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Regular paper

Competitive binding of circCCDC6 to microRNA-128-3p activates TXNIP/NLRP3 pathway and promotes cerebral ischemia-reperfusion defects

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Objective: Circular RNAs (circRNAs) are enriched in the brain and involved in various central nervous system diseases. The potential role of circCCDC6 in cerebral ischemia-reperfusion defects was partly elucidated in the work. Methods: A middle cerebral artery occlusion/reperfusion (MCAO/R) rat model and an oxygen-glucose deprivation and re-oxygenation (OGD/R)-treated SH-SY5Y cell model were constructed. CircCCDC6 expression in the two models was examined, and circCCDC6-involved mechanisms in neuronal pyroptosis and inflammation were analyzed through loss- and gain-of-function assays. Results: MCAO/R rat brain tissues and OGD/R-treated SH-SY5Y cells exhibited upregulated circCCDC6. Silencing circCCDC6 attenuated neuronal pyroptosis and inflammation in the brain tissue of MCAO/R rats. Overexpressing circCCDC6 or inhibiting miR-128-3p stimulated OGD/R-induced pyroptosis and inflammation in SH-SY5Y cells, while upregulating miR-128-3p attenuated OGD/R injury. CircCCDC6 silencing-induced effects on SH-SY5Y cells were antagonized by TXNIP overexpression. Conclusion: Mechanistically, circCCDC6 mediates miR-128-3p and activates TXNIP/NLRP3, thereby promoting OGD/R-induced neuronal pyroptosis and inflammation. CircCCDC6 may provide a new strategy for the treatment of MCAO/R.

Keywords: Cerebral ischemia-reperfusion, Circular RNA CCDC6, microRNA-128-3p, TXNIP/NLRP3, Pyroptosis, Inflammation

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Abbreviations: AKT, Protein kinase B; ASC, Apoptosis-associated speck-like protein containing a CARD; BCA, Bicinchoninic acid; CIR, Cerebral ischemia-reperfusion; circCCDC, 6Circular RNA Coiled-coil domain containing 6; circRNAs, Circular RNAs; DMEM, Dulbecco's modified essential medium; ELISA, Enzyme-linked immunosorbent assay; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GSDMD, Gasdermin D; IgG, Immunoglobulin G; IL-1β, Interleukin-1beta; Interleukin-18 (IL-18); IS, Ischemic stroke; LDH, Lactate dehydrogenase; LPS, Lipopolysaccharide; MCAO, Middle cerebral artery occlusion; miRNAs, microRNAs; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MUT, Mutant type; NF-κB, Nuclear factor-kappaB; NLRP, 3NOD-like receptor family pyrin domain containing 3; OA, Osteoarthritis; OGD/R, Oxygen-glucose deprivation/re-oxygenation; PI3K, Phosphoinositide 3-kinase; RIP, RNA immunoprecipitation assay; RIPA, Radio-Immunoprecipitation Assay; ROS, Reactive oxygen species; TGFBR2, Transforming Growth Factor-beta Receptor 2; TUNEL, Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling staining; TXNIP, Thioredoxin interacting protein; WT, Wild type

INTRODUCTION

Ischemic stroke (IS) is a severe neurological response syndrome that leads to damage to neurovascular units, inflammation, and neuronal death (Zhu *et al.*, 2020). Reperfusion after cerebral ischemia often leads to a series of cellular and biochemical consequences, including the production of reactive oxygen species (ROS) and inflammatory cytokines, and brain cell damage, collectively referred to as cerebral ischemia-reperfusion (CIR) injury (Lim *et al.*, 2021), which are also key factors attributing to the poor prognosis of IS patients (Zuo *et al.*, 2018). It is unable to effectively protect the neurological function of IS patients despite great progress in the treatment options. Therefore, an urgent need for new therapeutic approaches is on the agenda to protect CIR-induced neurological deficits.

Pyroptosis is a special way of cell death, which is different from necrosis and apoptosis (Yu *et al.*, 2021). Pyroptosis plays an important role in CIR injury (Zhang *et al.*, 2022). Cerebral ischemia leads to the disruption of intracellular energy metabolism and oxidative stress, which activates the inflammatory response and intracellular inflammasome recombination (Zhang *et al.*, 2022). The inflammasome complex includes molecules such as ASC, NLRP3, and Caspase-1, which interact and activate the Caspase-1 enzyme. Activated Caspase-1 cleaves the precursor's IL-1β and IL-18, releasing active pro-inflammatory cytokines (Wang *et al.*, 2022; Luo *et al.*, 2022). These pro-inflammatory cytokines induce inflammation in CIR injury and promote the further development of pyroptosis.

Circular RNAs (circRNAs) are highly stable covalently closed endogenous non-coding RNAs that are specifically expressed in tissues and cells (Vo *et al.*, 2019). CircRNAs are enriched in multiple organs, especially the brain, and a large body of evidence indicates that circRNAs are involved in the physiological and pathological processes of the brain, including CIR (Yang *et al.*, 2018). In addition, circRNAs exert their biological functions as molecular decoys of microRNAs (miRNAs) (Zhang *et al.*, 2020). In a circRNA expression profile after transient focal ischemia, circCCDC6 is confirmed to be upregulated in the brain of a model of middle cerebral artery occlusion (MCAO) rats (Mehta *et al.*, 2017). However, its related mechanism in CIR awaits further investigation.

MiRNAs, a group of post-transcriptional regulators of gene expression (Brennan *et al.*, 2020) are involved in the regulation of various cellular activi-

ties (Saliminejad *et al.*, 2019). miRNAs are emerging regulators of neuronal survival during CIR injury (Liu *et al.*, 2020). For instance, promoting miR-34c-5p expression is able to suppress inflammation and apoptosis, thereby preventing CIR injury (Tu *et al.*, 2021). The anti-inflammatory and anti-apoptotic properties of miR-128-3p have been accepted previously. miR-128-3p overexpression could protect against chronic constrictive injury to suppress neuroinflammation (Zhang *et al.*, 2020) and could restrain sepsis-induced apoptosis and inflammation (Yang *et al.*, 2021). However, not much was known about miR-128-3p-involved mechanism in CIR injury.

Hence, the function of circCCDC6 in neurological deficits following CIR was probed through *in vivo* and *in vitro* experiments. Speculation was put up that circCCDC6 activates TXNIP/NLRP3 inflammasome pathway by miR-128-3p to exacerbate CIR.

MATERIALS AND METHODS

MCAO/R animal model

Sixty male Sprague-Dawley rats, with a body weight of 220-280 g (Hunan SJA Laboratory Animal Co., Ltd., Changsha, China) were housed in an environment of 24-26°C with an adequate supply of water and food. The MCAO/R model was established as previously described (Yan *et al.*, 2020). Briefly, after anesthetization with 5% (v/v) isoflurane at a flow rate of 2 L/min, a monofilament nylon suture was inserted into the right common carotid artery through an incision over the head, and passed through the internal carotid artery as possible. After blocking the common carotid artery for 1 h, the suture was slowly retracted for reperfusion. Monitored by Laser Doppler flowmetry, and blood flow falling below 30% of baseline flow suggested the success of MCAO. Restoration of cerebral blood flow to >80% of baseline indicated the success of reperfusion. For the sham group, the same procedure was performed without suture insertion. After 24 h of reperfusion, neurological function was evaluated. After that, the rats were euthanized by overdosed CO_2, and 10 successfully modeled rats were selected from each group, of which the brain tissue of 5 rats was used for histopathological analysis, and that of the remaining 5 rats was for RNA or protein extraction. The success rate of modeling was 80%, and some rats died during the experiment. Authorization of animal care and methods procedures was by the Animal Ethics Committee of Dalian Municipal Central Hospital.

Lentiviral vector injection

circ shRNA-GFP (shRNA-Con) lentivirus or circCCDC6 shRNA-GFP (shRNA-circ CCDC6) lentivirus (2 μl, 1×10^9 TU/ml) was microinjected into the left ventricle of the rat to knock down circ CCDC6 at the following coordinates: AP, −0.3 mm; lateral, 1.0 mm; and ventral, 2.2 mm. The lentiviral vector was injected 2 weeks before MACO/R surgery. All lentiviral vectors mentioned above were purchased from GenePharma.

Neurological function scoring

Neurological function was assessed 7 d after MACO/R surgery (Tatlisumak *et al.*, 1998). 0-no obvious defect, 1-failure to fully extend the left front paw when stimulating tails, 2-circling to the left contralateral side when stimulating tails, 3-turning or walking left, 4-walking only when stimulating tails, 5-no response to stimuli, and unconsciousness.

Nissl staining

Brain tissues that were fixed with 4% paraformaldehyde were made into coronal fragments of 10 μm after routine paraffin embedding (Meng *et al.*, 2018). Each fragment was observed by light microscopy after staining with Nissl staining kit (Beyotime, Shanghai, China).

Immunofluorescence staining

After antigen retrieval through citrate buffer, brain slices were incubated with primary antibody GSDMD (20770-1-AP, Proteintech), followed by the addition of a secondary fluorescent antibody. Afterward, 4',6-diamidino-2-phenylindole-stained slices were observed under a fluorescence microscope (Olympus. Tokyo, Japan).

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining

Pyroptosis was assessed using the TUNEL staining kit (Roche, Mannheim, Germany) following the protocols, and the percentage of pyroptotic cells was calculated.

Cell culture

Dulbecco's modified essential medium (DMEM, Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin was the culture system for SH-SY5Y cells (Cell Bank Type Culture Collection, Shanghai, China). For oxygen-glucose deprivation/reoxygenation (OGD/R) treatment, SH-SY5Y cells were cultured in glucose-free DMEM for 4 h in an anoxic environment (95% N_2 and 5% CO_2), and maintained in a normoxic environment with normal DMEM for 24 h (Xin *et al.*, 2017).

Cell transfection

Small interfering RNAs targeting circCCDC6 and TXNIP and negative controls (si-CCDC6, si-TXNIP, si-NC), pcDNA 3.1 overexpression vector and empty vector (pcDNA 3.1-circCCDC6, pcDNA 3.1-TXNIP, pcDNA 3.1) were purchased from Ribobio (Guangzhou, China), whereas miR-128-3p mimic/inhibitor and mimic/inhibitor NC were from Invitrogen (CA, USA). Transient transfection of SH-SY5Y cells was implemented by Lipofectamine 2000 (Invitrogen), followed by OGD/R treatment.

ELISA

Concentrations of IL-1β and IL-18 in cell supernatants or brain tissue homogenates were quantified using ELISA kits (Thermo Fisher Scientific).

Viability analysis

SH-SY5Y cells, along with MTT solution (20 μL/well, Sigma-Aldrich) were incubated for 4 h. Then, the original medium was replaced with dimethyl sulfoxide (150 μL/well), and absorbance was measured at 490 nm using a microplate reader (Bio-Rad, CA, USA).

Table 1. PCR primers

Genes	Sequences
circCCDC6	Forward: 5′-AGCCGAACTAGAACAGCATCT-3′
	Reverse: 5′-TCTCCTTCTGCAAAGCCTGA-3′
miR-128-3p	Forward: 5′-TCACAGTGAACCGGTC-3′
	Reverse: 5′-CAGTGCGTGTCGTGGAGT-3′
GAPDH	Forward: 5′-CTGCCAACGTGTCAGTGGTG-3′
	Reverse: 5′-TCAGTGTAGCCCAGGATGCC-3′
U6	Forward: 5′-CGAATTTGCGTGTCATCCTT-3′
	Reverse: 5′-CGAATTTGCGTGTCATCCTT-3′

Note: circCCDC6, circular RNA CCDC6; miR-128-3p, microRNA-128-3p; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Colorimetry

Lactate dehydrogenase (LDH) release from cell supernatants was quantified colorimetrically to determine cytotoxicity using the kit (Clontech, CA, USA).

Flow cytometry

Using the FAM FLICATM Caspase-1 kit (AbD Serotec, Oxford, UK), pyroptosis of SH-SY5Y cells was tested. SH-SY5Y cells after trypsinization were made into cell suspension for staining with 5 μL caspase-1 FLICA and 5 μL propidium solution, followed by analysis on a flow cytometer (BD Company, NJ, USA). Data quantification was done by FlowJo software (TreeStar, CA, USA).

Quantitative PCR

From brain tissues and cells, total RNA extracts were collected *via* Trizol reagent (Invitrogen), and made into first-strand cDNA through PrimeScript RT kit (Takara, Tokyo, Japan). On the ABI7500 system, PCR was conducted by the SYBR Green method. Primers for all genes (Table 1) were from GeneCreate (Wuhan, Hubei, China). Standard controls for mRNA and miRNA were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6, respectively. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting

Total protein extracts were obtained using RIPA buffer (CST, USA), which was then quantified by BCA kit (Abbkine, USA) and separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis. After that, proteins were covered with polyvinylidene fluoride membrane (Millipore, USA), followed by blockade with 5% nonfat milk and incubation with primary antibodies β-actin (sc-47778, Santa Cruz), IL-1β (12242, CST), IL-18 (10663-1-AP, Proteintech), cleaved caspase-1 (4199, CST), TXNIP (14715, CST), NLRP3 (15101, CST), ASC (sc-514414, Santa Cruz) and the horseradish peroxidase-conjugated secondary antibody (CST). Through visualization by an enhanced chemiluminescence kit (GE Healthcare), band plots were further analyzed using ImageJ.

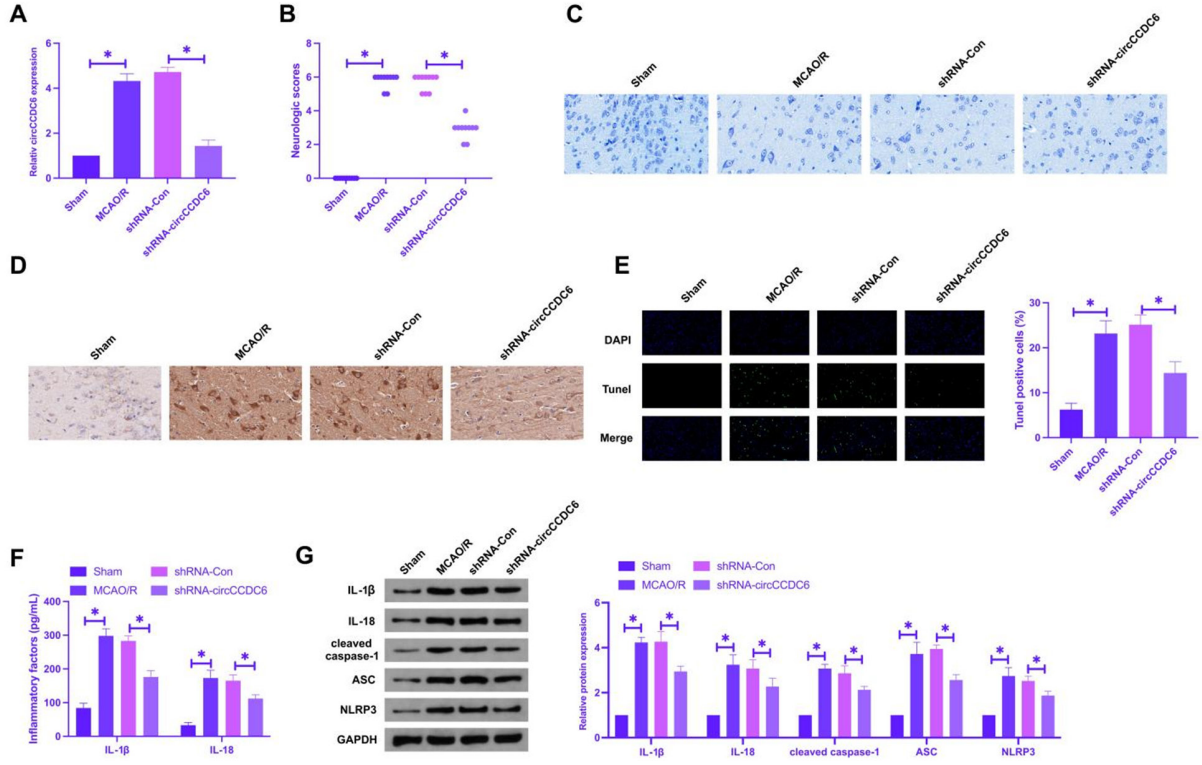

Figure 1. Effects of injection of shRNA-circCCDC6 lentiviral vector on biochemical indexes and pathological tissue damage of MCAO/R rat brain.
circCCDC6 expression in brain tissue (**A**), neurological function score (**B**), Nissl-stained cerebral cortex (**C**), immunofluorescence staining of GSDMD (**D**), TUNEL-stained brain tissue (**E**), IL-1β and IL-18 contents in brain tissue (**F**), protein expressions of IL-1β, IL-18, cleaved caspase-1, ASC and NLRP3 in brain tissue (**G**); data were reported as mean ± S.D. (C, n=10; rest, n=5).

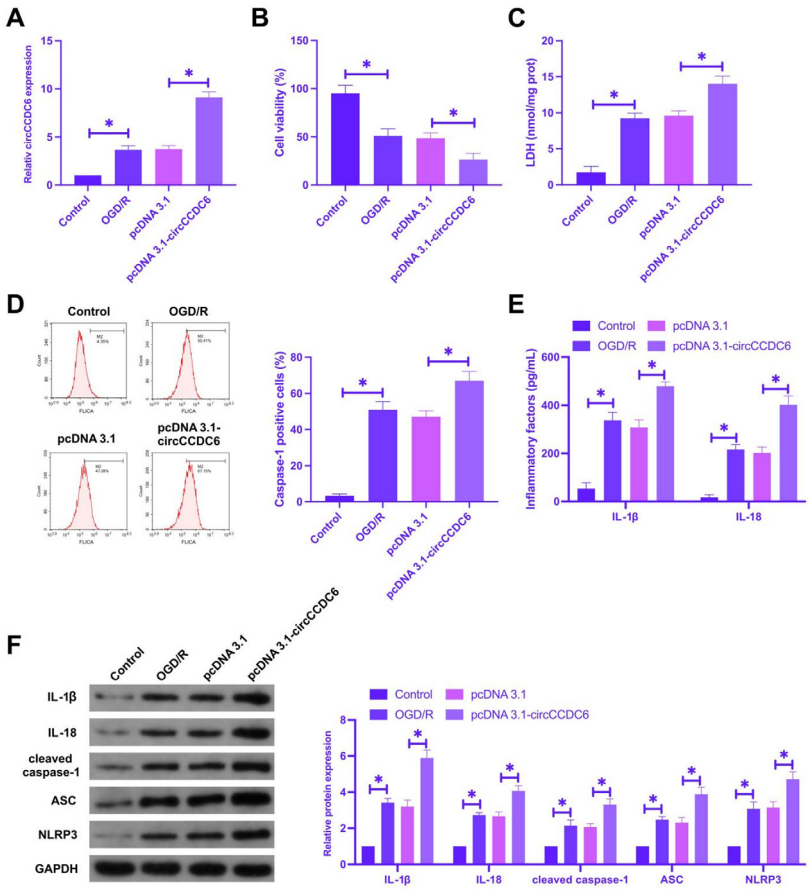

Figure 2. Effect of transfection of pcDNA 3.1-circCCDC6 on the biological behavior and biochemical indexes of OGD/R-treated SH-SY5Y cells.
circCCDC6 expression (**A**), cell viability (**B**), toxicity (**C**), caspase-1 positive cells (**D**), IL-1β and IL-1β contents (**E**), protein expression of IL-1β, IL-18, cleaved caspase-1, ASC and NLRP3 (**F**); data were reported as mean ± S.D. (N=3).

Dual-luciferase reporter assay

The putative binding site of circCCDC6 or TXNIP 3'UTR was amplified by PCR and cloned into a pmirGLO vector to generate pmirGLO-circCCDC6-WT and pmirGLO-TXNIP-3'UTR WT. Mutants of circCCDC6 or TXNIP 3'UTR were created based on QuickChange Lighting Multi Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA) to produce pmirGLO-circCCDC6-Mut and pmirGLO-TXNIP-3'UTR-Mut. The generated reporter, in combination with mimic NC or miR-128-3p mimic was co-transfected into SH-SY5Y cells to determine luciferase activity 48 h later using a dual-luciferase reporter gene assay system (Promega, WI, USA).

Antibody enrichment analysis

RNA immunoprecipitation assay was performed using the Magna RNA-Binding Protein Immunoprecipitation Kit (Millipore). Cell lysates were incubated with RIP buffer containing magnetic beads conjugated to mouse IgG or human anti-Ago2 antibody. After digestion with proteinase K, the resulting immunoprecipitated RNA was purified and utilized for quantitative PCR.

Statistics

Data were presented as mean ± standard deviation (S.D.), and statistical analysis was performed using GraphPad Prism 9.0. Unpaired Student's *t*-test was utilized for bilateral comparison whereas one-way analysis of variance for multi-data comparison. $P<0.05$ was considered statistically significant.

RESULTS

Silencing circCCDC6 ameliorated MCAO/R-induced pyroptosis and inflammation

CircCCDC6 is abnormally expressed after MCAO/R injury. To examine the biological role of circCCDC6 in CIR-induced pyroptosis, we interfered with circCCDC6 expression in MCAO/R rats by injecting lentivirus. It was demonstrated that the promoted expression of circCCDC6 induced by MCAO/R treatment was suppressed after injection of shRNA-circCCDC6 (Fig. 1A). Assessment of neurological function presented that MCAO/R rats had severe nerve damage, which could be alleviated by knocking down circCCDC6 (Fig. 1B). Nissl staining further pictured that neurons in MCAO/R rats were swollen, vacuolated in the cytoplasm, and the number of Nissl bodies was reduced, and knockdown of circCCDC6 alleviated these symptoms (Fig. 1C). During pyroptosis, GSDMD mediates the formation of membrane pores (Wang *et al.*, 2020). Here, we performed immunofluorescence staining of GSDMD and ultimately report-

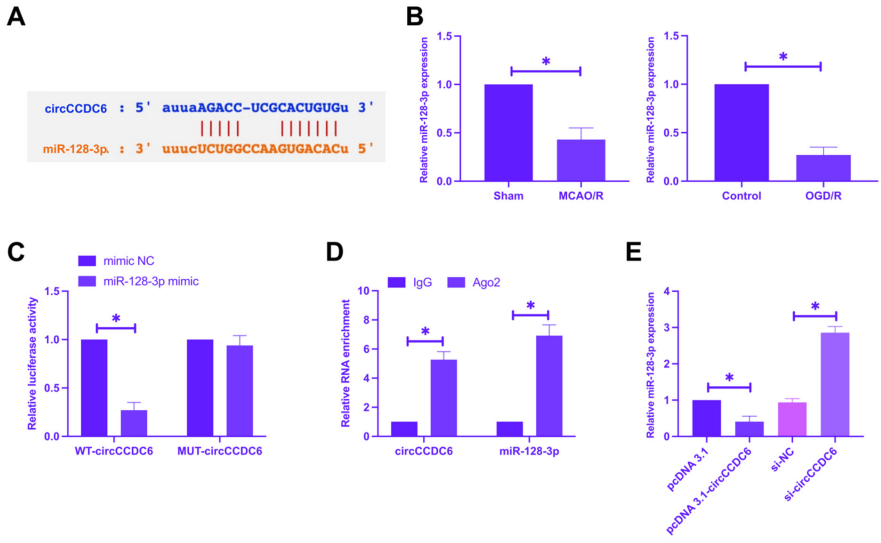

Figure 3. Binding relation of circCCDC6 and miR-128-3p.
Potential binding sites of miR-128-3p and circCCDC6 (**A**), miR-128-3p expression in *in vivo* and *in vitro* models (**B**), verification of targeting relationship between circCCDC6 and miR-128-3p (**C–D**), effects of low or overexpression of circCCDC6 on miR-128-3p expression in SH-SY5Y cells (**E**). data were reported as mean ± S.D. (N=3).

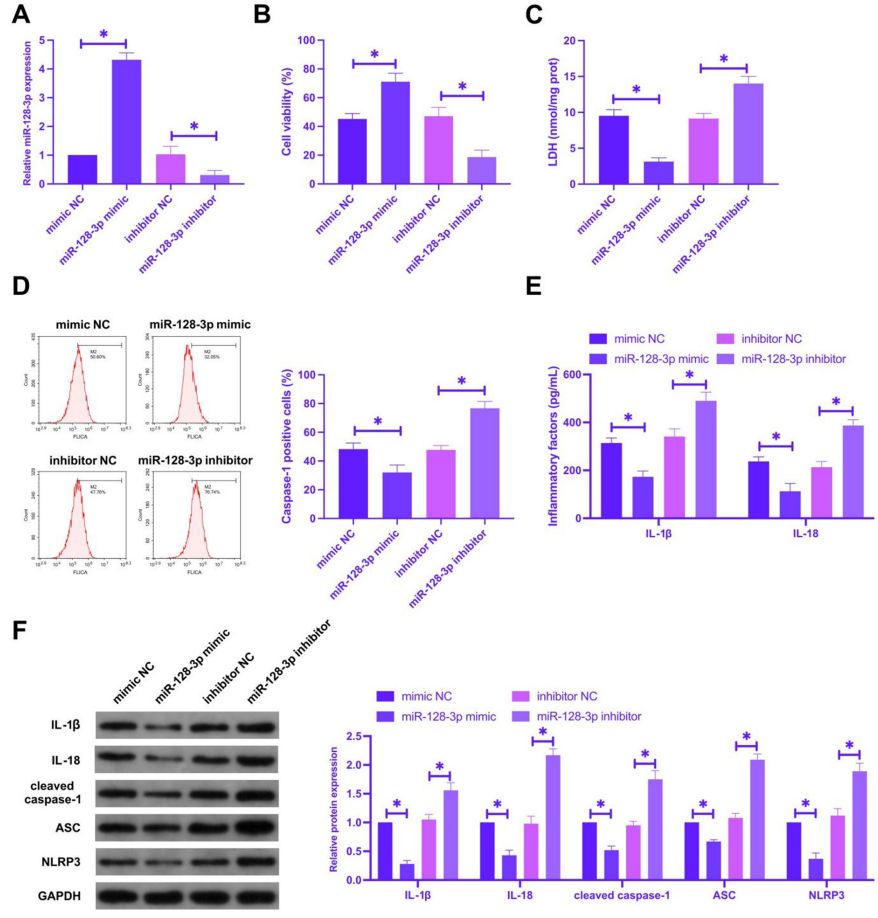

Figure 4. Effects of transfection of miR-128-3p mimic/inhibitor on the biological behavior and biochemical indexes of SH-SY5Y cells treated with OGD/R. miR-128-3p expression (A), cell viability (B), toxicity (C), caspase-1 positive cells (D), IL-1β and IL-1β contents (E), protein expression of IL-1β, IL-18, cleaved caspase-1, ASC and NLRP3 (F); data were reported as mean ± S.D. (N=3).

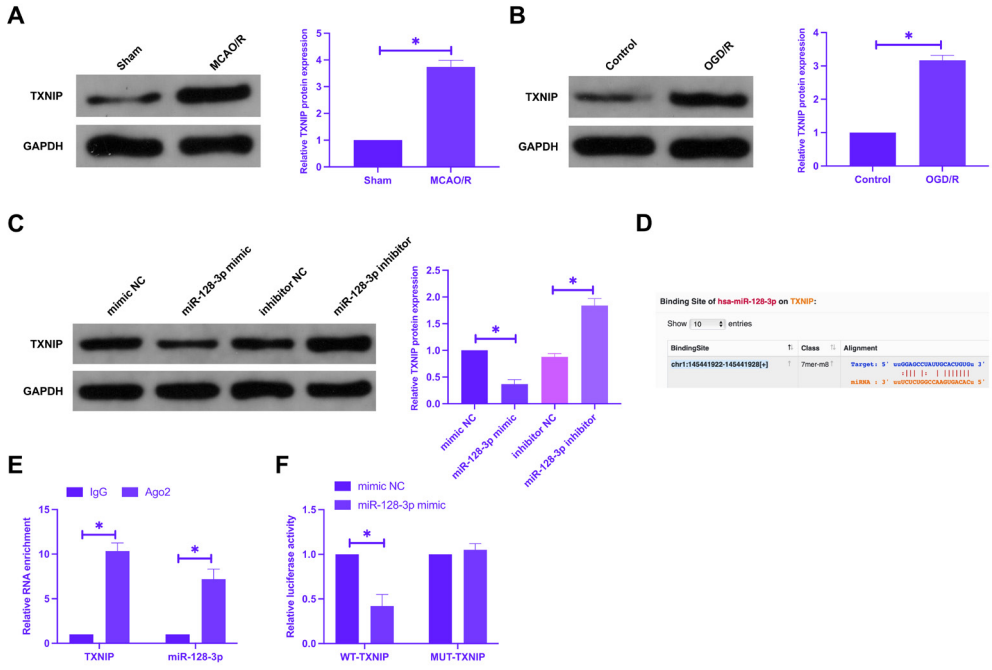

Figure 5. Targeting relation between miR-128-3p and TXNIP. TXNIP expression in *in vivo* and *in vitro* models (A–B), effect of miR-128-3p mimic/inhibitor on TXNIP protein expression in SH-SY5Y cells (C), potential binding sites of miR-128-3p and TXNIP (D), verification of the targeting relationship between miR-128-3p and TXNIP (E–F); data were reported as mean ± S.D. (N=3).

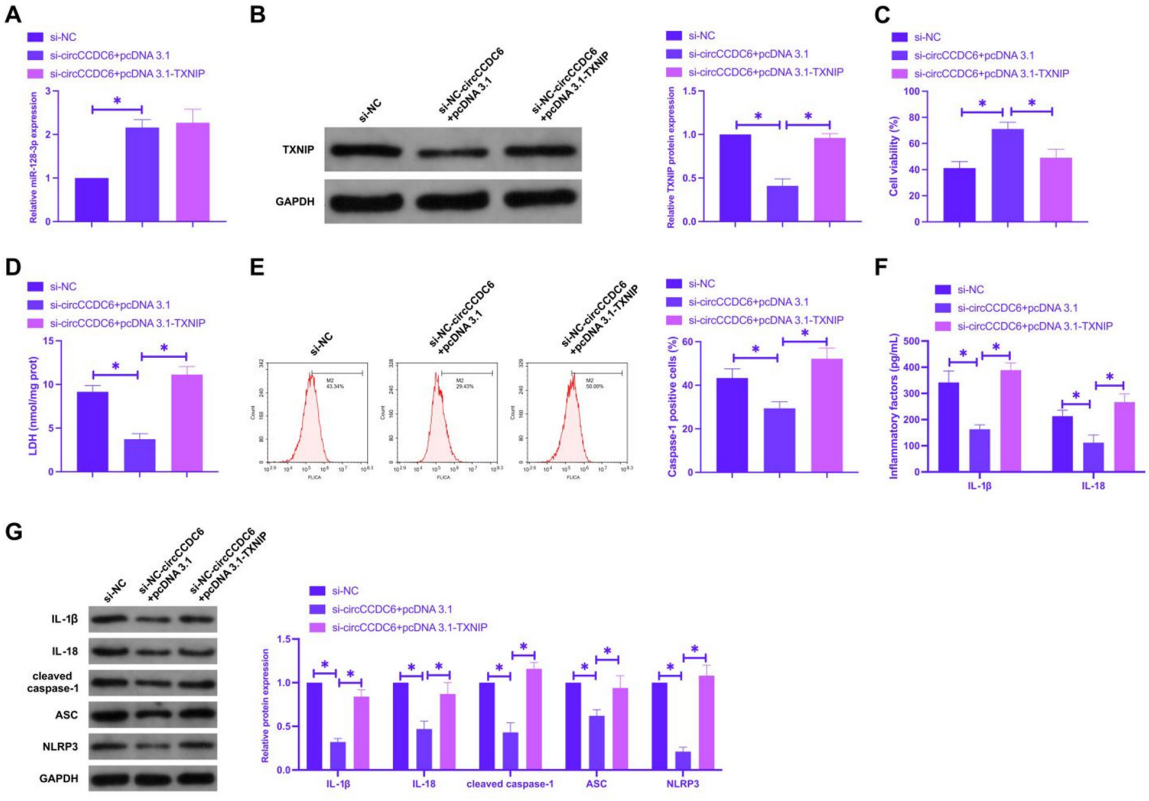

Figure 6. Effects of transfection of si-circCCDC6 and pcDNA 3.1-TXNIP on the biological behavior and biochemical indicators of OGD/R-treated SH-SY5Y cells.
miR-128-3p expression (**A**), TXNIP protein expression (**B**), viability (**C**), toxicity (**D**), caspase-1 positive cells (**E**), contents of IL-1β and IL-18 (**F**), protein expression of IL-1β, IL-18, cleaved caspase-1, ASC and NLRP3 (**G**) in SH-SY5Y cells; data were reported as mean ± S.D. (N=3).

ed that MCAO/R treatment increased the fluorescence intensity of GSDMD, but this trend was restricted in response to knockdown of circCCDC6 (Fig. 1D). Additionally, there were an increased number of TUNEL-positive cells (Fig. 1E), promoted release of IL-1β and IL-18 (Fig. 1F), and elevated protein expression of IL-1β, IL-18, cleaved caspase-1, ASC and NLRP3 (Fig. 1G) in the brain tissue of rats receiving MCAO/R surgery; the changes induced by MCAO/R were all reversed when circCCDC6 was knocked down (Fig. 1E). Clearly, silencing circCCDC6 ameliorated MCAO/R-induced pyroptosis and inflammation.

Overexpression of circCCDC6 aggravates OGD/R-induced neuronal pyroptosis and inflammation

Next, the molecular mechanism of circCCDC6 affecting neuronal pyroptosis and inflammation was explored through cellular experiments. It was checked that OGD/R treatment-induced elevation of circCCDC6 expression in neurons was further raised after transfection of pcDNA 3.1-circCCDC6 (Fig. 2A). Neuronal viability and toxicity were examined by MTT and colorimetry, respectively. The collected data displayed that after OGD/R treatment, neuronal viability was suppressed and LDH release was increased, and these changes were enhanced by overexpression of circCCDC6. Besides, it was further examined that OGD/R treatment induced the increase in the percentage of caspase-1 positive cells (Fig. 2D), contents of IL-1β and IL-18 (Fig. 2E), and protein expression of IL-1β, IL-18, cleaved caspase-1, ASC and NLRP3 in neurons (Fig. 2F); moreover, circCCDC6 upregulation further facilitated all of these alternations. Evidently, overexpression of circCCDC6 aggravates OGD/R-induced neuronal pyroptosis and inflammation.

CircCCDC6 competitively binds miR-128-3p

Next, we explored the downstream effectors bound by circCCDC6. Through the bioinformatics website https://starbase.sysu.edu.cn/, we predicted 20 miRNAs with potential binding sites for circCCDC6, among which miR-128-3p was particularly focused. miR-128-3p and circCCDC6 had binding sites at chr10:61564232-61564251[-] (Fig. 3A). miR-128-3p has been reported to be down-regulated in spinal cord I/R injury, and its overexpression is capable of ameliorating neuroinflammation and apoptosis (Wang *et al.*, 2020). Similar results were obtained in this study, with miR-128-3p downregulated in both MACO/R rats and OGD/R neurons (Fig. 3B). Dual-luciferase reporter experiments showed that co-transfection of WT-circCCDC6 with miR-128-3p mimic reduced luciferase activity (Fig. 3C), and RIP experiment further supported that circCCDC6 and miR-128-3p were increased in the Ago2 group (Fig. 3D). Subsequently, we examined that knockdown or overexpression of circCCDC6 upregulated and downregulated miR-128-3p expression, respectively. To conclude, circCCDC6 competitively binds miR-128-3p.

MiR-128-3p is involved in the process of pyroptosis and inflammation of OGD/R-conditioned neurons

Subsequently, we explored the role of miR-128-3p in OGD/R-induced neuronal pyroptosis and inflammation. Transfection of miR-128-3p mimic and inhibitor upregulated and down-regulated miR-128-3p expression in SH-SY5Y cells, respectively (Fig. 4A). Responded to miR-128-3p upregulation, neuronal viability increased and LDH release reduced, percentage of caspase-1 positive cells, contents of IL-1β and IL-18, and protein expressions of IL-1β, IL-18, cleaved caspase-1, ASC and NLRP3 decreased; while knockdown of miR-128-3p had completely opposite effects (Fig. 4B–F). Anyway, miR-128-3p is involved in the regulation of neuronal pyroptosis and inflammation.

A binding of miR-128-3p TXNIP 3′ UTR

TXNIP has been shown to be a pro-inflammatory factor in I/R injury (Jia *et al.*, 2020), and activation of the TXNIP/NLRP3 inflammasome pathway is a key factor in pyroptosis (Kong *et al.*, 2021). In both *in vivo* and *in vitro* models of CIR, TXNIP expression was found to be increased (Fig. 5A, B). Regarding miR-128-3p-mediated TXNIP expression change, quantitative PCR was performed to prove that transfection of miR-128-3p mimic or miR-128-3p inhibitor decreased and increased the protein level of TXNIP in neurons, respectively (Fig. 5C). Bioinformatics website searched for the complementary sequences of miR-128-3p and TXNIP 3'UTR (Fig. 5D), and their targeting relationships were subsequently by dual-luciferase reporter assays and RIP experiment (Fig. 5E, F). In short, a binding of miR-128-3p TXNIP 3' UTR is ensured.

CircCCDC6 affects neuronal pyroptosis and inflammation by regulating the miR-128-3p/TXNIP/NLRP3 pathway

Finally, rescue experiments were implemented to identify the effect of the circCCDC6/miR-128-3p/TXNIP/NLRP3 axis on OGD/R-induced neuronal pyroptosis and inflammation. It was examined that knockdown of circCCDC6 promoted miR-128-3p expression, while overexpression of TXNIP had no effect on miR-128-3p expression (Fig. 6A); circCCDC6 knockdown-induced suppression of TXNIP protein expression was reversed by overexpressing TXNIP (Fig. 6B). Functionally, circCCDC6 silencing-mediated protection against pyroptosis and inflammation was prevented when TXNIP was upregulated (Fig. 6C-G). CircCCDC6 affects neuronal pyroptosis and inflammation by regulating the miR-128-3p/TXNIP/NLRP3 pathway.

DISCUSSION

The key to the treatment of IS is blood reperfusion, but this can cause I/R injury (Wen *et al.*, 2019). Permanent damage to neurons with concomitant cell death happens in the hours following an IS (Zhang *et al.*, 2020). Acute neuroprotective agents attract much attention, however, clinical outcomes are often unsatisfactory. A genome-wide RNA sequencing report on the subcortical structure of CIR-injured rats shows that the expression of nearly 400 circRNAs is significantly altered 24 hours after tMCAO (Filippenkov *et al.*, 2021). This study reported a potential mechanism by which circCCDC6, regulates CIR injury-induced neurological deficits.

CircRNAs can interact with miRNAs, and their interaction is key in human diseases, including IS. For example, circTTC3 silencing could upregulate miR-372-3p, thereby suppressing CIR injury and neural stem cell apoptosis (Yang *et al.*, 2021). Moreover, depletion of circ_008018 in MCAO/R mice could prevent CIR damage by targeting miR-99a (Yang *et al.*, 2018). Also, in the model of MCAO/R, circHECTD1 knockdown induces neuroprotection against IS through binding to miR-133b

(Dai *et al.*, 2021). In this study, we found for the first time that circCCDC6 was upregulated in MCAO/R mouse model and OGD/R-treated neurons, and that circCCDC6 depletion alleviated MCAO/R-induced neuronal pyroptosis and inflammation, while overexpression circCCDC6 exacerbates OGD/R-induced neuronal pyroptosis and inflammation. Importantly, we investigated the possible miRNA sponge mechanism of circCCDC6 in CIR injury, paid great attention to the binding of circCCDC6 to miR-128-3p, and eventually confirmed that circCCDC6 may be involved in the pathogenesis of CIR injury by targeting miR-128-3p to promote neuronal pyroptosis and inflammation.

More attention is currently focused on the neuroinflammatory response triggered by I/R, and anti-inflammatory is likely to be a potential therapeutic strategy after IS (Wang *et al.*, 2020). miR-128-3p can exert anti-inflammatory effects by regulating gene expression and multiple signaling pathways. For example, overexpressing miR-128-3p could inhibit OA progression through PI3K/Akt/NF-κB pathway (Chen *et al.*, 2020), and restrain apoptosis and inflammation in LPS-induced sepsis by targeting TGFBR2. In both MACO/R rats and OGD/R neurons, we examined the downregulation of miR-128-3p and validated the protective actions of overexpressed miR-128-3p on increasing neuronal viability and reducing LDH release as well as inflammatory factors. Notably, MCAO/R treatment and OGD/R treatment promoted TXNIP and NLRP3 expression, while their expression could be regulated by circCCDC6 and miR-128-3p, suggesting that the role of circCCDC6 in CIR injury may be related to TXNIP/NLRP3 axis-mediated inflammation and pyroptosis.

TXNIP/NLRP3 signaling pathway is the key to pyroptosis and is activated in a variety of inflammatory diseases. It has been documented that (Chen *et al.*, 2021) in a rat model of myocardial I/R injury, the promoted expression of TXNIP reverses the inhibition of cardiomyocyte pyroptosis by overexpression of miR-200a-3p by promoting NLRP3 expression. (Yao *et al.*, 2022) have explained that cerebral ischemia leads to the accumulation of ROS, driving TXNIP overexpression and NLRP3 activation to induce pyroptosis. Furthermore, TXNIP/NLRP3 pathway has been shown to mediate pyroptosis by activating inflammatory factors such as IL-1β (Liu *et al.*, 2019). In CIR, we found increased expression of TXNIP and NLRP3 in animal and cell experiments, and confirmed that TXNIP was a downstream target of miR-128-3p, a result similar to the previous report (Liu *et al.*, 2021). Furthermore, rescue experiments obtained a conclusion that overexpression of TXNIP reversed the effects of silencing circCCDC6 on neuronal pyroptosis and inflammation.

However, there are some limitations as to the study design: 1, no detection of circCCDC6 expression in IS patients; 2. the correlation of circCCDC6 expression with clinicopathological features and whether it can be used as a diagnostic biomarker for IS has not yet been determined; 3. further systematic investigation of the relevant mechanism of circCCDC6 in MCAO-induced neurological deficits is required to further define the circRNA-miRNA gene network.

CONCLUSION

To sum up, circCCDC6 mediates neuronal pyroptosis and inflammation through the miR-128-3p/TXNIP/NLRP3 axis. This finding provides new insights into the mechanism by which circCCDC6 regulates CIR injury-induced neurological deficits and provides a potential target for CIR injury therapy.

Declarations

Acknowledgments. Not applicable.

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Competing interests. The authors have no conflicts of interest to declare.

Availability of data and materials. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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