

MicroRNA-424 alleviates neurocyte injury by targeting PDCD4 in a cellular model of cerebral ischemic stroke

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Abstract. Cerebral ischemic stroke is the primary cause of stroke-associated mortality and disability, and current therapeutic options are limited and ineffective. The present study aimed to investigate the potential of apoptotic therapy and the role of microRNA (miR)-424 in cerebral ischemic stroke. PC12 cells, a cloned cell line from rat adrenal pheochromocytoma, were treated with CoCl₂ to construct a cellular ischemia model. mRNA and protein levels of programmed cell death protein 4 (PDCD4), Bcl-2, Bax, caspase-3, PI3K and AKT were evaluated using reverse transcription-quantitative PCR and western blot analyses, respectively. Cell Counting Kit-8 assays were performed to examine cell viability in the ischemia model. Flow cytometry was conducted to evaluate the apoptosis of ischemic cells. Furthermore, a luciferase assay was performed to verify the target gene of miR-424. It was revealed that the expression level of miR-424 was downregulated in the ischemia model, while the expression of PDCD4 was upregulated. Moreover, the expression of miR-424 was increased after treatment with miR-424 mimics. The mRNA and protein expression of PDCD4 was upregulated after transfection with pcDNA3.1-PDCD4. PDCD4 was predicted and demonstrated to be a target of miR-424. Notably, overexpression of miR-424 increased cell viability and inhibited apoptosis in the ischemia model, which was reversed by co-treatment with pcDNA3.1-PDCD4. Furthermore, overexpression of miR-424 regulated the expression of PDCD4, Bax, Bcl-2, phosphorylated-PI3K/AKT and caspase-3, which was restored after co-transfection with pcDNA3.1-PDCD4. Collectively, the results indicated that miR-424 regulated the

progression of cerebral ischemic stroke in a cellular model by targeting PDCD4.

Introduction

Stroke is characterized with high morbidity, mortality and disability (1). Strokes can be stratified into ischemic and hemorrhagic categories, which account for ~80% and 20% of all strokes, respectively (2). Cerebral ischemic stroke is induced by the development of a thrombus within the major cerebral artery, contributing to the apoptosis of neuronal cells (3,4). The only efficacious therapies for cerebral ischemic stroke are dissolving the thrombus or using tissue plasminogen activators (5). However, the number of patients with cerebral ischemic stroke receiving these treatments is ≤4.5% (6). Additionally, secondary neuronal death may occur due to cerebral infarction, resulting in long-term neurological deficits (7). The incidence and mortality rate of ischemic stroke has been increasing annually in China (8), exerting a notable threat to public health. In 2017, a survey of 155 urban and rural centers in 31 provinces in China showed that the age-standardized prevalence, incidence and mortality rates were 1,114.8 per 100,000, 246.8 and 114.8 per 100,000 person-years, respectively (9). Therefore, there is a requirement to explore novel and effective treatment options for cerebral ischemic stroke. It has been reported that neuronal cell apoptosis following cerebral ischemia/reperfusion (I/R) is the primary cause of brain injury (10,11). Prevention of neuronal cell apoptosis is a potential treatment aim for ischemic stroke (12). Therefore, it is important to explore the underlying mechanisms of ischemic neuronal death.

MicroRNAs (miRNAs/miRs) are a family of small single-stranded non-coding RNAs (13). miRNAs serve important roles in various cellular processes, including cell proliferation, differentiation and apoptosis (14). Dysregulation of miRNAs influences various diseases, including stroke (15). Various studies have reported that aberrant expression of miRNAs influences cerebral ischemic stroke. For example, upregulation of miR-130b mediates cerebral ischemic injury in astrocytes (16). Furthermore, upregulation of miR-145 alleviates primary astrocyte injury following cerebral ischemic stroke *in vitro* (17). Notably, the abnormal expression

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of miRNAs is associated with neurological disorders and regulates the expression of apoptosis-associated genes (18). Dysregulated expression of miR-525-5p ameliorates I/R injury-induced neuronal cell death in a study in adult rats (19). Upregulation of miR-9 has been reported to suppress neuronal apoptosis in rats with ischemic stroke (20). A previous study reported that lower levels of circulating miR-424 in patients with acute cerebral infarction may predict poor prognosis, where the expression of circulating miR-424 is negatively associated with the serum levels of certain pro-inflammatory cytokines (21). This suggests that circulating miR-424 may have the potential to serve as a prognostic marker and therapeutic target for the management of acute cerebral infarction. However, the potential mechanisms of miR-424 in cerebral infarction have not been fully elucidated.

The PC12 cell line is a cloned cell line from rat adrenal pheochromocytoma that is widely used as a model to study neuronal cell differentiation and function (22,23). This cell line is favored due to its high stability, homogeneity and differentiation (24,25). Hypoxia of brain tissue after cerebral infarction causes a large amount of hypoxia inducible factor (HIF)-1 accumulation, mitochondrial dysfunction, free radical eruption, which leads to nerve cell damage (26). Chronic hypoxia occurs in nerve cells due to the inability to efficiently transport and utilize oxygen after CoCl₂ treatment (27). CoCl₂ induced a series of changes in nerve cells similar to the effects of hypoxia (28). Therefore, CoCl₂ is an ideal compound for establishing a chronic hypoxia model.

Programmed cell death factor 4 (PDCD4) is originally described as a tumor suppressor gene that exerts antineoplastic effects by promoting apoptosis (29). The present findings suggested that miR-424 may represent a promising biomarker for cerebral ischemic stroke. However, the mechanism of miR-424 in acute cerebral infarction remains unclear. In the present study, the regulatory role of miR-424 was investigated in an *in vitro* model of acute cerebral infarction.

Materials and methods

Cell culture. PC12 cells were obtained from American Type Culture Collection. Cells were incubated at 37°C in DMEM containing 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 5% CO₂ until they reached 60-70% confluence. The cells were then incubated either in medium supplemented with 200 µM CoCl₂ (cat. no. 618519; Sigma-Aldrich; Merck KGaA) or in normal medium. After 24 h, cells were collected for subsequent experiments.

Transfection. miR-424-5p mimic (sense, 5'-CAGCAGCAAUUCAUGUUUUGAA-3' and antisense, 5'-CAAACAUGAAUUGCUGCUGUU-3'), mimic negative control (NC; sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3') were purchased from Shanghai GenePharma Co., Ltd. PC12 Cells were cultured (8x10⁵ cells/well) in 12-well plates. After cells reached 70-80% confluence, they were transfected with 100 nM miR-424 mimics or miR-negative control (NC) mimics (Shanghai GenePharma Co., Ltd.) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). For programmed

cell death protein 4 (PDCD4) transfection, cells were treated with 1 µg pcDNA3.1-PDCD4 or pcDNA3.1 (Addgene, Inc.) for 48 h using Lipofectamine 2000 according to manufacturer's instructions. After transfection, cells were subjected to ischemic treatment for 12 h.

Cell Counting Kit-8 (CCK-8). At 0, 12, 24 and 48 h post-transfection, cells were re-seeded in a 96-well plate (5x10³ cells/well). Each well was supplemented with 10 µl CCK-8 solution (Dojindo Molecular Technologies, Inc.) and incubated at 37°C for another 2 h according to the manufacturer's protocol. The absorbance value at 450 nm was determined (Bio-Rad Laboratories, Inc.).

Flow cytometry. Cells were harvested and washed with pre-cooled PBS. Then cells were resuspended in 200 µl binding buffer. Subsequently, cells were stained with FITC-Annexin V and propidium iodide (PI; BD Biosciences) in the dark for 20 min at room temperature. Subsequently, the data were evaluated with a BD FACSCalibur[™] flow cytometer (Beckman Coulter, Inc.) using FlowJo software (version 10; FlowJo LLC). Sums of percentages of cells in early and late apoptosis were added as the total apoptosis ratio from each dot plot.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from cells was isolated with TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the concentration of total RNA was determined with NanoDrop[™] 1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). A total of 1 µg RNA was reversely transcribed using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reverse transcription were conducted under the following conditions: 42°C for 60 min, 72°C for 15 min. Amplification of cDNA was conducted using a RAPA3G SYBR Green qPCR Mix (cat. no. A2250A; HaiGene Biotech Co., Ltd.; <https://www.haigene.cn/html/show-226.html>). The PCR reactions were conducted under the following conditions: 95°C for 10 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 35 sec, with a final extension at 72°C for 10 min. U6 and GAPDH were used to normalize miRNA and mRNA, respectively. The sequences of the primers used for qPCR were as follows: miR-424 forward, 5'-TTCAAACATGAATTGCTGCTG-3' and reverse, 5'-CTCAACTGGTGTCTGGGA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACG AATTTGCGTGTTCAT-3'; PDCD4 forward, 5'-TCCCTAATTCCTCATGGTGTCT-3' and reverse, 5'-CGCCTTTTGCCTTGGCATT-3'; Bax forward, 5'-TGAAGGACGCAC GTTCAG-3' and reverse, 5'-CCTGCTCGATCCTGGATG AAA-3'; Bcl-2 forward, 5'-CTTTGAGTTTCGGTGGGG TCA-3' and reverse, 5'-GGGCCGTACAGTTCACAAA-3'; caspase-3 forward, 5'-GGAACCATCATACTGGAAGCG-3' and reverse, 5'-TTCCCTGAGGTTTGCTGCAT-3'; GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'. mRNA expression was determined using the 2^{-ΔΔC_q} method (30). Each experiment was performed in triplicate.

Western blotting. Total protein was extracted from PC12 cells using pre-cold RIPA buffer (Beyotime Institute of

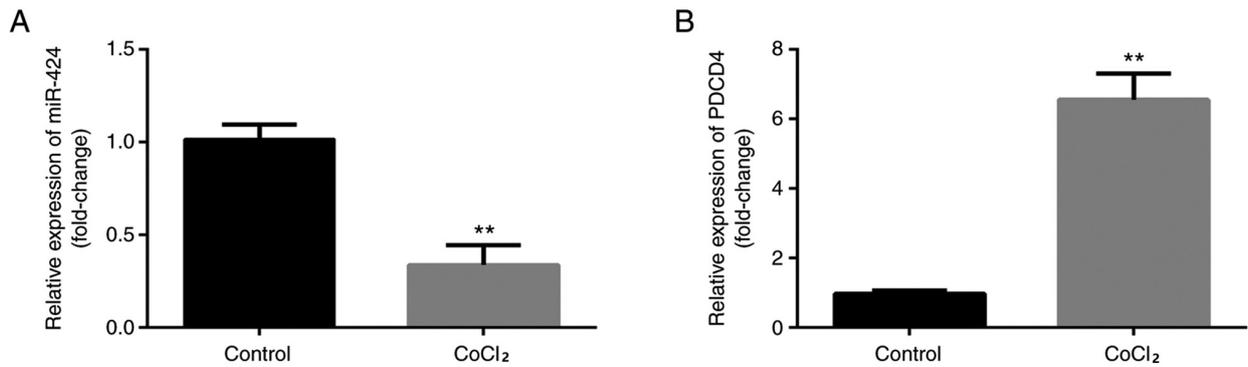


Figure 1. Expression of miR-424 and PDCD4 in a neuronal cell model of ischemia. (A) Expression of miR-424 was significantly decreased after treatment with CoCl₂. (B) Expression of PDCD4 was significantly higher after treatment with CoCl₂. **P<0.01 vs. Control. miR, microRNA; PDCD4, programmed cell death protein 4.

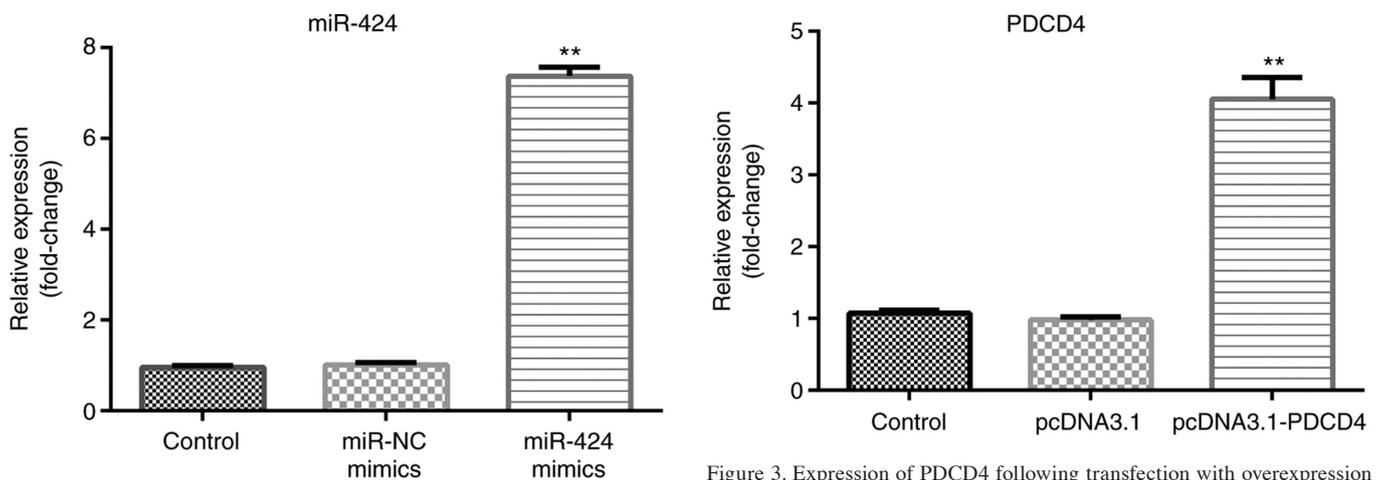


Figure 2. Expression of miR-424 following transfection with mimics. Expression of miR-424 was significantly increased after treatment with miR-424 mimics, while there was no significant difference between the control and miR-NC mimics groups. **P<0.01 vs. miR-NC mimics. miR, microRNA; NC, negative control.

Figure 3. Expression of PDCD4 following transfection with overexpression vector. Expression of PDCD4 was significantly upregulated after transfection with pcDNA3.1-PDCD4. The difference between the control and pcDNA3.1 groups was not significant. **P<0.01 vs. pcDNA3.1. PDCD4, programmed cell death protein 4.

Biotechnology) including protease inhibitor. Total protein was quantified using a bicinchoninic acid kit (Sigma-Aldrich; Merck KGaA). Proteins (40 µg) were then separated via 10% SDS-PAGE, proteins were transferred onto PVDF membranes. The membranes were then blocked with 5% skimmed milk at room temperature for 1 h. The membranes were incubated overnight at 4°C with anti-PDCD4 (1:1,000; cat. no. ab80590; Abcam), PI3K (1:1,000; cat. no. ab32089; Abcam), p-PI3K p85 (1:1,000; cat. no. ab278545; Abcam), AKT (1:1,000; cat. no. ab8805; Abcam), p-AKT (1:1,000; cat. no. ab81283; Abcam), Bax (1:1,000; cat. no. ab32503; Abcam), caspase-3 (1:1,000; cat. no. ab13847; Abcam) and GAPDH (1:1,000; cat. no. ab9485; Abcam) primary antibodies in the absence of light. Then cells were incubated with HRP-labeled anti-rabbit IgG secondary antibody (1:4,000; cat. no. ab6721; Abcam) or anti-mouse IgG secondary antibody (1:5,000, cat. no. ab6789, Abcam) for 2 h at room temperature. The bands were imaged with an Pierce™ ECL Western Blotting Substrate kit (cat. no. 32109; Thermo Fisher Scientific, Inc.) and band density was quantified using Quantity One v.6.2 software (Bio-Rad Laboratories, Inc.).

Dual-luciferase reporter assay. Bioinformatics analysis predicted using TargetScan 7.2 (http://www.targetscan.org/vert_71/) revealed that PDCD4 was a target of miR-424. For analysis of the predicted miRNA binding site, GCUGC UAA, located at the c*392-c*399 site of PDCD4 (NM_145341.4), a dual-luciferase reporter assay was performed to determine whether miR-424 directly targeted PDCD4. Complementary 50-60-bp DNA oligonucleotides consisting of the test sequence flanked by single-stranded overhangs encoding restriction enzyme sites *SacI* and *HindIII* in the multiple cloning sites of the pMIR-REPORT Luciferase plasmid (Ambion; Thermo Fisher Scientific, Inc.) were designed and synthesized. Synthetic oligonucleotides (Thermo Fisher Scientific, Inc.) annealing and ligation into the plasmid were performed according to the manufacturer's protocols. The DNA oligos used were as follows: Wild-type sense, 5'-CTATTTAGGGGGTAAAGTTAAGCTGCTAAAACCC CATGTTGGCTGCTGCTA-3' and antisense, 5'-AGCTTAGCAGCAGCCAACATGGGGTTTTAGCAGCTTAACTTT ACCCCCTAAATAGAGCT-3' and mutant sense, 5'-CTATTT AGGGGGTAAAGTTAACAGAAGAAAACCCCATGTTG GCT GCTGCTA-3' and antisense, 5'-AGCTTAGCAGC

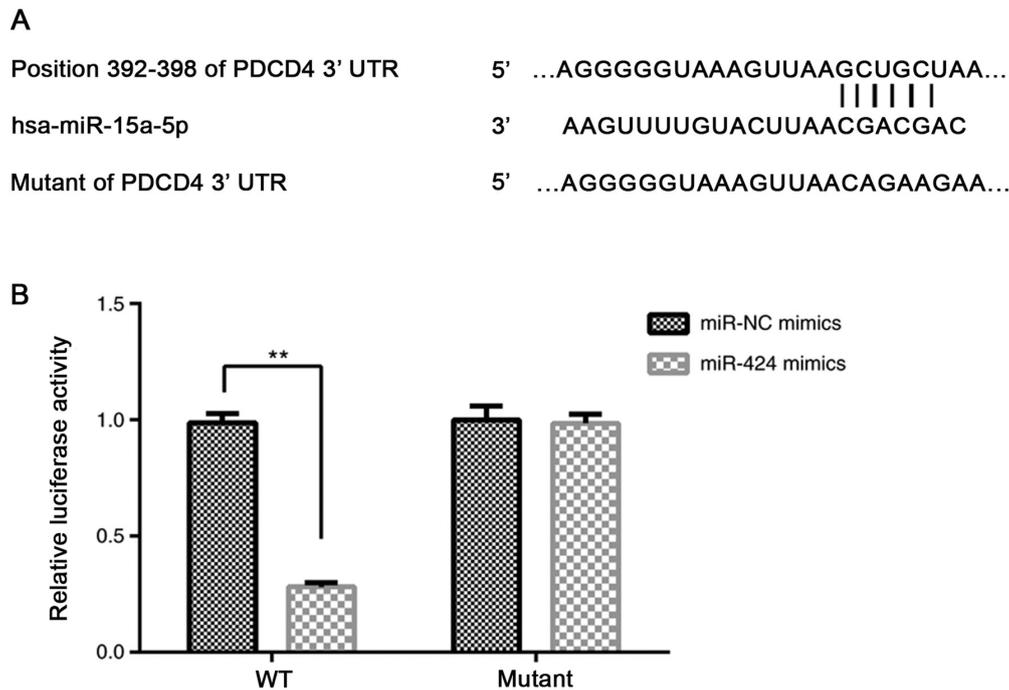


Figure 4. PDCD4 is a target of miR-424. (A) Sequence alignment revealed a binding site for miR-424 in the 3'UTR of PDCD4. (B) Luciferase activity of cells was significantly decreased after co-transfection with miR-424 mimics and PDCD4-3'UTR-WT, while there was no significant difference in the mutant groups. ** $P < 0.01$. miR, microRNA; PDCD4, programmed cell death protein 4; NC, negative control; UTR, untranslated region; WT, wild-type.

AGCCAACATGGGGTTTTCTTCTGTAACTTTACCCCTAAATAGAGCT-3'. PC12 cells were cultured (8×10^4 cells/cm²) into 12-well plates. After the cells reached 70-80% confluence, PC12 cells were co-transfected with 100 ng firefly luciferase constructs and 100 nM miR-424 mimic or miR-NC mimic using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). *Renilla* luciferase was used as the internal control for normalization. The luciferase activity was calculated using a dual-luciferase reporter assay (Promega Corporation) at 48 h post-transfection.

Statistical analysis. All experiments were repeated \geq three times. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.). Data are presented as the mean \pm SD. Comparisons between two groups were performed using an unpaired Student's *t*-test. Comparisons of >2 groups were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-424 and PDCD4 in ischemia model. RT-qPCR was performed to examine the RNA expression of miR-424 and PDCD4. Compared with normal PC12 cells, the expression of miR-424 was significantly decreased following CoCl₂ treatment, whereas the expression of PDCD4 was significantly upregulated (Fig. 1A and B).

Transfection of miR-424 mimics in PC12 cells. Cells were transfected with miR-NC mimics or miR-424 mimics. The expression of miR-424 was significantly increased after the treatment with miR-424 mimics compared with miR-NC mimics, while there

were no significant differences between the non-transfected control group and the miR-NC mimics group (Fig. 2).

Transfection of PDCD4 in PC12 cells. Cells were transfected with pcDNA3.1-PDCD4 or pcDNA3.1. As presented in Fig. 3, the expression level of PDCD4 in cells transfected with pcDNA3.1-PDCD4 was significantly upregulated compared with cells transfected with pcDNA3.1. There was no significant difference in expression between the pcDNA3.1 group and the non-treated control group.

PDCD4 is a target of miR-424. PDCD4 was predicted as a target gene of miR-424 using TargetScan 7.2 (Fig. 4A). The results of the luciferase assay demonstrated that for the PDCD4-3'UTR-WT transfected cells, the luciferase activity was significantly decreased after transfection with miR-424 mimics compared with that in the miR-NC mimics group, whereas for the PDCD4-3'UTR-mutant transfected cells, transfection with miR-424 mimics exerted no significant effects on the luciferase activity (Fig. 4B).

Overexpression of miR-424 inhibits the apoptosis of ischemia-exposed cells. The apoptosis of ischemic PC12 cells was examined via flow cytometry. As presented in Fig. 5A and B, there was no significant difference between the control and NC group; however, the apoptosis rate significantly decreased after transfection with miR-424 mimics, which was reversed by co-transfection with pcDNA3.1-PDCD4.

Overexpression of miR-424 promotes the viability of ischemia-exposed cells. The viability of ischemic PC12 cells was determined using a CCK-8 assay. Overexpression of miR-424 significantly increased the viability of ischemic

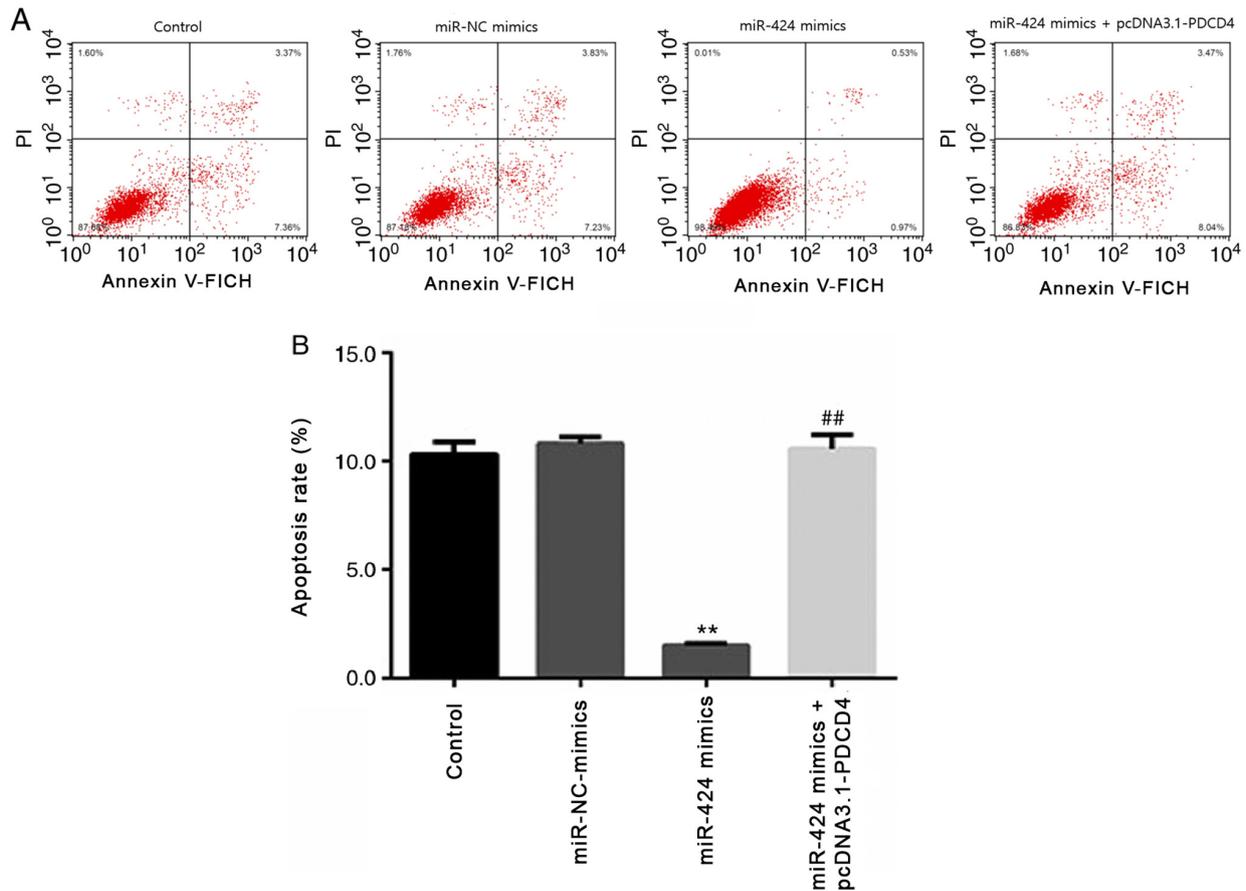


Figure 5. Overexpression of miR-424 inhibits the apoptosis of CoCl₂-treated PC12 cells. (A) Overexpression of miR-424 significantly suppressed the apoptosis of CoCl₂-treated PC12 cells compared with the miR-NC mimics group, which was significantly reversed after co-transfection with pcDNA3.1-PDCD4. There was no significant difference between the control and NC groups. (B) Quantification of flow cytometry assay. **P<0.01 vs. miR-NC mimics; ##P<0.01 vs. miR-424 mimics. miR, microRNA; PDCD4, programmed cell death protein 4; NC, negative control; PI, propidium iodide.

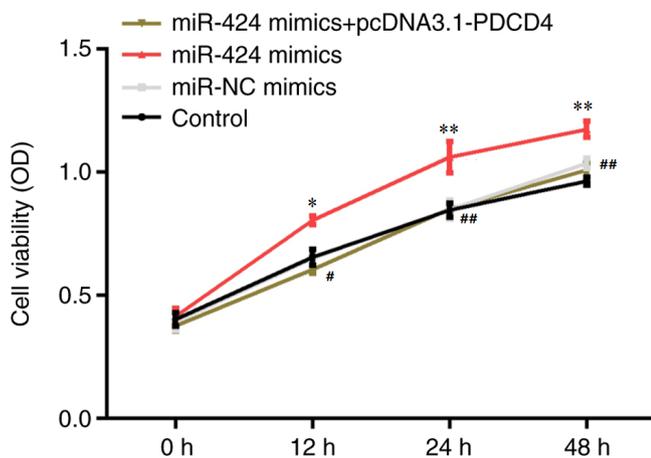


Figure 6. Overexpression of miR-424 promotes the viability of CoCl₂-treated PC12 cells. Increased miR-424 expression promoted the viability of CoCl₂-treated PC12 cells, which was significantly reversed after co-transfection with pcDNA3.1-PDCD4. There was no significant difference between the control and NC groups. *Between miR-424 mimics and miR-424 mimics+pcDNA3.1-PDCD4. #Between miR-424 mimics and miR-NC mimics. *P<0.05, **P<0.01, #P<0.05, ##P<0.01 vs. miR-424 mimics. miR, microRNA; PDCD4, programmed cell death protein 4; NC, negative control; OD, optical density.

PC12 cells, which was reversed following co-transfection with pcDNA3.1-PDCD4 at 12, 24 and 48 h (Fig. 6)

miR-424 regulates the expression of PDCD4, Bcl-2, Bax, caspase-3, PI3K/AKT and phosphorylated (p)-PI3K/AKT. RT-qPCR and western blotting were conducted to evaluate mRNA and protein expression, respectively. As presented in Fig. 7A-D, the mRNA expression of PDCD4, Bax and caspase-3 was downregulated following miR-424 overexpression, whereas Bcl-2 expression was increased; these effects were significantly reversed by co-transfection with pcDNA3.1-PDCD4. There was no significant difference between the control and miR-NC mimics groups. Protein expression exhibited the same trends as mRNA expression. Furthermore, the levels of p-PI3K and p-AKT in cells transfected with miR-424 mimics were significantly increased, which was significantly reversed following co-transfection with pcDNA3.1-PDCD4. The protein levels of PI3K/AKT were not significantly affected by any transfections (Fig. 7E and F).

Discussion

Increasing evidence has indicated that aberrant expression of miRNAs in the human brain serves a crucial role in various brain diseases, including cerebral ischemic stroke (31,32). In the present study, the roles of miR-424 in cerebral ischemic stroke were investigated. The results indicated that miR-424 was downregulated in CoCl₂-treated PC12 cells. Overexpression of miR-424 increased cell viability and inhibited apoptosis in

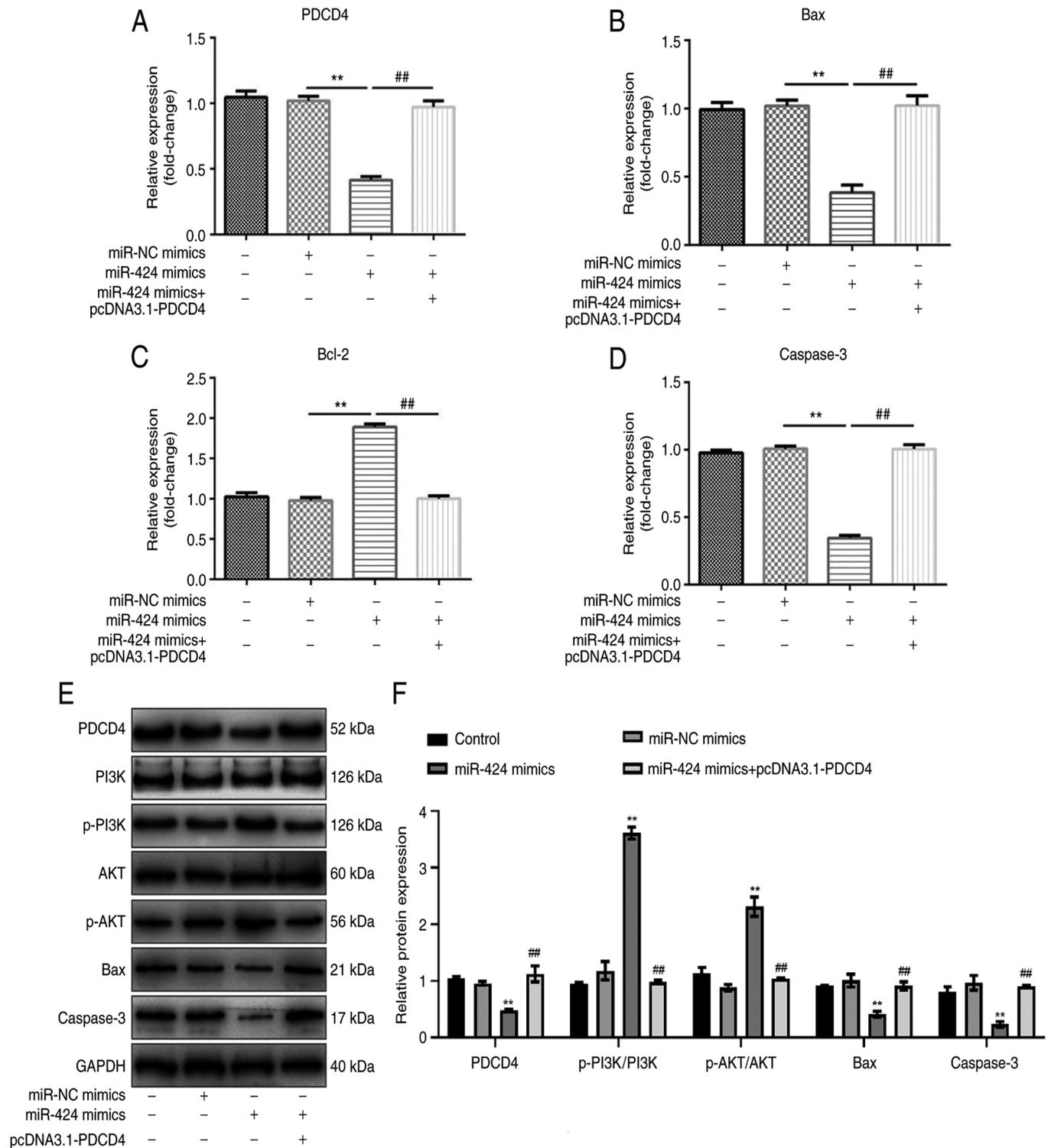


Figure 7. miR-424 regulates the expression of PDCD4, Bcl-2, Bax, caspase-3, PI3K/AKT and p-PI3K/AKT. Overexpression miR-424 altered the mRNA expression of (A) PDCD4, (B) Bax, (C) Bcl-2 and (D) caspase-3; its effects were attenuated by pcDNA3.1-PDCD4 co-transfection. (E) miR-424 overexpression increased the relative phosphorylation of PI3K and AKT, and suppressed PDCD4, Bax and caspase-3; its effects were attenuated by pcDNA3.1-PDCD4 co-transfection. (F) Qualitative analysis of western blotting. ** $P < 0.01$ vs. miR-NC mimics or as indicated; ## $P < 0.01$ vs. miR-424 mimics or as indicated. miR, microRNA; PDCD4, programmed cell death protein 4; NC, negative control; p, phosphorylated.

the ischemia model, which suggested that miR-424 may be a neuroprotective miRNA in cerebral ischemic stroke. This was consistent with a previous study that demonstrated that miR-424 mediated neuronal damage (33). However, the potential underlying mechanisms remain to be elucidated.

Neuronal cell death is considered to be the primary cause of stroke pathophysiology (34). This process can be slowed down by miRNAs via the regulation of apoptosis in neuronal cells (35). For example, overexpression of miR-455 exerted a positive role in protecting neuronal cells from

ischemic injury-induced death (36). Notably, downregulation of miR-155 serves a protective role in ischemic stroke (37). In the present study, overexpression of miR-424 inhibited the apoptosis of neuronal cells. Furthermore, increased miR-424 promoted the viability of CoCl₂-treated PC12 cells, suggesting that miR-424 may serve a neuroprotective role in cerebral ischemic stroke.

miRNAs regulate gene expression via binding sites in the 3'UTR of target genes (38). PDCD4 was predicted to be a target gene of miR-424. In the present study, overexpression of miR-424 decreased the expression of PDCD4, which was reversed after co-transfection with pcDNA3.1-PDCD4. Moreover, luciferase activity was decreased after co-transfection with miR-424 mimics and PDCD4-3'UTR-WT compared with miR-NC mimics, suggesting that PDCD4 is a target of miR-424. PDCD4 is an apoptotic protein and serves an important role in short-term responses to ischemic stroke (39). Additionally, associations between PDCD4 and miRNAs have previously been reported in cerebral ischemia (40). In the present study, co-transfection with PDCD4 attenuated the decrease in apoptotic rate induced by overexpression of miR-424, and inhibited cell viability. However, the underlying mechanisms remain unclear. The possible effects of PDCD4 on the expression of various genes and proteins were examined. It was revealed that miR-424 overexpression increased the expression of Bcl-2 and the phosphorylation of PI3K and AKT, and decreased Bax and caspase-3 expression; these effects were reversed by co-transfection with pcDNA3.1-PDCD4. Taken together, the present results indicated that miR-424 may regulate neuronal apoptosis by targeting PDCD4.

In conclusion, miR-424 was downregulated in ischemia-injured cells. miR-424 may serve a neuroprotective role by inhibiting the apoptosis and promoting the viability of ischemia-injured cells, potentially by targeting PDCD4. This may be a novel therapeutic target for cerebral ischemic stroke.

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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

HWR performed the majority of experiments and wrote the manuscript. BG, YZZ, TG, QW and YQS performed the remaining experiments and statistically analyzed the data. JW designed the present study. HWR and JW can authenticate the raw data in this study. All authors read and approved the final manuscript.

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.

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