Suppression of microRNA-141 suppressed p53 to protect against neural apoptosis in epilepsy by SIRT1 expression

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Abstract
We investigated that microRNA (miRNA)-141 protects against epilepsy-induced apoptosis and its reaction mechanism. The serum expression of miRNA-141 in epilepsy model mice and control volunteer was measured by quantitative reverse-transcription polymerase chain reaction. We found that miRNA-141 serum expression was upregulated in patients with epilepsy. Overexpression of miRNA-141 induced nerve cell apoptosis, suppressed proliferation, promoted caspase-3/9, Bax and p53 protein expression, and reduced silent information regulator 1 (SIRT1) protein expression in vitro model. In addition, the downexpression miRNA-141 using si-miRNA-141 reduced nerve cell apoptosis and increased proliferation, suppressed caspase-3/9, Bax and p53 protein expression, induced SIRT1 protein expression. SIRT1 inhibitor (nicotinamide) decreased SIRT1, reduced the effects of miRNA-141 on nerve cell apoptosis in vitro model of epilepsy through SIRT1/p53. SIRT1 agonist also reduced the effects of miRNA-141 overexpression on nerve cell apoptosis in vitro model of epilepsy through SIRT1/p53. Our preliminary findings indicate that anti-miRNA-141 protects against epilepsy-induced apoptosis via SIRT1/p53 expression.

KEYWORDS
apoptosis, epilepsy, microRNA-141, p53, SIRT1

1 | INTRODUCTION

Epilepsy is a kind of common disease in neurology, which is related to the abnormal synchronization of neuronal discharges in the brain. The repeated-epileptic seizure has severely damaged human health and has caused tremendous physical and mental damage to patients, and its pathogenesis has not been completely understood at present, which may be related to structural and functional injuries of the hippocampus and the limbic system induced by pathological changes such as neuronal apoptosis, mossy fiber sprouting, and synaptic plasticity. Neuronal apoptosis is one of the major forms of postepilepsy neuronal death, the mechanism of which can be attributed to the substantial production of free radicals as well as the activation of cell–death associated protease after epilepsy, thus leading to death and deletion of a large number of neurons. Although novel antiepilepsy agents have been developed in recent years, about one-third

Abbreviations: IE, intractable epilepsy; LSD, least significant difference; MUT, mutated; RIPA, radioimmunoprecipitation; TLE, temporal lobe epilepsy; WT, wild-type.

Ding Liu and Shu Li are co-first authors.
of patients with epilepsy develop intractable epilepsy (IE). Neuronal apoptosis plays an extremely important role in the genesis and development of epilepsy, therefore, drug intervention of the postepilepsy neuronal apoptosis will become a new pathway for treating epilepsy.5

MicroRNA (miRNA) is a kind of endogenous noncoding small RNA, which has a regulatory function, with about 18 to 23 nucleotides in length.6 miRNA is extensively distributed in animals and plants, which can degrade the target messenger RNA (mRNA) or blocking the translation of target mRNA through the formation of RNA-induced silencing complex, and the regulated target gene is frequently involved in multiple biological processes, such as cell proliferation, apoptosis, and tumor genesis; for instance, miR-430 which is related to brain development of zebrafish, miR-181 which participates in hemopoietic cell differentiation of mammals, and miR-143 that takes part in adipose tissue differentiation.7,9 It has been indicated in numerous studies that diseases of multiple human systems are closely related to miRNA, such as tumor, cardiovascular disease, blood disease, brain development, and nervous system disease.10

Research on miRNAs and their target genes in the central nervous system has become the hotspot at present.6 So far, about 50% of miRNAs are found to be expressed in mammal brains, among which many miRNAs have played important roles in the genesis of nervous system diseases, including stroke, Parkinson’s disease, and epilepsy.11 It has been discovered in recent research that miRNA is closely associated with the genesis and development of epilepsy, and its negative regulation of posttranscription gene level may participate in the molecular pathological process of forming the status epilepticus and chronic epilepsy.12

Previous experiments have demonstrated that multiple miRNAs have taken part in cell apoptosis, such as the let-7 family, and more and more apoptosis-associated miRNAs will be discovered and verified as the studies advance increasingly.13 The miRNA-141 family is one of the apoptosis-associated miRNAs that have attracted wide attention in recent years, which is studied as a tumor suppressor gene because it can induce neural tumor cell apoptosis.14 It is found in subsequent research that it plays a critical role in the biological process of tumor cells through treating the tumor suppressor gene p53 as the regulatory target; after that numerous studies have been carried out to reversely study the functions of miRNA-141 as well as its target mRNA.15 In numerous cancers, the expression quantities of miRNA-141 are downregulated, which may affect tumor cell apoptosis, aging, proliferation, and infiltration.16

Acetylation and deacetylation are one of the important forms to regulate gene expression and posttranscriptional protein modification, and silent mating-type information regulation 2 (Sir2) is the first NAD-dependent histone deacetylase that is found to prolong the replication lifetime of yeast.17 Silent information regulator 1 (SIRT1), a kind of histone deacetylase found in mammals that is homologous to Sir2, is highly conserved during genetic process and extensively expressed in all organs in the body, catalyzes histone as well as diversified nonhistones and thus regulates gene expression and protein activity; as a key enzyme in all physiological processes, it participates in multiple biological effects, such as chromatin remodeling, transcription inhibition, inflammatory response, energy metabolism, as well as cell survival and apoptosis.18 Therefore, we hypothesized that the suppression of miRNA-141 would protect against epilepsy-induced apoptosis via SIRT1/p53 expression. In this study, we investigated that protective effect of miRNA-141 against epilepsy-induced apoptosis and its reaction mechanism.

2 | MATERIALS AND METHODS

2.1 | Quantitative reverse-transcription polymerase chain reaction

Total cellular RNA was isolated with the miRNeasy Mini Kit (Qiagen, Redwood, CA) and 1-μg total RNA was carried out to compound complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). PCR reactions were run on a StepOne Plus Real-Time PCR machine (Applied Biosystems) using TaqMan quantitative reverse-transcription polymerase chain reaction (qRT-PCR; Invitrogen). Reactions were incubated at 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds and 60°C for 30 minutes. The relative expression was calculated using the 2^(-ΔΔCt) method.

2.1.1 | Gene expression profiling

For microarray analysis, cDNA was hybridized onto Affymetrix rat Genome U133 Plus 2.0 Array at 45°C for 24 hours. Fluidic Station-450 and GeneChips are scanned with Affymetrix GeneChip Scanner 7G (Silicon Genetics, Redwood, CA). Raw microarray data were analyzed using the GeneSpring GX 10 software (Silicon Genetics).

2.2 | In vivo model

Seven-week-old Wistar Han male rats were housed in a 12-hour light/dark cycle in a temperature of 141°C ± 2°C with food and water ad libitum. All animal experiments were approved by the Experimental Animal Ethical Committee of Xiangya Third Hospital of Middle South University. Rats were administered subcutaneous injections of kainic acid (5 mg/kg) for 10 minutes and
subcutaneous injections of kainic acid (2.5 mg/kg) for 45 minutes. Behavioral seizure responses were scored according to the Racine grading standard: 0, no reaction; 1, facial twitches (lips, nose, and eyes); 2, head nodding; 3, unilateral forelimb clonus; 4, earing with bilateral forelimb clonus; 5, imbalance and falling on side or back.

### 2.3 Immunohistochemistry

The rat was killed using decollation under 35 mg/kg pentobarbital sodium. The brains were removed and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. The brains were transferred to 30% sucrose for 24 hours at 4°C and sectioned at 10 μm. Sections were incubated with glial fibrillary acidic protein (GFAP) antibody (antirabbit, 1:100; Cell Signaling Technology, Danvers, MA) overnight at 4°C. The brain sections were viewed by confocal microscopy (Olympus Inc, Center Valley, PA).

### 2.4 Cell culture, transfections

C6 glioma cell line was obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium ( Gibco, Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (Gibco, Life Technologies), 5% CO₂ and 95% air. Negative mimics, anti-miRNA-141, miRNA-141 mimics were acquired from Sangon Biotech (Shanghai, China), and transfected in the cell using Lipofectamine 2000 Reagent (Life Technologies). After transfection for 24 hours, cells were treated with 6-μm kainic acid for other study.

### 2.5 Luciferase reporter assays

pGV306 luciferase reporter plasmids containing either a wild-type (WT) or mutated (MUT) Sirt1 3’UTR were cotransfected with miRNA-141 mimic using Lipofectamine 2000 (Invitrogen). Cells were plated in 24-well plates 24 hours before transfection for 48 hours. Dual Luciferase Assay System from Promega (Madison, WI) was used to measure the luciferase reporter activities.

### 2.6 Caspase-3/9 activity

Cells were obtained and washed with PBS, and lysed by radioimmunoprecipitation (RIPA; Beyotime, Ningbo, China) and protein concentrations were determined using BCA protein assay kit (Beyotime). 5-μg proteins were incubated with caspase-3/9 activity kits for 2 hours at 37°C. Absorbance was measured at 405 nm.

### 2.7 Western blot analysis

Cells were obtained and washed with PBS, and lysed by RIPA (Beyotime), and protein concentrations were determined using BCA protein assay kit (Beyotime). Proteins (20 μg) were separated on 8% to 12% sodium dodecyl sulfate polyacrylamide gels and electrotherapeutically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour at room temperature. The sections were colored using 3,3’-Diaminobenzidine (DAB) assay for 15 minutes and washed with water. The sections were stained using hematoxylin for 1 minute and washed with water. The sections were viewed by confocal microscopy (Olympus Inc, Center Valley, PA).

### 2.8 Immunofluorescence

Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized with 0.3% Triton X-100 for 15 minutes at room temperature and blocked with 5% BSA in TBST for 1 hour at room temperature. Cells were incubated with primary SIRT1 (1:200; Santa Cruz Biotechnology) at 4°C overnight and incubated with Alexa Fluor 488-conjugated secondary Antibody for 1 hour at room temperature. The cells were stained with 4’,6-diamidino-2-phenylindole assay for 15 minutes at darkness and viewed by confocal microscopy (Olympus Inc, Center Valley, PA).

### 2.9 Statistical analyses

Data are presented as the mean ± SD. Differences among three or more than three groups were compared by Student’s t test or one-way analysis of variance followed by least significant difference post-hoc test. Differences were considered statistically significant when P < 0.05.

### 3 RESULTS

#### 3.1 miRNA-141 expression in epilepsy rats

To investigate the expression levels of miRNA-141 in epilepsy rats, we analyzed miRNA-141 using gene chip
and qPCR. Behavioral seizure responses and seizure duration of IE rats were higher than those of normal group (Figure 1A, B). Meanwhile, GABA protein expression was effectively inhibited in epilepsy mice compared with control rat (Figure 1C-E). As shown in Figure 1F, G, miRNA-141 expression levels were increased in epilepsy mice, compared with control negative group.

3.2 Overexpression miRNA-141 reduced proliferation and promoted cell death of vitro model

To investigate the effects of miRNA-141 on cell death of nerve cell in epilepsy, we used miRNA-141 to increase miRNA-141 expression in nerve cell (Figure 2A). As expected, overexpression increased miRNA-141 expression, inhibited cell proliferation, and increased lactate dehydrogenase (LDH) activity and apoptosis rate in nerve cell compared with control group (Figure 2B-E). Caspase-3, caspase-9 and Bax protein expressions were increased in vitro model, compared with negative group (Figure 2F-I).

3.3 Downexpression miRNA-141 promoted nerve cell proliferation in vitro model

To further investigate whether the roles of miRNA-141 on epilepsy happen, LDH activity and cell proliferation were
measured. There was inhibition of miRNA-141 expression in vitro model, compared with negative group (Figure 3A). Downexpression miRNA-141 promoted cell proliferation, and reduced LDH activity and apoptosis rate in nerve cell compared with control group (Figure 3B-E). Downexpression miRNA-141 reduced caspase-3, caspase-9, and Bax protein expressions were increased in vitro model, compared with negative group (Figure 3F-I). So, there results showed that miRNA-141 regulates nerve cell proliferation and death in vitro model of epilepsy.

### 3.4 miRNA-141 regulates SIRT1/p53 signaling pathway in nerve cell

To identify the mechanism of miRNA-141 on apoptosis of nerve cell in epilepsy, SIRT1/p53 signaling pathway
were measured in this study. miRNA-141 targets the 3′-untranslated region (3′-UTR) of SIRT1, and luciferase reporter activities of miRNA-141 Overexpression was inhibited in WT group, however, luciferase reporter activities of miRNA-141 overexpression was inhibited in Mut group (Figure 4A,B). Immunofluorescence of Figure 4C showed that miRNA-141 overexpression suppressed SIRT1 protein expression in vitro model of epilepsy, compared with negative group. Overexpression of miRNA-141 suppressed SIRT1 protein expression and induced p53 protein expression in vitro model of epilepsy, compared with negative group (Figure 4D-F).
Downregulation of miRNA-141 induced SIRT1 protein expression and suppressed p53 protein expression in vitro model of epilepsy, compared with negative group (Figure 4G-I). These results suggest that miRNA-141 indirectly regulates p53 through SIRT1 in vitro model of epilepsy.
3.5 | SIRT1 participated in the effects of miRNA-141 in epilepsy

To investigate possible effects of SIRT1 on the role of miRNA-141 in apoptosis of epilepsy, SIRT1 inhibitor, nicotinamide 10 mM, was used to inhibit SIRT1 expression in vitro model of epilepsy by miRNA-141 downregulation. As shown in Figure 5A-D, SIRT1 inhibitor suppressed the protein expression of SIRT1, and induced p53 protein expression in vitro model of epilepsy by miRNA-141 downregulation, compared with miRNA-141 downregulation group. Then, CAY10602, SIRT1 agonist, 10 μM, was used to induce the protein expression of SIRT1 in vitro model of epilepsy by miRNA-141 overexpression. As shown in Figure 5E-G, SIRT1 agonist induced SIRT1 protein expression, and suppressed p53 protein expression in vitro.

**FIGURE 5** SIRT1 participated in the effects of microRNA-141 on the SIRT1/p53 signaling pathway in epilepsy. Bax, p53, and SIRT1 expression by statistical analysis (A-C) and Western blot analysis assays of p53 and SIRT1 expression (D) by SIRT1 inhibitor. Bax, p53, and SIRT1 expression by statistical analysis (E-G) and Western blot analysis assays of p53 and SIRT1 expression (H) by SIRT1 agonist. Negative, control negative group; miRNA-141, overexpression of miR-141 group; anti-miRNA-141, downexpression of miR-141 group. SIRT1 (I), SIRT1 inhibitor and downexpression of miR-141 group; SIRT1, SIRT1 agonist and overexpression of miR-141 group. **P < 0.01 compared with control negative group, *P < 0.01 compared with overexpression of miR-141 or downexpression of miR-141 group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SIRT1, silent information regulator 1.
model of epilepsy by miRNA-141 overexpression, compared with miRNA-141 overexpression group. Then, we also found that SIRT1 inhibitor inhibited the effects of miRNA-141 downregulation on the inhibition of cell proliferation, induction of LDH activity and apoptosis rate, activation of caspase-3, caspase-9, and Bax protein expressions in vitro model of epilepsy by miRNA-141 downregulation, compared with miRNA-141 downregulation group (Figure 6). Next, SIRT1 agonist also reduced the effects of miRNA-141 overexpression on induction of cell proliferation, inhibition of LDH activity and apoptosis rate, activation of caspase-3, caspase-9, and Bax protein expressions in vitro model of epilepsy by miRNA-141 overexpression, compared with miRNA-141 overexpression group (Figure 7). These findings suggested that suppression of miRNA-141 suppressed p53 to protect against neural apoptosis in epilepsy by SIRT1 expression.

4 | DISCUSSION

Epilepsy is a kind of chronic cerebral disease that is characterized by recurrent transient brain disorders induced by abnormal neuronal discharges in the brain. Patients with epilepsy are extensively distributed around the world, with the involved population of over 50 million, and about 30% of the newly discovered patients with epilepsy will develop into IE. Focal epilepsy occurring in the temporal lobe is referred to as temporal lobe epilepsy (TLE), and about 70% to 80% of patients with TLE will further develop into IE, which is a common type of IE. Epileptic seizure is not the only damage of epilepsy, instead, longterm and repeated-epileptic seizure usually severely affects the memory, intelligence, language, and executive function of patients, posing tremendous economic and psychological burdens on both the society and
patients with epilepsy. However, the molecular mechanism of the pathogenesis of epilepsy remains unclear, therefore, searching for the pathogenesis of epilepsy and developing novel antiepilepsy agents are the urgent issues to be solved at present. In this study, miRNA-141 serum expression was upregulated in epilepsy mice in epilepsy rats. Xiong et al showed that miRNA-141 may be a functional cancer regulator in glioblastoma.

SE is a repeated or continuous pathological status with epileptic seizure as its clinical feature, and it is also one of the common acute critical diseases in clinic. SE will not only induce acute neuron injury, but can also lead to everlasting central nervous system damage. SE at the acute stage will result in ischemia, hypoxia, and edema of hippocampus; induces cells to release excitatory amino acids (such as glutamic acid); initiates sodium and calcium influx; activates caspase protein consequent reaction; and thus produces the free radicals, neuronal nitric oxide synthase, as well as apoptosis core execution protein kinase caspase-3 to degrade or inactivate the important proteins in cytoplasm, cell nucleus, and cytoskeleton, eventually leading to substantial selective death of neurons in hippocampus, which becomes the foundation for the repeated seizure of chronic epilepsy. We confirmed overexpression miRNA-141 reduced proliferation and promoted cell death of in vitro model epilepsy.

As the major form of neuronal death, apoptosis directly induced postSE neuron loss and brain damage. SE-induced apoptosis triggers the apoptosis pathway through activating the endogenous proapoptotic proteins, such as the Bcl-2 and
caspase families. In addition, a large number of exogenous apoptosis pathways are activated after SE. P53 gene locates in human chromosome 17p13.1, which is the most common proapoptotic gene that plays an important role during the process of neuronal apoptosis. High expression of p53 gene can frequently be seen in the case of neuronal apoptosis; in contrast, administering p53 gene blocker can inhibit cell apoptosis. It is discovered in research that the immunoreactivity of p53 protein is notably increased in the hippocampal tissue removed from patients with intractable TLE. Our study identified the downexpression miRNA-141 reduced caspase-3, caspase-9, Bax, and p53 protein expressions were increased in vitro model. Zhou et al suggest that miRNA-141-3p promotes cell growth and temozolomide resistance by directly targeting p53 in glioma. In the nervous system, SIRT1 is mainly expressed in neurons in cortex, hippocampus, cerebellum, and hypothalamus, which plays an important role in neuronal apoptosis and differentiation, cognitive function, and synaptic plasticity. SIRT1 has neuroprotective effects in multiple acute and chronic nervous system diseases, such as cerebral ischemia, Wallerian degeneration, Huntington’s disease, axonal injury, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and amyotrophic lateral sclerosis. Increasing evidence has indicated that SIRT1 is closely associated with epilepsy, and SIRT1 agonist resveratrol has antiepilepsy effect on kainic acid and FeCl3-induced epilepsy animal models. Thus, we found that miRNA-141 overexpression was suppressed SIRT1 protein expression in vitro model of epilepsy. Yang et al demonstrated that miRNA-141 targets SIRT1 and inhibits autophagy to reduce HBV replication. The limitation of it was that in this study, only in vitro experiments were performed to confirm our hypothesis. Further study with in vivo experiment was still needed to further support the conclusion that anti miRNA 141 could be a novel pathway to treat epilepsy.

Our study confirms the novel role of anti-miRNA-141 protects against epilepsy-induced apoptosis via SIRT1/p53 expression (Figure 8). Our findings might provide evidence for miRNA-141 as novel biomarkers for epilepsy-induced apoptosis.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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