MicroRNA-194 Inhibition Promotes SUMO-2-Dependent Neuro-Protection Against Oxygen-Glucose Deprivation

Xiao-Hua Wang
Xuanwu Hospital
Si-Yuan Zhang
Daxing Hospital Affiliated to Capital Medical
Yi Huang
Xuanwu Hospital Department of Radiology
Yunqian Guan
Xuanwu Hospital Department of Neurosurgery
Yi Zheng
Massachusetts General Hospital Department of Neurology
Wenwei Qi
The Second Hospital of Tianjin Medical University
Guoguang Zhao
Xuanwu Hospital Department of Neurosurgery
Tian-Long Wang (w_tl5595@yahoo.com)
Xuanwu Hospital Department

Research

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Abstract

Background

Hypothermia is a powerful neuroprotectant. However, clinical translation has been difficult partly because the underlying mechanisms remain to be fully elucidated. Recently, it has been suggested that hypothermic neuroprotection may be linked with specific microRNA signatures, specifically the downregulation of miR-194-5p. Here, we attempt a reverse translation study to define the novel neuroprotective mechanism of miR-194-5p downregulation.

Methods

Our research designed experiments to determine the expression of miR-194-5p in hypothermic neuroprotection. After transfected with miRNA-194-5p inhibitor or control miRNA, neuron cells were performed Oxygen-glucose deprivation (OGD) and reoxygenation. Cell viability was determined by staining with propidium iodide (PI) and expression of SUMO-2 were quantified using Western blot. Luciferase reporter assays were performed to verify the direct binding of miR-194-5p to SUMO2 transcripts. Puromycin and recombinant lentivirus (pLKD-CMV-eGFP-sumo2-shRNA) were used to suppressed SUMOylation non-specifically and specifically.

Results

First, we documented that miR-194-5p was highly expressed in rat primary cortical neurons and astrocytes, compared with other glial and vascular cell types. Blockade with anti-miR-194-5p did not affect astrocytes, but significantly protected neurons against oxy-glucose deprivation. Using a miRNA target prediction algorithm, we found that SUMO-2, a known endogenous neuroprotective regulator, possessed the binding site for miR-194-5p. When miR-194-5p was inhibited, SUMO-2 mRNA was increased in neurons along with the enhancement of SUMO-2 conjugation. Finally, downregulation of SUMO-2 with none-special puromycin and special lentiviral shRNA-mediated knockdown of SUMO2 both canceled the neuroprotection mediated by miR194-5p inhibition.

Conclusion

Taken together, this study provides the first proof-of-concept that miR194-5p may be a negative controller of endogenous SUMO-2 protective mechanisms, and therefore miR194-5p inhibition may provide a novel approach for leveraging hypothermic neuroprotection against ischemic stress.

Background

Many neuroprotective treatments are effective in cell and animal models. However, the translation of experimental studies to humans has not been broadly successful. Within the wide spectrum of neuroprotective approaches that have been assessed to date, hypothermia appears to be one of the most efficient treatments for protecting brain, including resuscitation after cardiac arrest, complex cardiovascular surgery, and protection against cerebral ischemia (1–3). Although the potential of hypothermia to protect human brain is well-accepted, little is known about the underlying mechanisms. Thus, understanding the fundamental mechanism of hypothermic neuroprotection will enable this approach to be more widely and effectively applied.

In our previous study using a piglet model of deep hypothermic circulatory arrest, we identified significant microRNA signatures in the hippocampus CA1 region, specifically the downregulation of miR-194-5p as the response with the highest *q* value and biggest fold-change (4), suggesting that miR-194-5p may be responsible for the mechanisms of hypothermia. In this study, we attempted a reverse translation study to define the role of miR-194-5p in neuroprotection against oxygen-glucose deprivation (OGD). Our findings showed that inhibition of miR-194-5p promotes neuronal survival via the enhancement of endogenous neuroprotective machinery involving SUMO-2 activation.

Methods

Cell cultures

Rat primary cell cultures: Primary cultures for rat cortical neurons, rat cortical astrocytes, and rat cortical microglia were prepared as described previously(20-22). All cells were cultured at 37 °C in a humidified chamber of 95% air and 5% CO_2. Primary neuron cultures. Primary neuron cultures were prepared from cerebral cortices of embryonic day (E)17 Sprague–Dawley rat embryos. In brief, cortices were dissected and dissociated using papain dissociation system (Worthington Biochemical Corporation, LK003150). Primary neuron were spread on plates coated with poly-d-lysine (Sigma, P7886) and cultured in DMEM (NBM, Life Technology, 11965-084) containing 25 mM glucose, 4 mM glutamine, 1 mM sodium pyruvate, and 5% FBS at a density of 2×10^5 cells ml−1 (1 ml for 12-well format, 0.5 ml for 24-well format). At 24 h after seeding, the medium was changed to Neurobasal medium (Invitrogen, 21103-049) supplemented with B-27 (Invitrogen, 17504044) and 0.5 mM glutamine. Primary neurons were cultured at 37 °C in a humidified chamber of 95% air and 5% CO2. Primary neuron cultures were used for experiments from 7 to 10 days after seeding. Rat cortical astrocytes Primary astrocyte cultures were prepared from cerebral cortices of 2-day-old neonatal Sprague–Dawley rats. In brief, dissociated cortical cells were suspended in DMEM (Life Technology, 11965-084) containing 25mM glucose, 4mM glutamine, 1mM sodium pyruvate, and 10% FBS and plated on uncoated 25cm2 flasks at a density of 6×105 cells cm^{-} 2. Monolayers of type 1 astrocytes were obtained 12–14 days after plating. Non-astrocytic cells such as microglia and neurons were detached from the flasks by shaking and removed by changing the medium. The microglia were collected for further culture. Astrocytes were dissociated by trypsinization and then reseeded on uncoated T75 flasks. After the astrocytes reached 70–80% confluence, were used for experiments.

Rat brain endothelial cells: A rat brain microendothelial cell line, RBE.4 were maintained in EBM-2 containing EGM-2MV Single Quots kit onto collagen-coated 25 cm^2 flasks at a density of 2×10^5 cells/cm^2 incubated in a 5% CO_2 incubator at 37°C.

RNA extraction and real-time PCR.

Total RNA was extracted from the primary cultured cells using RNeasy Plus Mini Kit (50) (QIAGEN, Cat. No. 74134) in accordance with the manufacturer's instructions. Total RNA was reverse-transcribed with hairpin-loop primers designed to target the specific miRNA at a concentration of 600 ng/μL used for cDNA synthesis PrimeScript^{TM} 1st strand cDNA Synthesis Kit (TaKaRa Clontech, Cat#6110A). Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction was performed with 20 ng cDNA in a 20 μL volume on the ABI 7500 System. Semi-quantitative real-time PCR, using RT_2 SYBR Green ROX qPCR Mastermix (QIAGEN, Cat. No. 330520), For each of the selected miRNAs, real-time PCR measurements were performed to obtain a mean CT value for each sample. The CT values of the different samples were compared using the $2^{-\Delta\Delta CT}$ method, and U6 expression levels were used as an internal reference. For mRNA assays total RNA was reverse- transcribed with oligoDT primers at a concentration of 600 ng/μL used for cDNA synthesis PrimeScript^{TM} 1st strand cDNA Synthesis Kit (TaKaRa Clontech, Cat#6110A). TaqMan mRNA assays (Applied Biosystems Inc., Carlsbad, CA, USA) were used to quantify SUMO-2 mRNA (Rn00821719-g1, invitrogen) expression levels, in accordance with the manufacturer's protocol. and B2m (Rn00560865-m1, invitrogen) expression levels were used as an internal reference.

Transfection of miRNA-194-5p inhibitor or control miRNA.

The Rno-miR-194-5p Anti-miR-miRNA Inhibitor ID:AM10004 (Ambion, AM17000) or MiR-Con ID:AM10004 (Ambion, AM17000) was mixed with Lipofectamine RNAiMAX Reagent (Invitrogen, 13778-075). Each mixture was added into primary rat neuron (7 days in vitro) or rat cortical astrocytes as manufacturer's instructions.

Oxygen-glucose deprivation (OGD) and reoxygenation

OGD experiments were performed using a specialized, humidified chamber (Heidolph, incubator 1000, Brinkmann Instruments) kept at 37 °C, which contained an anaerobic gas mixture (90% N2, 5% H2, and 5% CO2) as we descripted before (22). To initiate OGD, culture medium was replaced with deoxygenated, glucose-free DMEM (Life Technology, 11966-025). After 2 h challenge, cultures were removed from the anaerobic chamber, and the OGD solution in the cultures was replaced with maintenance medium. Cells were then allowed to recover for 18 h (for neurotoxicity assay) in a regular incubator.

Determination of cell viability

Neuronal injury was measured by standard cell cytotoxicity assays such as Cell Counting Kit 8 cytotoxicity assay (DOJINDO, CK04-13) and PI (Propidium Iodide Incorporation) staining. Cell viability

was quantified using a Cell Counting kit-8 (CCK-8, Dojindo) according to the manufacturer's instructions. CCK-8 solution (10 μl) was added to each well of the plate, and the cells were incubated at 37 ℃ for 2 hours. The optical density at a wavelength of 450 nm was measured with microplate reader. The relative assessments of neuronal injury were normalized by comparison with control cell as 100% cell survival (CCK8). For the PI staining, Cell viability was assessed after staining of naive cell cultures with propidium iodide (PI) to distinguish between living and dead cells (0.001mg/mL for 10minutes with subsequent rinsing) and fixed with 4% paraformaldehyde in phosphate buffered saline. Five images per well were taken using microscope. Viable neurons not incorporating PI were counted in transmission images and quantified.

Western blot analysis

Pro-PREP Protein Extraction Solution (iNtRON Biotechnology, 17081) was used to collect samples. Each sample was loaded onto 4–20% Tris-glycine gels. After electrophoresis and transferring to nitrocellulose membranes, the membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 0.2% I-block (Tropix, T2015) for 90 min at room temperature. Membranes were then incubated overnight at 4 °C with the following primary antibodies, anti-β -actin (1:1,000, Sigma-Aldrich, A5441), SUMO2/3-specific antibody (1:1,000, Abcam, ab3742). After incubation with peroxidase-conjugated secondary antibodies, visualization was enhanced by chemiluminescence (GE Healthcare, NA931 (anti-mouse), NA934 (anti-rabbit)). Optical density was assessed using the NIH Image analysis software.

Luciferase reporter assays were performed to verify the direct binding of miR-194-5p to SUMO2 transcripts.

Construction of 3'-UTR reporter plasmids and luciferase assays synthesized double-strand oligonucleotides containing 20 bases upstream and 15 bases downstream of miR-194-5p seed sequences. We constructed pmiR-GLO-194-5p SUMO2-TS (targeting sequence) reporter vector, containing the 3'-UTR of SUMO2. The pmiRGLO-194-5p-SUMO2-WTS (with wide targeting sequence) also generated in this cloning step. The pmiRGLO-194-5p-SUMO2-MTS (with mutated targeting sequence) also generated in this cloning step, by reversing the seed targeting sequence of SUMO2. The 293T cells were co-transfected with either pmiRGLO-194-5p-SUMO2-MTS or pmiRGLO-194-5p-SUMO2-WTS with miR-Con or miR-194. Luciferase reporter assay cells were lysed in passive lysing buffer and then analyzed for the firefly and renilla luciferase activities using the commercial Dual-Luciferase reporter assay system on the GloMax-multi Detection Luminometer (Promega Corporation, E7031, Madison, WI, USA), and the firefly luciferase activity was normalized to the renilla luciferase activity. In all, 24 h after transfection, the firefly and Renilla luciferase activities were detected consecutively using Dual-Luciferase Kit (Promega Corporation). Relative protein levels were expressed as Firefly/Renilla luciferase ratios.

SUMO2 knockdown Lentiviral vector contraction and transduction in primary neurons.

The SUMO2 knockdown by shRNA infection Lentiviral vectors encoding shRNAs, Recombinant lentivirus (pLKD-CMV-eGFP-sumo2-shRNA) against rat SUMO2 and control vector (pLKD-CMV-eGFP-U6-

shRNA) were designed and reconstructed by Obio Technology Co., Ltd, (Shanghai, China). The target sequences for rat SUMO2-shRNA are Seq1: 5'-GCGAGATCAGCATCTGCAT- 3', Seq2: 5'-GCAGCTCATGGTACATGCT-3'. Four siRNA oligonucleotide sequences targeting sumo2 (NCBI accession Gene ID NM_133594) were Seq1: 5'-GTTTGTCAATGAGGCAGATCA-3' (Y6228), and Seq2:5'-GGCATACACCACTTAGTAA-3'(Y6229), Seq3: 5'-GGATGAAGATACGATTGAT-3' (Y6230) The NC sequence was 5'-TTCTCCGAACGTGTCACGT-3' (Y004) whose knockdown effect has been demonstrated previously. The vector clones were constructed pcDNA-sumo2 and transformed into competent Escherichia coli. The sense and antisense oligomers were cloned into SUMO2 restriction sites of lentiviral vector (pLenti-U6-shRNA-CMV-EGFP) to drive the shRNA expression under the U6 promoter. The SUMO2 knockdown lentivirus was packaged with pLKD-CMV-eGFP plasmid and transfected into C6 cell line. The viral supernatant was harvested, filtered, and concentrated. For lentivirus infection, primary cultured rat neurons were seeded in 24-well plate. After neurons were cultured for 3 days, cells were infected with recombinant SUMO2 shRNA-lentivirus or or NC-shRNA-lentivirus control vector (as negative control) at a multiplicity of infection (MOI) of 20 in medium (Supplementary Fig. A). To determine the transduction efficiency in primary neurons, GFP expression was examined by fluorescence microscopy (ZEISS, German) at different multiplicities of infection (MOIs) on day 3 after infection. Primary cultured rat neurons were transfected with the NC or recombinant vectors. After 8 h infection, the medium was replaced by fresh complete medium for another 4 days prior to further experiments, neurons were harvested at 4 days after transfection. GFP-positive cells were confirmed under a fluorescence microscope (Supplementary Fig. B). Image acquisition was executed using an inverted fluorescence microscope (Olympus) equipped with a 60× oil immersion objective. Sustained SUMO2 downregulation levels were confirmed by western blot analysis neurons after transfection (Supplementary Fig. C).

Statistical analysis.

All values are represented as mean± SD of at least three independent experiments. When only two groups were compared, Student’s t-test was used. Multiple comparisons were evaluated by one-way ANOVA followed by Tukey's tests. *P*-values of <0.05 were deemed to be significantly different.

Results

First, we assessed the expression levels of miR-194-5p in CNS cells. qRT-PCR analysis showed that miR-194-5p was highly expressed in neurons and astrocytes, compared to microglia and cerebral endothelial cells (Figure 1A). Next, control (miR-Con) or the miR-194-5p inhibitor (anti-miR-194-5p) was transfected into neurons and astrocytes (Figure 1B). Anti-miR-194-5p significantly protected neuronal viability against oxygen-glucose deprivation (Figure 1C). No effects were seen in astrocytes (Figure 1D). To further confirm these findings, experiments were repeated with propidium iodide staining; anti-miR-194-p5 effectively reduced propidium iodide-stained neuron death as well (Figure 1E).

Next, we proceeded to investigate the mechanism regulated by miR-194-5p. Using a molecular target prediction algorithm based on Target Scan, miRanda, and Pictar analysis (5), we found that the small

ubiquitin-related modifier-2 (SUMO-2) possessed a well conserved binding site for miR-194-5p within its 3`-untranslated region (UTR) in most species including human (Figure 2A). Therefore, we tested the effect of miR-194-5p inhibition on SUMO-2. qRT-PCR analysis demonstrated that SUMO-2 mRNA was significantly increased by miR-194-5p inhibition (Figure 2B). Furthermore, western blot showed that accumulation of SUMO-2 conjugates were clearly increased by anti-miR-194-5p (Figure 2C). Using luciferase assays we determine miR-194 can directly target the 3'UTR of SUMO-2, miR-194 can bind to the SUMO-2 3'UTR to regulate gene expression. (Figure 2D).

To test this hypothesis, we performed a SUMOylation blockade experiment with puromycin (6). Western blot analysis demonstrated that puromycin successfully suppressed SUMOylation induced by anti-miR-194-5p (Figure 3A, B). Concomitantly, inhibiting SUMOylation with puromycin canceled the neuroprotective effect induced by miR-194-5p inhibition after oxygen-glucose deprivation in neuronal cultures (Figure 3C, D). To test the inhibition of the specific SUMOylation blockade with recombinant lentivirus (pLKD-CMV-eGFP-sumo2-shRNA). Western blot result confirmed the anti-miR-194-5p cannot elevate the SUMO2 after recombinant lentivirus (pLKD-CMV-eGFP-sumo2-shRNA) application (Figure 4A, B). Specifically blocking the SUMOylation with specific Recombinant lentivirus (pLKD-CMV-eGFP-sumo2-shRNA), there was no neuroprotective effect observation (Figure 4C, D).

Discussion

Taken together, these data suggest a novel mechanism that miR-194-5p inhibitor may up-regulated SUMO-2, thus blocking miR-194-5p may be a potential therapy for protecting neurons against ischemic stress. microRNAs (miRNAs) are short, non-coding RNAs that bind to mRNAs to inhibit translation and/or promote mRNA degradation by imperfect base-pairing between the seed region in miRNAs (7). Emerging evidence suggest that miRNAs play important roles in CNS. For example, miR-124, the most abundant miRNA in the CNS, regulates neuroprotection by preventing neuronal apoptosis (8) and modulating inflammatory response through inducing anti-inflammatory phenotype in microglia/macrophage (9) after cerebral ischemia. More recently, it has been reported that hypothermia may alter miRNA response after traumatic brain injury, suggesting the presence of temperature-sensitive miRNAs in the brain (10). From our previous study using a piglet model of deep hypothermic circulatory arrest, we identified miR-194-5p as a leading candidate for hypothermia-sensitive miRNA and neuroprotection (4). Here, we demonstrated the miR-194-5p may specifically recognize SUMO-2 mRNA and downregulate gene expression by translational repression or mRNA cleavage posttranscriptional mechanisms.

SUMO-2/3 conjugation is indeed involved in the protective effects induced by deep hypothermia and may play a role in protein quality control such as subcellular localization, transcription regulation, DNA replication, and repair (11–13). SUMO-2 conjugation and levels of SUMO-conjugated proteins markedly increase in hibernating animal brain during the torpor phase. Therefore, it has been suggested that this may be a protective response shielding neurons from damage induced by low blood flow and substrate deprivation (14). Moreover, accumulating studies verified that global increases in SUMO- 2/3 and simultaneous SUMOylation are endogenous neuroprotective response to ischemic stress (15–17). Our

current study showed that SUMO-2 conjugation was significantly increased when miR-194-5p was blocked in neurons, suggesting that inhibition of miR-194-5p-SUMO-2 interaction may be a key mechanism in hypothermia-mediated brain protection.

Nevertheless, there are several caveats that should be considered. Our data implicate miR-194-5p as a candidate for hypothermia-sensitive miRNA. But other miRNAs such as miR-874 and miR-451 are also known to be temperature sensitive (10). How miR-194-5p interacts with other miRNA networks remain to be fully dissected. A second caveat involves the role of miR-194-5p in other brain cells. We found that miR-194-5p was also highly expressed in astrocytes. Many miRNAs have been described to explain astrocytic functional activation including astrogliosis, induction of inflammation, and enhancement of neuroplasticity (18). Although miR-194-5p inhibitor did not affect cell survival or proliferation in astrocytes following ischemic stress, miR-194-5p may still affect neuron-glia crosstalk in other ways. The miR-194-5p-SUMO-2 interaction occurs in ischemic brain should be eventually addressed with in vivo studies. Finally, based on our previous our findings that hypothermia inhibited miR-194-5p expression, we tested the effect of miR-194-5p inhibition on neuroprotection. Of course, hypothermia inhibits many different pathways including excitotoxicity, neuroinflammation, apoptosis and free radical production (19). Further studies are required to define how miR-194-5p inhibition may affect these pathways.

Conclusions

The present study demonstrates that miR-194-5p inhibition promotes neuronal viability following oxygen-glucose deprivation via upregulating SUMO-2. These findings provide a novel mechanism for interaction between miR-194-5p and SUMO-2, also provide a potential strategy for miRNA-targeted therapies that further leverage hypothermic neuroprotection.

List Of Abbreviations

OGD: oxygen-glucose deprivation; RBE: Rat brain endothelial cells; PI: Propidium Iodide Incorporation; TS: targeting sequence; WTS: with wide targeting sequence; MTS: with mutated targeting sequence; MOI: multiplicity of infection; SUMO-2: small ubiquitin-related modifier-2; UTR : untranslated region.

Declarations

Acknowledgements

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Authors' contributions

The research was conceived and designed by XHW. The experiments were performed by SYZ, YH and YQG. The data was analyzed by WWQ. The manuscript was draft by SYZ and the manuscript was

modified by YZ. GGZ and TLW contributed to the final approval of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Ethics approval and consent to participate

All laboratory animals were in strict accordance with Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. All experimental procedures were approved by the Animal Care and Use Committee of Capital Medical University, China.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Anesthesiology, Xuanwu Hospital, Capital Medical University, Beijing 100053, China. [2] National Clinical Research Center for Geriatric Disorders, Beijing, China. [3] Daxing Hospital Affiliated to Capital Medical, Xingfeng Street, Daxing District, Beijing 100026, China. [4] Department of Radiology, Xuanwu Hospital, Capital Medical University, Beijing 100053, China. [5] Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, Beijing, 100053, China. [6] Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, Beijing, 100053, China. [7] Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA. [8] Department of Institute of Cardiology, The Second Hospital of Tianjin Medical University, Tianjin, 300211, China.

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Figures

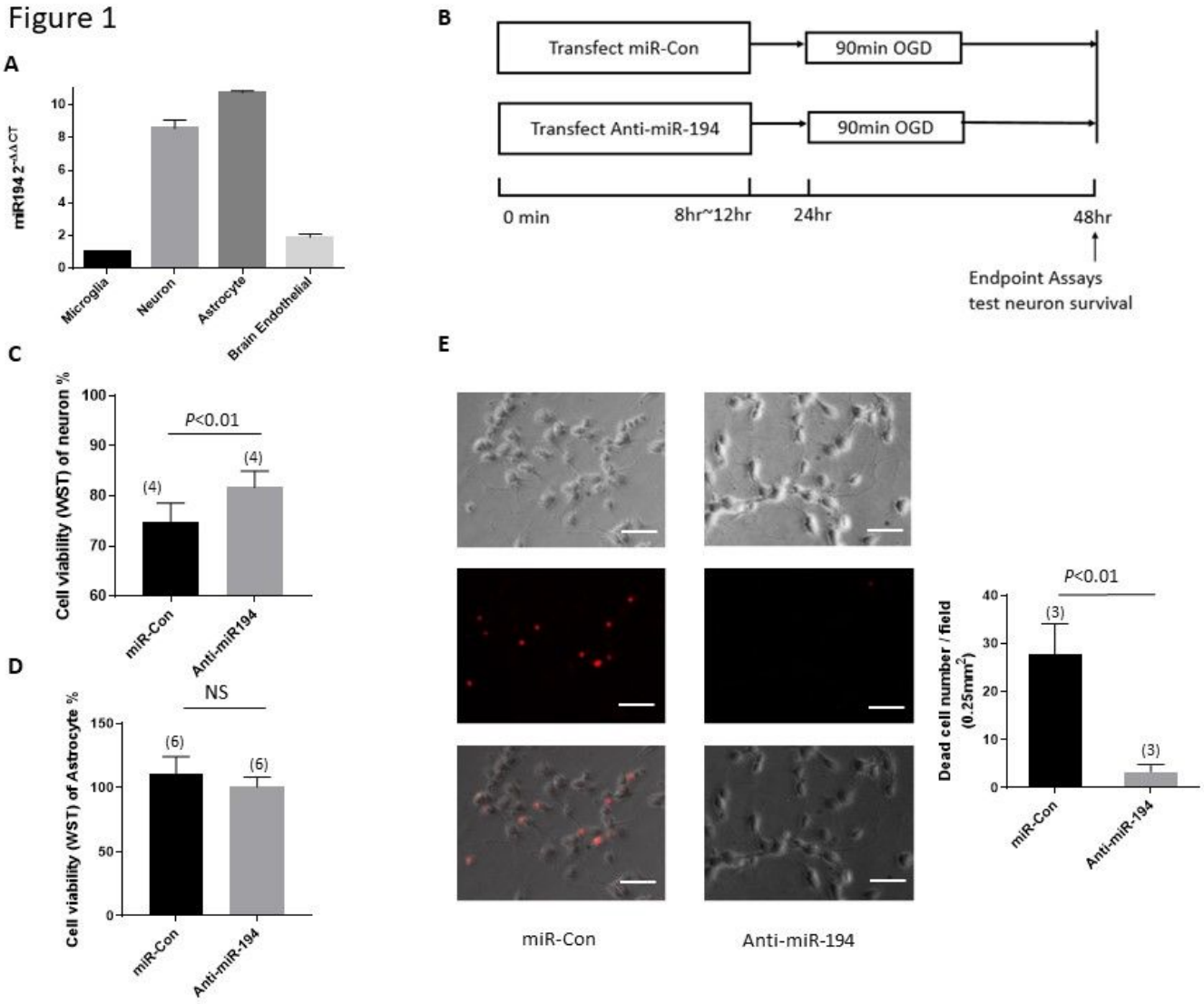

Figure 1

MiR-194-5p inhibition promotes neuronal viability after oxygen-glucose deprivation (OGD). A. MiR-194-5p expression was assessed in brain-derived cells such as microglia, neuron, astrocyte, and brain endothelial cell. qRT-PCR analysis showed that miR-194-5p was highly expressed in neurons and astrocytes compared to microglia (n=3). B. Experimental schedule for miR-Con or miR-194-5p inhibitor transfection, followed by oxygen-glucose deprivation (OGD) and reoxygenation. C. Anti-miR-194-5p significantly improved neuronal survival after OGD. D. But, cell viability or proliferation in astrocytes was not affected by miR-194-5p inhibition (n=4 or 6). E. PI staining demonstrated that neuronal cell death after OGD was remarkably decreased by miR-194-5p inhibition (n=3).

Figure 2

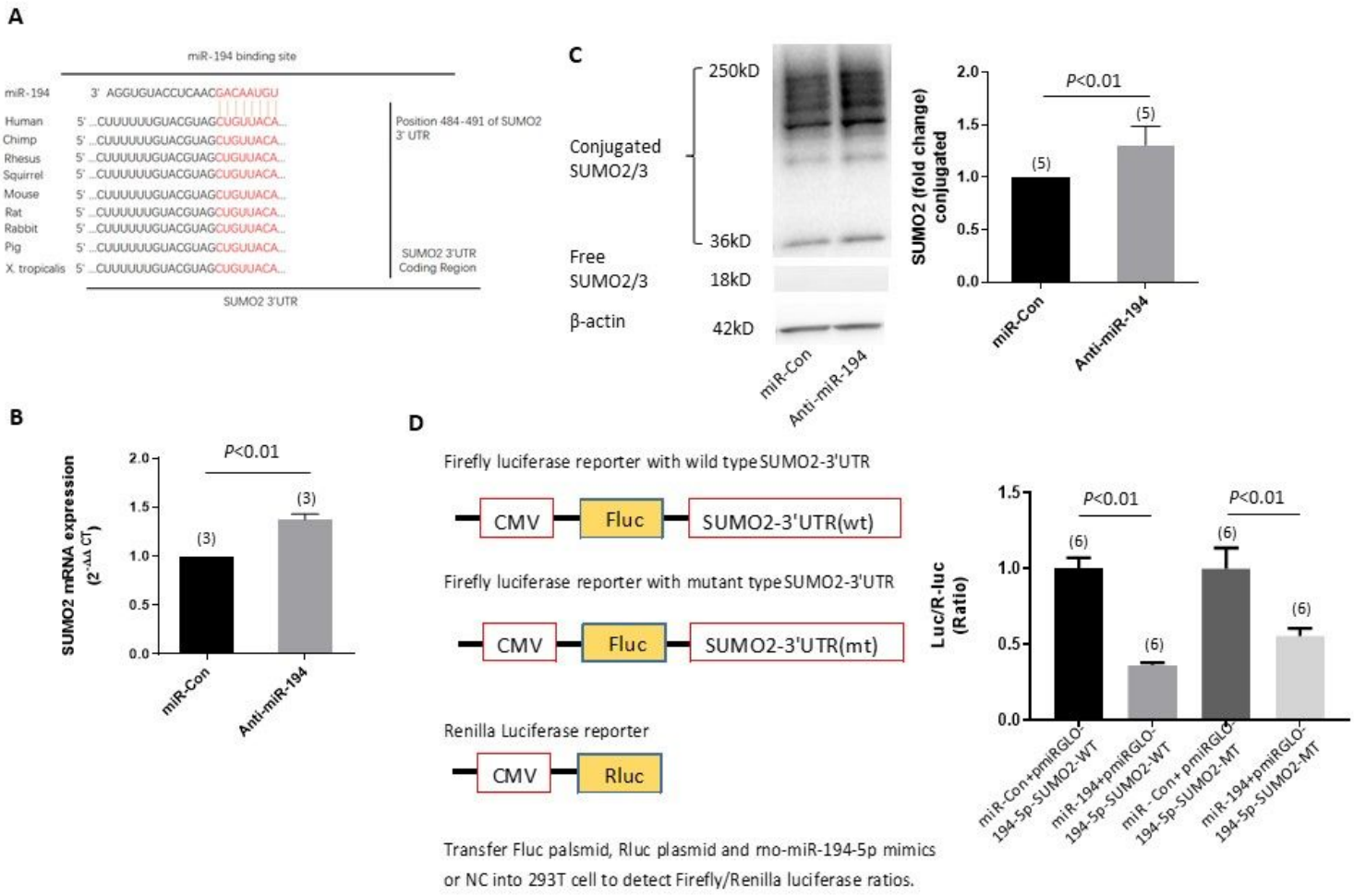

Figure 2

SUMO-2 may be a target of miR-194-5p in rat cortical neurons. A. In miRNA target prediction algorithm, we found that SUMO-2 had the binding site for miR-194-5p within its 3`-untranslated region (UTR) in most species including human. B. qRT-PCR analysis demonstrated that SUMO-2 mRNA was significantly increased by miR-194-5p inhibition (n=3). C. Western blot showed that accumulation of SUMO-2 conjugates were clearly increased by the inhibition (n=5). D. Using luciferase assays we determine miR-194 can directly target the 3'UTR of SUMO-2. miR-194 can bind to the SUMO-2 3'UTR to regulate gene expression.

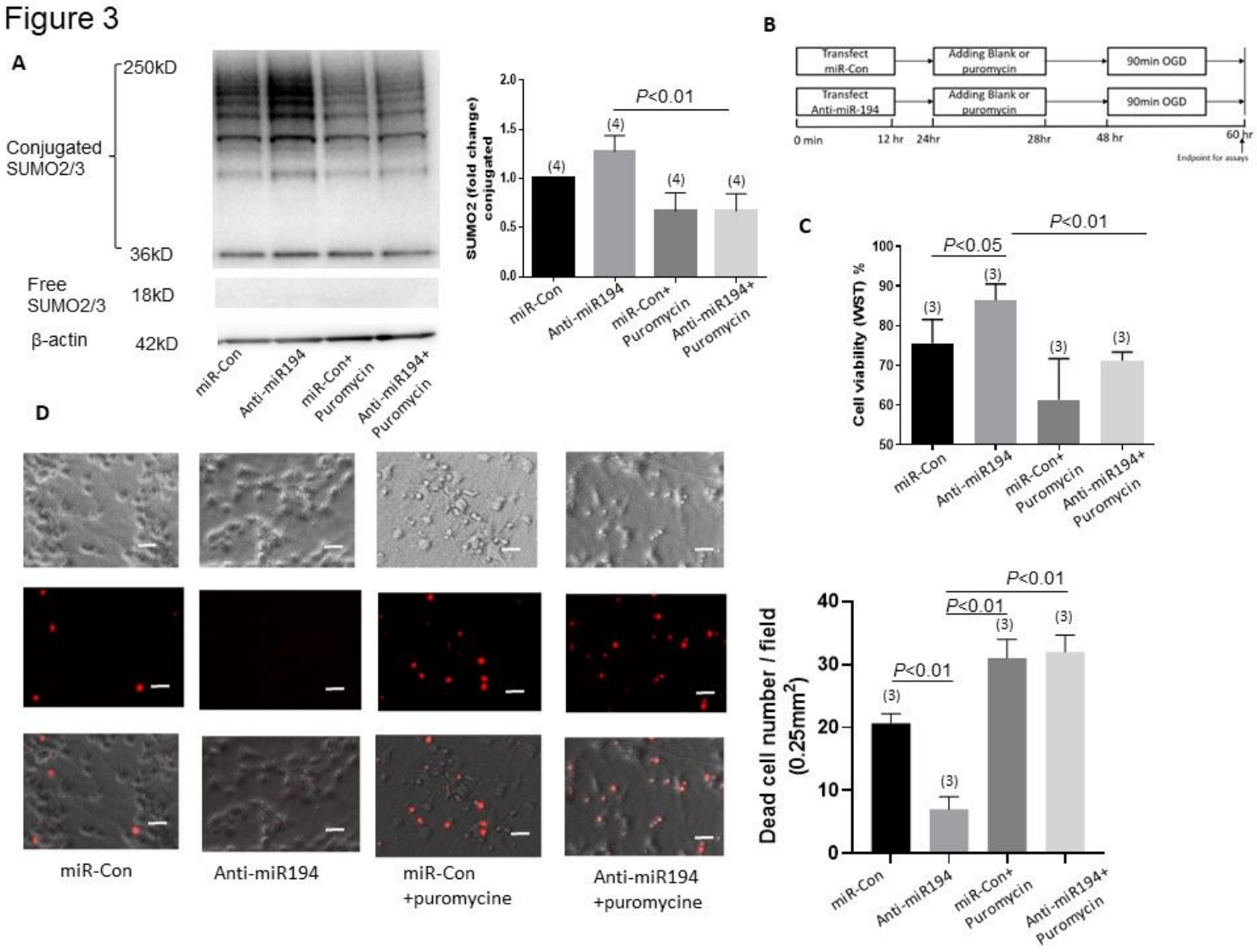

Figure 3

None-special suppression of SUMO-2 reversed neuroprotection mediated by miR-194-5p inhibition after OGD. A.Western blot analysis revealed that puromycin successfully suppressed SUMOylation induced by inhibiting miR-194-5p (n=4). B. Inhibition of SUMOylation with puromycin canceled neuroprotective effect induced by miR-194-5p inhibition following OGD (n=6). C.Anti-miR194 reduce the neuronal cell death after OGD, but puromycin reverse the Anti-miR194 neuron protective effect after OGD (n=3) D. Propidium iodide (PI) demonstrated that neuronal cell death after OGD was remarkably decreased by miR-194-5p inhibition. But puromycin reverse the Anti-miR194 neuron protective effect after OGD (n=3)

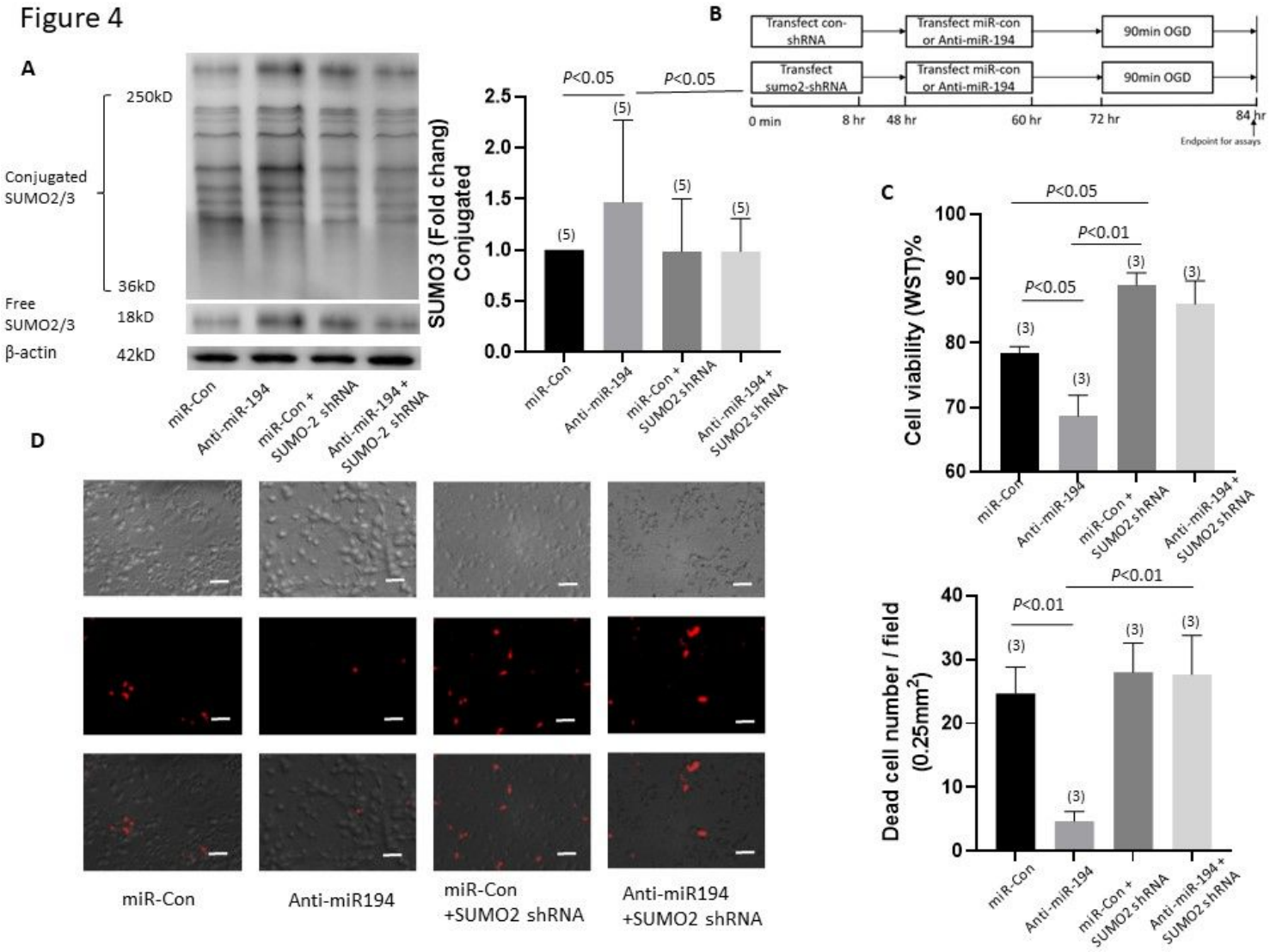

Figure 4

Special suppression of SUMO-2 reversed neuroprotection mediated by miR-194-5p inhibition after OGD. A. Western blot analysis revealed that Recombinant lentivirus pLKD-CMV-eGFP-sumo2-shRNA successfully specially suppressed SUMOylation induced by inhibiting miR-194-5p (n=5). B. Experimental schedule for Recombinant lentivirus pLKD-CMV-eGFP-sumo2-shRNA transfection. C. Inhibition of SUMOylation with Recombinant lentivirus pLKD-CMV-eGFP-sumo2-shRNA canceled neuroprotective effect induced by miR-194-5p inhibition following OGD (n=5). D. Propidium iodide (PI) demonstrated that neuronal cell death after OGD was remarkably decreased by miR-194-5p inhibition. Recombinant lentivirus pLKD-CMV-eGFP-sumo2-shRNA reverse the Anti-miR194 neuron protective effect after OGD (n=5)

Supplementary Files

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- SupplenmentaryFigure1.jpg