA-AS2 silencing in a cellular model of epilepsy, the pcDNA-STAT3 expression vector was employed to overexpress STAT3 in CTX-TNA2 cells (Figure 4A). As revealed by CCK-8 assay, the protective effect of HOXA-AS2 silencing was impaired by miR-372-3p inhibitor and STAT3 overexpression in CTX-TNA2 cells treated with KA (Figure 4B). Similarly, co-transfection of STAT3 expression vector and miR-372-3p suppressor also abolished the protective function of HOXA-AS2 silencing against KA-induced apoptosis (Figure 4C, D), as well as the effect on the production of inflammatory cytokines (Figure 4E). These findings suggested that the miR-372-3p/STAT3 signaling pathway acted as a mediator of HOXA-AS2's activity in KA-induced damage.

DISCUSSION

Epilepsy is a frequently occurring chronic neurological disease characterized by repeated and unpredictable stroke attacks. This neurological condition is often associated with metabolic and nervous system disorders caused by neuronal overstimulation or brain damage (2,3). The status of epilepticus seriously affects the quality of life of patients (2), and understanding the molecular mechanisms underlying the pathophysiological progression of epilepsy is key for the development of novel therapies. Dysregulation of lncRNAs is implicated in the development of epilepsy by regulating

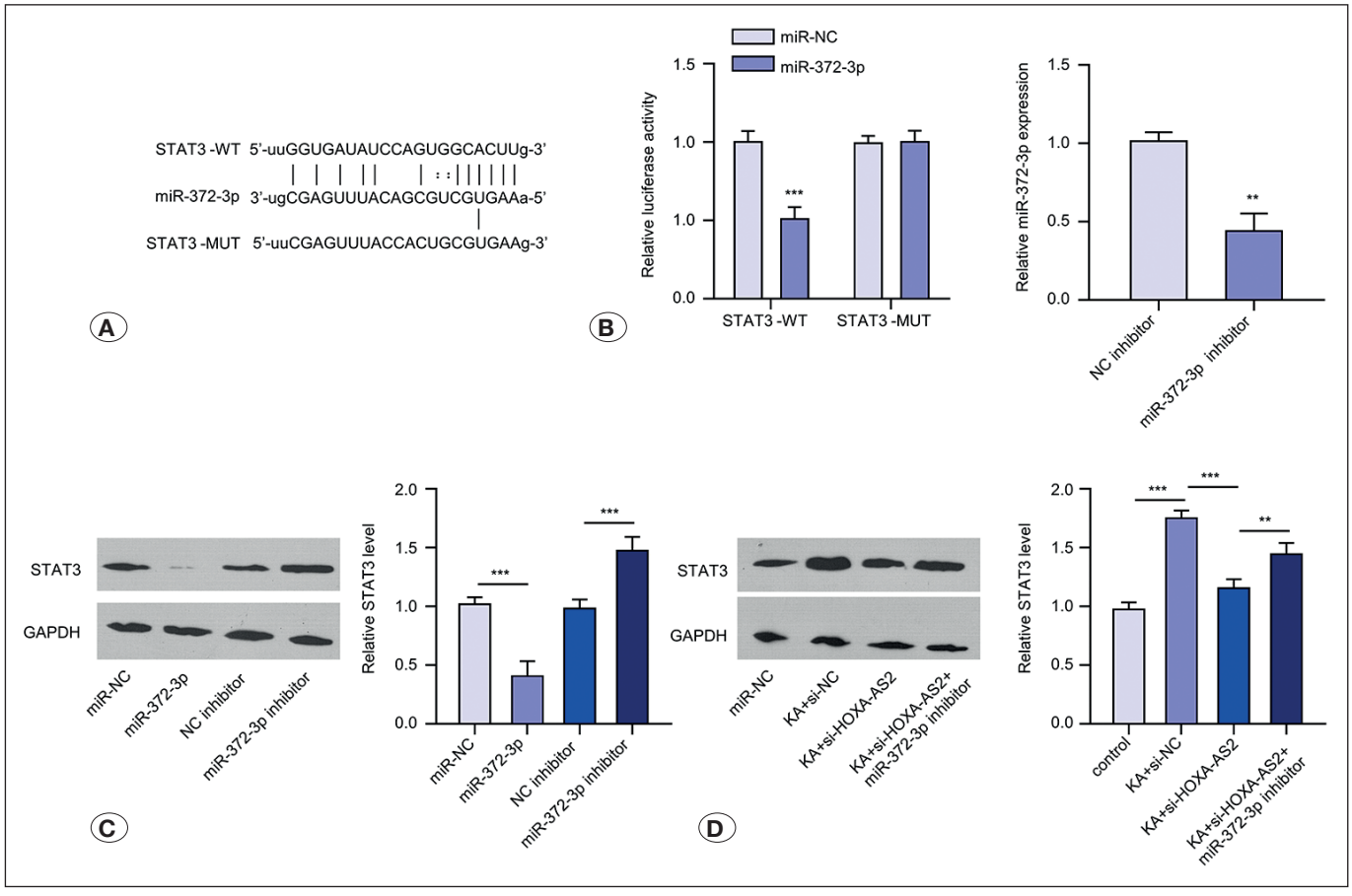

Figure 3: Knockdown of HOXA-AS2 increases STAT3 levels by regulating miR-372-3p. **A)** The online tool starBase was employed to predict the complementary sites between the 3' untranslated region of STAT3 mRNA and miR-372-3p. The interaction between STAT3 mRNA and miR-372-3p in CTX-TNA2 cells was verified by dual luciferase reporter assay. **B)** The effect of miR-372-3p inhibitor on miR-372-3p expression level was determined by reverse transcription-quantitative PCR. **C)** The level of STAT3 after transfection of miR-372-3p inhibitor or mimic was determined by western blotting. **D)** In CTX-TNA2 cells treated with kainic acid, the levels of STAT3 following transfection of si-HOXA-AS2 and miR-372-3p inhibitor+si-HOXA-AS2 were measured by western blotting. *p<0.05, **p<0.01, ***p<0.001. HOXA-AS2, HOXA cluster antisense RNA; miR, microRNA; si, small interfering.

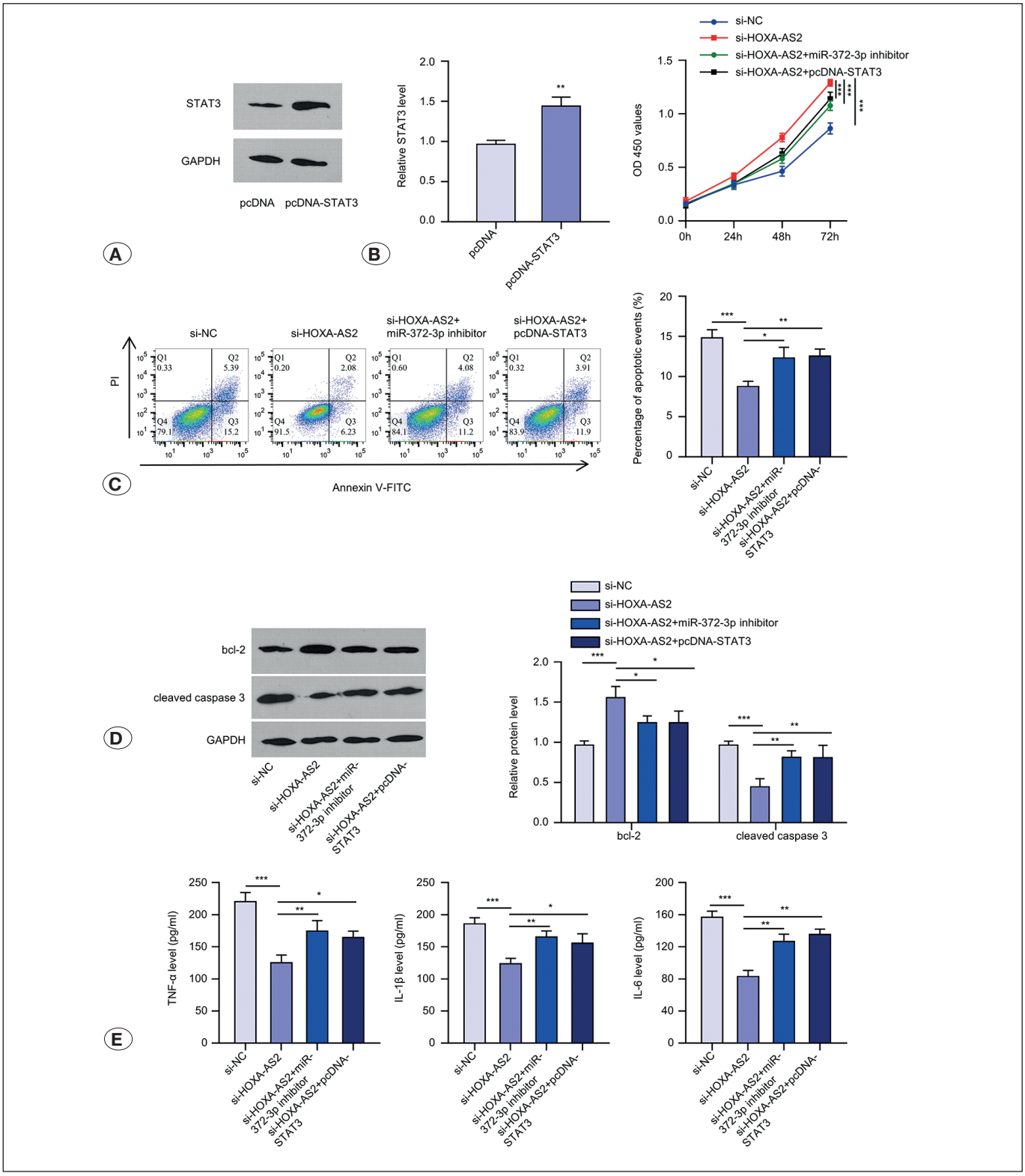

Figure 4: miR-372-3p silencing or STAT3 overexpression partially reverse the effects of HOXA-AS2 silencing. **A)** Overexpression of STAT3 in CTX-TNA2 cells was measured by western blotting. The following experiments were carried out in kainic acid-treated cells, which were transfected with si-NC, si-HOXA-AS2, si-HOXA-AS2+miR-372-3p inhibitor and si-HOXA-AS2+pcDNA-STAT3. **B)** Cell Counting Kit-8 assay was performed to detect cell proliferation. **C)** Flow cytometry was employed to determine cell apoptosis. **D)** The levels of apoptosis-related protein were detected by western blotting. **E)** The levels of inflammatory factors in cell culture supernatants were detected by ELISA. *p<0.05, **p<0.01, ***p<0.001. HOXA-AS2, HOXA cluster antisense RNA; miR, microRNA; si, small interfering.

neuronal dysfunction (5,13,15). A recent study suggested that HOXA-AS2 was upregulated in male patients with epilepsy (6). According to the present results, the HOXA-AS2 expression level in KA-treated CTX-TNA2 cells showed significant upregulation, and silencing HOXA-AS2 alleviated the inflammatory response and apoptosis induced by KA treatment. These results indicated that elevated HOXA-AS2 levels may contribute to the development and progression of epilepsy.

miRNAs are involved in different biological processes and pathogenic conditions such as cell proliferation, inflammation progression, neuron progenitor genesis, immature neuron growth and neuronal differentiation (1). The present study identified miR-372-3p as a downstream target of HOXA-AS2. In the context of cerebral infarction, miR-372-3p inhibitor could alleviate ischemia-reperfusion injury in neural stem cells (16). The current findings showed that the miR-372-3p level was reduced upon KA treatment, and miR-372-3p mimic could diminish the inflammatory response and reduce apoptosis in the KA group.

Previous studies indicated that STAT3 promoted neuronal apoptosis, and the inhibition of STAT3 expression could reduce brain damage and neuroinflammation (10,11,18). Consistently, the present study observed the regulation of STAT3 levels by the HOXA-AS2/miR-372-3p signaling pathway in a KA-induced epileptic model. The expression of STAT3 in the KA group was markedly increased, while silencing HOXA-AS2 or overexpressing miR-372-3p could suppress the STAT3 levels. The current functional assays further showed that miR-372-3p/STAT3 mediated the protective effect of HOXA-AS2 silencing in the epileptic cell model, including the inhibition of inflammatory factor production and cell apoptosis. However, the mechanisms underlying the upregulation of HOXA-AS2 in an epileptic cell model need to be further evaluated.

CONCLUSION

To conclude, upregulation of HOXA-AS2 promoted inflammation and apoptosis in an epileptic cell model through sponging miR-372-3p. Silencing HOXA-AS2 showed protective effects in an epileptic cell model, while miR-372-3p inhibitor transfection or STAT3 overexpression attenuated the protective effects. These data demonstrated that HOXA-AS2 may play act as a potential anti-epilepsy therapeutic target, which requires to be confirmed in animal models.

AUTHORSHIP CONTRIBUTION

Study conception and design: LS, ZS
Data collection: JL, LS
Analysis and interpretation of results: JL, LS
Draft manuscript preparation: LS, ZS
Critical revision of the article: LS, ZS
Other (study supervision, fundings, materials, etc...): JL, LS
All authors (LS, JL, ZS) reviewed the results and approved the final version of the manuscript.

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