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Original Article

Overexpression of lncRNA Gm43050 alleviates apoptosis and inflammation response induced by sevoflurane treatment by regulating miR-640/ZFP91

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Abstract: Aims: The present study investigated the function and mechanism of lncRNA Gm43050 in sevoflurane-induced abnormal cognition. Methods: Primary hippocampal neurons were used to establish the model of abnormal cognitive disorder. Overexpression and knockdown experiments were performed to analyze cell death rates, proliferation, apoptosis and the inflammatory response. The dual-luciferase reporter assay was used to analyze the potential binding targets of lncRNA Gm43050. Rescue experiments were used to assess the downstream targets of Gm43050. Results: We found that lncRNA Gm43050 was in the cytoplasm. Overexpression of lncRNA Gm43050 had no impact on proliferation but significantly reduced the cell death rates and apoptosis. The inflammation markers IL-6, IL-1β, IL-8 and TNF-α were manifestly downregulated in the overexpression group. Opposite effects were detected in the lncRNA Gm43050 knockdown group. Bioinformatics analysis showed that miR-640 may be the potential target of Gm43050. Additionally, we found that ZFP91 was the downstream target of miR-640. Conclusion: We provided comprehensive data of the function and mechanism of lncRNA Gm43050 in abnormal cognition. Our study showed that lncRNA Gm43050 exerted its important role via the regulation of miR-640 and ZFP91.

Keywords: Sevoflurane, Gm43050, miR-640, ZFP91

Introduction

Abnormal cognition after anesthesia, which is also named postoperative cognitive dysfunction (POCD), is a common complication following anesthesia [1, 2]. It is defined as a decline in cognitive function, especially in memory and executive function, which may last for weeks or years [3-5]. From November 1, 1994 to May 31, 1996, Moller performed the famous ISPOCD1 study in 13 hospitals with a sample size of 1218 patients aged at least 60 years and found that POCD occurred in 25.8% of patients 1 week after surgery and 9.9% of patients 3 months after surgery [6]. The more recent research of Johnson reported that the incidence of POCD was up to 19.2% 1 week after surgery, even in middle-aged patients [7]. It is well-known that the incidence of POCD correlates patient age. With the rapidly aging population, there is no doubt that the incidence of POCD will increase annually and command more social resources.

Previous research revealed that increasing age, the duration of anesthesia, and postoperative infections were risk factors for early postoperative cognitive dysfunction, which indicates that hypoxia and inflammatory responses may play critical roles in the development of POCD [8-10]. For example, Cao and colleagues found that hypoxia-inducible factor-1α (HIF-1α) and HIF-1α-dependent neuroinflammation were involved in isoflurane-induced POCD [11]. Zhu and colleagues found that peripheral immune cell-induced inflammation worsened isoflurane-induced POCD [12]. Although many studies were performed to elucidate the mechanisms of POCD, the molecular mechanism are not clear.

Noncoding RNAs (ncRNAs) are RNA molecules that cannot be translated into proteins [13], and long noncoding RNAs (lncRNAs) are generally longer than 200 nt [14]. LncRNAs participate in almost every cell life activity and the development of human diseases, including POCD. For example, Zhang and colleagues identi-

fied 68 dysregulated lncRNAs in a POCD group compared with a non-POCD group [15]. Li found 868 differentially expressed lncRNAs in aged mice with POCD [16]. Our previous research found that the overexpression of lncRNA Gm-15621 alleviated cell apoptosis and inflammation response in sevoflurane-treated primary hippocampal neurons via the lncRNA Gm15-621/miR-133a/Sox4 axis [17]. Our current research demonstrated that lncRNA Gm43050 was downregulated in neurons treated with sevoflurane. We found that the reduced expression of lncRNA Gm43050 correlated with increased cell death rate, apoptosis rate and inflammation response, which may influence the process of POCD. Bioinformatics analysis revealed that miR-640 may be the potential target of Gm43050 because the base sequences were partially complementary according to the base pairing rules. Bioinformatics analysis also revealed that miR-640 may target the 3'-UTR of the mRNA of zinc finger protein 91 (ZFP91), which positively regulated the production of IL-1β and plays a role in the inflammation response.

We hypothesized that lncRNA Gm43050 played a protective in sevoflurane-induced neurotoxic effects via the sponging of miR-640, which increased the expression of ZFP91 and its downstream signaling. The findings of this research reveal a new target for inhibiting or reversing POCD.

Materials and methods

Cell culture and sample preparation

Primary hippocampal neurons were prepared as described previously [17]. Different concentrations of sevoflurane were used for six hours to establish the neurotoxicity model. The 293T cells were stored in our laboratory. Tissue samples were harvested from sacrificed C57BL/6 mice.

Transfection

SiRNAs of lncRNA Gm43050 and the overexpression vectors of lncRNA Gm43050 were constructed by Gene Pharma (Gene Pharma, China). Vectors with the wild-type (WT) or mutant (mut) binding sites for miR-302b were constructed by Gene Pharma (Shanghai, China). The 3'-untranslated region (UTR) of ZFP91, with the wild-type (WT) or mutant (mut) binding sites for miR-302b, was amplified and cloned into the pGL3 vector (Promega, USA) to generate the vector pGL3-WT-ZFP91-3'-UTR or pGL3-mut-ZFP91-3'-UTR. The miR-640 mimics, NC mimics, miR-640 inhibitor, and NC inhibitor were purchased from Shanghai Gene Pharma. Primary hippocampal neuronal cells and 293T cells were transfected with vectors, miR-640 mimics, NC mimics, miR-640 inhibitor, and NC inhibitor when cell confluence reached 70%. Transfection was performed using Lipo3000 reagent (Invitrogen, USA) according to the manufacturer's protocol. Cells were incubated for 36-48 h before further use.

CCK-8 assay

Primary hippocampal neurons were seeded into a 96-well plate for the CCK-8 assay. CCK-8 reagent (Dojindo, Japan) was added to each well at 0 h, 24 h, 48 h, and 72 h and incubated at 37°C for 2 h. The optical density was measured at 450 nm.

Cell death assay

Cell death rates were measured using Trypan blue (Beyotime, China) staining. Cells were digested for 3 min and pipetting thoroughly.

TUNEL staining

Terminal deoxytransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assays were performed to detect cell apoptosis. The apoptotic cells were visualized using TUNEL staining according to the manufacturer's instructions (Beyotime, China). The fluorescence density was assessed using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed using the interleukin-6 (IL-6), IL-1β, IL-8, and tumor necrosis factor-α (TNF-α) ELISA kit (Elabscience, China). After the cells were transfected with siRNA, vectors or microRNA mimics, the supernatants of cell cultures were collected and concentrated to remove cellular debris. The secretion levels of IL-6, IL-1β,

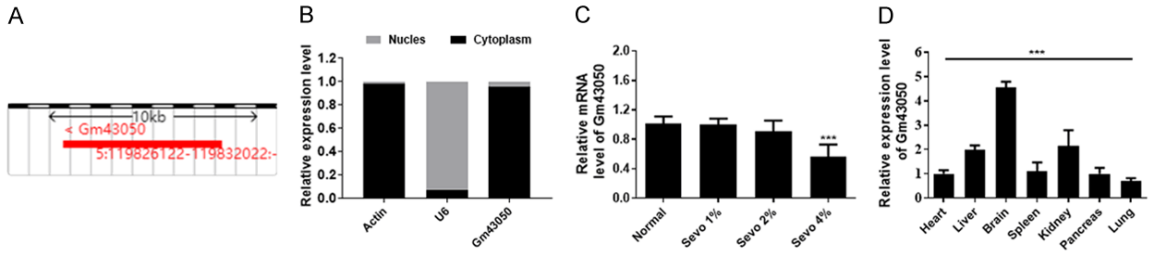

Figure 1. Bio-characteristics of lncRNA Gm43050. A. NCBI showed the location of lncRNA Gm43050. B. lncRNA Gm43050 was mainly expressed in cytoplasm. C. The expression of lncRNA Gm43050 was reduced with the increasing concentrations of sevo. D. The expression of lncRNA Gm43050 was highest in brain tissues.

IL-8, and TNF-α in primary hippocampal neurons were detected.

Dual-luciferase reporter assay

The dual-luciferase reporter assay was performed using the Dual-Luciferase® Reporter Assay System (Promega, USA). LncRNA Gm43050 vectors with wild-type or mutant binding sites for miR-640 were cotransfected with miR-640 mimics or NC mimics in 293T cells. Luciferase activity was analyzed using multiscan spectrum (Thermo Fisher Scientific, USA).

Real-time PCR

Total RNA was extracted by using TRIzol reagent (Thermo Fisher Scientific, USA). RNA reverse transcription was performed using a PrimeScript™ RT reagent Kit with a gDNA eraser (Takara, Japan), and real-time PCR was performed using SYBR® Premix Ex Taq™ (Takara, Japan). The data were normalized to GAPDH levels and further analyzed using the 2-ΔΔCT method.

Western blotting

Cells were harvested and lysed using RIPA lysis buffer containing proteinase inhibitor (Roche, USA). Total protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples were resolved in a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. Membranes were blocked and incubated with primary antibodies against ZEP91 (Abcam, USA) and GAPDH (Abcam, USA) at 4°C overnight, followed by incubation with a peroxidase-conjugated goat anti-rabbit (or mouse) IgG antibody. Immunopositive bands were analyzed using a FluorChem M system (ProteinSimple, USA).

RNA binding protein immunoprecipitation (RIP)

Primary hippocampal neurons were transfected with miR-640 mimics or NC mimics, incubated for one day and lysed. An antibody against mouse Ago2 (Abcam, USA) was used for protein immunoprecipitation. RIP was performed using the Magna RIP Kit (Millipore, USA) according to the manufacturer's instructions. After Ago2 immunoprecipitation and protein-RNA complex digestion, the RNA was isolated using TRIzol reagent, and the expression levels of lncRNA Gm43050 and the mRNA of ZFP91 were detected using qRT-PCR.

Data analysis

We used SPSS 25.0 to calculate the values (means ± standard error of the mean). Statistical analyses were performed using two-sided Student's t-test or one-way ANOVA. The statistical significance was $P<0.05$.

Results

Biological characteristics of lncRNA Gm43050

As shown in **Figure 1A**, we found the location of lncRNA Gm43050 on the chromosome. We separated the cytoplasmic and nuclear components, and qRT-PCR of these two components revealed that lncRNA Gm43050 was primarily located in the cytoplasm (**Figure 1B**). We further investigated the expression of lncRNA Gm43050 in primary hippocampal neurons treated with different concentrations of sevoflurane and found that the expression of lnc-

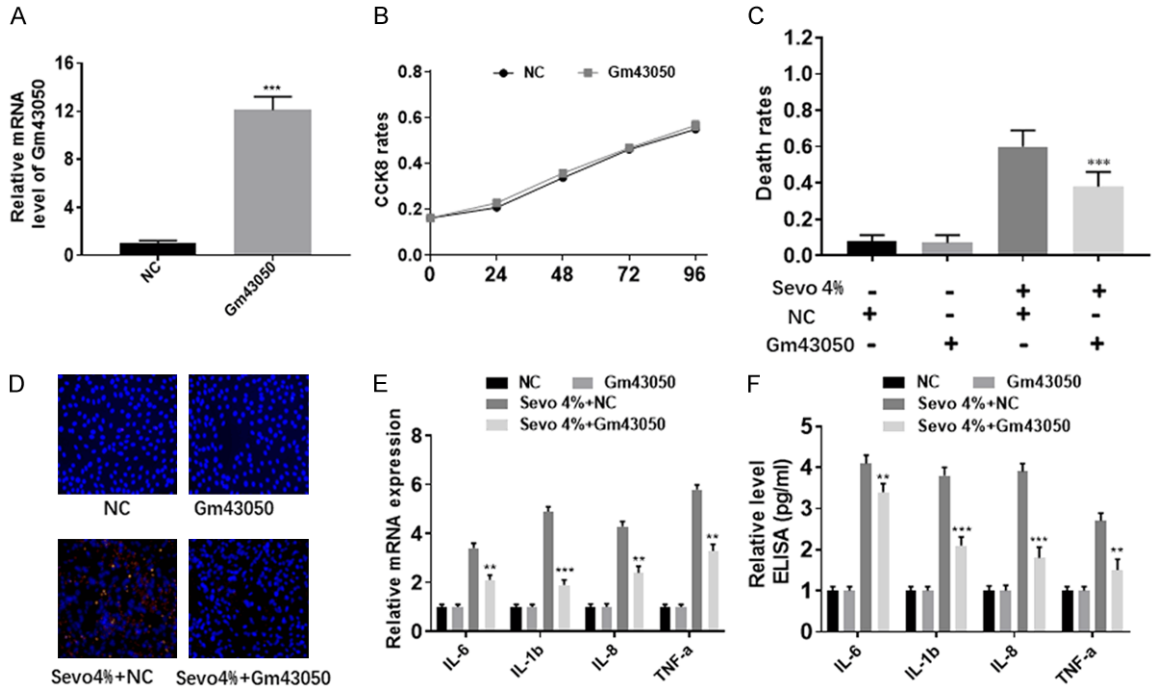

Figure 2. Function of overexpression of lncRNA Gm43050. A. The overexpression efficiency was verified by qRT-PCR. B. Overexpression of lncRNA Gm43050 has no impact on proliferation. C. Overexpression of lncRNA Gm43050 significantly reduced the cell death rates. D. Tunel assay showed that overexpression of lncRNA Gm43050 significantly decreased the apoptosis cell rates. E. Real-time PCR results showed that overexpression of lncRNA Gm43050 significantly decreased the expression of inflammation markers. F. ELISA results showed that overexpression of lncRNA Gm43050 significantly decreased the expression of inflammation markers.

RNA Gm43050 decreased with increasing concentrations of sevoflurane, especially in 4% sevoflurane-treated neurons (**Figure 1C**). We also detected the expression of lncRNA Gm43050 in different tissues derived from C57BL/6 mice and found that lncRNA Gm43050 was primarily expressed in brain (**Figure 1D**).

Effects of the overexpression of lncRNA Gm43050 in neurotoxicity

To investigate the biological function of lncRNA Gm43050, we constructed an overexpression vector of lncRNA Gm43050 and transfected the vector into primary hippocampal neurons. The expression of lncRNA Gm43050 was significantly upregulated in neurons transfected with overexpression vectors compared to empty vectors (**Figure 2A**). CCK-8 assays were performed to detect the influence of lncRNA Gm43050 on cell proliferation activity, and the results showed that lncRNA Gm43050 had no impact on cell proliferation (**Figure 2B**). The cell death and TUNEL staining assays revealed that the overexpression of lncRNA Gm43050 in neurons treated with 4% sevoflurane significantly reduced the cell death rate and cell apoptosis rate, which indicated that lncRNA Gm43050 played a protective role in cell survival (**Figure 2C**, **2D**). We used qRT-PCR and ELISA to detect the expression of inflammatory markers in neurons treated with 4% sevoflurane and found a significant suppression of inflammatory markers, such as IL-6, IL-1β, IL-8, TNF-α, in neurons transfected with overexpression vectors (**Figure 2E**, **2F**).

Effect of knockdown of lncRNA Gm43050 in neurotoxicity

SiRNAs was used to suppress the expression of lncRNA Gm43050. qRT-PCR of neurons transfected with siRNAs revealed that siRNA-3 significantly reduced the expression of lncRNA Gm43050 (**Figure 3A**). Trypan blue staining and the TUNEL assay revealed that the inhibition of lncRNA Gm43050 expression in 4% sevoflurane-treated neurons significantly increased cell death and apoptosis rate (**Figure 3B-D**). qRT-PCR and ELISA revealed that inhibiting the expression of lncRNA Gm43050 in

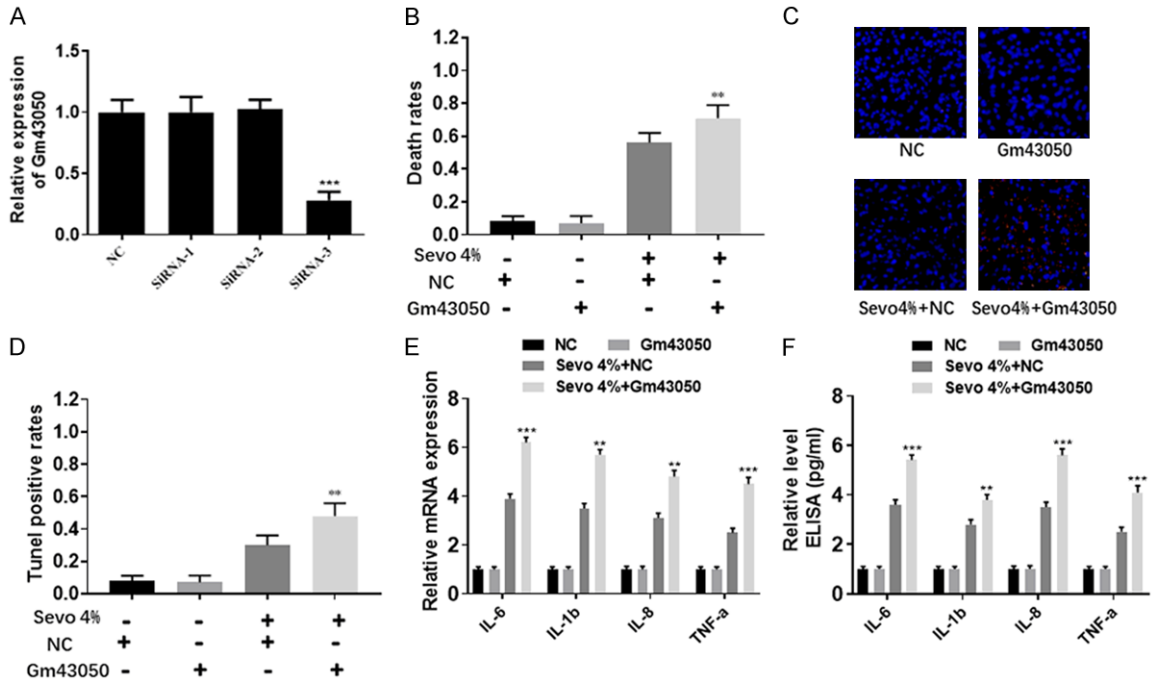

Figure 3. Function of knockdown of lncRNA Gm43050. A. The knockdown efficiency was verified by qRT-PCR. B. Knockdown of lncRNA Gm43050 significantly increased the cell death rates. C. Tunel assay showed that knockdown of lncRNA Gm43050 significantly increased the apoptosis cell rates. D. Quantification results showed knockdown of lncRNA Gm43050 promotes cell apoptosis. E. Real-time PCR results showed that knockdown of lncRNA Gm43050 significantly increased the expression of inflammation markers. F. ELISA showed that knockdown of lncRNA Gm43050 significantly increased the expression of inflammation markers.

4% sevoflurane-treated neurons significantly increased the expression of inflammatory markers, such as IL-6, IL-1β, IL-8, TNF-α (**Figure 3E**, **3F**).

miR-640 is the direct target of lncRNA Gm43050

Bioinformatics analysis revealed that miR-640 was a potential target of Gm43050 because the base sequences were partially complementary according to the base-pairing rules. The potential binding sequence is shown in **Figure 4A**. The dual-luciferase reporter assay revealed that the relative luciferase activity was significantly reduced in 293T cells cotransfected with miR-640 mimics and lncRNA Gm43050 vectors with the wild-type binding site for miR-640 mimics compared to the NC group and mutant group (**Figure 4B**), which indicated the direct combination of miR-640 and lncRNA Gm43050. We detected the expression of miR-640 in primary hippocampal neurons treated with different concentrations of sevoflurane and found that miR-640 was upregulated with increasing concentrations of sevoflurane, especially in cells treated with 4% sevoflurane (**Figure 4C**). We further investigated the expression of miR-640 in neurons transfected with siRNA or the overexpression vector of lncRNA Gm43050 and found that the overexpression of lncRNA Gm43050 reduced the expression of miR-640 and vice versa (**Figure 4D**). Taken together, our results showed that lncRNA Gm43050 negatively regulated the expression of miR-640 (**Figure 4E**).

miR-640 negatively regulates ZFP91

Bioinformatics analysis showed that ZFP91 may be the potential target of miR-640 (**Figure 5A**). Dual-luciferase reporter assay results revealed that the relative luciferase activity was significantly reduced in 293T cells cotransfected with miR-640 mimics and the pGL3-WT-ZFP91-3'UTR vector compared to the 293T cells cotransfected with pGL3-WT-ZFP91-3'UTR and mimic NC or pGL3-mut-ZFP91-3'UTR and miR-640 mimics (**Figure 5B**), which indicated that the 3'-UTR of ZFP91 mRNA was the direct

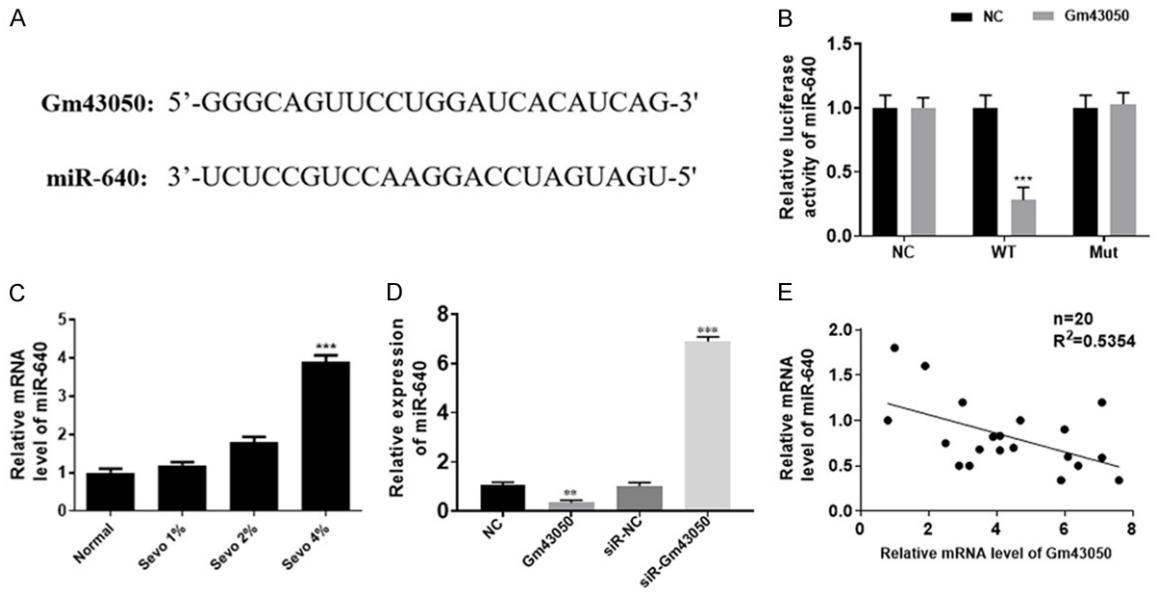

Figure 4. miR-640 is the direct target of lncRNA Gm43050. A. The potential binding sequence was showed. B. Dual-luciferase reporter assay demonstrated that miR-640 is the direct target of lncRNA Gm43050. C. The expression of miR-640 was increased in a dose-dependent manner. D. Overexpression of lncRNA Gm43050 significantly reduced the expression of miR-640 and vice versa. E. lncRNA Gm43050 negatively regulate the expression of miR-640.

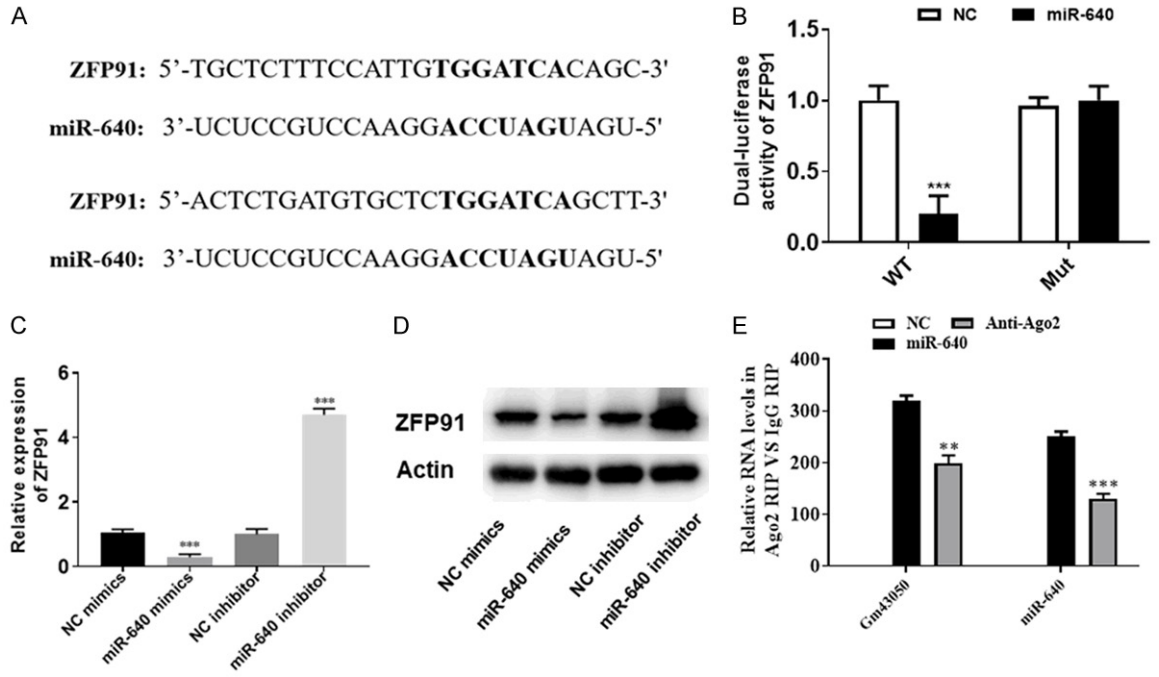

Figure 5. miR-640 negatively regulate ZFP91. A. Bioinformatics analysis showed that ZFP91 may be the potential target of miR-640. B. Dual-luciferase reporter assay demonstrated that ZFP91 is the direct target of miR-640. C. Overexpression of miR-640 inhibited the expression of ZFP91 and vice versa via qRT-PCR. D. Overexpression of miR-640 inhibited the expression of ZFP91 and vice versa via western blot. E. RIP experiment showed the binding relationship between miR-640 and ZFP91 and lncRNA Gm43050.

target of miR-640. We found that the expression of ZFP91 was significantly reduced in primary hippocampal neurons transfected with miR-640 mimic compared to neurons trans-

fected with NC mimics, miR-640 inhibitors, and NC inhibitors (**Figure 5C**, **5D**). We further performed an RNA-binding protein immunoprecipitation experiment and found that lncRNA Gm43050 and ZEP91 mRNA were enriched in Ago2 immunoprecipitation in neurons transfected with miR-640 mimics (**Figure 5E**).

lncRNA Gm43050/miR-640/ZFP91 axis in neurotoxicity

We found that the expression of lncRNA Gm43050 positively correlated with the expression of ZFP91, at the mRNA and protein levels, in primary hippocampal neurons transfected with the overexpression vector and siRNA of lncRNA Gm43050 (**Figure 6A**, **6B**). We performed the rescue experiment and found that transfection with the miR-640 mimic in neurons transfected with the overexpression vector of lncRNA Gm43050 significantly rescued the lncRNA Gm43050-induced upregulated expression of ZFP91 (**Figure 6C**, **6D**). The cell death rate, apoptosis rate, and the expression of inflammatory markers, such as IL-6, IL-1β, IL-8, and TNF-α, were also reversed in 4% sevoflurane-treated neurons cotransfected with the overexpression vector of lncRNA Gm43050 and miR-640 mimics (**Figure 6E-H**).

Discussion

Postoperative cognitive dysfunction (POCD) is a common complication following anesthesia [18-20]. Patients with POCD are at an increased risk of death in the first year after surgery [21]. With the rapid aging of the population, the discussion of POCD is getting increasingly fierce.

The mechanism of POCD is not clear. Previous research indicated that the body's inflammatory response to surgery may play an important role in the development of POCD [1, 3]. LncRNA is a newly emerging regulatory factor of the inflammatory response [22]. For example, Bai and colleagues reported that lncRNA NEAT1 induced corneal neovascularization via promotion of the inflammatory response [23]. Sun and colleagues found lncRNA NRON alleviated atrial fibrosis via suppression of M1 macrophage activation [24]. The current research demonstrated that lncRNA Gm43050 was overexpressed in sevoflurane-treated primary hippocampal neurons, which indicated that lncRNA Gm43050 may play an important role in anesthesia-induced neurotoxicity. We found that the expression of lncRNA Gm43050 correlated with reduced cell death, apoptosis and inflammatory markers, such as IL-6, IL-1β, IL-8, and TNF-α. LncRNA Gm43050 likely plays a protective role in anesthesia-induced neurotoxicity. LncRNAs may act via various methods, and their function depends on their location [25, 26]. For example, nucleus lncRNAs establish and maintain the conformation of the chromosome and regulate alternative splicing, and lncRNAs in the cytoplasm modulate mRNA stability and translation via the sequestering of microRNAs [27]. The current research demonstrated the lncRNA Gm43050 was primarily located in the cytoplasm, and we hypothesized that lncRNA Gm43050 acted as a microRNA sponge that influenced the expression the microRNA-targeted mRNA, which is the most widely investigated mechanism of lncRNAs located in cytoplasm. We performed bioinformatics analysis to test this hypothesis and found that miR-640 may be the direct target of lncRNA Gm43050 because the base sequences were partially complementary.

MicroRNAs are also members of the noncoding RNA family that mediate cell activities via combination with the 3'-UTR of the targeted mRNA, which curbs the function of the downstream signaling. MiR-640 may participate in the development of human diseases, such as chronic lymphocytic leukemia and ovarian carcinoma. Dong and colleagues recently found that miR-640 aggravated intervertebral disc degeneration via the NF-κB and WNT signaling pathway, which indicates that miR-640 may also play a role in the inflammatory response [28]. The current research used bioinformatics analysis and revealed that the mRNA of ZFP91 may be the direct target of miR-640, and we demonstrated their combination using a dual-luciferase reporter assay. qRT-PCR and Western blotting revealed that miR-640 negatively regulated the expression of ZFP91 at the RNA and protein levels. We performed the RIP experiment and found that lncRNA Gm43050 and the mRNA of ZEP91 were enriched in Ago2 immunoprecipitation in neurons transfected with miR-640 mimics. These results further confirmed the interaction between lncRNA Gm43050 and miR-640 and miR-640 and the 3'-UTR of ZFP91 mRNA.

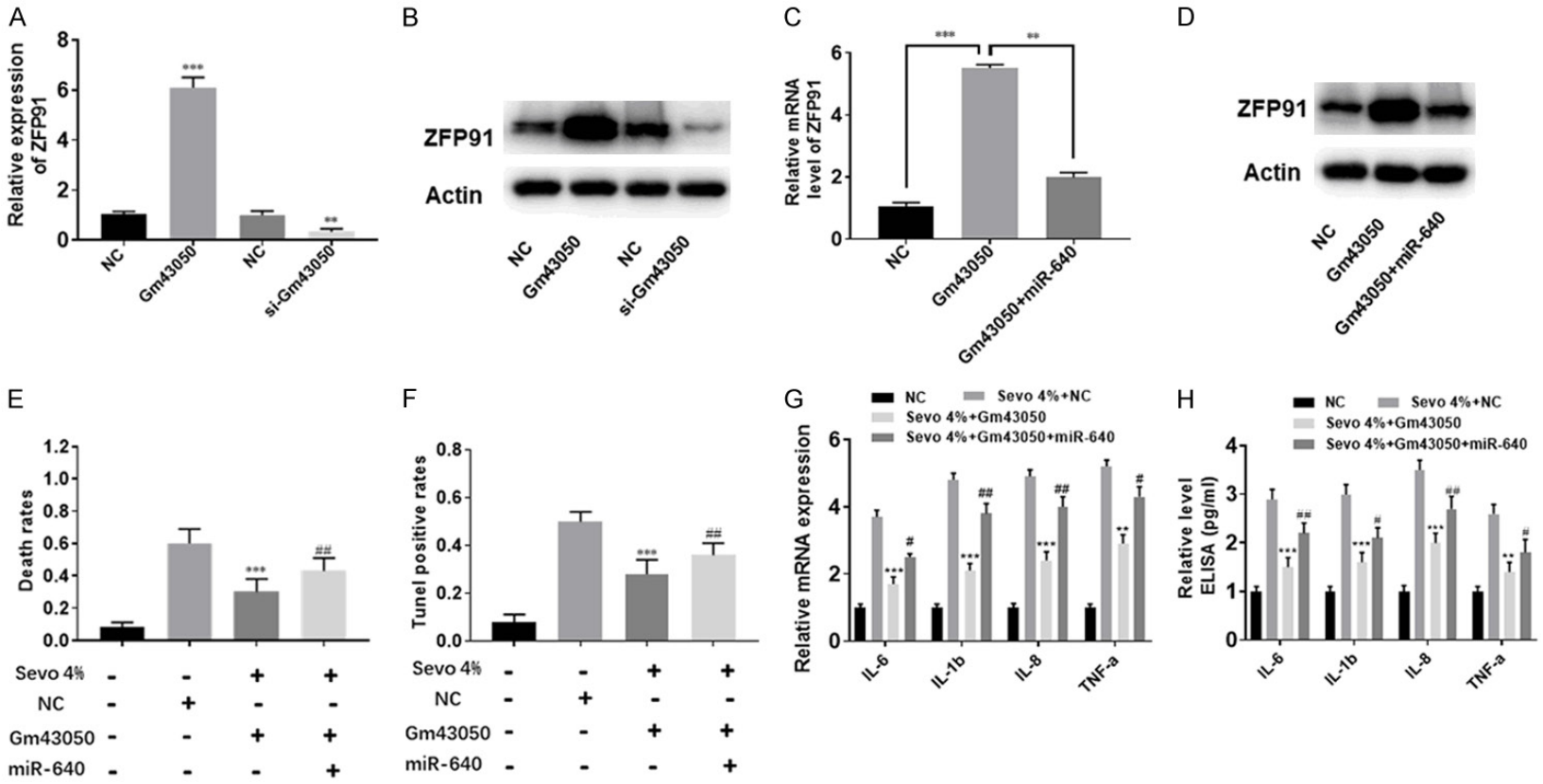

Figure 6. lncRNA Gm43050 exerts its important role through regulating miR-640 and ZFP91. A. Overexpression of lncRNA Gm43050 significantly increased the expression of ZFP91 and vice versa by real-time PCR. B. Overexpression of lncRNA Gm43050 significantly increased the expression of ZFP91 and vice versa by western blot. C. Co-transfection of Gm43050 and miR-640 significantly reduced the expression of ZFP91 compared with Gm43050 group via real-time PCR. D. Co-transfection of Gm43050 and miR-640 significantly reduced the expression of ZFP91 compared with Gm43050 group by western blot. E. Rescue experiment showed that overexpression of miR-640 can block the anti-apoptosis effect of Gm43050. F. Tunel assay revealed that overexpression of miR-640 can reverse the effect of Gm43050. G. Inflammation markers were assessed via real-time PCR. H. ELISA experiment was performed to further confirm the relationship between miR-640 and Gm43050.

We also performed the rescue experiment to validate the axis of lncRNA Gm43050/miR-640/ZFP91. We detected the expression of ZFP91 in primary hippocampal neurons transfected with the siRNA and overexpression of lncRNA Gm43050 and found that lncRNA Gm43050 expression positively regulated the expression of ZFP91, which was consistent with our hypothesis. We transfected the miR-640 mimics in neurons that were already treated with the lncRNA Gm43050 overexpression vector. The results revealed that transfection of miR-640 mimics in the previously treated neurons significantly rescued the lncRNA Gm-43050-induced protective role on cell death and apoptosis and the suppressed inflammatory response.

In summary, the current research demonstrated that lncRNA Gm43050 was downregulated in 4% sevoflurane-treated neurons and that lncRNA Gm43050 reduced cell death rate and apoptosis rate and the inflammatory response. Bioinformatics analysis suggested that lncRNA Gm43050 sponged miR-640, which may also target the 3'-UTR of ZFP91 mRNA. To verify this axis, dual-luciferase reporter assay, cell transfection and RIP experiments were performed, and the results were consistent with our previous hypothesis. We also performed a rescue experiment and found that transfection with miR-640 mimics in lncRNA Gm43050 overexpression vector-treated neurons significantly increased the cell death rate, apoptosis rate and inflammatory response. The current results validated the role of the lncRNA Gm43050/miR-640/ZFP91 axis for further investigation of these new targets for the inhibition or reversal of POCD.

Acknowledgements

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Disclosure of conflict of interest

None.

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