

RETRACTED ARTICLE: Transcription Factor E2F1 Aggravates Neurological Injury in Ischemic Stroke microRNA-122- Targeted Sprouty2

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Yunxia Wu
Zhiqiang Gao
Jiang Zhang

Department of Neurology, Linyi Central
Hospital, Linyi, Shandong 276400,
People's Republic of China

Background: It has been documented that microRNAs (miRs) assume a pivotal role in the development of ischemic stroke (IS). However, we remain poorly identified about the role of miR-122 in IS. Herein, this study was intended to explore the mechanism of E2F1-orchestrated miR-122 in IS.

Patients and Methods: E2F1, miR-122, and SPRY2 expression in serum from patients with IS and oxygen-glucose deprivation (OGD) treated N2a cells was detected by RT-qPCR. After gain- and loss-of-function approaches in OGD-induced N2a cells, GFP staining, flow cytometry, and Western blot analysis were adopted to assess neuronal viability, cell cycle and apoptosis, and expression of apoptosis and autophagy-related proteins, respectively. Meanwhile, mice with IS were induced in which E2F1, miR-122, and SPRY2 were over-expressed, followed by evaluation of neurological deficit and cerebral infarction area. The MAPK pathway activity in tissues of mice and cells was determined.

Results: miR-122 was down-regulated, and E2F1 and SPRY2 were up-regulated in IS patients and OGD-induced N2a cells. E2F1 inhibited miR-122 transcription, while miR-122 targeted SPRY2. Overexpression (OE) of miR-122 or down-regulation of E2F1 or SPRY2 increased viability but decreased apoptosis, cell cycle arrest, and autophagy in OGD-induced N2a cells. In IS mice, the neurological deficit score and cerebral infarction area were elevated, which was aggravated by up-regulating E2F1 or SPRY2 but attenuated by overexpressing miR-122. E2F1/miR-122/SPRY2 axis mediated the MAPK pathway in vivo and in vitro.

Conclusion: Collectively, E2F1 reduced miR-122 transcription to up-regulate SPRY2, which inactivated MAPK pathway and promoted neurological deficit in IS.

Keywords: ischemic stroke; IS, E2F1, microRNA-122, SPRY2, MAPK pathway, neurological injury

Introduction

Ischemic stroke (IS) results from transient or permanent cerebrovascular occlusion, eventually contributing to cerebral infarction.¹ IS occupies 85% of all stroke cases, which is one of the main causes of human disability and death, accounting for over 5.5 million human deaths every year on a global scale.^{2,3} IS exhibits an upward incidence rate in recent decades due to the increase in the global population of the elderly.⁴ The clinical manifestations of IS are mainly focal neurological deficits secondary to ischemic events.⁵ Current major therapeutic approaches for IS aim to achieve reperfusion, neuroprotection, and neurological recovery.⁶ However, only a small proportion of patients with IS can benefit from timely initiation of acute

Correspondence: Jiang Zhang
Department of Neurology, Linyi Central
Hospital, No. 17, Jiankang Road, Yishui
County, Linyi 276400, Shandong, People's
Republic of China
Tel/Fax +86-0539-22260223
Email Jiangzhang3241@163.com

reperfusion therapy, while the most promising neuroprotective drugs are documented to be ineffective in the reduction of infarction size.⁷ Therefore, it is pivotal to explore the molecular mechanism underlying neurological deficits of IS to figure out more effective therapies for IS.

As a transcription factor, E2F transcription factor 1 (E2F1) can bind DNA to dimerization partner (DP) proteins through E2 recognition site (5'-TTTC[CG]CGC-3') in the promoter region of various genes.⁸ The crucial involvement of E2F1-regulatory pathway has been elucidated in stroke injury by a prior study, where E2F1 activated Cited2 transcription to orchestrate stroke.⁹ E2F1 modulates numerous genes, such as those encoding S-phase-related proteins and microRNAs (miRNAs/miRs), which participate in cell self-renewal and differentiation, DNA synthesis and replication, DNA damage repair, and mitotic checkpoint control.¹⁰ Moreover, the role of miRNAs has been elaborated in development of IS.¹¹ Additionally, miR-122 upregulation alleviated stroke following middle cerebral artery occlusion in rats.¹² Interestingly, a previous research identified the neuroprotective role of miR-122 against IS by protecting neuronal cells from death.¹³ However, it remains unclear how miR-122 was regulated in IS. In the present study, we identified that E2F1 directly modulate the transcription of miR-122 in IS. More importantly, the targeting relationship between miR-122 and Sprouty2 (SPRY2) in osteoblasts was detected by a research conducted by Liao et al.¹⁴ It has been documented that SPRY2 is expressed in neopallial cortex, cingulate flexure, and cerebellum and assumes a critical role in neuronal diseases.¹⁵ SPRY2 silencing resulted in reduction of ischemic brain injury through limiting neuronal cell death and lesion size.¹⁶ On basis of this ground, a hypothesis could be proposed that E2F1 might be involved in IS by mediating SPRY2 via miR-122. Therefore, experiments were conducted in clinical, cell, and animal levels to confirm this hypothesis, thus exploring the novel mechanism for IS treatment.

Patients and Methods

Ethical Approval

The study regarding animals was conducted in strict accordance with the approval of the Institutional Review Board and the Animal Care and Use Committee of Linyi Central Hospital as per the ARRIVE guidelines. All human experiments were performed following the *Declaration of Helsinki* (2013). Specific experimental methods were ratified by the Ethics Committee of Linyi Central Hospital,

and each participant signed written informed consent prior to experiments.

Patient Information

From February 2017 to February 2019, 81 patients with IS confirmed by clinical and imaging treated in Linyi Central Hospital were consecutively enrolled. All patients were diagnosed as IS from cerebral arterial vascular obstruction by magnetic resonance imaging and angiography. Patients were free of history of chronic disease, any infections, history of cardiovascular disease and pregnancy, history of substance abuse, nor chronic diseases other than IS. Meanwhile, we recruited 81 age- and sex-matched controls who had no history of chronic illness, any infections, history of cardiovascular disease and pregnancy, no history of substance abuse, physical and neurological examination showing good health, no brain or vascular lesions detected by computed tomography or magnetic resonance imaging scan. None of participant received thrombolytic therapy or suffered from liver and kidney failure, tumors, infectious disease or hematological diseases. The severity of IS was assessed with National Institute of Health Stroke Scale 24 (NIHSS-24) 3 months after stroke onset. The volume of cerebral infarction was calculated by ABC/2 using computed tomography or magnetic resonance imaging. Patients were classified into five subtypes based on clinical characteristics, imaging, and laboratory findings, with reference to the Trial of Org 10,172 in Acute Stroke Treatment (TOAST) classification criteria proposed by Adams et al in 1993.¹⁷ Patient data are listed in Table 1.

Table 1 Demographic Characteristics of the Healthy Control and is Patients

Demographic Characteristics	CON	IS
Total number	81	81
Age (Years)	61.23 ± 7.12	61.23 ± 7.12
Sex (male/female)	48/33	48/33
NIHSS score	NA	5.33 ± 6.12
Infarct volumes	NA	2.82 ± 2.16
TOAST subtype		
LAA	NA	17
CE	NA	9
SAA	NA	27
SOE	NA	0
SUE	NA	28

Abbreviations: CON, control; IS, ischemic stroke; NIHSS, National Institute of Health Stroke Scale; NA, not applicable; TOAST, Trial of Org 10,172 in Acute Stroke Treatment; LAA, large-artery atherosclerosis; CE, cardioembolism; SAA, small-artery occlusion; SOE, stroke of other determined etiology; SUE, stroke of undetermined etiology.

Table 2 Demographic Characteristic of Three is Patients and Controls for Microarray Analysis

Demographic Characteristics	CON	IS
Total number	3	3
Age (years)	61.00 ± 1.0	61.00 ± 1.0
Sex	Male	Male
NIHSS score	NA	5.33 ± 0.61
Infarct volumes	NA	2.82 ± 0.42
TOAST subtype	LAA	LAA

Abbreviations: CON, control; IS, ischemic stroke; NIHSS, National Institute of Health Stroke Scale; NA, not applicable; TOAST, Trial of Org 10,172 in Acute Stroke Treatment; LAA, large-artery atherosclerosis.

Microarray Analysis

Peripheral blood (5 mL) was harvested from three IS patients of similar age and the same sex and TOAST subtype and three control volunteers (Table 2) and plated in a test tube containing ethylenediaminetetraacetic acid, followed by a 10-min centrifugation at 1500 g and 4°C. Total RNA was then extracted using TRIzol reagent (Thermo Fisher Scientific, Worcester, MA, USA) and reversely transcribed into cDNA using Prime Script II reverse transcriptase (Takara Bio., Tokyo, Japan). Following fragment of cDNA, cDNA fragments were hybridized with Human miRNA Expression Microarray V4.0 and Human mRNA Expression Microarray V4.0 (Arraystar, Rockville, MD, USA), respectively. Afterwards, the microarrays were scanned with a GeneChip™ Scanner 3000 7B system (Thermo Fisher Scientific). Expression data were collected and normalized using the Affy Package (Bioconductor) of R software (R-project). Non-specific screening was performed using linear model-empirical Bayesian statistics in the Limma Package (Bioconductor), and differentially expressed RNAs were displayed in combination with the commonly used statistical unpaired t-test, followed by plotting of heatmaps using the Pheatmap Package.

Expression Detection

Following total RNA isolation using TRIzol reagent (Thermo Fisher Scientific), the quality and yield of extracted RNA were analyzed by agarose gel electrophoresis and spectrophotometry, and genomic DNA contamination was deleted using DNA ASE I treatment (Takara Bio.). Then, cDNA was synthesized using Prime Script II reverse transcriptase (Takara Bio.). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted following standard procedure on an ABI PRISM 7500 instrument (Applied

Biosystems, Carlsbad, CA, USA). Primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China): glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F: 5'-ATGGGGAAGGTGAAGGTCTG-3' and R: 5'-GGGGTCATTGATGGCAACAATA-3'), U6 (F: 5'-CTCGCTTCGGCAGCACA-3' and R: 5'-CTCGCTTCGGCAGCACA-3'), E2F1 (F: 5'-TGCTCTCCGAGGACACTGACAG-3' and R: 5'-TCTTGCTCCAGGCTGAGTAGAGAC-3'), SPRY2 (F: 5'-CTCGGCCCA GAACGTGATT-3' and R: 5'-GGCAAAAAGAGGGACATGACAC-3'), and miR-122 (F: 5'-GTGACAATGGTGGAATGTGG-3' and R: 5'-AAAGCAACGATGCCAAGAC-3'). Relative changes in gene expression were calculated by $2^{-\Delta\Delta Ct}$ using GraphPad Prism software normalized to U6 (for miR-122) and GAPDH (for E2F1 and SPRY2).

Cell Treatment

Mouse neuroblastoma N2a cells (American Type Culture Collection, Manassas, VA, USA) were seeded in Dulbecco's Modified Eagle Medium (DMEM, Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS) and 100 U/L penicillin/streptomycin, followed by culture at 37°C with 5% CO₂. For oxygen glucose deprivation (OGD) treatment, stably growing N2a cells were transferred to glucose-free DMEM and cultured in a hypoxic incubator with 95% N₂ and 5% CO₂ for 12 h at 37°C for subsequent experiments.

Neuronal Viability Assay

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and then blocked with 5% goat serum at 37°C for 40 min, followed by overnight incubation with rabbit monoclonal glial fibrillary acidic protein (GFAP) antibody (ab114149, 1: 100, Abcam, Cambridge, USA) at 4°C. After 3 phosphate buffer saline (PBS)-washes, secondary antibody (ab205718, 1: 5000, Abcam) was supplemented into cells for a 1-h incubation at 25°C. The nuclei were stained with 4',6-diamidino-2-phenylindole (Sigma, St Louis MO, USA), followed by observation and photographing using a fluorescence microscope (IX81, Olympus, Tokyo, Japan).

Cell Cycle Detection

After transfection for 48 h, cells were harvested, and centrifuged at 1200 rpm for 5 min. After 2 PBS-washes, cells were added with 1 mL ice 70% ethanol (Shanghai Sangon Biotechnology Co., Ltd.) for a 30-min fixation. Cell suspension was incubated with 500 µL propidium

iodide (PI, Sigma) for 30 min at room temperature. The number of cells at each cell cycle stage was determined by Fluorescence Activated Cell Sorting Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), followed by analysis using cell Quest software (BD Biosciences).

Cell Apoptosis Assay

Apoptosis was assessed using Annexin V-fluorescein isothiocyanate (FITC)/PI double staining kit (Beyotime Biotechnology, Shanghai, China). After 10× binding buffer was diluted at 1: 9 to prepare 1× binding buffer, cells were centrifuged at 1200 rpm for 5 min, and resuspended with pre-cooled PBS. Cells were mixed with 5 µL FITC staining solution and incubated for 15 min at room temperature. After PBS washing, 5 µL PI staining solution was added into cells for a 5-min incubation. FITC fluorescence was detected at 515 nm and PI fluorescence was determined at 560 nm using a flow cytometer (BECKMAN, Brea, CA, USA).

Western Blot Analysis

Phenylmethanesulfonyl fluoride containing Radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology) was adopted for lysis of the transfected cells. The protein was electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gel following evaluation of the protein concentration by a bicinchoninic acid quantitative kit (Solarbio). Proteins were electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) by wet transfer method, and 5% bovine serum albumin (Thermo Fisher Scientific) was supplemented to seal the membranes for 1 h at room temperature. Then, overnight culture of the membranes was conducted with primary antibodies to BCL2 Associated X Protein [Bax, #14,796, 1: 1000, Cell Signaling Technology (CST), Beverly, MA, USA], Active-caspase-3 (#89,332, 1: 700, CST), Beclin-1 (ab207612, 1: 1000, Abcam), light chain 3B-II (LC3B-II, ab192890, 1: 200, Abcam), phosphorylated extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (sc-514,302, 1: 2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK 1/2 (sc-514,302, 1: 2000, Santa Cruz Biotechnology), phosphorylated p38 (sc-166,182, 1: 500, Santa Cruz Biotechnology), p38 (sc-271,120, 1: 1200, Santa Cruz Biotechnology), and β-actin (ab5694, 1: 1000, Abcam) at 4°C. The membranes were incubated at room temperature for 1 h following the addition of goat anti-rabbit secondary antibody (ab205718, 1: 10,000, Abcam).

After washing, the membranes were soaked in electrogenerated chemiluminescence solution (Beyotime Biotechnology) and analyzed by Bio-Rad gel imaging system (MG8600; Thmorgan Biotechnology, Beijing, China).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was carried out using a commercially available kit (Beyotime). N2a cells were crosslinked with 1% formaldehyde, and RNA fragments were generated by ultrasound treatment on ice. The ultrasound products were cultured with 60 µL E2F1 antibody (Abcam) or isotype control Immunoglobulin G (IgG) antibody overnight at 4°C. After the immune complexes were eluted, 20 µL NdeI solution (5 M) was supplemented for de-crosslinking overnight, and the precipitated chromatin DNA was recovered using the Omega gel Recovery Kit (Solarbio). The DNA was dissolved in 100 µL deionized water and analyzed using RT-qPCR.

Dual-Luciferase Reporter Gene Assay

The SPRY2 3'-translated region (3'-UTR) fragments containing the conserved miR-122 binding sites [wild type (WT) and the corresponding mutant binding sites (MUT)] were amplified by PCR and cloned into the pmirGLO vector (Promega, Madison, WI, USA). In the luciferase reporter assay, N2a cells were seeded into 24-well plates and cultured to 80% confluence. The 100 ng WT or MUT plasmids were co-transfected with 50 nM miR-122 Agomir into the cells for 48 h. Luciferase activity was measured by a dual-luciferase reporter assay system (Promega) using Exfect transfection reagent (Vazyme Biotech, Nanjing, China), with Renilla luciferase reporter gene luciferase activity as a control.

IS Mouse Model

Totally 24 male C57BL/6 mice (aged 7 weeks, weighing 20–25 g; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were kept at 25 ± 2°C with normal circadian rhythm and free access to food and water. Twenty-one male mice were anesthetized by an intraperitoneal injection of 1% sodium pentobarbital at 50 mg/kg. The right common carotid artery (CCA), external carotid artery and internal carotid artery were exposed surgically through a neck incision under an operating microscope. Silicon-coated nylon wire (Doccol Corporation, Redlands, CA, USA) was introduced into the CCA and advanced into the internal carotid artery

until the tip reached the starting point of the middle cerebral artery (MCA) to occlude the MCA. The wire was removed after 60-min occlusion. Control mice underwent the same experimental procedure without wire insertion into MCA.¹⁸ Neurological deficits were assessed in mice by Modified Neurological Severity Score (mNSS). Mice were intraperitoneally injected with 1% pentobarbital sodium at 90 mg/kg to remove the orbits, and peripheral blood was collected for subsequent experiments.

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

After intraperitoneal injection of 1% sodium pentobarbital at 150 mg/kg, the brains of mice were collected when the heartbeat, respiration and nerve reflex of mice disappeared and cut into 1.0-mm coronal sections. The sections were incubated in 0.5% TTC solution (Solarbio) at 37°C for 15 min. The stained images were analyzed with Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) software, followed by calculation of infarction volume.

Statistical Analysis

All data were statistically analyzed using SPSS 22.0 (IBM, Armonk, NY, USA). Mean $\pm$ standard deviation was utilized to summarize the measurement data, with $p < 0.05$ as a level of statistical significance. Unpaired *t*-test was conducted to compare two groups, whereas one-way analysis of variance (ANOVA) or two-way ANOVA was carried out to compare multiple groups, followed by Tukey's post hoc test. Log rank test was used for post-statistical analysis.

Results

miR-122 and E2F1 Were Differentially Expressed in IS

Serum from patients with IS was obtained clinically for microarray analysis and screening of differentially expressed genes. In order to clarify the specific mechanism and factors in IS, we selected serum samples from 3 patients for microarray analysis of miRNAs and mRNAs, and the differential expression heat-maps were plotted with $p < 0.01$ and multiple of differential expression ($|\text{Foldchange}| > 2$) as screening criteria. In the microarray analysis of miRNAs, it was found that there were 11 up-regulated and 3 down-regulated miRNAs in the serum of IS patients, with the greatest difference multiples of miR-99b among the up-regulated miRNAs and the highest downregulation degree of miR-122 among the down-regulated miRNAs (Figure 1A). When analyzing the results of the mRNA

microarray, we found that there were 8 up-regulated and 2 down-regulated mRNAs, among which E2F1 was the most significant in IS (Figure 1B). To test whether the microarray results were correct, we examined the expression of differentially expressed genes in microarray samples by RT-qPCR, which verified the microarray results (Figure 1C and D). These results revealed the differential expression of miR-122 and E2F1 in IS.

miR-122 Expression Was Poor in Serum of IS Patients and OGD-Treated Neuronal Cells

Since miR-122 expression was the most significant in the results of microarray analysis, we explored the role of miR-122 in IS. Quantitative analysis of miR-122 expression in serum samples from IS patients revealed that miR-122 was significantly down-regulated in IS (Figure 2A). Examination of the expression of miR-122 in the serum of patients with different TOAST subtypes revealed that patients in all subtypes had lower levels of miR-122 than that in the control group (Figure 2B). The correlation between miR-122 and stroke status was analyzed by scoring stroke scale, and a negative correlation was found between the two (Figure 2C). By assessing the infarction size of the patients and also performing correlation analysis between the infarction size and miR-122, it was found that there was also an inverse correlation between the two (Figure 2D). GFP staining of N2a cells and OGD-treated N2a displayed that OGD resulted in a decrease in cell activity, indicating that cell injury model was successfully developed (Figure 2E). A decrease of miR-122 expression was detected in OGD-treated N2a cells (Figure 2F). Therefore, we elevated miR-122 expression in cells using three miR-122 Agomir fragments to transfect OGD-N2a cells, and selected the Agomir with the highest efficiency for subsequent experiments (Figure 2G). Therefore, miR-122 down-regulation was associated with IS onset.

miR-122 Overexpression Promoted Proliferation and Inhibited Apoptosis and Autophagy in OGD-Treated Neuronal Cells

In order to explore the role in miR-122 in IS, miR-122 Agomir was transfected into OGD-induced N2a cells. GFP immunofluorescence staining and flow cytometry manifested that miR-122 Agomir transfection increased viability (Figure 3A) but decreased apoptosis (Figure 3B) in OGD-treated N2a cells. Meanwhile, it was observed that the number of cells at S phase was elevated, but

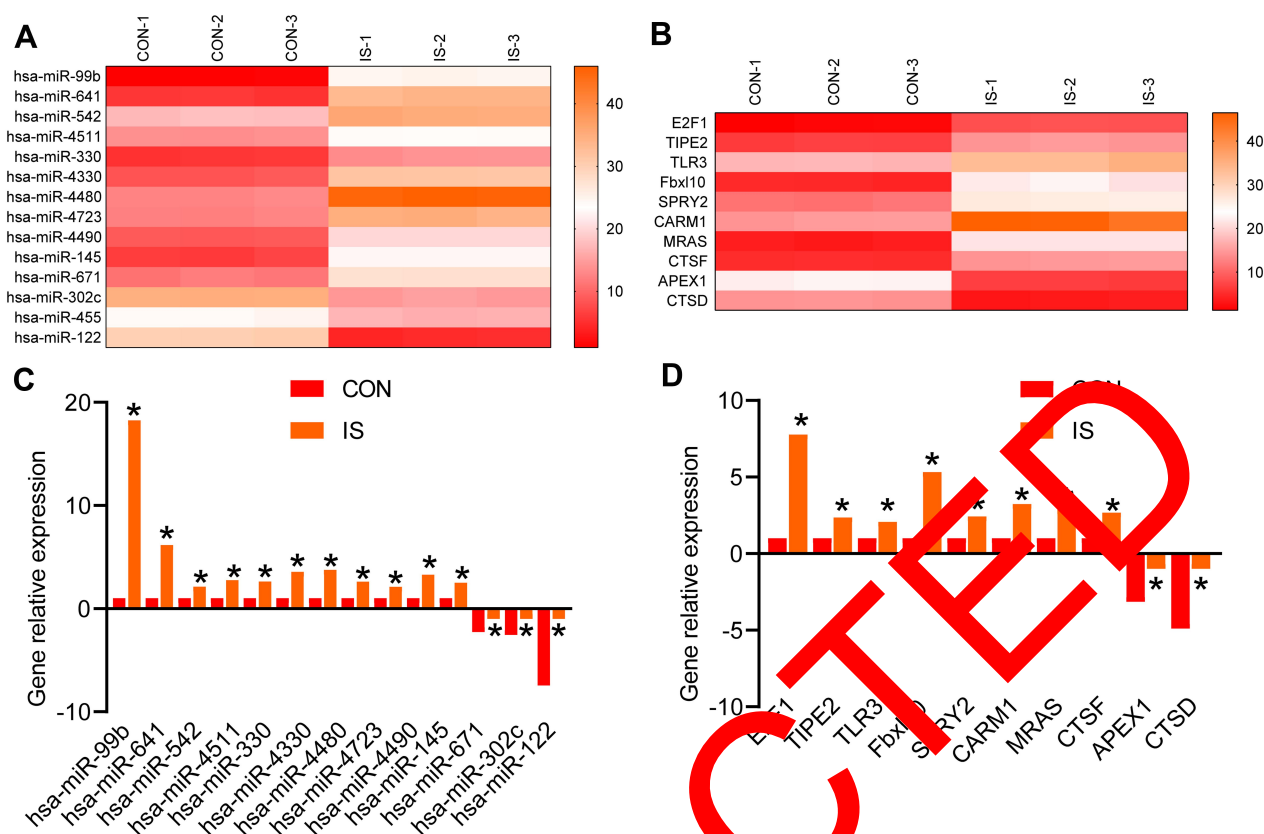


Figure 1 Differentially expressed genes in serum of clinical IS patients are evaluated. (A and B) Microarray analysis to detect differentially expressed miRNAs and mRNAs. (C and D) RT-qPCR detection of the expression of differentially expressed miRNAs and mRNAs to verify the microarray results. Two-way ANOVA was carried out to compare multiple groups, followed by Tukey's post hoc test. * $p < 0.05$ vs. controls.

cells at G0/G1 phase were reduced in OGD-treated N2a cells after transfection with miR-122 Agomir (Figure 3C). Western blot analysis was performed to assess the expression of apoptosis- (Bax and active-caspase-3) (Figure 3D) and autophagy-related proteins (Beclin-2 and LC3B-II) (Figure 3E), which illustrated declines in Bax, active-caspase-3, Beclin-2, and LC3B-II expression in OGD-treated N2a cells after transfection with miR-122 Agomir. In summary, OGD-treated neuronal cell proliferation was induced by which apoptosis and autophagy were repressed by overexpressing miR-122.

E2F1 Decreased miR-122 Expression to Upregulate SPRY2 in OGD-Treated Neuronal Cells

In mRNA microarray analysis, we found that E2F1 was also highly expressed in IS, so we wondered whether E2F1 acted as a transcription factor to regulate miR-122 in IS. Firstly, RT-qPCR documented that E2F1 expression was augmented in serum of all patients (Figure

4A), and was inversely correlated with miR-122 expression (Figure 4B). Detection of E2F1 expression in OGD-treated N2a cells reported a significant increase of E2F1 expression, but its expression showed no significant change in the OGD-treated N2a cells induced by miR-122 (Figure 4C). Therefore, OGD-treated N2a cells overexpressing E2F1 were established. Overexpression (OE) of E2F1 caused a reduction in miR-122 expression (Figure 4D). The promoter sequence of miR-122 was searched in UCSC and the binding relationship between E2F1 and miR-122 promoter region from -482 to -489 bp was found by TransmiR prediction. According to ChIP assay, E2F1 was enriched in the promoter region from -482 to -489 bp of miR-122 (Figure 4E). Meanwhile, we intersected the microarray results (TIPE2, TLR3, Fbxl10, SPRY2, CARM1, MRAS and CTSE) and the predicted target genes of miR-122 by Targetscan using a Venn map, which depicted that SPRY2 was not only upregulated in the serum of IS patients, but also a target gene of miR-122 (Figure 4F). The targeting relationship between SPRY2 and miR-122

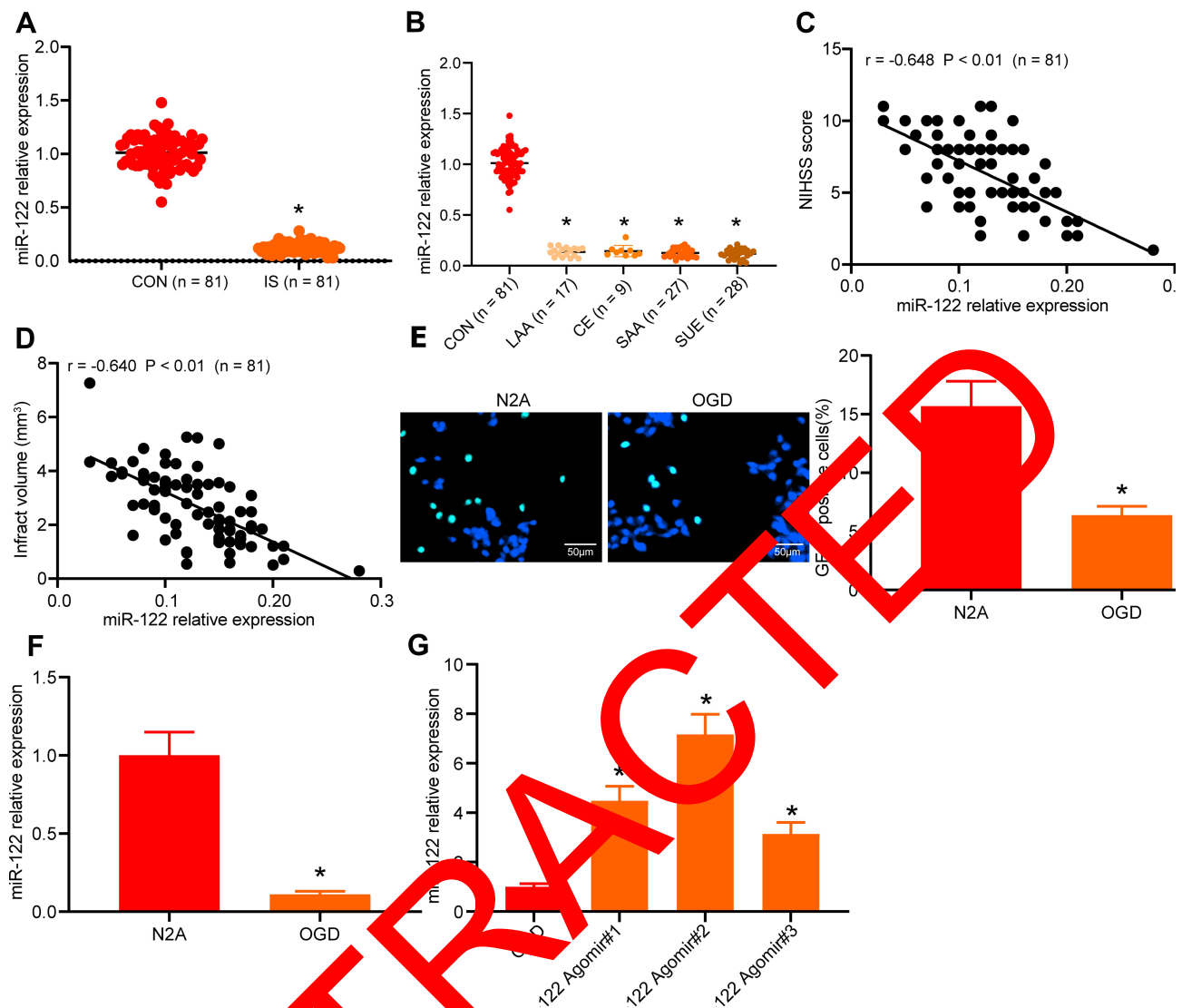


Figure 2 miR-122 is downregulated in serum of IS patients and OGD-treated neuronal cells. **(A)** RT-qPCR detection of miR-122 expression in serum of IS patients. **(B)** Differences in miR-122 expression between serum of patients with each TOAST subtype and control normal subjects were detected by RT-qPCR. **(C)** Analysis of the correlation between miR-122 and NIHSS score. **(D)** Analysis of the correlation between miR-122 and infarction size. **(E)** N2a cell viability after OGD treatment detected by GFP staining. **(F)** miR-122 expression in N2a cells after OGD treatment detected by RT-qPCR. **(G)** miR-122 expression in OGD-treated N2a cells after transfection with miR-122 Agomir determined by RT-qPCR. Unpaired t-test (Panel **E** and **F**) was conducted to compare two groups, whereas one-way (Panel **G**) or two-way ANOVA (Panel **A** and **B**) was carried out to compare multiple groups, followed by Tukey's post hoc test. * $p < 0.05$ vs. controls, N2a cells without treatment or exposed to OGD.

in N2a cells were verified by dual-luciferase reported assays (Figure 4G). By analyzing the mRNA expression of SPRY2, it was depicted that SPRY2 was up-regulated in IS (Figure 4H), which was positively correlated with E2F1 expression, and negatively correlated with miR-122 expression (Figure 4I). OE of miR-122 in N2a cells resulted in down-regulation of SPRY2, and treatment with OGD and E2F1-OE resulted in up-regulation of SPRY2 expression (Figure 4J). These results suggested that E2F1 contributed to upregulation of SPRY2 by decreasing miR-122 expression in OGD-treated neuronal cells.

E2F1 Overexpression Reduced Viability but Elevated Apoptosis and Autophagy of OGD-Treated Neuronal Cells via the miR-122/SPRY2 Axis

Subsequently, OGD-induced N2a cells were transfected with E2F1-OE alone or with miR-122 Agomir, miR-122 Agomir alone or with SPRY2 to explore the role of E2F1/miR-122/SPRY2 axis in vitro. Firstly, miR-122 expression after OE of E2F1 and miR-122, and SPRY2 expression after OE of miR-122 and SPRY2 were determined in N2a cells by RT-qPCR (Figure 5A). As depicted in Figure 5B, miR-122 Agomir

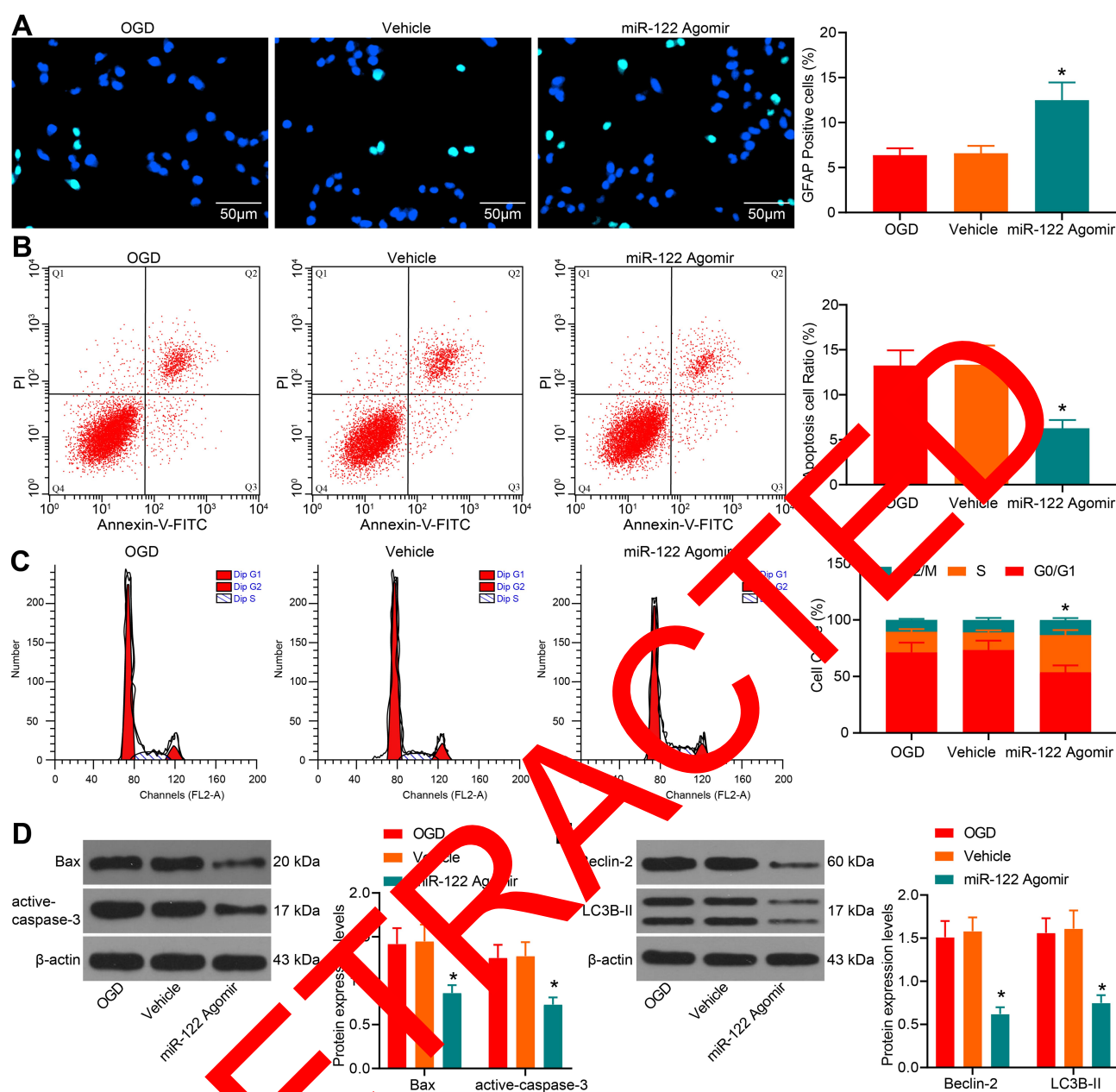


Figure 3 miR-122 upregulation enhances proliferation and diminishes apoptosis and autophagy in OGD-treated neuronal cells. OGD-treated N2a cells were transfected with miR-122 Agomir or Vehicle or without transfection. **(A)** N2a cell viability measured by GFAP staining. **(B)** N2a cell apoptosis assessed by flow cytometry. **(C)** Cell cycle distribution of N2a cells evaluated by flow cytometry. **(D)** Western blot analysis of protein expression of apoptosis-related proteins (Bax and active-caspase-3) in N2a cells. **(E)** Western blot analysis of protein expression of autophagy-related proteins (Beclin-2 and LC3B-II) in N2a cells. One-way (Panel **A** and **B**) or two-way ANOVA (Panel **C**) was carried out to compare multiple groups, followed by Tukey's post hoc test. * $p < 0.05$ vs. N2a cells exposed to OGD.

negated decreased cell viability, increased cells at G0/G1 phase, reduced cells at S phase, and elevated apoptosis caused by E2F1 OE in OGD-treated N2a cells. Additionally, in the presence of miR-122 Agomir, cell activity was diminished (Figure 5B), cells in G0/G1 phase (Figure 5C) and apoptosis (Figure 5D) were enhanced in OGD-treated N2a cells by overexpressing SPRY2. Therefore, E2F1/miR-122/SPRY2 axis orchestrated OGD-treated neuronal cell proliferation and apoptosis.

E2F1 Promoted Neurological Deficit and Cerebral Infarction in IS Mice via SPRY2 by Downregulating miR-122

In order to investigate the role of E2F1/miR-122/SPRY2 axis in vivo, IS mouse model was established. Neurological deficits in mice were assessed by mNSS (Figure 6A) and infarction size was measured by TTC staining (Figure 6B). It was found that compared with

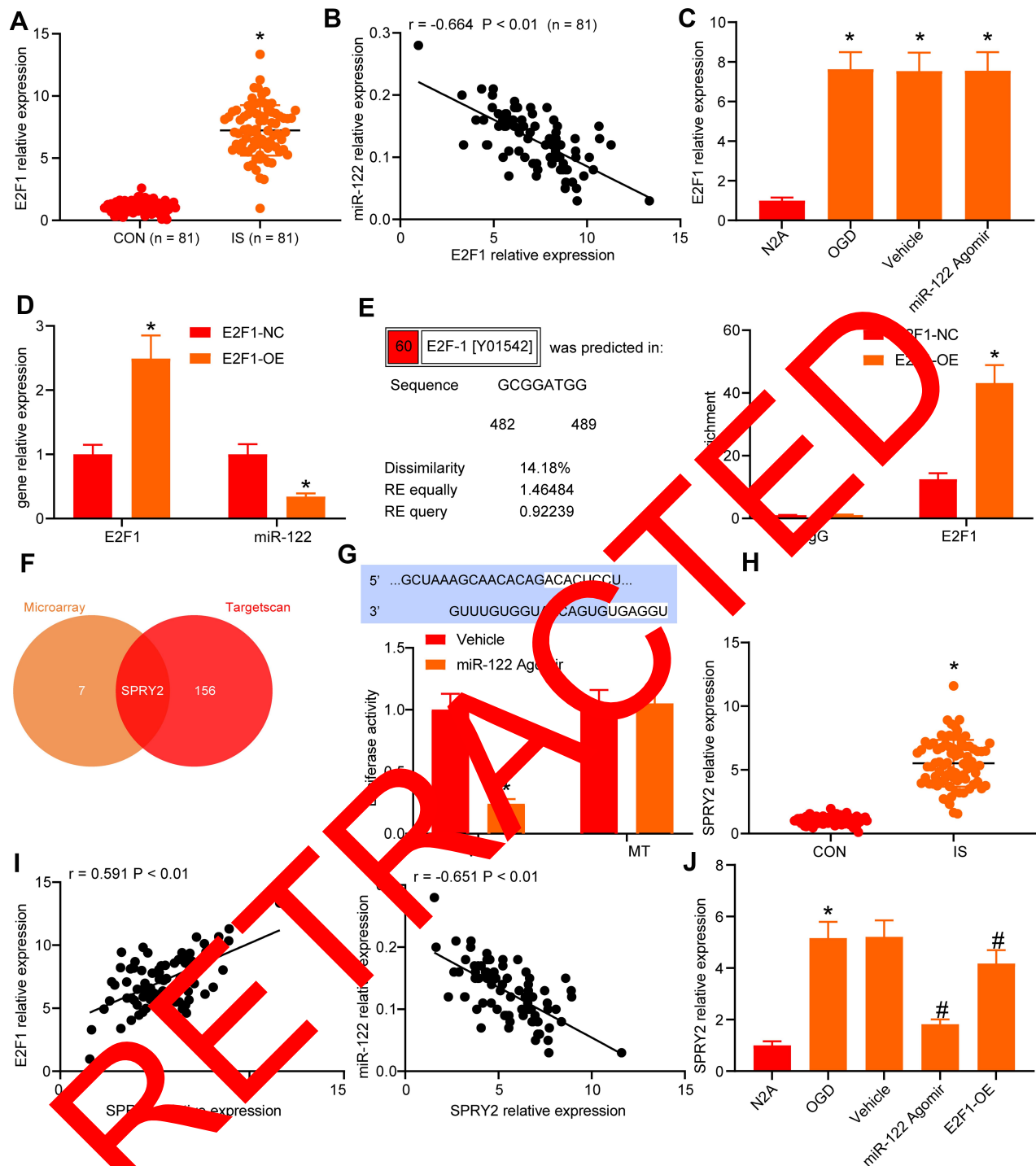


Figure 4 E2F1-downregulated miR-122 enhances SPRY2 expression in OGD-treated neuronal cells. (A) RT-qPCR detecting E2F1 expression in serum of all patients. (B) The correlation between E2F1 and miR-122 expression. (C) RT-qPCR detection of E2F1 expression in N2a cells after treatment with OGD and miR-122 Agomir. (D) The changes of E2F1 and miR-122 expression in OGD-induced N2a cells after E2F1 overexpression. (E) ChIP detecting the binding of E2F1 to the promoter of miR-122. (F) Venn map of predicted targeted genes of miR-122 and the upregulated genes in serum of three patients with IS and controls. (G) Dual-luciferase reporter assay of targeting relationship of miR-122 and SPRY2. (H) RT-qPCR detection of SPRY2 expression in serum of IS patients. (I) Correlation analysis of SPRY2 with E2F1 and miR-122. (J) SPRY2 expression in N2a cells after treatment with OGD and miR-122 Agomir or E2F1-OE assessed by RT-qPCR. One-way (Panel C and J) or two-way ANOVA (A, D, E, G, H) was carried out to compare multiple groups, followed by Tukey's post hoc test. **p* < 0.05 vs. controls, N2a cells without treatment or transfected with E2F1-NC; #*p* < 0.05 vs. N2a cells transfected with Vehicle.

normal mice, IS mice had neurological injury and cerebral infarction. The serum of IS and control mice were harvested. The expression of E2F1, SPRY2 and miR-122 was

measured by RT-qPCR, and it was revealed that the expression of E2F1 and SPRY2 was expedited and the expression of miR-122 was reduced in the serum of IS mice, which was

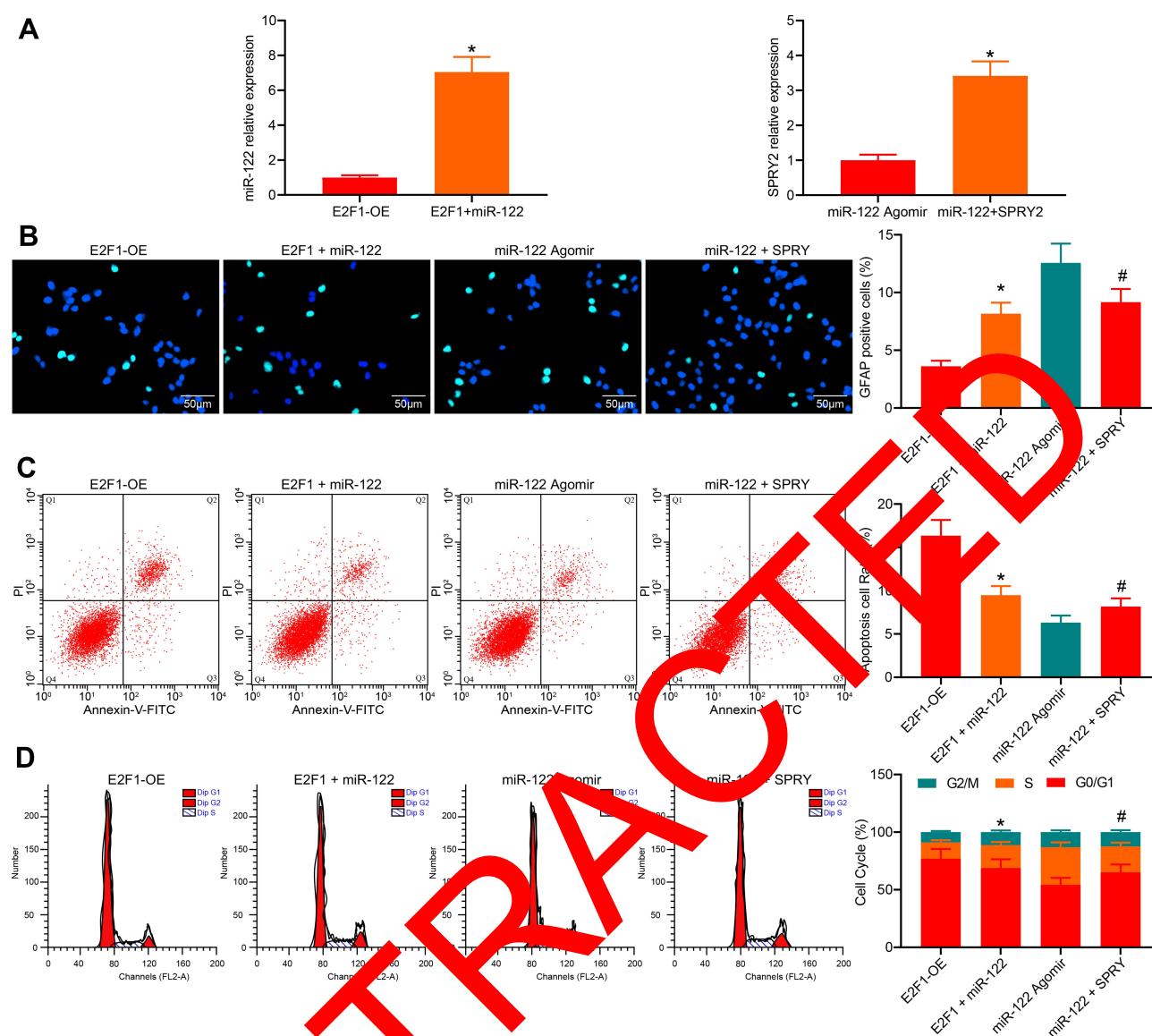


Figure 5 OGD-treated neuronal proliferation and apoptosis are modulated by E2F1/miR-122/SPRY2 axis. OGD-induced N2a cells were transfected with E2F1-OE, E2F1-OE + miR-122 Agomir, miR-122 Agomir, or miR-122 Agomir + SPRY2. (A) miR-122 expression after overexpression of E2F1 and miR-122, and SPRY2 expression after overexpression of miR-122 and SPRY2 determined in N2a cells by RT-qPCR. (B) N2a cell viability measured by GFAP staining. (C) N2a cell apoptosis assessed by flow cytometry. (D) Cell cycle distribution of N2a cells evaluated by flow cytometry. Unpaired t-test (Panel A) was conducted to compare two groups, whereas one-way (Panel B and C) or two-way ANOVA (Panel D) was carried out to compare multiple groups, followed by Tukey's post hoc test. * $p < 0.05$ vs. N2a cells transfected with E2F1-OE, # $p < 0.05$ vs. N2a cells transfected with miR-122 Agomir.

consistent with the results of clinical analysis (Figure 6C). RT-qPCR analysis of changes in gene expression in rat brain tissues found that E2F1 and SPRY2 expression was up-regulated and miR-122 expression was down-regulated in the brain tissue of IS mice (Figure 6D). IS mice were injected with Vehicle, E2F1-OE, miR-122 Agomir, SPRY2-OE, E2F1 + miR-122, miR-122 + SPRY2 plasmids. According to RT-qPCR results, the transfection efficiency was confirmed (Figure 6E). Based on mNSS score, IS mice overexpressing E2F1 or SPRY2 had severer neurological

deficits, while IS mice overexpressing miR-122 had partial recovery from neurological deficits. Moreover, miR-122 Agomir improved neurological deficits in E2F1-overexpressed IS mice, while SPRY2 OE aggravated neurological deficits in miR-122-overexpressed IS mice (Figure 6F). TTC staining demonstrated that IS mice exhibited obvious cerebral infarction, which was deteriorated by the OE of E2F1 or SPRY2 but alleviated by miR-122 Agomir. Also, miR-122 attenuated the cerebral infarction caused by E2F1 OE in IS mice, while SPRY2 reversed the repressed

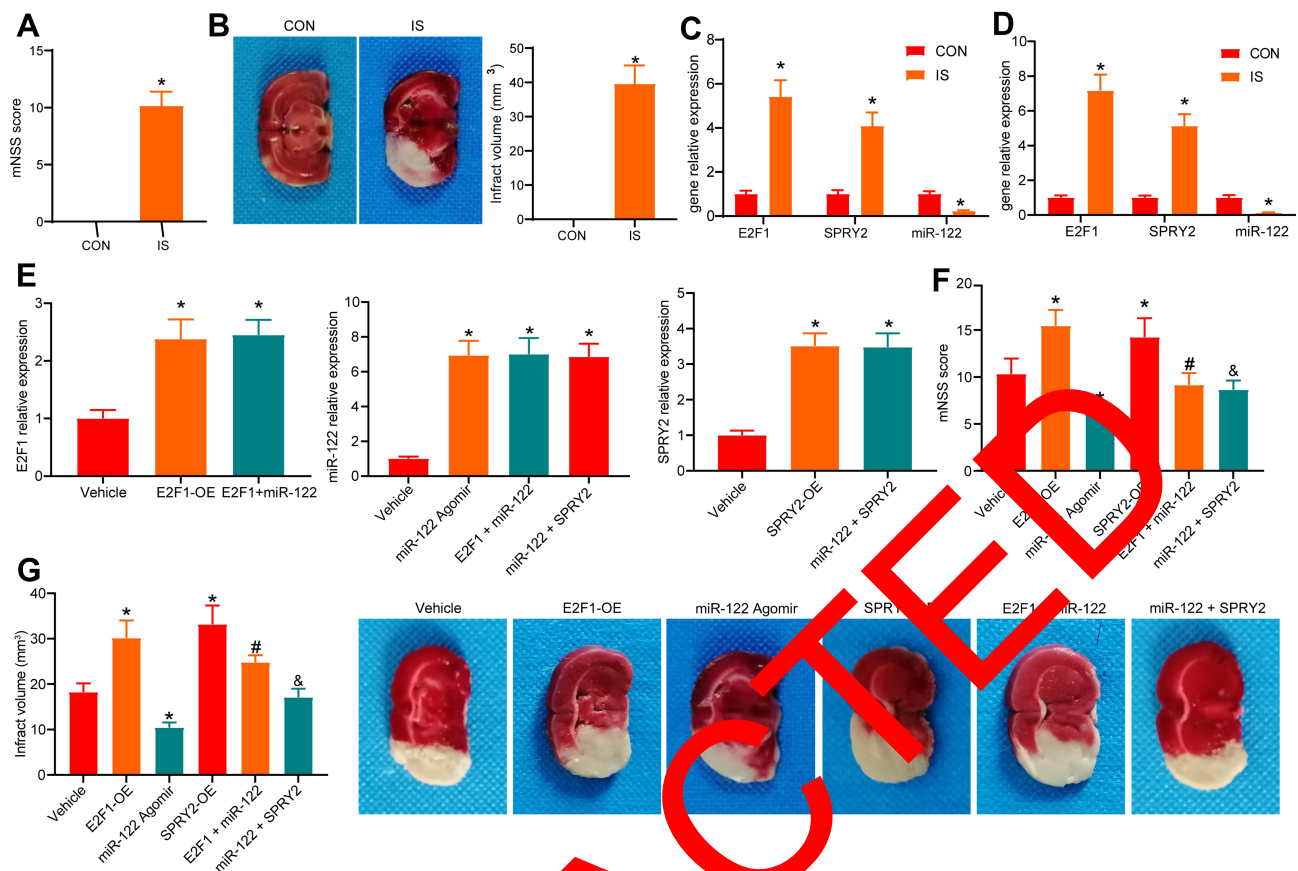


Figure 6 E2F1 stimulates neurological deficits and cerebral infarction in IS mice by upregulating SPRY2. (A) Neurological deficits in mice after modeling evaluated by mNSS score. (B) Infarction size in mice after modeling measured by TTC staining. (C) E2F1, miR-122, SPRY2 expression in the serum of mice after modeling assessed by RT-qPCR. (D) E2F1, miR-122, SPRY2 expression in the brain tissues of mice after modeling assessed by RT-qPCR. IS mice were injected with Vehicle, E2F1-OE, miR-122 Agomir, SPRY2-OE, E2F1 + miR-122, miR-122 + SPRY2 plasmids. (E) E2F1, miR-122, SPRY2 expression in IS mice assessed by RT-qPCR. (F) Neurological deficits in IS mice evaluated by mNSS score. (G) Infarction size in IS mice measured by TTC staining. Unpaired t-test (Panel A and B) was conducted to compare two groups, whereas one-way (Panel E) or two-way ANOVA (Panel C and D) was carried out to compare multiple groups, followed by Tukey's post hoc test. * $p < 0.05$ vs. controls, N2a cells treated with Vehicle, # $p < 0.05$ vs. N2a cells transfected with E2F1-OE and & $p < 0.05$ vs. N2a cells transfected with miR-122 Agomir.

cerebral infarction induced by miR-122 Agomir (Figure 6G). Therefore, E2F1/miR-122/SPRY2 axis mediated neurological deficits and cerebral infarction in IS mice.

E2F1 Overexpression Blocked the MAPK Pathway in IS via the miR-122/SPRY2 Axis

Finally, we explored the pathway mediated by the E2F1/miR-122/SPRY2 axis in IS. On the basis of Western blot analysis, the extent of ERK and p38 phosphorylation decreased in cells overexpressing E2F1 or SPRY2, but increased in cells treated with miR-122 Agomir (Figure 7A). Meanwhile, the extent of ERK and p38 phosphorylation reduced in mice overexpressing E2F1 and SPRY2, and elevated in mice treated with miR-122 Agomir (Figure 7B). Therefore, it was suggested that the E2F1/miR-122/SPRY2 axis mediated the MAPK pathway during IS process.

Discussion

As a principle cause of death and disability on a global scale, IS is featured by episodes of neurological dysfunction attributed to cerebral infarction in perfused areas of stenotic or occluded arteries.¹⁹ It is widely acknowledged that miRNAs post-transcriptionally orchestrate gene silencing in both the physiology of brain development and pathology of IS.²⁰ Thus, targeting miRNAs may be beneficial to therapies for IS. In this study, we investigated the effect of miR-122 on neurological deficits in IS and its potential mechanism by conducting experiments at clinical, cell, and animal levels. We provide evidence that E2F1 disrupted the MAPK pathway and then aggravated neurological deficit in IS by upregulating SPRY2 via miR-122 downregulation.

Initially, our study revealed that miR-122 expression was poor in serum of IS patients. The involvement of miRNAs in

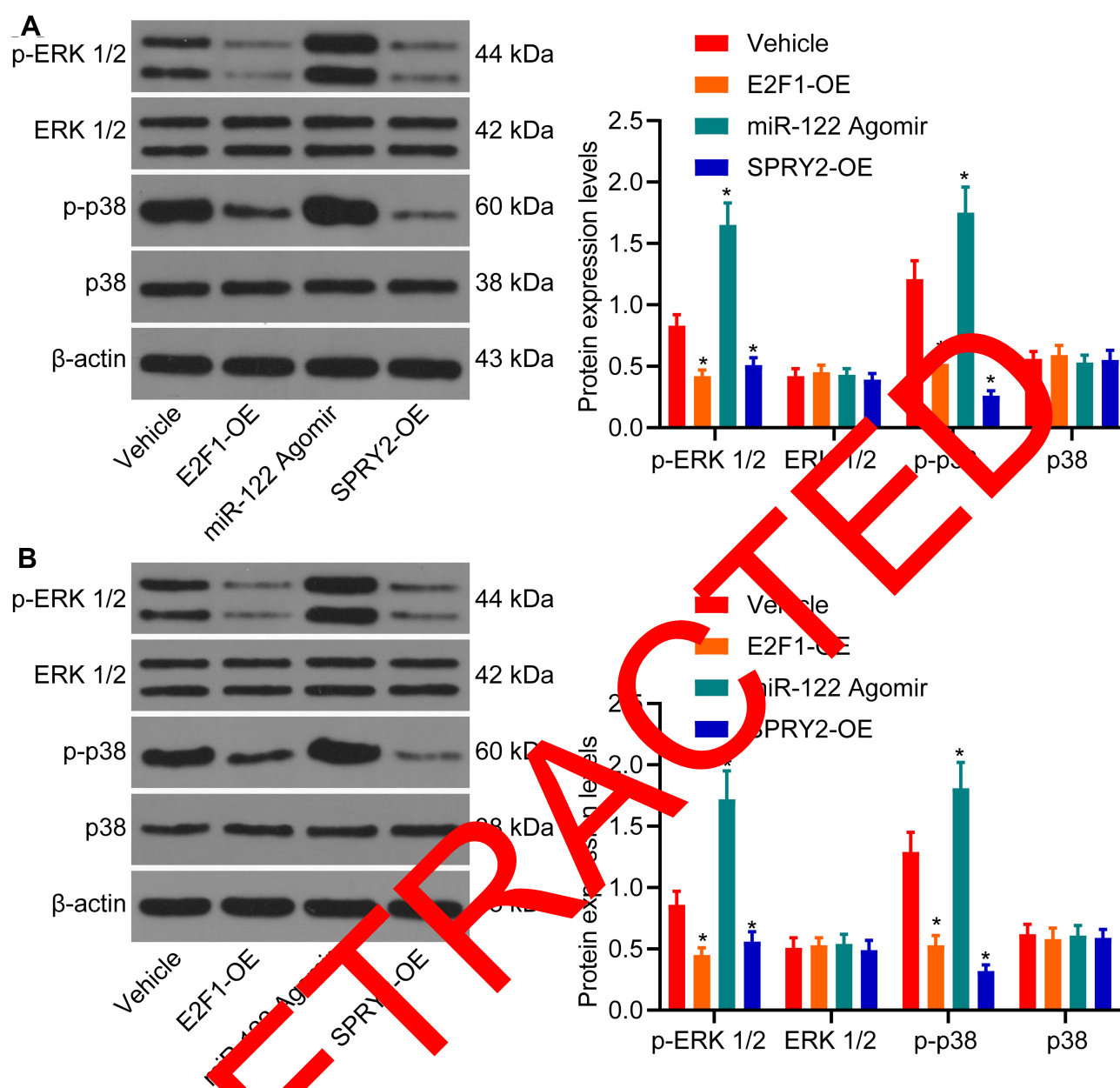


Figure 7 E2F1-downregulation and miR-122 blocks MAPK pathway in IS via SPRY2. (A) Western blot analysis of the extent of ERK and p38 phosphorylation and expression of ERK and p38 in neuronal cells after transfection. (B) Western blot analysis of the extent of ERK and p38 phosphorylation and expression of ERK and p38 in mice after delivery. * $p < 0.05$ according to the two-way ANOVA.

IS has been identified.²¹ For instance, Zhang et al observed downregulation of miR-126 in OGD-treated brain microvascular endothelial cells.²² Also, Kolosowska et al found the microglial activation by miR-669c OE using lentiviral vectors exerted protection in a mouse model against transient focal IS.²³ In addition, the results obtained from this study manifested that miR-122 OE increased viability but decreased apoptosis and autophagy of OGD-treated N2a cells, and alleviated neurological deficit and cerebral infarction in IS mice. In line with our findings, miR-122 OE

promoted cell viability and inhibited cell apoptosis and autophagy in renal cancer.²⁴ More specifically, a prior study illustrated that ectopic expression of miR-122 repressed OGD-treated N2a cell death, and reduced neurological deficit and ischemic injury caused by middle cerebral artery occlusion in mice.¹³ However, the upstream mechanism of action in miR-122 downregulation remains unclear. mRNA-based microarray revealed that E2F1 was upregulated in IS patients. Our further validation substantiated that E2F1 directly mediated the miR-122 transcription.

It is well acknowledged that there is feedback loops between E2F1 and miRNAs, where miRNAs can be modulated by E2F1.²⁵ For example, E2F1 acted as a repressor of miR-520/372/373 family transcriptional activity by directly binding to their promoter in renal tumor cells.²⁶ These reports supported our finding that E2F1 decreased miR-122 expression by binding to miR-122 promoter. Moreover, the findings presented by our study elaborated the upregulation of E2F1 in serum of IS patients and mice, and that E2F1 OE resulted in reduction of viability but elevation of apoptosis and autophagy of OGD-treated N2a cells, and promotion of neurological deficit and cerebral infarction in IS mice. Consistently, a previous research clarified that E2F1 expression was highly expressed in the rat intracerebral hemorrhage (ICH) model and the hemin-induced injury model in rat cortical cultures, and that E2F1 downregulation diminished hemin-induced neuronal damage and ICH-induced brain injury.²⁷ Interestingly, E2F1 knockout alleviated brain injury and improved behavior in mice with focal ischemia.²⁸ Another research unraveled that overexpressing E2F1 contributed to neuronal cell death after ischemic insult both in vivo and in vitro.⁹ It was well established that pRb/E2F1 pathway increased cell autophagy by promoting number of GFP-LC3 puncta.²⁹

In the following experiments, we also observed that SPRY2 was a target gene of miR-122. As reported, miRNAs reduce gene expression posttranscriptionally by binding to their targets.³⁰ More importantly, a prior

study supported our results that SPRY2 was directly targeted by miR-122 in keratinocytes.³¹ Furthermore, another critical finding in our study was that SPRY2 was upregulated in serum of IS patients, and that viability was reduced but apoptosis and autophagy were enhanced in OGD-treated N2a cells, and neurological deficit and cerebral infarction were elevated in IS mice after overexpressing SPRY2. Consistent with our findings, Klimaschewski et al reported that downregulation of SPRY2 in mice conferred neuroprotective effects and induced injury-induced astrogliosis which limited neuronal cell death and lesion size.¹⁶ Moreover, we also found that E2F1-downregulated miR-122 and disrupted the MAPK pathway in IS via SPRY2. A prior study revealed that E2F1 depletion increased enrichment of MAPK pathway in esophageal squamous cell carcinoma cells.³² Vahdati Hassani et al discovered that miR-122 inhibition-inactivated MAPK pathway was involved in protective effect of crocin on BPA-induced liver toxicity in rats.³³ Consistently, another research also manifested that inhibition of SPRY2 resulted in activation of MAPK pathway in hepatocellular carcinoma cells.³⁴

Conclusion

In conclusion, our major findings confirm the mechanism whereby up-regulated E2F1 increased SPRY2 expression by downregulating miR-122, thereby decreasing viability but increasing apoptosis and autophagy of OGD-treated neuronal cells and enhancing neurological deficit and

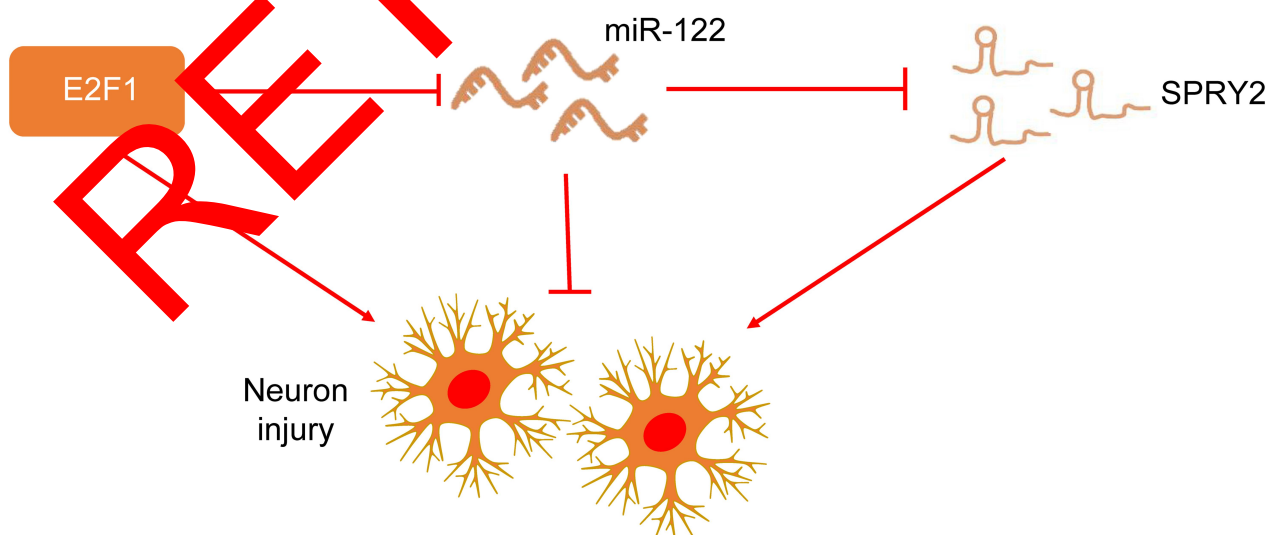


Figure 8 Mechanism. E2F1 inhibits the transcription of miR-122 to promote the occurrence of neuronal injury. miR-122 targets SPRY2 to inhibit the occurrence of neuronal injury.

cerebral infarction in IS mice (Figure 8). This study also validated our claim that targeting miR-122 could prevent neurological deficit caused by IS. This result offers a novel theoretical basis for developing miRNA-based therapeutics against IS. Nevertheless, further studies are warrant to perform microarray analysis and validation on patients with different clinical characteristics.

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Disclosure

The authors declare no conflicts of interest.

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