miR-363-3p attenuates the oxygen-glucose deprivation/reoxygenation-induced neuronal injury *in vitro* by targeting PDCD6IP

YIHAN WANG[1*], JIAHUI JIN[2*], ZONGXIN XIA[3] and HUISHENG CHEN[1]

[1]Department of Neurology, General Hospital of Northern Theater Command, Shenyang, Liaoning 110013; [2]Department of Neurology, Jilin City Central Hospital, Jilin, Jilin 132011; [3]Department of Neurology, Baishan City Central Hospital, Baishan, Jilin 134399, P.R. China

Received July 9, 2021; Accepted February 14, 2022

DOI: 10.3892/mmr.2022.12838

Abstract. The purpose of the present study was to explore the functional role of microRNA (miR)-363-3p and related regulatory mechanisms in cerebral ischemia/reperfusion (I/R) injury. The neuronal cell line SH-SY5Y was exposed to 4 h of oxygen and glucose deprivation (OGD), followed by 6, 12, 24 and 48 h of re-oxygenation to mimic I/R injury *in vitro*. Cell viability, apoptosis and inflammation were assessed by CCK-8, lactate dehydrogenase (LDH), flow cytometry and ELISA assays. The association between miR-363-3p and programmed cell death 6-interacting protein (PDCD6IP) was further confirmed using luciferase reporter assay. Our data revealed that the expression level of miR-363-3p was significantly downregulated after OGD/R induction. Overexpression of miR-363-3p markedly suppressed OGD/R-induced cell injury, as reflected by attenuated cell viability, reduced apoptosis, LDH activity and pro-inflammatory cytokine levels. Mechanistically, PDCD6IP was confirmed as the target of miR-363-3p. Furthermore, PDCD6IP knockdown imitated, while overexpression reversed the effects of miR-363-3p overexpression on OGD/R-induced cell injury. Collectively, miR-363-3p could attenuate OGD/R-induced cell injury by alleviating apoptosis and inflammation, which may be mediated, at least in part, via inhibition of PDCD6IP.

Correspondence to: Dr Huisheng Chen, Department of Neurology, General Hospital of Northern Theater Command, 83 Wenhua Road, Shenhe, Shenyang, Liaoning 110013, P.R. China
E-mail: chen_huisheng02@163.com

*Contributed equally

Key words: Cerebral ischemia/reperfusion injury, microRNA-363-3p, oxygen and glucose deprivation/reperfusion, programmed cell death 6-interacting protein

Introduction

Ischemic stroke represents the most common type of stroke threatening human health, which is characterized by the sudden loss of blood circulation to an area of the brain (1). Currently, re-perfusing the ischemic area via drugs or early thrombolysis has been considered to be effective for the treatment of this disease, but restoration of blood reperfusion may aggravate brain injury and dysfunction, a condition termed cerebral ischemia/reperfusion (I/R) injury (2,3). Accumulating studies have demonstrated that apoptosis and inflammation are the main factors in I/R-induced nerve cell injury (4-7). Hence, exploring the molecular biology underlying I/R injury may be a remedy target to moderate the deterioration of ischemic stroke.

MicroRNAs (miRNAs/miRs) are a subtype of highly conserved endogenous single-stranded, non-coding small RNAs with a length of 22-25 nucleotides that can negatively regulate the expression of their target genes via binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs (8). It is well known that miRNAs participate in diverse biological processes, including proliferation, apoptosis, survival and inflammation (9,10), which are implicated in the adjustment of neurodegenerative diseases (11). With the increasing studies on ischemic stroke, related investigation on miRNAs regulating cerebral I/R injury has been increased. For instance, Wang *et al* (12) elucidated that miR-186-5p was upregulated and induced apoptosis in an oxygen and glucose deprivation/reperfusion (OGD/R) model by targeting insulin-like growth factor (IGF)-1. Conversely, Ren *et al* (13) reported that overexpression of miR-195-5p efficiently enhanced cell viability, while it reduced lactate dehydrogenase (LDH) release and the apoptotic rate in OGD-treated endothelial cells by targeting PTEN. In addition, miR-124-5p could reduce ROS production and improve the inflammatory microenvironment to attenuate cerebral I/R injury by targeting NOX2 (14). The attention of the authors was aroused by studies by Sohrabji and Selvamani (15,16) which revealed miR-363-3p as a target for ischemic stroke by improving ischemic stroke outcomes in female rats. Moreover, miR-363-3p has been reported to be intensively involved in cell proliferation (17), apoptosis (18)

and inflammation (19) in different diseases. Nevertheless, the expression level and regulatory role of miR-363-3p in cerebral I/R injury remain unclear.

Programmed cell death 6-interacting protein (PDCD6IP), known as apoptosis-linked gene-2-interacting protein 1 (ALIX), is one of the most intensely studied multifunctional cytosolic and multi-domain scaffold proteins (20), which usually binds to the pro-apoptotic protein PDCD6 (21). In addition, it was revealed that ALIX was correlated with inflammation reaction in response to different stimuli, including cytokines (22,23) and glutaminase 1 (GLS1) (24) during neuroinflammation. This evidence indicated that PDCD6IP may play an important role in cerebral I/R injury by regulating apoptosis and inflammation status.

Based on the online prediction that PDCD6IP is a target of miR-363-3p, the present study hypothesized that miR-363-3p regulated apoptosis and inflammation in cerebral I/R injury by targeting PDCD6IP. To validate this hypothesis, an OGD/R cell injury model was first established and this model was assessed by analyzing cell apoptosis, inflammation and cell viability. Furthermore, the effects of miR-363-3p on OGD/R-induced injury were analyzed and it was explored whether PDCD6IP was the downstream regulator involved in the miR-363-3p-mediated effects.

Materials and methods

Cell culture and transfection. Human neuronal cell line SH-SY5Y (CRL-2266) was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; Cytiva) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. The miR-363-3p mimics (5'-AAUUGCACG GUAUCCAUCUGUA-3'), small interference RNA targeting PDCD6IP (si-PDCD6IP, 5'-GCTCCTGAGATATTATGA TCA-3') and their corresponding negative controls, miR-NC (5'-CAGUACUUUUGUGUAGUACAA-3') and si-NC (5'-GTGTTACGTTACCAACTAGAT-3'), respectively, were purchased from Shanghai GenePharma Co., Ltd. To overexpress PDCD6IP, the full length of PDCD6IP was cloned into the pcDNA3.1 vector (Sangon Biotech Co., Ltd.) to generate the overexpression plasmid PDCD6IP, and the empty vector was used as the negative control. All of miRNA mimics, siRNA and NCs were diluted into 50 nM for use and 0.5 μg recombinant plasmid was transfected into SH-SY5Y cells seeded in six-well plates ($3x10^5$ cells/well) for 48 h at 37˚C before OGD/R treatment in accordance with the instructions of Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

OGD/R establishment. After 48 h of transfection, SH-SY5Y cells were exposed to glucose-free DMEM and cultured for 4 h with $N_2/CO_2/O_2$ (94/5/1%) at 37˚C, as previously reported (25,26). Thereafter, the glucose-free DMEM was replaced by standard culture medium and the cells were transferred to normal conditions to receive re-oxygenation. Cells in the control group were still cultured in normal DMEM medium in a normoxic atmosphere. Subsequently, the cells received 4 h of OGD followed by 6, 12, 24 and 48 h of re-oxygenation.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from SH-SY5Y cells using the TRIzol reagent (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. For detection of miR-363-3p, cDNA was synthesized using the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 37˚C for 5 min. Next, RT-qPCR was performed with Express SYBR GreenER miRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). For detection of PDCD6IP, reverse transcription was conducted with EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd.) and RT-qPCR was performed using SYBR Premix Ex Taq (Takara Bio, Inc.). The expression levels of miR-363-3p and PDCD6IP were normalized against U6 and GAPDH, respectively. Relative quantification of target genes was conducted with the $2^{-\Delta\Delta Cq}$ method (27). The primers used in the present study were as follows: miR-363-3p forward, 5'-GCCGAGAATTGCACGGTAT-3' and reverse, 5'-CTC AACTGGTGTCGTGGA-3' (28); PDCD6IP forward, 5'-CTG CCTTAAGTCGAGAGCCG-3' and reverse, 5'-CAGGGA ACACCTCCTGGAAATA-3'; GAPDH forward, 5'-GGTGAA GGTCGGAGTCAACG-3' and reverse, 5'-GCATCGCCCCAC TTGATTTT-3'; and U6 forward, 5'-TCTTCGTCATCACAT ATACTAAAAT-3' and reverse, 5'-CTCTTCACGAATTTT CGTGTCAT-3'.

Cell viability assay. Cells at a density of $5x10^3$ cells/well were seeded into 96-well plates and cultured overnight at 37˚C. The following day, 10 μl of Cell Counting Kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc.) was added to each well. Following incubation for 2 h at 37˚C, the optical density at 450 nm was measured by a microplate reader. Subsequently, cell viability was calculated as the percentage of the OD value in the experimental group/control group cells. The experiment was performed in triplicate.

Detection of LDH release. Cell cytotoxicity was detected using the LDH Cytotoxicity Assay Kit (cat. no. A020-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol (29). In brief, cells at a density of $5x10^3$ cells/well were plated into 96-well plates and incubated with 150 μl of LDH release reagent for 1 h at 37˚C. According to the absorbance at 490 nm in each well, the LDH release was expressed as concentration units per liter. The experiment was performed in triplicate.

Cell apoptosis. Cell apoptosis was assessed by an Annexin V-FITC/propidium (PI) Apoptosis Detection Kit (Nanjing Keygen Biotech Co. Ltd.) according to the manufacturer's instructions. Briefly, cells were digested and obtained cell lysates were incubated with 5 μl Annexin V/PI in 200 μl binding buffer for 15 min in the dark at room temperature. Subsequently, the proportion of apoptotic cells was determined by FACSCalibur flow cytometer and BD Accuri C6 Plus software (both BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA). ELISA assay (30) was performed to investigate the alteration of inflammatory status in cell-free supernatants in response to the indicated treatments. In brief, the concentration levels of

pro-inflammatory cytokines, including interleukin (IL)-1β (ab214025), IL-6 (ab178013) and tumor necrosis factor (TNF)-α (ab181421) in the supernatants of SH-SY5Y cells were measured using commercially available ELISA kits (Abcam) in accordance with the standard methods. The experiment was performed in triplicate.

Luciferase reporter assay. The putative binding site between miR-363-3p and the 3'-UTR of PDCD6IP was predicted by TargetScan v.7.1 (http://www.targetscan.org/vert_71/). For the luciferase reporter assay, the wild-type (WT) and mutant (MUT) of PDCD6IP, containing the putative binding site with miR-363-3p, were inserted into the pmirGLO luciferase reporter vector (Promega Corporation) to generate recombinant reporter constructs termed as WT PDCD6IP or MUT PDCD6IP, respectively. SH-SY5Y cells were incubated in 24-well plates and co-transfected with 200 ng of the luciferase reporter plasmid and 50 nM of miR-363-3p mimics or miR-NC using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, firefly and *Renilla* luciferase activities were determined and relative luciferase activity was calculated with *Renilla* luciferase used for normalization (Promega Corporation).

Western blot analysis. Total protein sample was extracted from cells with ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified by BCA assay (Beyotime Institute of Biotechnology) according to the standard protocols. Equal amounts of protein sample (30 μg) were separated by 10% SDS-PAGE gels and then transferred onto PVDF membranes (EMD Millipore). After being blocked with 5% skim milk containing 0.1% TBST for 2 h, the membranes were incubated with primary antibodies against PDCD6IP (cat. no. ab225555, 1:500; Abcam) and GAPDH (cat. no. ab245355, 1:5,000; Abcam) at 4˚C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. ab97051; Abcam) for 2 h at room temperature, followed by protein visualization using ECL detection reagent (Bio-Rad Laboratories, Inc.). The band intensities were quantified using ImageJ v1.8.0 (National Institutes of Health).

Statistical analysis. Statistical analyses were performed by GraphPad Prism 6.0 (GraphPad Software, Inc.). The data are expressed as the means ± standard deviation (SD) of three independent experiments. Differences between two groups were analyzed by Student's t-test, while differences between multiple groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MiR-363-3p is downregulated following OGD/R-induced neuronal injury in vitro. To explore the potential role of miR-363-3p in cerebral I/R injury, SH-SY5Y cells were used to establish the *in vitro* I/R injury model by exposing the cells to 4 h of OGD followed by 6, 12, 24 and 48 h of re-oxygenation. The results of RT-qPCR revealed that the expression level of miR-363-3p was significantly lower in the OGD-4 h/R model than that of the normoxic control group and gradually reached the lowest expression level at 24 h reoxygenation compared with the normoxic control (Fig. 1A). Subsequently, the constructed *in vitro* cerebral I/R model was evaluated through evaluation of cell survival. As revealed in Fig. 1B, cell viability was significantly impaired in the OGD-4 h/R model compared with the normoxic condition and reduced cell viability reached the lowest level at 24 h reoxygenation. Consistently, the release of LDH in the culture supernatant was significantly increased when the cells were under OGD-4 h/R conditions, which reached its peak at 24 h reoxygenation compared with the normoxic condition (Fig. 1C). Thus, 4-h OGD/24-h reoxygenation was selected as the optimal time-point for subsequent experiments. Flow cytometric analysis further demonstrated that OGD/R treatment significantly induced apoptosis of SH-SY5Y cells compared with that in the control group (Fig. 1D). In addition, the inflammatory status of SH-SY5Y cells under OGD/R treatment was examined by ELISA assay. As expected, the levels of pro-inflammatory cytokines, including IL-1β (Fig. 1E), IL-6 (Fig. 1F) and TNF-α (Fig. 1G) were significantly increased when the cells were under OGD/R conditions. These results suggested that miR-363-3p may play an important role in OGD/R induced neuronal injury.

Overexpression of miR-363-3p attenuates OGD/R-induced apoptosis and inflammation in SH-SY5Y cells. To further determine the functional role of miR-363-3p in the constructed OGD/R model, miR-363-3p mimics were transfected into SH-SY5Y cells, followed by OGD/R treatment. The results from the RT-qPCR assay showed that the expression of miR-363-3p in SH-SY5Y cells after exposure to OGD/R was significantly increased after transfection with miR-363-3p mimics compared with that after miR-NC transfection (Fig. 2A). It was then observed that miR-363-3p overexpression significantly increased cellular viability (Fig. 2B) and reduced LDH release (Fig. 2C) induced by OGD/R exposure in SH-SY5Y cells. Moreover, the percentage of apoptotic cells was significantly lower in the miR-363-3p mimic-transfected OGD/R SH-SY5Y cells than in the miR-NC-transfected group (Fig. 2D). ELISA assay further confirmed that transfection of miR-363-3p mimics significantly suppressed the levels of IL-1β (Fig. 2E), IL-6 (Fig. 2F) and TNF-α (Fig. 2G) in OGD/R-treated SH-SY5Y cells.

MiR-363-3p directly targets the 3'-UTR of PDCD6IP. The mechanisms of how miR-363-3p attenuated OGD/R-induced injury were further explored by searching its possible targets. Among the predicted target genes, PDCD6IP was screened as a candidate target for being reported to be involved in apoptosis (31). As shown in Fig. 3A, there was a putative binding site between miR-363-3p and the 3'-UTR of PDCD6IP. Luciferase reporter assay further demonstrated that transfection with miR-363-3p mimics significantly reduced the relative luciferase activity of WT PDCD6IP compared with that of the control in SH-SY5Y cells, while the vector containing the site-mutated sequence was not influenced by miR-363-3p mimics (Fig. 3B). Additionally, RT-qPCR analysis indicated that the increased PDCD6IP mRNA expression induced by OGD/R exposure could be significantly reversed by transfection with miR-363-3p mimics in SH-SY5Y cells (Fig. 3C).

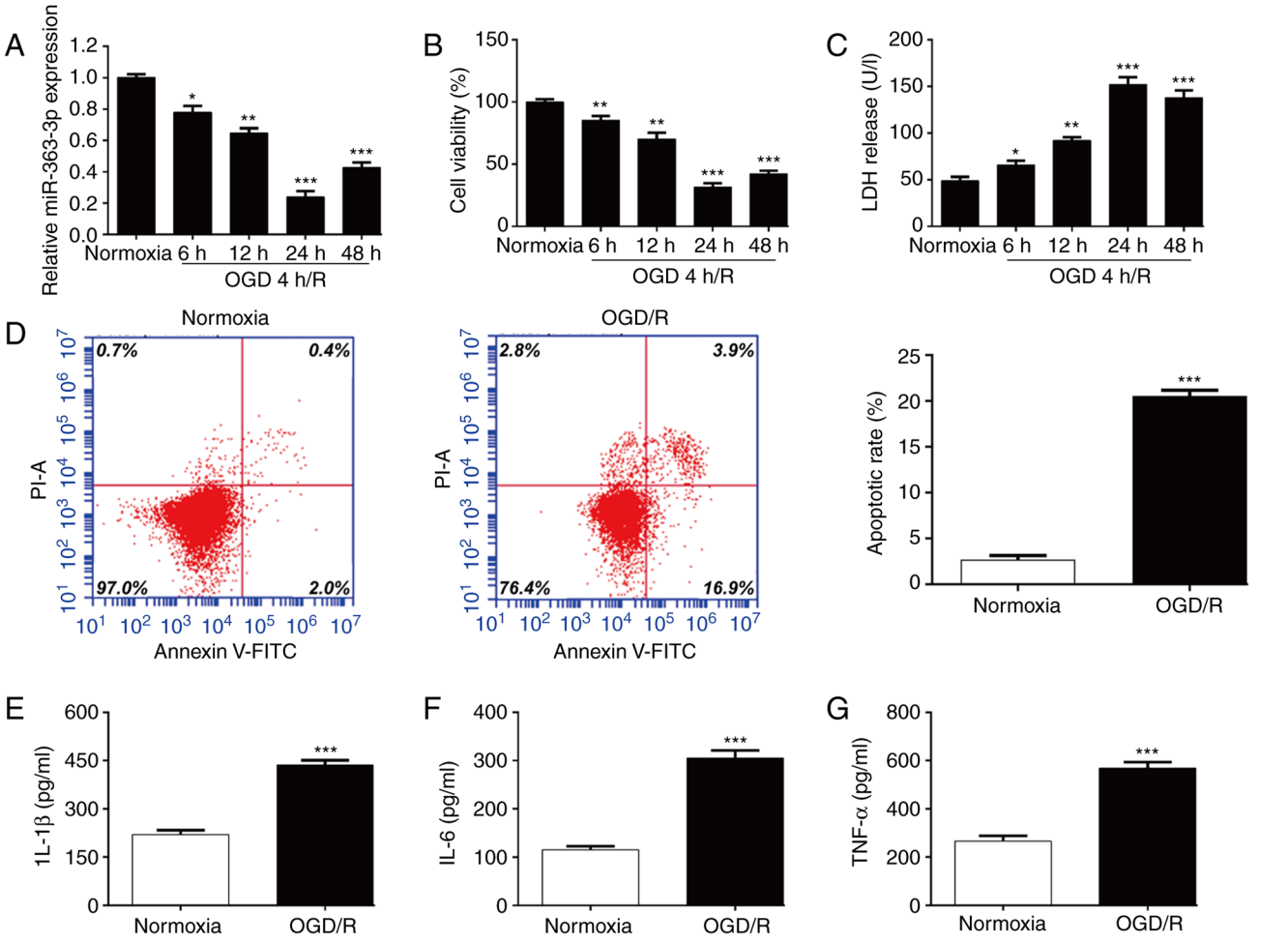

Figure 1. MiR-363-3p expression levels in an OGD/R model. SH-SY5Y cells were exposed to 4 h OGD, followed by 6, 12, 24 and 48 h of re-oxygenation. (A) The expression of miR-363-3p was determined by RT-qPCR. (B) Cell viability and (C) cytotoxicity were analyzed using CCK-8 and LDH assays, respectively. (D) Cell apoptosis was analyzed in the 4-h OGD/24-h R model and in the normoxic control group of SH-SY5Y cells by flow cytometry. ELISA assays were applied to determine the levels of (E) IL-1β, (F) IL-6 and (G) TNF-α in the 4-h OGD/24-h R model and in the normoxic control group of SH-SY5Y cells. Data are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 compared with normoxia. MiR, microRNA; OGD/R, oxygen and glucose deprivation/re-oxygenation; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor.

Western blot analysis further confirmed that the protein level of PDCD6IP was upregulated in the OGD/R treatment group (Fig. 3D) and this increased protein level of PDCD6IP was attenuated after miR-363-3p overexpression (Fig. 3E). These results indicated that miR-363-3p may directly target the expression of PDCD6IP by binding to its 3'UTR in OGD/R-induced SH-SY5Y cells.

PDCD6IP knockdown suppresses OGD/R-induced apoptosis and inflammation. Since increased PDCD6IP expression induced by OGD/R treatment was reversed by miR-363-3p overexpression, it was thus theorized that PDCD6IP could promote OGD/R-induced cell injury. To confirm this, loss-of-function assays were performed in OGD/R-treated SH-SY5Y cells. First, western blot analysis confirmed that si-PDCD6IP transfection caused a significant decrease in PDCD6IPO expression in OGD/R-induced SH-SY5Y cells (Fig. 4A). Next, the effects of PDCD6IP knockdown on cell viability, apoptosis and inflammation were examined. As revealed in Fig. 4B, knockdown of PDCD6IP could increase cell viability in OGD/R-induced cells. In line with miR-363-3p overexpression, knockdown of PDCD6IP significantly alleviated OGD/R-induced LDH release (Fig. 4C) and apoptosis (Fig. 4D), and suppressed proinflammatory cytokines, including the levels of IL-1β (Fig. 4E), IL-6 (Fig. 4F) and TNF-α (Fig. 4G).

Overexpression of PDCD6IP counteracts miR-363-3p-mediated inhibitory effects against OGD/R-induced injury. To determine whether PDCD6IP was the downstream regulator involved in miR-363-3p exerting protection against OGD/R-induced injury, the overexpression of PDCD6IP in SH-SY5Y cells after transfection with pcDNA3.1-PDCD6IP was first confirmed (Fig. 5A). Rescue experiments were then performed in SH-SY5Y cells by co-transfection with miR-363-3p mimics and PDCD6IP overexpression plasmid, followed by OGD/R treatment. It was first confirmed that the downregulation of PDCD6IP induced by transfection with miR-363-3p mimics was markedly reversed after PDCD6IP transfection (Fig. 5B). It was then observed that overexpression of PDCD6IP counteracted the promoting effect of miR-363-3p overexpression on the viability of OGD/R-induced SH-SY5Y

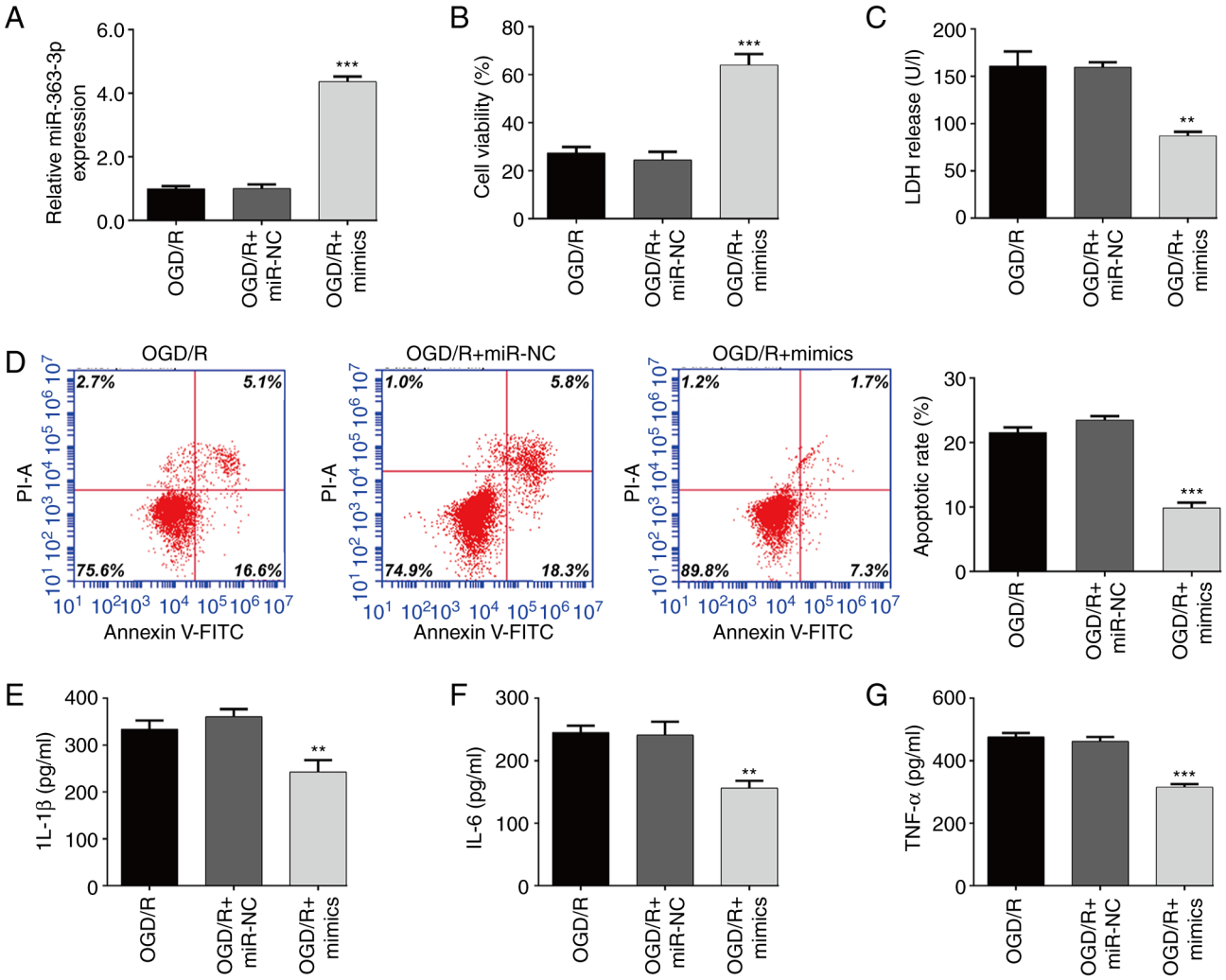

Figure 2. Overexpression of miR-363-3p attenuates OGD/R-induced apoptosis and inflammation in SH-SY5Y cells. SH-SY5Y cells were transfected with miR-363-3p mimics and miR-NC, and then received 4 h of OGD followed by 24 h of re-oxygenation. (A) The transfection efficiency was determined by RT-qPCR. (B) Cell viability was evaluated by CCK-8 assay. (C) The release of LDH was detected by specific cytotoxicity assay kit. (D) Cell apoptosis was evaluated by flow cytometer. ELISA assay was applied to determine the levels of (E) IL-1β, (F) IL-6 and (G) TNF-α. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001 compared with OGD/R + miR-NC. miR, microRNA; OGD/R, oxygen and glucose deprivation/re-oxygenation; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor.

cells (Fig. 5C). PDCD6IP overexpression reversed the miR-363-3p mimics-induced decrease in LDH release (Fig. 5D) and apoptosis (Fig. 5E) of OGD/R-induced SH-SY5Y cells. Furthermore, the decrease in the levels of IL-1β (Fig. 5F), IL-6 (Fig. 5G) and TNF-α (Fig. 5H) induced by miR-363-3p overexpression were rescued after PDCD6IP overexpression. These findings ascertained that the suppressive effects of miR-363-3p on OGD/R-induced injury may be mediated via PDCD6IP inhibition.

Discussion

Cerebral I/R injury, a common event that occurs in patients with acute ischemic stroke, is a complex systemic process that involves inflammation, protein synthesis inhibition and impaired mitochondrial function, causing irreversible dysfunctions and structural damage (32). In the last decades, miRNAs have become promising therapeutic targets in ischemic stroke (33). When studying the role of certain miRNAs in the pathogenesis of ischemic stroke, the *in vitro* OGD/R procedure has usually been applied to mimic cerebral I/R injury (34,35). Moreover, SH-SY5Y cells have been frequently selected as the most commonly used cell line in five models of ischemia-related injury, including OGD, H_2O_2-induced oxidative stress, oxygen deprivation, glucose deprivation and glutamate excitotoxicity because of its human origin, catecholaminergic neuronal properties, and ease of maintenance (36). In the present study, an OGD/R injury model was successfully constructed by exposing SH-SY5Y cells to OGD for 4 h, followed by 24 h of re-oxygenation. Before establishing 24 h of re-oxygenation, it was determined that re-oxygenation for 48 h had different effects compared with 24 h, including cell viability and LDH release. In fact, OGD/R is an *in vitro* model that mimics the *in vivo* process of a series of pathological reactions initiated by I/R, which is a dynamic process previously described (12,25,37). Similar with the aforementioned studies (15,16), the expression of miR-363-3p was decreased gradually and reached the lowest expression

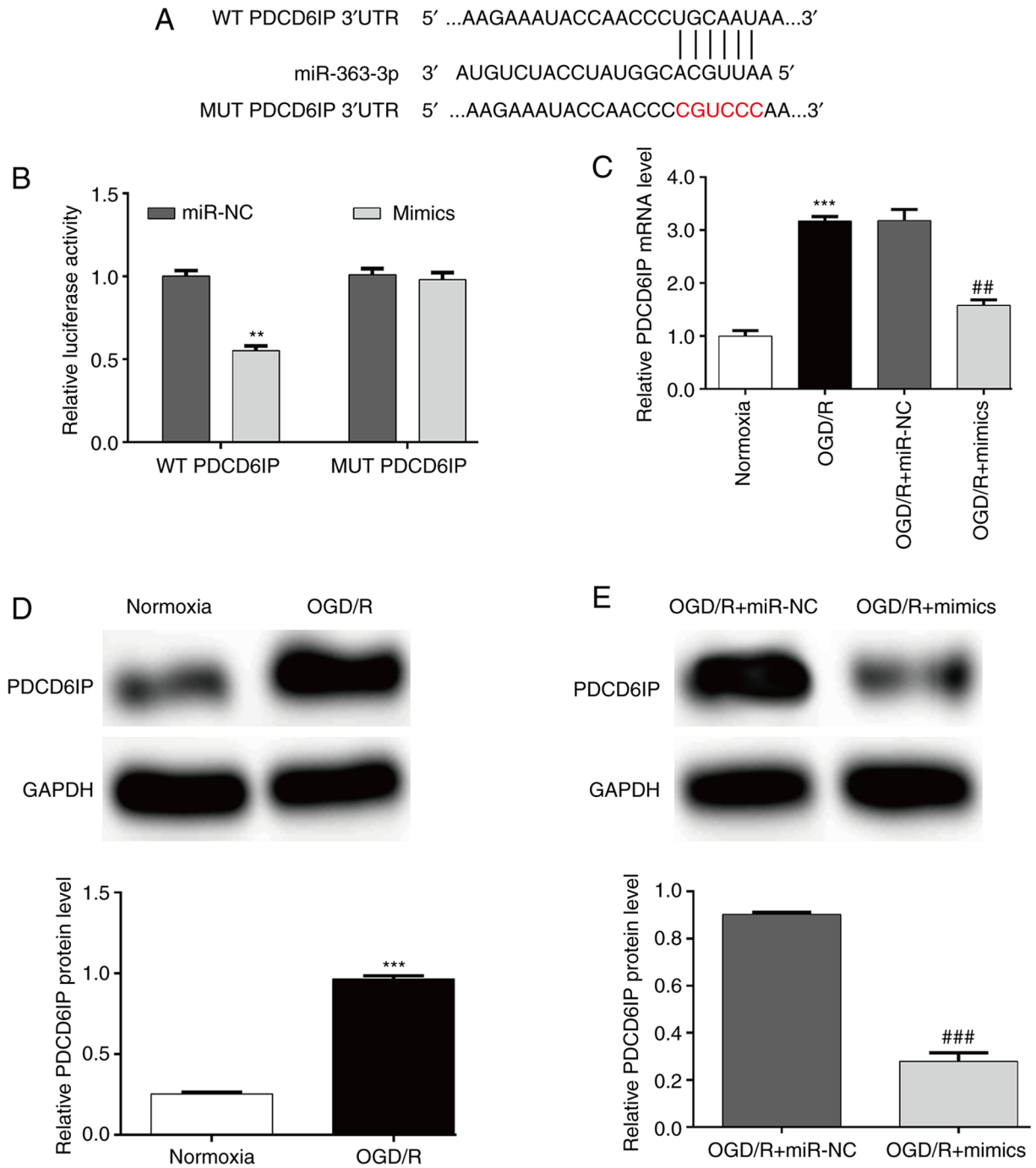

Figure 3. MiR-363-3p directly targets the 3'-UTR of PDCD6IP. (A) The potential interaction between miR-363-3p and PDCD6IP was predicted by TargetScan. (B) Investigation of the regulatory effects of miR-363-3p on PDCD6IP expression using luciferase reporter assay in SH-SY5Y cells. **P<0.01 compared with miR-NC; SH-SY5Y cells were transfected with miR-363-3p mimics or miR-NC, followed by OGD/R treatment. (C) The expression level of PDCD6IP mRNA was detected by RT-qPCR. Data are presented as the mean ± SD. (D and E) Western blot analysis was performed to assess the protein level of PDCD6IP in (D) the normoxic and OGD/R groups, as well as that in (E) the miR-363-3p mimics/miR-NC-transfected OGD/R groups of SH-SY5Y cells. ***P<0.001 compared with normoxia; and $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with OGD/R + miR-NC. miR, microRNA; 3'-UTR, 3'-untranslated region; PDCD6IP, programmed cell death 6-interacting protein; NC, negative control; OGD/R, oxygen and glucose deprivation/re-oxygenation; RT-qPCR, reverse transcription-quantitative PCR.

at 24 h reoxygenation. The difference of cell viability and LDH release between 24 and 48 h may be associated with the corresponding altered miR-363-3p expression level, although these differences were not significant. To sum up, the significantly decreased miR-363-3p expression at OGD 4 h, followed by 24 h of re-oxygenation may be associated with the pathogenesis of I/R injury (15).

To the best of our knowledge, miR-363-3p has been revealed to play an important role in the pathogenic processes of malignant tumors, including proliferation, apoptosis, and epithelial-mesenchymal transition in colorectal cancer (38), lung cancer (39), retinoblastoma (40), oral squamous cell carcinoma (41), and acute myeloid leukemia (42). Recent studies have reported that miR-363-3p as a stroke neuroprotectant could improve ischemic

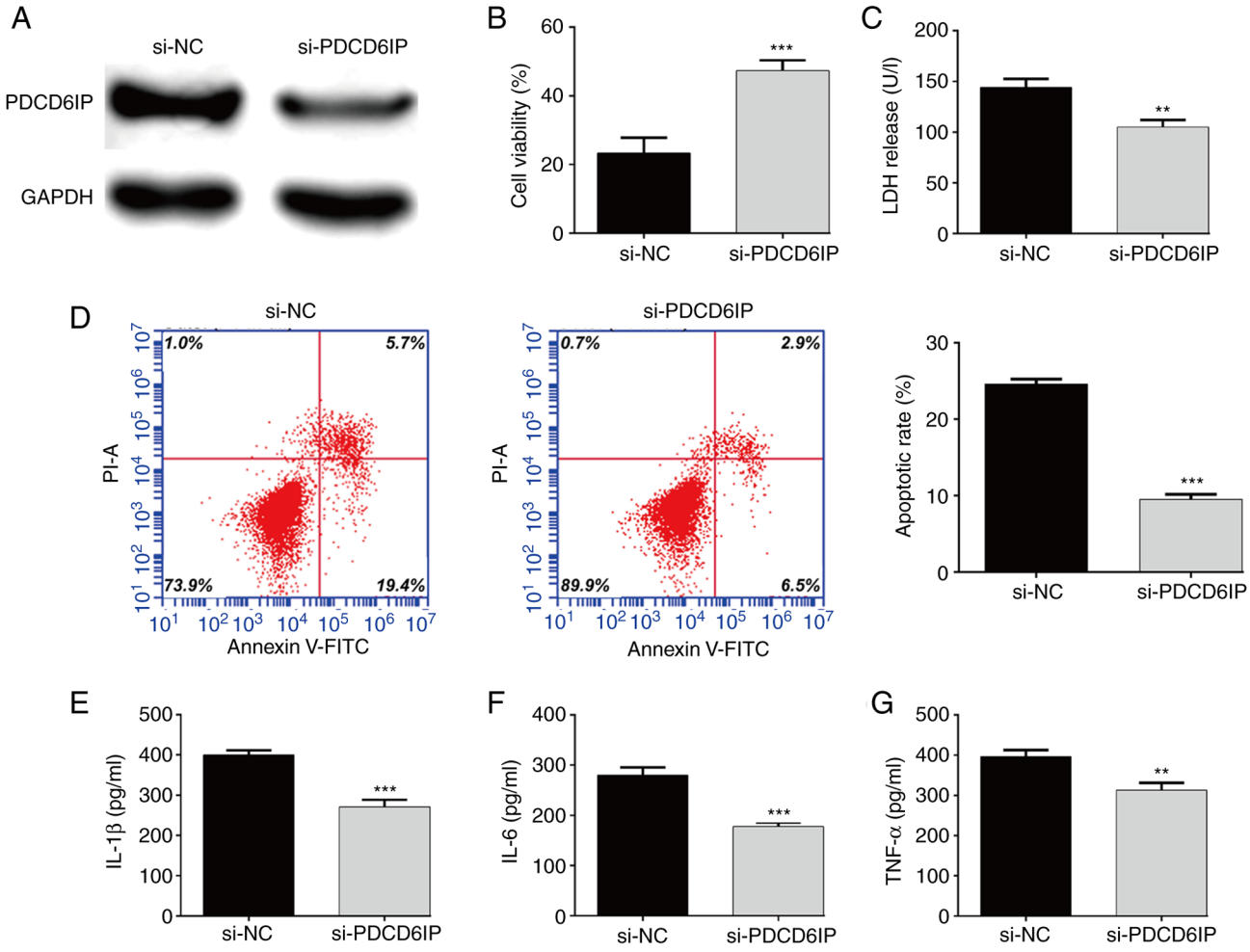

Figure 4. PDCD6IP knockdown suppresses OGD/R-induced apoptosis and inflammation. SH-SY5Y cells were transfected with si-PDCD6IP, followed by OGD/R treatment. (A) The protein expression level of PDCD6IP in SH-SY5Y cells was detected. (B) The viability of SH-SY5Y cells was evaluated using the CCK-8 assay. (C) Cell cytotoxicity was analyzed using LDH assay. (D) Flow cytometric analysis was conducted to detect apoptosis of SH-SY5Y cells. ELISA assay was applied to determine the levels of (E) IL-1β, (F) IL-6 and (G) TNF-α. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001 compared with si-NC. PDCD6IP, programmed cell death 6-interacting protein; OGD/R, oxygen and glucose deprivation/re-oxygenation; si-, small interfering; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor; NC, negative control.

stroke outcomes in female rats (15,16). However, the regulatory role of miR-363-3p in cerebral I/R-induced injury remains largely unclear. In agreement with a previous study which reported that miR-363-3p plays a protective role in renal fibrosis (RF) *in vitro* by regulating the TGF-β2/Smad3 signaling pathway (43), our results revealed that overexpression of miR-363-3p could significantly promote cell viability, and suppress the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and cell apoptosis in OGD/R-induced SH-SY5Y cells. Considering that excitotoxicity, apoptosis and inflammation are the primary pathological causes of neuronal loss following cerebral I/R (44,45), it was thus inferred that miR-363-3p may have neuroprotective effects on simulated cerebral ischemia *in vitro*. Moreover, miR-363-3p was revealed to reduce inflammatory response and cell apoptosis in coronary arterial endothelial cells (CAECs) but increase their viability, which acts as a promising therapeutic target for coronary heart disease (CHD) (19). By contrast, miR-363-3p was significantly upregulated in osteoarthritis (OA) model rats and in LPS-induced chondrocytes, which could promote chondrocyte injury and apoptosis (46). In addition, downregulation of miR-363-3p improved acute myocardial infarction-associated endothelial injury by targeting KLF2 (47). These opposite regulatory roles of miR-363-3p on cell apoptosis and inflammation may be ascribed to different disease factors.

Mechanistically, our findings revealed that miR-363-3p bound to the 3'-UTR region of PDCD6IP mRNA to inhibit its expression, subsequently resulting in the suppression of apoptosis and inflammation in OGD/R-induced SH-SY5Y cells. PDCD6IP encodes for a protein that is known to bind to the products of the PDCD6 gene, which is involved in the apoptosis pathway and was demonstrated to decrease the risk of breast cancer in a sample of Iranian women (21). PDCD6IP, also named ALIX (20), was previously reported to promote neuronal death (48,49). On the other hand, PDCD6IP has been shown to be associated with inflammation reaction in response to different stimuli, including cytokines (22,23) and glutaminase 1 (GLS1) during neuroinflammation (24). Hence, targeting PDCD6IP may be a potential therapeutic aim for preventing apoptosis and inflammation causing neuronal cell injury. Our study provided a new regulatory axis of miR-363-3p and PDCD6IP in cerebral I/R injury, which may contribute to understanding of the pathogenesis of ischemic stroke. However, there were some limitations

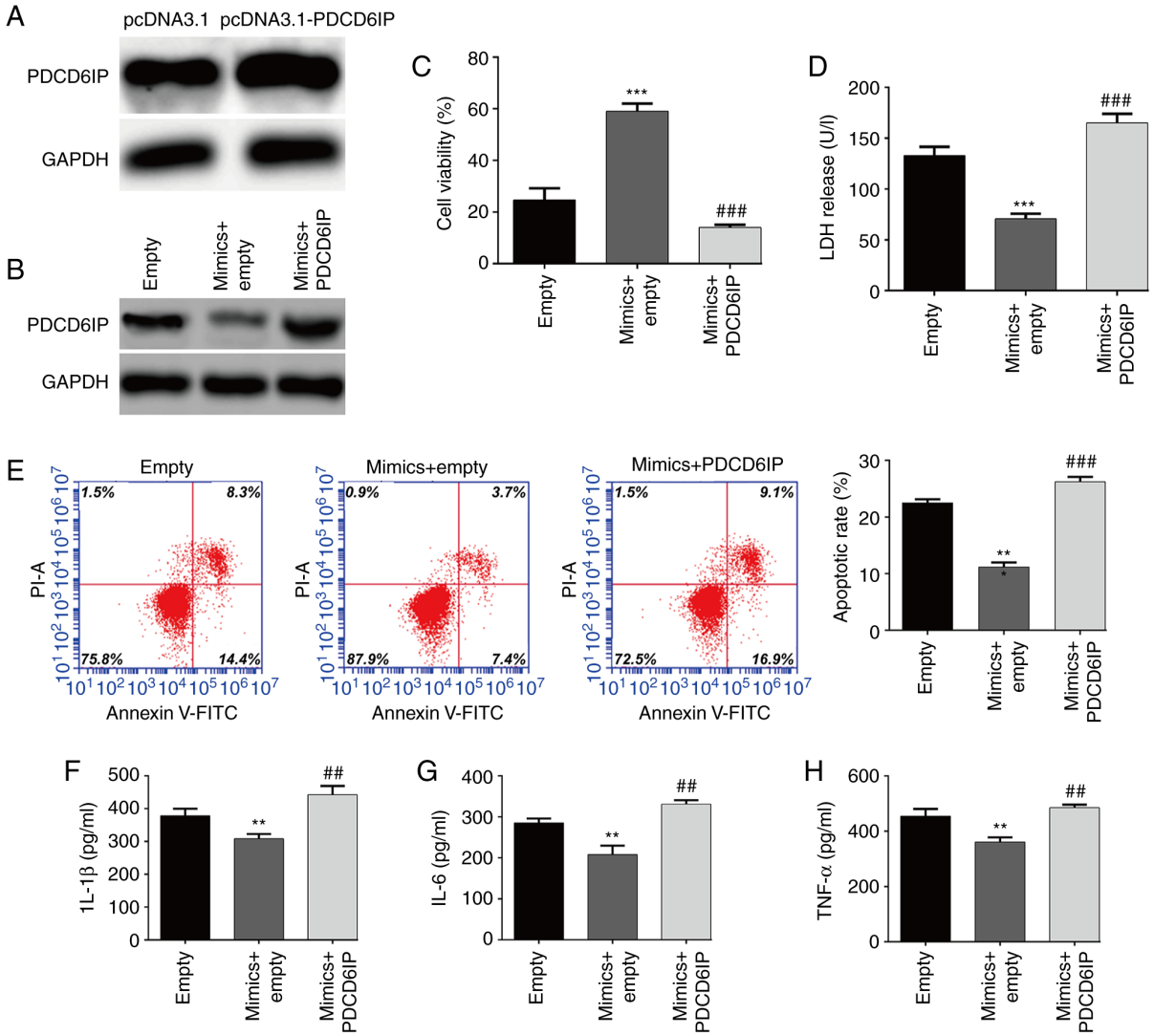

Figure 5. Overexpression of PDCD6IP counteracts the miR-363-3p-mediated protective effects against OGD/R-induced injury. SH-SY5Y cells were divided into the following groups according to different transfections: pcDNA3.1, pcDNA3.1-PDCD6IP, empty vector, empty + miR-363-3p mimics, and miR-363-3p mimics + PDCD6IP, followed by OGD/R treatment. (A and B) The protein expression level of PDCD6IP was detected in SH-SY5Y cells from different groups. (C) Cell viability, (D) LDH release and (E) apoptosis were evaluated by CCK-8 assay, LDH assay and flow cytometric analysis, respectively. (F-H) ELISA assays were applied to determine the levels of IL-1β, IL-6 and TNF-α. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001 compared with empty; and $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with mimics + empty. PDCD6IP, programmed cell death 6-interacting protein; miR, microRNA; OGD/R, oxygen and glucose deprivation/re-oxygenation; LDH, lactate dehydrogenase; CCK-8, Cell Counting Kit-8; IL, interleukin; TNF, tumor necrosis factor.

in the present study including i) lack of further experiments to confirm how PDCD6IP can completely counteract the effects of miR-363-3p; ii) lack of an *in vivo* I/R injury rat model to further confirm the role of the miR-363-3p/PDCD6IP axis in cerebral I/R injury; and iii) more targets of miR-363-3p need to be identified in cerebral 1/R injury.

In summary, our study revealed that miR-363-3p was significantly downregulated in OGD/R-induced neurons, and overexpression of miR-363-3p could attenuate OGD/R-induced neuron apoptosis and inflammation *in vitro*, which may be mediated, at least in part, via inhibition of PDCD6IP (Fig. S1). Our findings suggest that miR-363-3p may be a potential therapeutic target for cerebral I/R injury.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HC made substantial contributions to the conception of the present study. YW and JJ were involved in the acquisition, analysis and interpretation of data for the work. ZX dperformed the statistical analysis and wrote the manuscript. HC and YW confirm the authenticity of all the raw data. All authors agreed the final version of manuscript to be published.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chugh C: Acute ischemic stroke: Management approach. Indian J Crit Care Med 23 (Suppl 2): S140-S146, 2019.
2. Xu F, Ma R, Zhang G, Wang S, Yin J, Wang E, Xiong E, Zhang Q and Li Y: Estrogen and propofol combination therapy inhibits endoplasmic reticulum stress and remarkably attenuates cerebral ischemia-reperfusion injury and OGD injury in hippocampus. Biomed Pharmacother 108: 1596-1606, 2018.
3. Yan RY, Wang SJ, Yao GT, Liu ZG and Xiao N: The protective effect and its mechanism of 3-n-butylphthalide pretreatment on cerebral ischemia reperfusion injury in rats. Eur Rev Med Pharmacol Sci 21: 5275-5282, 2017.
4. Tuttolomondo A, Di Sciacca R, Di Raimondo D, Renda C, Pinto A and Licata G: Inflammation as a therapeutic target in acute ischemic stroke treatment. Curr Top Med Chem 9: 1240-1260, 2009.
5. Ornellas FM, Ornellas DS, Martini SV, Castiglione RC, Ventura GM, Rocco PR, Gutfilen B, de Souza SA, Takiya CM and Morales MM: Bone marrow-derived mononuclear cell therapy accelerates renal ischemia-reperfusion injury recovery by modulating inflammatory, antioxidant and apoptotic related molecules. Cell Physiol Biochem 41: 1736-1752, 2017.
6. Yingjie K, Haihong Y, Lingwei C, Sen Z, Yuanting D, Shasha C, Liutong P, Ying W and Min Z: Apoptosis repressor with caspase recruitment domain deficiency accelerates ischemia/reperfusion (I/R)-induced acute kidney injury by suppressing inflammation and apoptosis: The role of AKT/mTOR signaling. Biomed Pharmacother 112: 108681, 2019.
7. Lv Z, Liu C, Zhai M, Zhang Q, Li J, Zheng F and Peng M: LPS pretreatment attenuates cerebral ischaemia/reperfusion injury by inhibiting inflammation and apoptosis. Cell Physiol Biochem 45: 2246-2256, 2018.
8. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
9. Rippe C, Blimline M, Magerko KA, Lawson BR, LaRocca TJ, Donato AJ and Seals DR: MicroRNA changes in human arterial endothelial cells with senescence: Relation to apoptosis, eNOS and inflammation. Exp Gerontol 47: 45-51, 2012.
10. Singh RP, Massachi I, Manickavel S, Singh S, Rao NP, Hasan S, Mc Curdy DK, Sharma S, Wong D, Hahn BH and Rehimi H: The role of miRNA in inflammation and autoimmunity. Autoimmun Rev 12: 1160-1165, 2013.
11. Ferrante M and Conti GO: Environment and neurodegenerative diseases: An update on miRNA role. Microrna 6: 157-165, 2017.
12. Wang R, Bao H, Zhang S, Li R, Chen L and Zhu Y: miR-186-5p promotes apoptosis by targeting IGF-1 in SH-SY5Y OGD/R model. Int J Biol Sci 14: 1791-1799, 2018.
13. Ren X, Wang Z and Guo C: MiR-195-5p ameliorates cerebral ischemia-reperfusion injury by regulating the PTEN-AKT signaling pathway. Neuropsychiatr Dis Treat 17: 1231-1242, 2021.
14. Wu Y, Yao J and Feng K: miR-124-5p/NOX2 axis modulates the ROS production and the inflammatory microenvironment to protect against the cerebral I/R injury. Neurochem Res 45: 404-417, 2020.
15. Sohrabji F and Selvamani A: Sex differences in miRNA as therapies for ischemic stroke. Neurochem Int 127: 56-63, 2019.
16. Selvamani A and Sohrabji F: Mir363-3p improves ischemic stroke outcomes in female but not male rats. Neurochem Int 107: 168-181, 2017.
17. Wang K, Yan L and Lu F: miR-363-3p inhibits osteosarcoma cell proliferation and invasion via targeting SOX4. Oncol Res 27: 157-163, 2019.
18. Chen J, Lai W, Deng Y, Liu M, Dong M, Liu Z, Wang T, Li X, Zhao Z, Yin X, *et al*: MicroRNA-363-3p promotes apoptosis in response to cadmium-induced renal injury by down-regulating phosphoinositide 3-kinase expression. Toxicol Lett 345: 12-23, 2021.
19. Zhou T, Li S, Yang L and Xiang D: microRNA-363-3p reduces endothelial cell inflammatory responses in coronary heart disease via inactivation of the NOX4-dependent p38 MAPK axis. Aging (Albany NY) 13: 11061-11082, 2021.
20. Missotten M, Nichols A, Rieger K and Sadoul R: Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein. Cell Death Differ 6: 124-129, 1999.
21. Hashemi M, Yousefi J, Hashemi SM, Amininia S, Ebrahimi M, Taheri M and Ghavami S: Association between programmed cell death 6 interacting protein insertion/deletion polymorphism and the risk of breast cancer in a sample of Iranian population. Dis Markers 2015: 854621, 2015.
22. Ajasin DO, Rao VR, Wu X, Ramasamy S, Pujato M, Ruiz AP, Fiser A, Bresnick AR, Kalpana GV and Prasad VR: CCL2 mobilizes ALIX to facilitate Gag-p6 mediated HIV-1 virion release. Elife 8: e35546, 2019.
23. Xu C, Sun G, Yang J, Sun Q and Tong Z: Interleukin-13 promotes expression of Alix to compromise renal tubular epithelial barrier function. Cell Biol Int 39: 548-553, 2015.
24. Wu B, Liu J, Zhao R, Li Y, Peer J, Braun AL, Zhao L, Wang Y, Tong Z, Huang Y and Zheng JC: Glutaminase 1 regulates the release of extracellular vesicles during neuroinflammation through key metabolic intermediate alpha-ketoglutarate. J Neuroinflammation 15: 79, 2018.
25. Li J, Yang C and Wang Y: miR-126 overexpression attenuates oxygen-glucose deprivation/reperfusion injury by inhibiting oxidative stress and inflammatory response via the activation of SIRT1/Nrf2 signaling pathway in human umbilical vein endothelial cells. Mol Med Rep 23: 165, 2021.
26. Zhang L, Cai Q, Lin S, Chen B, Jia B, Ye R, Weygant N, Chu J and Peng J: Qingda granule exerts neuroprotective effects against ischemia/reperfusion-induced cerebral injury via lncRNA GAS5/miR-137 signaling pathway. Int J Med Sci 18: 1687-1698, 2021.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Rong H, Chen B, Wei X, Peng J, Ma K, Duan S and He J: Long non-coding RNA XIST expedites lung adenocarcinoma progression through upregulating MDM2 expression via binding to miR-363-3p. Thorac Cancer 11: 659-671, 2020.
29. Zhang JF, Zhang L, Shi LL, Zhao ZH, Xu H, Liang F, Li HB, Zhao Y, Xu X, Yang K and Tian YF: Parthenolide attenuates cerebral ischemia/reperfusion injury via Akt/GSK-3β pathway in PC12 cells. Biomed Pharmacother 89: 1159-1165, 2017.
30. Tian M, Yang M, Li Z, Wang Y, Chen W, Yang L, Li Y and Yuan H: Fluoxetine suppresses inflammatory reaction in microglia under OGD/R challenge via modulation of NF-κB signaling. Biosci Rep 39: BSR20181584, 2019.
31. Chatellard-Causse C, Blot B, Cristina N, Torch S, Missotten M and Sadoul R: Alix (ALG-2-interacting protein X), a protein involved in apoptosis, binds to endophilins and induces cytoplasmic vacuolization. J Biol Chem 277: 29108-29115, 2002.
32. Schaller B and Graf R: Cerebral ischemia and reperfusion: The pathophysiologic concept as a basis for clinical therapy. J Cereb Blood Flow Metab 24: 351-371, 2004.
33. Vasudeva K and Munshi A: miRNA dysregulation in ischaemic stroke: Focus on diagnosis, prognosis, therapeutic and protective biomarkers. Eur J Neurosci 52: 3610-3627, 2020.
34. Xu S, Li Y, Chen JP, Li DZ, Jiang Q, Wu T and Zhou XZ: Oxygen glucose deprivation/re-oxygenation-induced neuronal cell death is associated with Lnc-D63785 m6A methylation and miR-422a accumulation. Cell Death Dis 11: 816, 2020.
35. Chai Z, Gong J, Zheng P and Zheng J: Inhibition of miR-19a-3p decreases cerebral ischemia/reperfusion injury by targeting IGFBP3 in vivo and in vitro. Biol Res 53: 17, 2020.
36. Liu Y, Eaton ED, Wills TE, McCann SK, Antonic A and Howells DW: Human ischaemic cascade studies using SH-SY5Y cells: A systematic review and meta-analysis. Transl Stroke Res 9: 564-574, 2018.
37. Xiang Y, Zhang Y, Xia Y, Zhao H, Liu A and Chen Y: LncRNA MEG3 targeting miR-424-5p via MAPK signaling pathway mediates neuronal apoptosis in ischemic stroke. Aging (Albany NY) 12: 3156-3174, 2020.

38. Dong J, Geng J and Tan W: MiR-363-3p suppresses tumor growth and metastasis of colorectal cancer via targeting SphK2. Biomed Pharmacother 105: 922-931, 2018.
39. Chang J, Gao F, Chu H, Lou L, Wang H and Chen Y: miR-363-3p inhibits migration, invasion, and epithelial-mesenchymal transition by targeting NEDD9 and SOX4 in non-small-cell lung cancer. J Cell Physiol 235: 1808-1820, 2020.
40. Ma X, Jin L, Lei X, Tong J and Wang R: MicroRNA-363-3p inhibits cell proliferation and induces apoptosis in retinoblastoma cells via the Akt/mTOR signaling pathway by targeting PIK3CA. Oncol Rep 43: 1365-1374, 2020.
41. Zhu L, Zhang L, Tang Y, Zhang F, Wan C, Xu L and Guo P: MicroRNA-363-3p inhibits tumor cell proliferation and invasion in oral squamous cell carcinoma cell lines by targeting SSFA2. Exp Ther Med 21: 549, 2021.
42. Chen Y, Chen S, Lu J, Yuan D, He L, Qin P, Tan H and Xu L: MicroRNA-363-3p promote the development of acute myeloid leukemia with RUNX1 mutation by targeting SPRYD4 and FNDC3B. Medicine (Baltimore) 100: e25807, 2021.
43. Dong X, Li Y, Cao R and Xu H: MicroRNA-363-3p inhibits the expression of renal fibrosis markers in TGF-β1-treated HK-2 cells by targeting TGF-β2. Biochem Genet 59: 1033-1048, 2021.
44. Dirnagl U, Iadecola C and Moskowitz MA: Pathobiology of ischaemic stroke: An integrated view. Trends Neurosci 22: 391-397, 1999.
45. Chamorro Á, Dirnagl U, Urra X and Planas AM: Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. Lancet Neurol 15: 869-881, 2016.
46. Zhang M, Wang Z, Li B, Sun F, Chen A and Gong M: Identification of microRNA-363-3p as an essential regulator of chondrocyte apoptosis in osteoarthritis by targeting NRF1 through the p53-signaling pathway. Mol Med Rep 21: 1077-1088, 2020.
47. Gao C, Qian H, Shi Q and Zhang H: MicroRNA-363-3p serves as a diagnostic biomarker of acute myocardial infarction and regulates vascular endothelial injury by targeting KLF2. Cardiovasc Diagn Ther 10: 421-430, 2020.
48. Hemming FJ, Fraboulet S, Blot B and Sadoul R: Early increase of apoptosis-linked gene-2 interacting protein X in areas of kainate-induced neurodegeneration. Neuroscience 123: 887-895, 2004.
49. Blum D, Hemming FJ, Galas MC, Torch S, Cuvelier L, Schiffmann SN and Sadoul R: Increased Alix (apoptosis-linked gene-2 interacting protein X) immunoreactivity in the degenerating striatum of rats chronically treated by 3-nitropropionic acid. Neurosci Lett 368: 309-313, 2004.