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LNC000152 Mediates Aluminum-Induced Proliferation of Reactive Astrocytes

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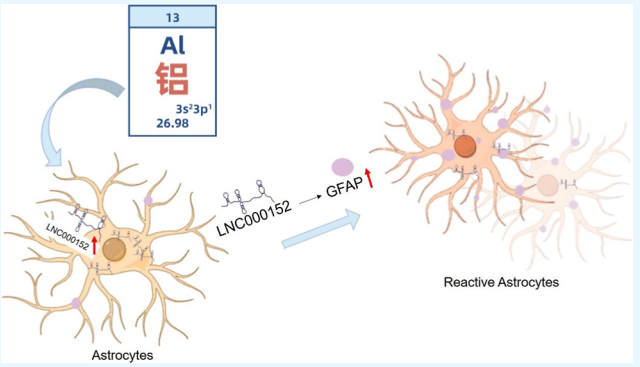
ABSTRACT: Aluminum is a metal element with significant neurotoxicity, and there is a substantial correlation between aluminum exposure and cognitive dysfunction. Glial fibrillary acidic protein (GFAP) is widely used as a marker of reactive astrocyte proliferation in response to pathological injury of the central nervous system. Studies of various neurodegenerative diseases have confirmed that the expression changes in GFAP are associated with nerve injury. We investigated the role of LNC000152 in the aluminum-induced reactive proliferation of astrocytes. By establishing two aluminum-exposed cell models of rat primary astrocytes and CTX-TNA2 cell lines, we examined the expression of LNC000152 and GFAP and detected cell proliferation with EdU and cell cycle changes with flow cytometry. The role of aluminum in promoting glial cell proliferation was verified; the expression levels of LNC000152 and GFAP increased with the concentration of aluminum exposure. Intervention of LNC000152 expression by siRNA technology revealed that LNC000152 affected glial cell responsive proliferation by influencing GFAP expression. These results suggest that LNC000152 plays a role in the reactive proliferation of astrocytes induced by aluminum.



1. INTRODUCTION

Aluminum (Al) is a natural metal element that is widely distributed in the environment. Because of its excellent characteristics, it is widely used in industrial production and our daily life, which leads to increased human exposure to aluminum.[1] Ultrafine particles or ions of aluminum can enter the human body through the respiratory system, digestive system, skin, and other ways. Current studies have not found that aluminum is involved in normal physiological activities in the human body. However, excessive exposure to aluminum can lead to increased intake, which can cause an aluminum overload and damage the health of the body.[2] Excessive accumulation of aluminum in mammals leads to pathological changes throughout the body[1] including pulmonary lesions,[3] skeletal abnormalities,[4] and neurological disorders.[5] Occupational exposure to aluminum has been found to be associated with cognitive impairment in occupational epidemiological studies. In studies of occupational aluminum workers, it was found that spatial perception and memory were impaired to varying degrees.[6] Exposure to aluminum has also been found to have significant adverse effects on short-term, spatial learning and memory abilities in animal studies.[2,7] Aluminum ions can enter the brain through the blood−brain barrier and are present in the extracellular fluid and cerebrospinal fluid, causing neurological damage to the brain. Aluminum exposure promotes oxidative stress in neural tissues, inducing neurodegeneration and neuronal cell death, which underlie the neurological disorders associated with aluminum toxicity.[8] Exposure to aluminum may produce neurodegenerative lesions similar to those found in Alzheimer's disease (AD). Increased levels of aluminum in the brain may be one of the key factors in the formation of neurofibrillary tangles and amyloid plaques, which is consistent with common neuropathology observed in the brains of Alzheimer's patients.[9] Aluminum is a widely used and ubiquitous element in contemporary life. As an unavoidable environmental factor, although scholars have been focusing on the molecular mechanisms of aluminum toxicity-induced body damage since the last century,[10] the biomolecular mechanisms of aluminum-induced neurological damage still need to be comprehensively explored sufficiently to completely and fully elucidate the mechanism of the damage and to find prevention and treatment of the relevant diseases[11] and also to bring new ideas for the study of other similar toxicity damage mechanisms.

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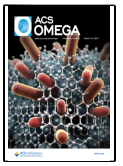

Most of the work on the toxic effects of aluminum has involved the examination of the direct effects of aluminum on neurons.[12,13] However, work on the toxic effects of aluminum on astrocytes has lagged relatively behind.[14] As the most numerous glial cells in the brain, astrocytes are essential for maintaining the normal function of the central nervous system (CNS). It is currently believed that astrocytes can respond rapidly to pathological changes in their surroundings through adjustments in their morphology, molecular expression, and function.[15] Astrocyte dysfunction or reactive proliferation may be a major cause or manifestation of neurological dysfunction and disease.[16] In a recent study of the progression of AD, it was noted that abnormal astrocyte reactivity can act as an early upstream event in neurological injury. Abnormal astrocyte signaling may lead to synaptic and network imbalances that result in cognitive dysfunction.[17]

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is mainly expressed in astrocytes and is a crucial component of the cytoskeleton, which is essential for maintaining the structure and shape of astrocytes.[18] GFAP also has a wide range of functions, from regulating astrocytes' cellular movements to determining their functional state and intracellular signaling. At the same time, it can function as a physical barrier between adjacent neurons of astrocytes and as a stabilizer of the extracellular environment.[19,20] Increased GFAP expression is considered as a typical response marker for astrocyte activation and gliosis.[21]

Long noncoding RNA (LncRNA) is a noncoding RNA with a more than 200 nt length. It is not usually translated into proteins and is considered an endogenous regulatory molecule.[22] LncRNA is involved in many biological functions and regulates gene expression through various mechanisms. As one of epigenetic components, LncRNA is a class of molecules with rich potential research value.[23] More and more studies have found that LncRNA is involved in different CNS gene expression regulation mechanisms.[23−25] Among them, the dysregulation of LncRNA expression has also been shown to play an essential role in the pathogenesis of a variety of neurodegenerative diseases, including AD, Parkinson's disease, and Huntington's disease. Even several LncRNAs have been demonstrated to be potential neurological biomarkers.[26−28] Our study group has been committed to studying the neurotoxicity of aluminum for many years and has made some progress in the study of epigenetic regulation in the mechanism of aluminum-induced nerve injury in recent years.[29−31] In our previous study, we performed complete transcriptome sequencing of hippocampal tissues from aluminum-exposed SD rats and found an increased expression of LNC000152 in the aluminum-exposed group. Combined with bioinformatics predictions, it was found that this aberrant expression may be associated with glial cell proliferation and differentiation.[32]

In summary, we speculate that the mechanism of aluminum toxicity may be to regulate the proliferation of glial cells through LncRNA, which, in turn, affects neurons. Therefore, in this study, we propose to investigate whether aluminum promotes the responsive proliferation and differentiation of astrocytes through LNC000152 by in vitro experiments on astrocytes.

2. MATERIALS AND METHODS

2.1. Cell Culture and Purification. Rat primary astrocyte culture: Primary astrocytes were extracted from SD rats and cultured in vitro.[33] SD neonatal mice (provided by the Experimental Animal Center of Shanxi Medical University) from 1 to 3 days old were selected. Both cerebral hemispheres of the neonatal mice were removed under sterile conditions, the hippocampus was separated, meninges and blood vessels were stripped, and digestion was performed at 37 °C for 10 min with 0.25% pancreatic enzyme. After digestion was terminated, the cells were gently beaten repeatedly and screened with 100 mesh nets to collect cells. DMEM/F12 complete culture medium (GIBCO, China) containing 20% fetal bovine serum (GIBCO, China) and 1% penicillin/streptomycin (Beijing Solaibo Technology, China) was inoculated into a 10 mm Petri dish and cultured in a 5% CO_2 incubator at 37 °C. The medium was renewed three times a week. Astrocytes were removed by shaking at 260 pm overnight at 37 °C in an orbital shaker (ZhiCheng, China). Astrocytes were collected and subcultured for 24 h before experiments. Cell passage was performed after cell culture for a period of time. After purification and passage for —two to three generations, the astrocyte-specific marker, GFAP, was detected. Follow-up experiments were performed using cultures with >95% GFAP-positive cells.

Culture of the immortalized rat astrocyte line CTX-TNA2: CTX-TNA2 cell line was obtained from the American Type Culture Collection, and cultured in DMEM complete medium with 1% penicillin/streptomycin (Beijing Solaibo Technology, China) and 10% fetal bovine serum (Gibco, USA). The medium was changed every 2−3 days.

2.2. Cytotoxic Exposures, Transfection, and Grouping. The corresponding masses of maltol powder (Sigma, USA) and anhydrous aluminum trichloride ($AlCl_3$) (Sigma, USA) powder were weighed, and they were dissolved in autoclaved purified water and formulated into maltol reserve solution at a concentration of 60 mM and aluminum trichloride reserve solution at a concentration of 20 mM, respectively; the concentration of maltol was adjusted to 1200 μM for preparing aluminum treatment solution, and aluminum trichloride was dissolved into the maltol solution. It was dissolved in the maltol solution and used as it was prepared. Aluminum maltol exposure concentrations were referenced from previous studies by our group.[34,35] The final concentrations of maltol aluminum solutions treating primary astrocytes were 10, 20, and 40 μM; those treating CTX TNA2 cells were 100, 200, and 400 μM; and the treatment time gradients were set at 12, 36, 24, and 48 h.

According to the experimental design, LNC000152 siRNA and NC siRNA (Shanghai Gima, China) were transfected into CTX-TNA2 cells when the cell fusion degree in the six-well plate was about 30%. The transfection efficiency was evaluated by a quantitative real-time polymerase chain reaction (qRT-PCR). Transfection was divided into a blank control group, transfection reagent control group (mock group), negative control group (NC group), siRNA group, $Al(mal)_3$ group, and siRNA + $Al(mal)_3$ group.

2.3. Cell Viability Assay. According to the CCK-8 assay kit (MCE, USA), the purified AST and CTX cells were inoculated into the 96-well plate at a density of about 1×10^5 cells per well. After 24 h, according to the group set above, the corresponding $Al(mal)_3$ solution was changed, each group was set with five boreholes, and the corresponding treatment times were 12, 24, 36, and 48 h). The working liquid was added to each well, cultured for 30 min, and then Bio-Rad (USA) was used to detect the absorbance value of each well at 450 nm.

2.4. Cell Cycle Analysis. A cell cycle and apoptosis detection kit (Wuhan Sevier Biotechnology, China) was used. Cells collected by washing with precooled PBS were precipitated and resuspended in cold 75% ethanol. The cells were fixed

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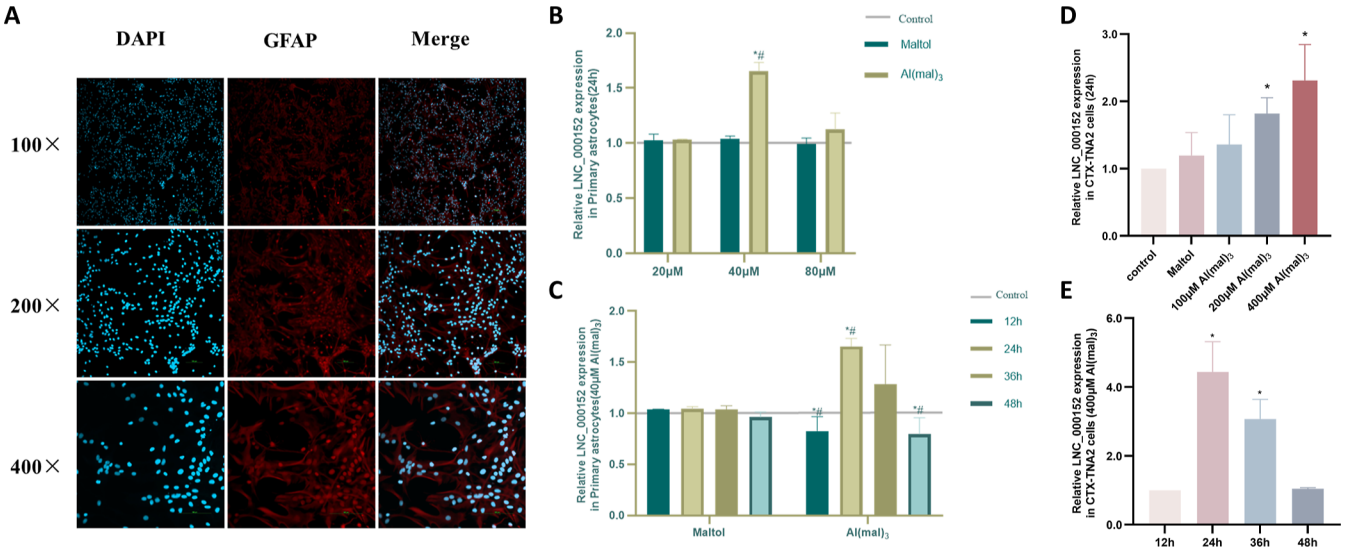

Figure 1. Aluminum stimulates LNC000152 expression in astrocytes. (A) Immunofluorescence staining of primary astrocytes with GFAP. Nuclei stained with DAPI. Scale bar: 100 μm. (B) The expression of LNC000152 in primary astrocytes was detected by RT-qPCR after 24 h exposure to $Al(mal)_3$ at different concentrations. (C) The expression of LNC000152 in primary astrocytes after exposure to 40 μM $Al(mal)_3$ for 12, 24, 36 and 48 h was detected by RT-qPCR. Control line: the blank control group was set as 1, and the other groups were standardized by the control group to get the results, compared with the control group, $^*P < 0.05$. (D) The expression of LNC000152 in CTX-TNA2 cells was detected by RT-qPCR after 24 h exposure to $Al(mal)_3$ at different concentrations. (E) The expressions of LNC000152 were detected by RT-qPCR in CTX-TNA2 cells after 400 μM Al(mal)3 exposure for 12, 24, 36, and 48 h, compared with the 12 h group, $^*P < 0.05$. Data are expressed as mean ± SD; images are representative of at least three experiments.

overnight at 4 °C, resuspended and washed with PBS buffer, and centrifuged at 1000*g* for 5 min, and the supernatant was discarded to collect the cell precipitate. After the dye working solution was prepared away from light, 500 μL was added to each tube. Incubation at 37 °C protected from light for 30 min was performed by flow cytometry (Ace biosciences Incorporated, USA), and the cell proliferation index (PI) (%) = $(S + G2/M)/(G0/G1 + S + G2/M)$.

2.5. Assessing Cell Proliferation. The kFluor555-EdU cell proliferation kit was used according to the reagent manufacturer's instructions (KGA336, KeyGEN Biotechnology, China). In short, the cells were inoculated and cultured into culture dishes, the final concentration of EdU was added, and the culture time was appropriate. 4% paraformaldehyde was fixed for 15 min, and 0.5% TritonX-100 permeabilization solution was used to penetrate the cells for 30 min. The Click-iT EdU reaction solution was prepared according to the product instructions; the reaction was incubated at room temperature for 30 min and then washed twice with 3% BSA. Three random areas were photographed using a confocal laser microscope (Nikon AR1MP) to count the number of EdU-positive cells.

2.6. Six Reverse Transcriptional Quantitative Polymerase Chain Reactions. TRIzol reagent (Takara Bioengineering, Japan) was added to the collected cell precipitate, and RNA samples were extracted by chloroform/isoamyl alcohol. Total ribonucleic acid (RNA) was extracted and quantified by a spectrophotometer. According to the protocol of the cDNA synthesis kit (Takara Bioengineering, Japan), an equivalent amount of RNA was reverse-transcribed to synthesize cDNA. RT-qPCR was performed using a TB Green Premix Ex *Taq*II (Takara Bioengineering, Japan) on a Step One real-time fluorescent RT-qPCR system (Thermo Fisher Technologies). The expression levels of LNC000152 and GFAP were measured. The results were normalized to the expression level of GAPDH and quantified by the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used in this study were produced by Servicebio Biotech and are presented as follows.

LNC000152-F, 5′-TGCCACATCTGGTGCTTGC-3′,
LNC000152-R, 5′-GACCGAGGCAACTTGTAAACC-3′,
GAPDH-F,5′-CTGGAGAAACCTGCCAAGTATG-3′,
GAPDH-R,5′-GGTGGAAGAATGGGAGTTGCT-3′,
GFAP-R,5′-CCACCAGTAACATGCAAGAAACA-3′, and
GFAP-F, 5′-AGTTGGCGGCGATAGTCATTAG-3′.

2.7. Western Blot Analyses. Cell samples were harvested and lysed with a protein extraction solution (Beijing Kangwei Century, China) consisting of protease and phosphatase inhibitors. According to the size of protein molecular weight, the appropriate concentration of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel was prepared for protein separation and then transferred to a poly(vinylidene difluoride) membrane (Millipore, USA), which was closed with 5% skimmed milk for 4 h; the membrane was incubated with GFAP monoclonal antibody (1:5000, Santa Cruz Biotechnology, USA) and control anti-GAPDH (1:3000, CWBIO, China) overnight at 4 °C; the bound antibodies were detected using HRP-labeled sheep antirabbit IgG (1:3000, CWBIO, China) and HRP-labeled sheep antimouse IgG (1:3000, CWBIO, China), and the bound antibodies were detected using ultrasensitive electrochemical luminescence chemiluminescence solution (1:3000, CWBIO, China) on a Universal Hood II gel imager (Bio-Rad, USA) for protein banding image acquisition.

2.8. Statistical Analyses. SPSS 22.0 software was used for statistical analysis. All data are measurement data expressed as mean ± SD. One-way ANOVA was used for comparison among multiple groups, the LSD test was used for pairwise comparison when variance was homogeneous, and the Games−Howell test was used for pairwise comparison when the variance was uneven. Test level $\alpha = 0.05$ (bilateral).

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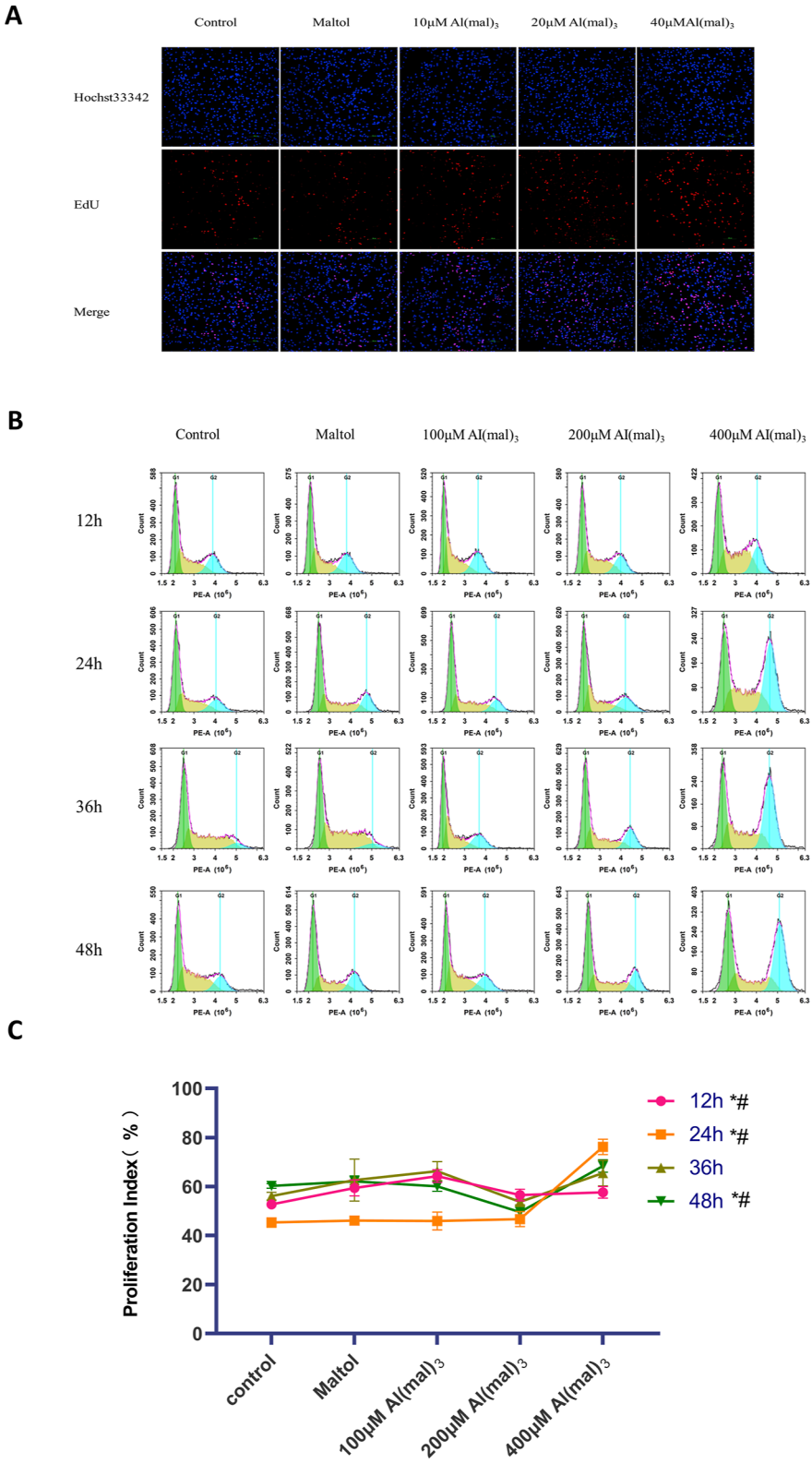

Figure 2. Al(mal)3 stimulates astrocyte proliferation. (A) EdU (red) and Hoechst 33,342 (blue) double staining to assess astrocyte proliferation in $Al(mal)_3$-exposed groups. Scale: 100 μm. The images represent at least three experiments. (B) The cell cycle of CTX-TNA2 cells in different groups was detected by flow cytometry. (C) The cell proliferation index (PI %) of CTX-TNA2 cells in different groups was expressed as mean ± SD, compared with the control group ($^{*}P < 0.05$) and compared with the 100 μM $Al(mal)_3$ exposure group ($^{\#}P < 0.05$). Data are expressed as mean ± SD; images are representative of at least three experiments.

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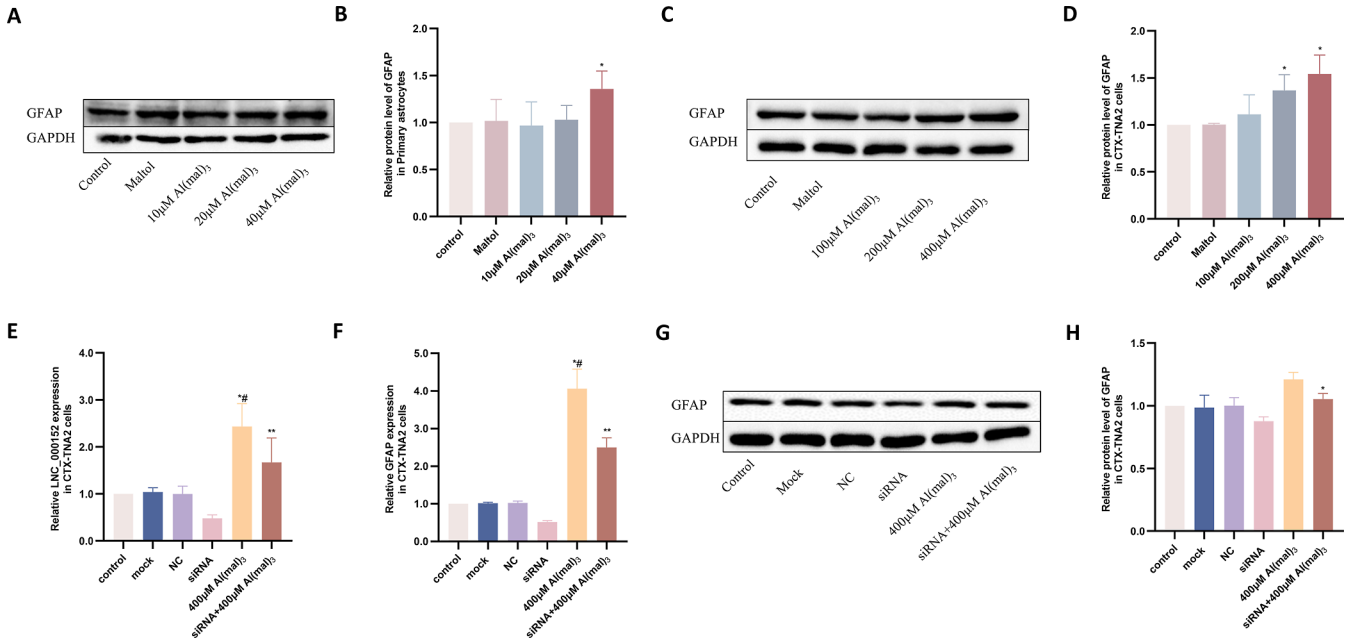

Figure 3. Al(mal)3 and siRNA intervention groups stimulate the expression of GFAP protein in CTX-TNA2 cells. (A) Results of western blotting for the expression of GFAP in primary astrocytes after 24 h of $Al(mal)_3$ treatment. (B) Blots in Figure 3A were analyzed by densitometric scanning using ImageJ image analysis software, and data are representative of at least three experiments. Compared with the control group, $^*P < 0.05$. (C) Results of western blotting for the expression of GFAP in CTX-TNA2 cells after 24 h of $Al(mal)_3$ treatment. (D) Blots in Figure 3C were analyzed by densitometric scanning using ImageJ image analysis software, and data are representative at least three experiments, compared with the control group, $^*P < 0.05$. (E) The expression of LNC000152 in CTX-TNA2 cells of each intervention group was detected by RT-qPCR. Compared with the control group, $^*P < 0.05$; compared with NC group, $^{\#}P < 0.05$; and compared with the 400 μM $Al(mal)_3$ group, $^{**}P < 0.05$. (F) The expression of GFAP protein in CTX-TNA2 cells of each intervention group was detected by western blot. Compared with the control group, $^*P < 0.05$; compared with the negative control group, $^{\#}P < 0.05$; and compared with the 400 μM $Al(mal)_3$ group, $^{**}P < 0.05$. (G) Results of western blotting for the expression of GFAP in CTX-TNA2 cells after 24 h of $Al(mal)_3$ and siRNA treatment. (H) Blots in Figure 3G were analyzed by densitometric scanning using ImageJ image analysis software, and data are representative of at least three experiments. Data are expressed as mean ± SD; images are representative of at least three experiments.

3. RESULTS

The purity of rat primary astrocytes cultured by immunofluorescence was more than 95%, which met the requirements of the following experiments (Figure 1A).

According to the previous conditions explored by our research group and combined with CCK-8, changes in cell viability after treatment with $Al(mal)_3$ at different times and doses were detected (Supporting Information A), and the treatment time and concentration of primary astrocytes and CTX-TNA2 cells were determined for subsequent experiments. RT-PCR was used to verify the expression of LNC000152 in astrocytes and the changes after exposure to aluminum. The results showed that LNC000152 gene expression in primary astrocytes of rats in the 40 μM $Al(mal)_3$ group was significantly increased after 24 h exposure compared with the blank control group and maltol group ($P < 0.05$). The most obvious increase in expression was observed in the 40 μM $Al(mal)_3$ group, and a decreasing trend in expression was observed in the 80 μM $Al(mal)_3$ group (Figure 1B). Therefore, we chose 40 μM $Al(mal)_3$ as the highest dose group for subsequent experiments. In addition, there was no statistically significant difference between the different concentrations of maltitol among the groups and the blank control group. Therefore, we concluded that the main changes in the experimental results were caused by aluminum, while maltol was only used as a solvent. In terms of time change, we observed a significant and statistically significant increase of LNC000152 after aluminum exposure for 24 h. With the extension of exposure time (48 h), the expression level of LNC000152 decreased (Figure 1C) Similarly, the expression level of LNC000152 in CTX-TNA2 cells was also positively correlated with the exposure concentration of aluminum (Figure 1D), and the differences in the higher dose group were statistically significant ($P < 0.05$). According to the analysis of the change of exposure time, compared with the control group, there was a statistically significant difference in LNC000152 expression between 24 and 36 h exposure ($P < 0.05$), among which LNC000152 expression increased most significantly at 24 h exposure (Figure 1E).

We used the EdU to detect changes in the proliferation of primary astrocytes exposed to aluminum. As shown in Figure 2A, the proliferation of astrocytes increased after treatment with $Al(mal)_3$. The EdU-positive cell rate of primary astrocytes treated with 40 μM $Al(mal)_3$ for 24 h was about 2.50 times that of the control group (Supporting Information B). The effect of aluminum on the proliferation of the two types of astrocytes was similar. The effect of aluminum on the cell cycle of CTX-TNA2 was detected by flow cytometry (Figure 2B). Compared with the control group, the increase of the cell PI in 400 μM $Al(mal)_3$ groups was statistically significant ($P < 0.05$) in each time group (12, 24, 36, and 48 h); in addition, CTX-TNA2 cells exposed to 400 μM $Al(mal)_3$ for 24, 36, and 48 h were significantly more than those captured in the control group at the s and G2 phases, indicating that aluminum affected the cycle distribution of astrocytes (Figure 2C). Western blot was used to detect the expression of the GFAP protein in primary astrocytes treated with different concentrations of $Al(mal)_3$ (10, 20, and 40 μM).

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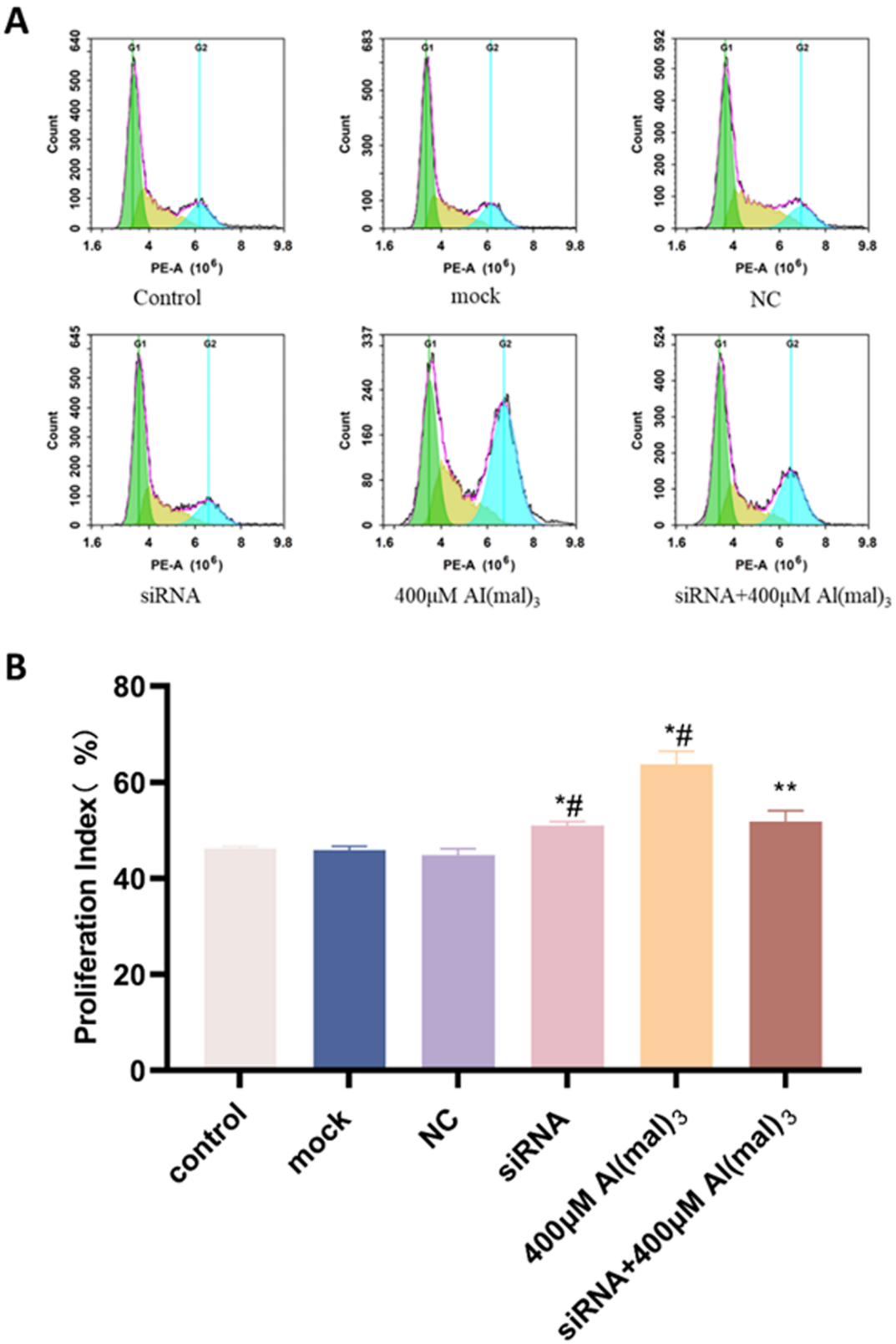

Figure 4. Al(mal)3 and siRNA intervention groups affect the cell cycle of CTX-TNA2 cells. (A) The cell cycle of CTX-TNA2 cells in different groups was detected by flow cytometry. (B) The cell proliferation index (PI %) of CTX-TNA2 cells in different groups was expressed as mean ± SD; compared with the control group, $^{*}P < 0.05$; compared with NC group, $^{\#}P < 0.05$; and compared with the 400 μM Al(mal)$_3$ group, $^{**}P < 0.05$. Data are expressed as mean ± SD; images are representative of at least three experiments.

As shown in Figure 3A,B, the expression of GFAP was significantly upregulated with the increase of aluminum exposure concentration. The level of GFAP protein in the 40 μM Al(mal)$_3$ group was 1.36 times that of the control group ($P < 0.05$). Similarly, the expression of GFAP protein in CTX-TNA2 cells in the high-dose aluminum exposure group [400 μM

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$Al(mal)_3$] was also significantly upregulated (Figure 3C,D). To explore the potential role of LNC000152 in astrocytes, we chose to use siRNA to interfere with the expression of LNC000152 in CTX-TNA2 cells and to verify the role of LNC000152 in aluminum-stimulated astrocytes by aluminum exposure. Based on the results of CCK8 detection of cell viability after intervention and PCR detection of LNC000152 expression, the gene sequences and intervention conditions were screened for intervention (Supporting Information A, C). Finally, we selected the optimal intervention conditions, selected the SIRNA-6306 interference sequence, transfected siRNA for 24 h, and then exposed 400 μM $Al(mal)_3$ for 24 h. RT-qPCR analysis showed that the relative expression of LNC000152 and GFAP genes did not change significantly between the control and NC groups, and the differences between the two groups were not statistically significant (Figure 3E−F). The expression of GFAP was significantly elevated in CTX-TNA2 cells after aluminum exposure, but the expression of GFAP was reduced again in the siRNA group with statistical differences (Figure 3F), suggesting that LNC000152 could affect GFAP mRNA expression in astrocytes after aluminum stimulation. In addition, western blot analysis revealed that there was also a tendency for the relative expression of the GFAP protein to decrease after LNC000152 silencing (Figure 3G,H).

Then, the effect of siRNA on the cell cycle of CTX-TNA2 cells was measured by flow cytometry (Figure 4A,B). The results showed that compared to the 400 μM $Al(mal)_3$ exposure group, more cells were captured in the G0/G1 phase and fewer cells were captured in the S phase after silting LNC000152. Moreover, the cell PI decreased by 12% ($P < 0.05$), indicating that the down-expression of LNC000152 could inhibit the proliferation of CTX-TNA2 cells (Figure 4B). These results suggest that LNC000152 may affect the proliferation of astrocytes by regulating the expression of GFAP.

4. DISCUSSION

In this study, we established two different aluminum exposure models regarding primary astrocytes and rat astrocyte CTX-TNA2 cells. The results all showed that the expression of LNC000152 was increased in the cells of the aluminum-exposed group compared with that in the control group, which was consistent with the results of presequencing. We confirmed the effect of aluminum exposure on astrocyte activation by detecting GFAP (a marker of activated astrocytes) and astrocyte proliferation phenomena. After reducing the expression of LNC000152 in CTX-TNA2 cells using siRNA intervention, we also observed that inhibition of LNC000152 reduced the proactivation effect of aluminum in CTX-TNA2 cells. It was shown that aluminum could affect the responsive proliferation of astrocytic glial cells by upregulating LNC000152.

Aluminum is widely available in the environment and is used in industrial production and our life, significantly increasing the opportunity for the body to absorb aluminum. Aluminum absorbed by the body is easy to accumulate in the body and produces toxicity.[1] It has been proved that aluminum ions can enter the brain quickly and is a metal element with obvious neurotoxicity.[8] Excessive exposure to aluminum can seriously damage the function of the body's nervous system.[2] Numerous studies on the aluminum-induced toxic mechanism have suggested that aluminum exposure promotes mitochondrial dysfunction, oxidative stress, neuroinflammation, and neuronal apoptosis in nervous tissue, which underlie the neurological diseases associated with aluminum toxicity (29, 31, 36). Notably, astrocytes are involved in many key functions in many physiological aspects of the brain, including providing trophic support to neurons and other neuronal cells, mediating neurotransmitter uptake and recycling as well as participating in the maintenance of the blood−brain barrier, and also mediating the formation and elimination of synapses during development, and it provides intrinsic neuroprotection to neurons.[19] Therefore, more and more studies have also begun to focus on the role of astrocytes in neurotoxicity.[36] Successive views have pointed out that astrocytes might also be the main target of aluminum toxic effects.[37,38]

In this study, two cell models, primary astrocyte and rat astrocyte CTX-TNA2, were used to investigate the neurotoxicity of aluminum. Primary cells are established by removing them directly from tissues and processing them for optimal culture conditions.[38] As they are tissue-derived and unmodified, they are more similar to the in vivo state and exhibit normal physiological characteristics. Primary astrocytes in the culture provide a good cellular model for assessing neurotoxic injury. However, they also suffer from the disadvantages of stringent cell culture conditions and limited cell lifespan compared with passaged cells. In addition, compared with other immortalized passaged cells, CTX-TNA2 cells are more characterized by the retention of relevant phenotypic features in vivo, as well as a better ability to respond to induction.[39] Therefore, we chose both cellular models for investigation in order to have a more comprehensive view of the neurotoxic effects of aluminum. In addition, considering the difference in toxicant tolerance between primary and passaged cells, our team respectively set up the rat primary astrocyte and CTX TNA2 cell line aluminum exposure concentration of cell models. Also, considering the difference in tolerance to poison between primary and secondary cells, our research group explored the aluminum exposure concentrations of primary rat astrocytes and CTX-TNA2 cell lines, respectively. For primary astrocytes, our previous data mainly focused on the mechanism of the effect of aluminum on the death of primary neurons,[35] whereas in this study, we were concerned with the damage of aluminum exposure to the cellular state of neuronal cells and their function prior to cell death and whether aluminum would be indirectly toxic to neurons by affecting the proliferation of glial cells. Therefore, on the basis of the preliminary experimental mapping, we chose the exposure conditions in which neuronal cell viability was above 80%, and the concentration range of aluminum exposure in primary astrocytes was set between 10 and 40 μM. As for CTX-TNA2 cells, the group did not carry out relevant studies in the previous period; therefore, this experiment referred to the general concentration of the previous group of passaged cells mapping, the CTX-TNA2 cells staining concentration was also mapped in the previous period, and the results are shown in Supporting Information A, with the final determination of its concentration range of 100−400 μM.

There is research evidence that various types of CNS injury commonly trigger complex changes known as reactive astrocyte proliferation. They are mainly characterized by reactive astrocyte proliferation and increased expression of GFAP, a specific marker of astrocyte proliferation, which is an important marker of astrocyte pathological dysfunction.[16] In this study, we used flow cytometry and EdU fluorescence to detect the cell cycle and the cell proliferation of the cells. Dynamic proliferation assessment by flow cytometry can be used to monitor cell cycle progression in vitro at high resolution, providing a wide range of information on cell cycle duration, transition points, and

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proliferative quiescence, and it is an indisputably powerful method for detecting changes in the cell cycle but with specific requirements for accurate preparation of cells and execution of the measurement.[40] In this study, we labeled primary astrocytes with 5-ethynyl-2′-deoxyuridine (EdU), a thymidine analogue that is detected by covalently cross-linking acetylene with fluorescent amide-linked chemistry. This method does not require denaturation of DNA, causes minimal damage to cells, and can replace thymidine (T) in penetrating DNA molecules synthesized during DNA replication, allowing for direct and accurate detection of DNA replication activity. It is one of the assays that can be used to accurately reflect cell proliferation.[41] Conclusively, this study found that aluminum exposure led to an increased proliferation of astrocytes in different aluminum-exposed cell models. A tendency toward an increase in the number of EdU-positive cells with increasing concentrations of aluminum exposure was observed by the EdU assay, and it was statistically significant in the high-dose maltol aluminum exposure group compared to that in the control group. Combined with flow cytometry to detect changes in the DNA content of CTX-TNA2 cells, it was found that the cell PI was greater than that of the control group in almost all aluminum-exposed groups under different conditions of exposure time and exposure concentration. It suggests that aluminum is a proliferation-promoting factor for astrocytes.

GFAP is commonly used as a marker for astrocytes.[42] In addition, its increased expression is often considered one of the indicators of reactive astrocyte proliferation associated with brain injury or neurological disease.[43] Elevated levels of GFAP expression have also been found in many experimental models involving the proliferation of reactive astrocytes. For example, Zhao's study[44] verified the proliferation of reactive glial cells in a cortically damaged cell model by detecting GFAP fluorescence intensity and cell cycle changes; Li's experiments[45] demonstrated the proliferation of reactive astrocytes in an LPS model by combining EdU detection with immunofluorescence and western blot. Similarly, in this study, the expression of GFAP in primary astrocytes and CTX TNA2 cells was also detected by qRT-PCR and western blot methods, which also increased with increasing concentrations of aluminum exposure. Altogether, we found an increased number of cells in the aluminum-exposed group that were in the proliferative phase of cellular proliferation. Meanwhile, under aluminum-exposed conditions, an increase in gene and protein expression of GFAP was also observed. From this, we can conclude that aluminum exposure promotes the reactive proliferation of astrocytes. However, for different CNS injuries, astrocyte proliferation is characterized differently.[46] For instance, with toluene exposure, astrocyte proliferation is characterized by increased GFAP expression and no increase in astrocyte proliferation.[47] Yet in cocaine[48] exposure models, it is cell proliferation that is the main manifestation. Exploration of the present model may help to define the properties of astrocyte injury induced by aluminum exposure, where increased GFAP expression is accompanied by an increased cell proliferation rate.[49]

In recent years, epigenomics of toxics has been extensively studied as a frontier area in environmental health sciences. Several independent experiments have demonstrated that aluminum mediates epigenetic changes in cells and interacts with genomic components such as DNA, RNA, transcription factors, and regulatory proteins in vivo, affecting gene transcription and expression in normal cells (26, 29, 49). Previous studies of our research group found that aluminum can regulate BACE1 expression by influencing miR-29 and eventually cause the increase and accumulation of Aβ production, resulting in the decline of learning and memory ability of animals and the damage of nerve cells;[50] LncRNA_001209 is involved in Al-induced neuronal apoptosis by regulating the PI3K/AKT/mTOR pathway.[51] Our group analyzed the transcriptome sequencing results of hippocampal tissues from SD rats exposed to aluminum and found that the hippocampus of the aluminum-exposed group of rats showed an increased expression of LNC000152. Combined with the results of bioinformatics prediction, we believe that this abnormal expression may also be related to the proliferation and differentiation of glial cells.[32] LncRNA are transcripts more than 200 nucleotides long that do not translate into proteins but are involved in many biological functions and regulate gene expression through multiple mechanisms.[22] Current research indicates that the mechanism of action of LncRNA can be categorized into (1) directly binding to DNA or transcription factors to achieve gene expression regulation at the transcriptional level; (2) targeting mRNAs, miRNAs, or proteins and regulating their activity and stability for post-transcriptional effects; and (3) interfering with chromatin complexes to epigenetically repress or activate gene expression.[22,26] The mechanism of action of LncRNA enables it to participate in almost all physiological activities in cells and has been implicated in the development of several diseases.[25,27] Moreover, several studies have also found that LncRNA is involved in the regulation of astrocyte activation. For example, upregulation of LncRNA MEG3 inhibited astrocyte activation in the hippocampus of AD;[52] knockdown of LncRNA PVT1 inhibited astrocyte activation in the rat hippocampus.[53] These results suggest that LncRNA may also play an important role in aluminum-induced astrocyte activation. Our study found that the level of expression of LNC000152 in primary astrocytes and CTX-TNA2 cells increased with aluminum exposure. In addition, mRNA expression of the GFAP gene was higher in aluminum-exposed cells than in control cells. We found that the expression of both GFAP protein and mRNA decreased in aluminum-exposed cells after the knockdown of LNC000152. These results suggest that in our cellular model, aluminum regulates the mRNA expression of GFAP by modulating the expression of LNC000152, thereby affecting the changes in GFAP protein expression in astrocytes.

In this study, we explored the effects of aluminum exposure on the reactive proliferation of astrocytes by establishing two different cellular models and verified that LNC000152 plays a role in this process. The results of this study provide new perspectives for the study of the mechanism of aluminum-induced cognitive dysfunction. At the same time, this study has some limitations. We investigated only the expression of total GFAP in cells and did not discuss the different isoforms of the GFAP molecule in more depth. It has also been pointed out that a single molecular marker of reactive glial cell proliferation is somewhat flawed.[43] Since this study focused on confirming whether the novel molecule LNC000152 plays a role in reactive glial cells due to aluminum exposure, the specific mechanisms of glial cell reactivity as well as the major signaling pathways of cell proliferation were not explored. This is one of our future research directions. There are further studies indicating that activated reactive astrocytes are considered to be one of the prominent neuropathological features of AD and that GFAP concentrations are associated with longitudinal decline in cognitive performance.[54] These findings suggest that there is a correlation between the proliferation of reactive astrocytes and

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cognitive dysfunction. This also suggests the need to focus on GFAP changes in the mechanism of aluminum-induced neurological damage. This also provides a prerequisite for further exploration of the correlation between reactive astrocyte proliferation and cognitive function.

5. CONCLUSIONS

In conclusion, our results suggest that aluminum can promote astrocyte responsiveness by upregulating LNC000152 and that LNC000152 affects the effect of aluminum on the expression of GFAP, which promotes glial cells, and on the phenomenon of cell proliferation. The study of the relationship between LNC000152 and astrocyte responsiveness and proliferation may provide a new idea to explore the role of lncRNA in the mechanism of aluminum-induced cognitive impairment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09702.

CTX-TNA2 cells viability test; primary astrocytes viability test; CTX-TNA2 cells transfected with si-LNCRNA_000152; EdU (red) and Hoechst 33,342 (blue) double stained to assess astrocytes' proliferation; primer sequences for RT-PCR; expression levels of LNC000152 in CTX TNA2 cells intervened by different transfection sequences; expression levels of LNC000152 in CTX TNA2 cells intervened by different transfection times (siRNA-6306); cell proliferation index of CTX-TNA2 cells exposed to different concentrations and times of Al(mal)3; CTX-TNA2 cells transfected with si-LNCRNA_000152, and cell proliferation index evaluated by flow cytometry (PDF)

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Author Contributions

Z.Z.: Data curation, formal analysis, and writing—original draft. X.L.: Data curation and formal analysis. L.M.: Investigation. S.W.: Methodology. J.Z.: Validation. Y.Z.: Validation. X.G.: Visualization. Q.N.: Conceptualization, writing—review and editing, and funding acquisition.

Notes

The authors declare no competing financial interest.

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