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DIXDC1 prevents oxygen-glucose deprivation/reoxygenation-induced injury in hippocampal neurons *in vitro* by promoting Wnt/β-catenin signaling

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Abstract. – OBJECTIVE: Dishevelled-Axin (DIX) domain containing 1 (DIXDC1), a novel DIX domain-containing protein and a positive regulator of Wingless (Wnt) signaling, has previously been reported to play multiple roles in neurodevelopment and neurological disorders. However, whether DIXDC1 plays a role during cerebral ischemia/reperfusion injury remains unknown. In this study, we investigated the potential role of DIXDC1 in neuronal injury induced by oxygen-glucose deprivation and reoxygenation (OGD/R), an *in vitro* model of cerebral ischemia/reperfusion injury.

MATERIALS AND METHODS: Neuronal injury was induced by OGD/R treatment. Relative mRNA expression of DIXDC1 was detected by real-time quantitative polymerase chain reaction (RT-qPCR). Protein expression of DIXDC1 and β-catenin was determined by Western blot. Cell viability was examined by the cell counting kit-8 assay. Cell cytotoxicity was detected by the lactate dehydrogenase assay. Cell apoptosis was detected by the caspase-3 activity assay. The activity of Wnt/β-catenin signaling was detected by the luciferase reporter assay.

RESULTS: TWe found that DIXDC1 expression was significantly upregulated in hippocampal neurons following OGD/R treatment. Small interfering RNA-mediated silencing of DIXDC1 significantly impaired viability and promoted cell injury and apoptosis in neurons with OGD/R treatment. In contrast, overexpression of DIXDC1 increased the viability and reduced cell injury and apoptosis in neurons with OGD/R treatment, showing protective effects against OGD/R injury. Furthermore, our results showed that DIXDC1 promoted the expression of β-catenin and activation of Wnt signaling. Notably, inhibition of Wnt signaling significantly abrogated DIXDC-mediated neuroprotective effects.

CONCLUSIONS: Our results demonstrate that DIXDC1 prevents OGD/R-induced neuronal injury by promoting Wnt/β-catenin signaling. Our study indicates that DIXDC1 may play an important role in cerebral ischemia and reperfusion serving as a potential target for the treatment of cerebral ischemia/reperfusion injury.

Key Words
Cerebral ischemia/reperfusion, DIXDC1, Oxygen-glucose deprivation and reoxygenation, Wnt.

Introduction

Cerebral ischemia/reperfusion injury is a major health concern and a serious socioeconomic problem caused by shock, stroke, or surgery anesthesia[1]. In addition to tissue damage and dysfunction, various biological changes, including excitotoxicity, apoptosis, calcium overload, and neuroinflammation, contribute to injury mechanisms induced

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by cerebral ischemia/reperfusion[2]. However, the complex pathological mechanisms of cerebral ischemia/reperfusion injury are largely unknown, which hinders the development of clinically effective therapies. Therefore, it is essential to seek potential and promising intervention targets to develop effective therapeutic options for the treatment of cerebral ischemia/reperfusion injury.

Dishevelled-Axin (DIX) domain containing 1 (DIXDC1) protein, containing a coiled-coil domain and a DIX domain, has been reported as an important regulator of various cellular processes and diseases[3]. DIXDC1 has been suggested as a positive regulator of the Wingless (Wnt) signaling pathway and to be involved in the regulation of embryonic development[4]. DIXDC1 acts as a novel branching component in the Wnt signaling pathway targeting the β-catenin/T-cell factor/lymphocyte enhancer factor complex for gene expression involved in regulating cytoskeleton formation and the cell cycle[5,6]. Dysregulation of DIXDC1 is associated with tumorigenesis in a variety of cancers, including colorectal cancer, gastric cancer, lung cancer, hepatocellular carcinoma, pancreatic cancer, and lymphomas[3,7-12], by functioning as an oncogene or a tumor suppressor. Importantly, accumulating evidence has documented[13] that DIXD1 is involved in regulating neuronal development and neurological disorders. DIXDC1 has been reported[14,15] to regulate cell survival during traumatic brain injury and sciatic nerve crush. However, whether DIXDC1 is involved in regulating cerebral ischemia/reperfusion injury remains unclear.

Wnt signaling has been suggested[16,17] to play a central role in the development and a variety of pathological conditions. Binding of Wnt to Frizzled receptors results in activation of Dishevelled and subsequent inhibition of glycogen-synthase-kinase-3β, which leads to stabilization of β-catenin and activation of the canonical Wnt pathway[16]. Wnt/β-catenin signaling pathway is involved in regulating various cellular processes, including proliferation, apoptosis, metastasis, and fibrosis[18,19]. The Wnt/β-catenin signaling pathway plays an important role in neurological disorders[20], including cerebral ischemia/reperfusion injury, and represents a promising therapeutic target[21]. Therefore, identification of a novel molecule involved in regulating the Wnt/β-catenin signaling pathway can aid in understanding the pathological development of cerebral ischemia/reperfusion injury.

Considering the role of DIXDC1 in regulating the Wnt/β-catenin signaling pathway and neurological disorders, our study aimed to investigate the potential role of DIXDC1 in regulating cerebral ischemia/reperfusion injury. Herein, we detected the effect of DIXDC1 on neuronal survival of hippocampal neurons following oxygen-glucose deprivation and reoxygenation (OGD/R), an *in vitro* model of cerebral ischemia/reperfusion. We found that DIXDC1 expression was significantly upregulated in hippocampal neurons following OGD/R treatment. Inhibition of DIXDC1 significantly impaired viability and promoted injury and apoptosis in neurons with OGD/R treatment, whereas overexpression of DIXDC1 increased viability and inhibited injury and apoptosis in neurons with OGD/R treatment. Furthermore, our results showed that DIXDC1 promoted the expression of β-catenin and activation of Wnt signaling. Notably, inhibition of Wnt signaling significantly abrogated the protective effect of DIXDC1 against OGD/R injury. Taken together, our results demonstrate that DIXDC1 prevents OGD/R-induced neuronal injury by regulating Wnt/β-catenin signaling. Our study suggests that DIXDC1 may play an important role in cerebral ischemia and reperfusion, serving as a presumptive target of preventive or therapeutic interventions in cerebral ischemia/reperfusion injury.

Materials and methods

Cell Culture

HT22, a mouse hippocampal cell line, was purchased from the BeNa Culture Collection (Kunshan, Jiangsu, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 4 mM L-glucose, and penicillin 100 U/mL-streptomycin 100 μg/mL. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Induction of Oxygen-Glucose Deprivation/Reoxygenation

HT22 hippocampal neurons were cultured in glucose-free DMEM in an anaerobic chamber (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 1% O_2, 94% N_2, and 5% CO_2 at 37°C for oxygen-glucose deprivation (OGD) treatment to mimic ischemia. After 3 h of OGD treatment, the medium was replaced by fresh normal culture medium, and the cells were cultured under normal conditions (95% air and 5% CO_2) at 37°C for 12 and 24 h of reoxygenation. Cells in the control group were not exposed to OGD/R treatment.

Cell Transfection

The small interfering RNA (siRNA) targeting DIXDC1 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and transfected into cells according to the manufacturer's instructions. For overexpression of DIXDC1, the full length of DIXDC1 cDNA was inserted into pcDNA3.0 plasmid followed by transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturers' protocols.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (TaKaRa, Dalian, Liaoning, China). Amplification of the cDNA template was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers specific to the target genes. β-actin was used as a housekeeping gene for normalization of gene expression. Relative gene expression was calculated using the classic $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Proteins were isolated from cells using radio immunoprecipitation assay (RIPA) lysis buffer, and protein concentrations were determined using the bicinchoninic acid (BCA) kit (Beyotime Biotechnology, Shanghai, China). Total protein (40 μg) from each sample was loaded onto 10% sodium dodecyl sulfate polyacrylamide gels for separation. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), which was then blocked with 5% nonfat milk for 1 h at 37°C. Afterwards, the membrane was incubated with primary antibodies including anti-DIXDC1, anti-β-catenin, and anti-β-actin (Abcam, Cambridge, MA, USA) at 4°C overnight. After incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, the protein bands were detected using the enhanced chemiluminescence (ECL) plus Western Blotting System (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The intensity of protein bands was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Cell Viability Assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured overnight. After the indicated treatments, the medium was refreshed, and 20 μL of MTT solution (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h at 37°C. Afterwards, 150 μL of dimethyl sulfoxide was added to each well to dissolve the crystals for 10 min. The optical density (OD) of the solubilization solution was measured at a wavelength of 570 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

Lactate Dehydrogenase (LDH) Assay

Cell injury was determined by measuring LDH release using an LDH Cytotoxicity Assay Kit (Beyotime Biotechnology, Shanghai, China). In brief, cells were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured overnight. After the indicated treatments, cells were centrifuged at 400 *g* for 5 min. A total of 120 μL cell culture medium was harvested and mixed with 60 μL LDH working solution. After incubation for 30 min in the dark at room temperature, the OD value at 490 nm was determined using a microplate reader (Bio-Rad, Hercules, CA, USA).

Caspase-3 Activity Assay

Following the indicated treatments, cells were lysed for measurement of caspase-3 activity. After centrifugation, a total of 50 μL of supernatant was collected and mixed with 40 μL of reaction buffer and 10 μL of L-Asparagine,N-acetyl-L-a-aspartyl-L-a-glutamyl-L-valyl-N-(4-nitrophenyl)-(9CI) (Ac-DEVD-pNA; 2 mM). After incubation for 1-2 h, the OD value at 405 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Luciferase Reporter Assay

The activity of Wnt signaling was detected using a TOPflash luciferase reporter assay. Briefly, cells were plated into 96-well plates and cotransfected with DIXDC1 siRNA or DIXDC1 expression vector, TOPflash vector (Promega, Madison, WI, USA) and pRL-TK vector (Promega, Madison, WI, USA). The luciferase activity was detected by using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocols.

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA), and differences were interpreted using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. A *p*-value less than 0.05 was defined as statistically significant.

Results

OGD/R Treatment Induces DIXDC1 in HT22 Cells In Vitro

To investigate the potential role of DIXDC1 in cerebral ischemia/reperfusion injury, we first determined the expression pattern in OGD/R-induced HT22 hippocampal neuron cell injury, a model to mimic cerebral ischemia/reperfusion injury *in vitro*. We found that both mRNA and protein expression of DIXDC1 were significantly increased in HT22 cells after OGD/R treatment (Figure 1A and B). The results indicate that DIXDC1 may play an important role in OGD/R-induced injury.

Knockdown of DIXDC1 Promotes OGD/R-Induced Injury in HT22 Cells

To explore the precise role of DIXDC1 in regulating OGD/R-induced injury, we performed gain-of-function experiments using DIXDC1 siRNA to knock down DIXDC1 expression. Our findings demonstrated that transfection of DIXDC1 siRNA significantly decreased the protein expression of DIXDC1 in HT22 cells (Figure 2A). Furthermore, we found that knockdown of DIXDC1 significantly decreased the viability of HT22 cells with OGD/R treatment (Figure 2B). In addition, OGD/R-induced cell injury and apoptosis were further exacerbated by DIXDC1 knockdown (Figure 2C and D). Collectively, these results suggest that inhibition of DIXDC1 aggravated OGD/R-induced injury in HT22 cells *in vitro*.

Overexpression of DIXDC1 Attenuates OGD/R-Induced Injury in HT22 Cells

As described above, inhibition of DIXDC1 aggravates OGD/R-induced injury. We suggested that overexpression of DIXDC1 may be protective against OGD/R-induced injury. To test our hypothesis, we upregulated DIXDC1 expression by transfecting HT22 cells with the DIXDC1 expression vector. Our results showed that transfection with the DIXDC1 expression vector significantly upregulated DIXDC1 expression in HT22 cells (Figure 3A). We then assessed the effect of DIXDC1 overexpression on OGD/R-induced injury by MTT, LDH, and caspase-3 activity assays. We found that overexpression of DIXDC1 significantly improved cell viability in HT22 cells following OGD/R treatment (Figure 3B). Furthermore, overexpression of DIXDC1 markedly attenuated OGD/R-induced cell injury and apoptosis in HT22 cells (Figure 3C and D). These data suggest that overexpression of DICDC1 prevents OGD/R-induced injury in HT22 cells *in vitro*.

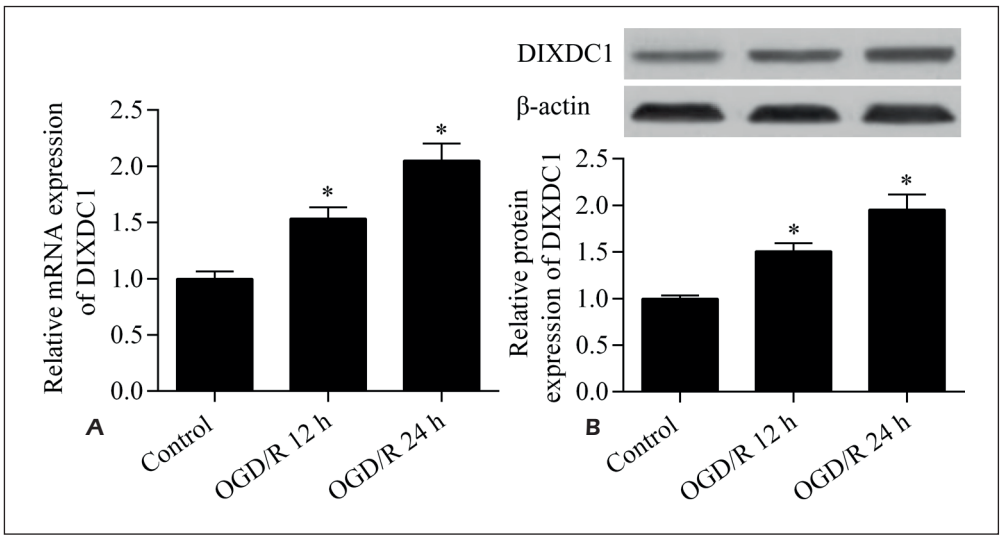

Figure 1. OGD/R treatment affects DIXDC1 expression in HT22 cells. HT22 cells were subjected to OGD for 3 h and then reoxygenation (R) for 12 and 24 h. **A**, Relative mRNA expression of DIXDC1 was detected by RT-qPCR. **B**, Relative protein expression of DIXDC1 was determined using western blot analysis. **p*<0.05 *vs*. control.

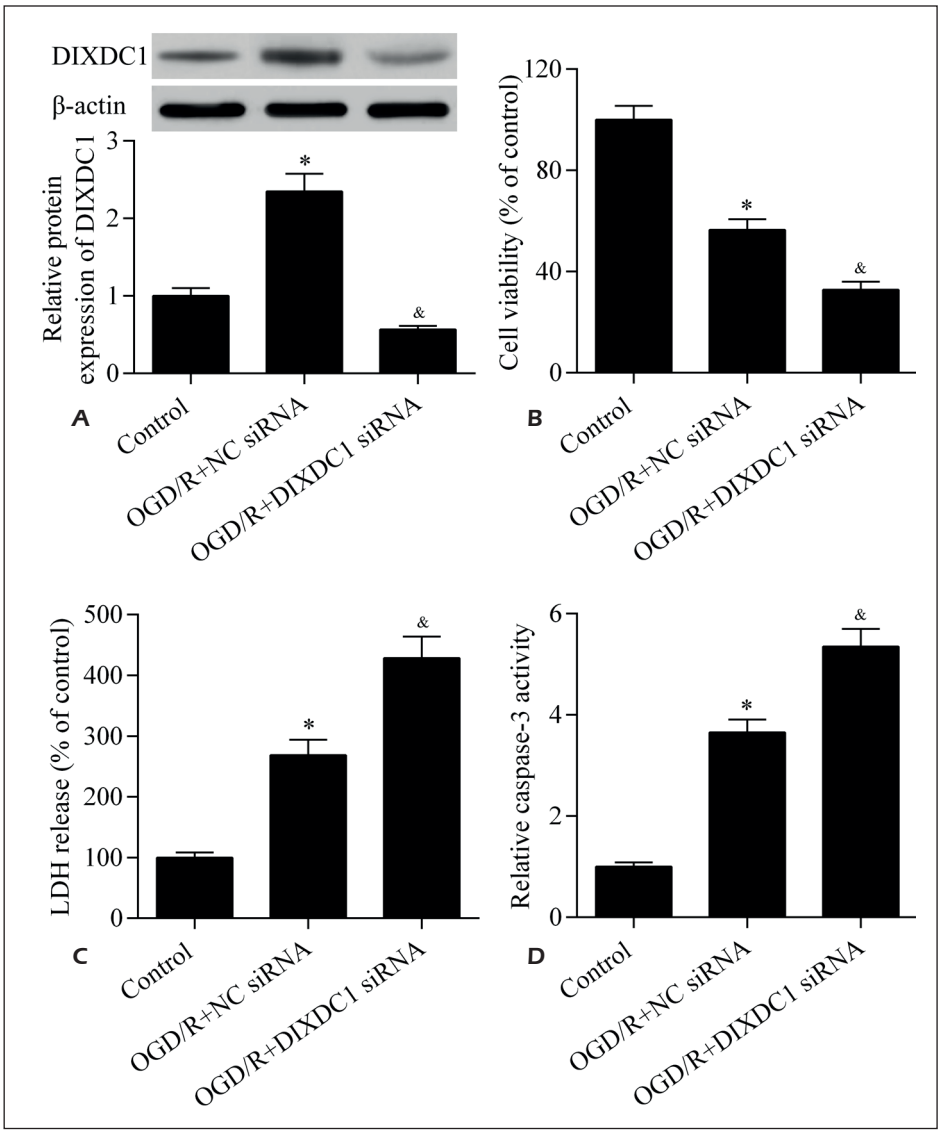

Figure 2. Knockdown of DIXDC1 promotes OGD/R-induced injury in HT22 cells *in vitro*. HT22 cells were transfected with DIXDC1 siRNA or negative control (NC) siRNA for 24 h and then subjected to OGD/R treatment. **A**, Relative protein expression of DIXDC1 was determined using western blot analysis. **B**, Effect of DIXDC1 knockdown on cell viability was detected by MTT assay. **C**, Effect of DIXDC1 knockdown on cell injury was assessed by measuring LDH release using LDH assay. **D**, Effect of DIXDC1 knockdown on cell apoptosis was evaluated by caspase-3 activity assay. *$p<0.05$ vs. control; &$p<0.05$ *vs*. OGD/R + NC siRNA.

DIXDC1 is Involved in Regulating Wnt/β-Catenin Signalin in HT22 Cells Following OGD/R Treatment

DIXDC1 has been recognized as a positive regulator of Wnt/β-catenin signaling[4]. Considering the important role of Wnt/β-catenin signaling in regulating cerebral ischemia and reperfusion injury[21], we next investigated whether DIXDC1 regulates Wnt/β-catenin signaling in HT22 cells following OGD/R treatment. Our results showed that β-catenin expression and Wnt activity were significantly lower in OGD/R-treated cells than in controls (Figure 4A-D). Notably, knockdown of DIXDC1 further decreased β-catenin expression and Wnt activity, whereas overexpression of DIXDC1 promoted β-catenin expression and Wnt activity in HT22 cells following OGD/R treatment (Figure 4-D). Overall, these results indicate that DIXDC1 regulates Wnt/β-catenin signaling in HT22 cells following OGD/R treatment.

Inhibition of Wnt/β-Catenin Signaling Reverses the Protective Effect of DIXDC1 Against OGD/R-Induced Injury

To validate whether DIXDC1 regulates OGD/R-induced injury through Wnt/β-catenin signaling, we examined the effect of Wnt/β-catenin signaling inhibition on the effect of DIXDC1 overexpression. We found that treatment of Wnt/β-catenin inhibitor ICG-001 (10 μM) significantly blocked the effect of DIXDC1 on the promotion of β-catenin expression and Wnt ac-

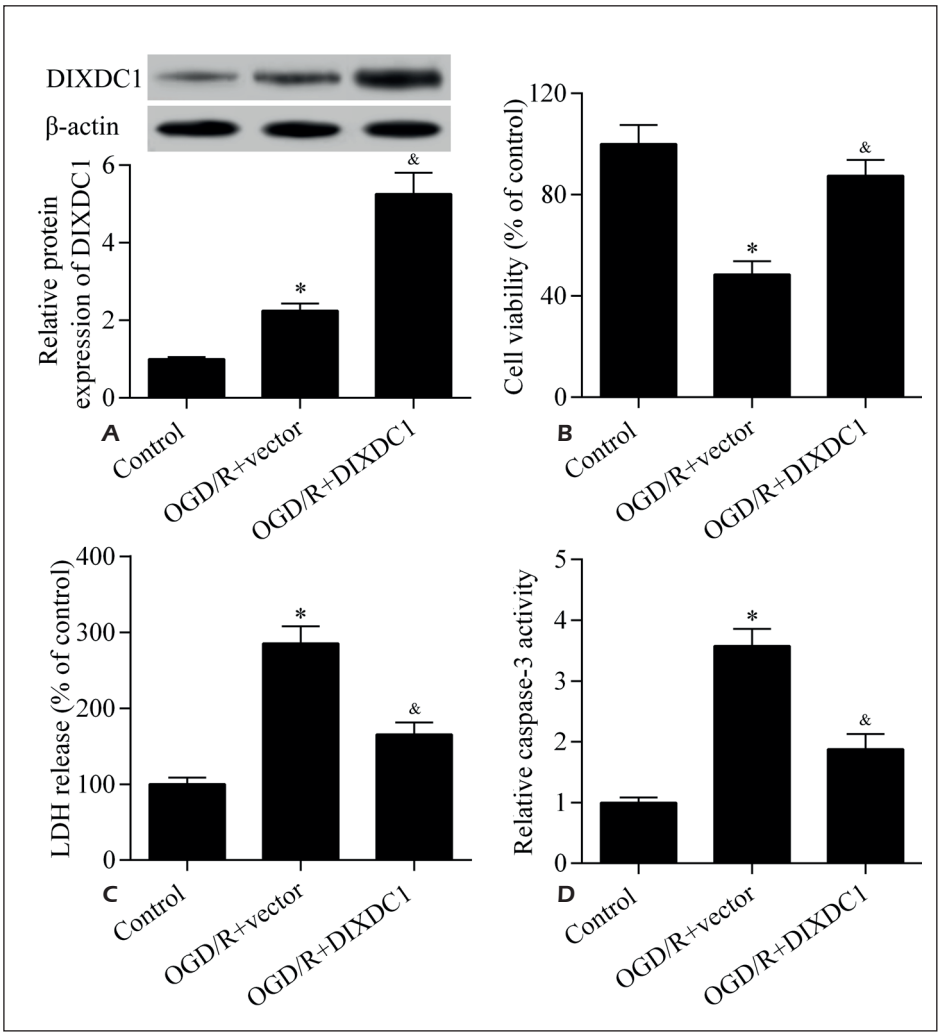

Figure 3. Overexpression of DICDC1 prevents OGD/R-induced injury in HT22 cells *in vitro*. HT22 cells were transfected with pcDNA3.0-DIXDC1 vector (DIXDC1) or pcDNA3.0 empty vector (vector) for 24 h and then subjected to OGD/R treatment. **A**, Relative protein expression of DIXDC1 was analyzed by western blot. **B**, Effect of DIXDC1 overexpression on cell viability was assessed by MTT assay. **C**, Effect of DIXDC1 overexpression on cell injury was determined by measuring LDH release. **D**, Effect of DIXDC1 overexpression on cell apoptosis was detected by caspase-3 activity assay. *$p<0.05$ vs. control; $^{\&}p<0.05$ *vs.* OGD + vector.

tivity (Figure 5A and B). Moreover, inhibition of Wnt/β-catenin signaling also inhibited the DIXDC1-mediated neuroprotective effect against OGD/R-induced injury in HT22 cells (Figure 5C and D). These results demostrated that DIXDC1 prevents OGD/-R induced injury through upregulation of Wnt/β-catenin signaling.

Discussion

In the present work, we reported an important role of DIXDC1 in regulating neuronal survival following OGD/R treatment. We found that DIXDC1 was upregulated in response to OGD/R treatment, indicating a possible role of DIXDC1 in OGD/R-induced injury. By using gain-of-function and loss-of-function experiments, we demonstrated that DIXDC1 could protect neurons against OGD/R-induced injury *in vitro*. Moreover, the underlying mechanism may involve a regulatory role of DIXDC1 in the Wnt/β-catenin signaling pathway.

DIXDC1 has been suggested as an important gene for maintaining normal functioning of the nervous system. Overexpression of DIXDC1 facilitates the neuronal differentiation of P19 cells induced by retinoic acid[22]. Singh et al[23] reported that DIXDC1 promoted the proliferation and migration of neural progenitor proliferation during embryonic cortical development. DIXDC1 is expressed in cortical neurons and localizes to dendrites and spines involved in regulating neuronal morphogenesis[24]. Missense variants in DIXDC1 have been identified in patients with autism spectrum disorders[24]. Mutations in DIXDC1 in mice result in abnormal behaviors such as schizophrenia or depression[25]. Consistently, Martin et

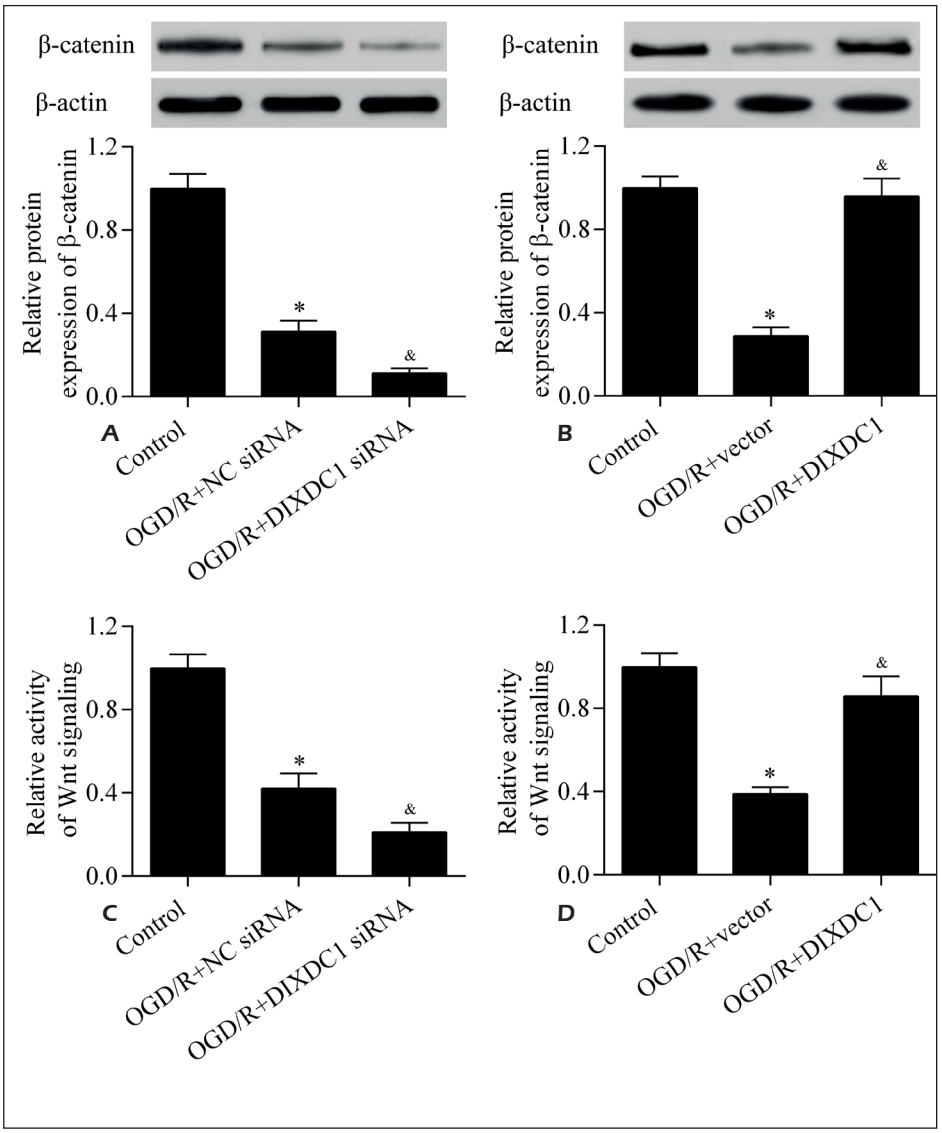

Figure 4. DIXDC1 regulates Wnt/β-catenin signaling in HT22 cells. The effect of DIXDC1 knockdown (**A**) or overexpression (**B**) on β-catenin protein expression was detected by Western blot. The effect of DIXDC1 knockdown (**C**) or overexpression (**D**) on Wnt activity was determined using the TOPflash luciferase reporter assay. *$p<0.05$ *vs.* control; $^{\&}p < 0.05$ *vs.* OGD/R + NC siRNA or OGD/R + vector.

al[26] reported that mice lacking DIXDC1 have abnormal measures of anxiety, depression, and social behaviors. Deficiency of DIXDC1 leads to reduced spines and glutamatergic synapses associated with impairment of the Wnt/β-catenin signaling pathway, suggesting that the DIXDC1/Wnt/β-catenin signal transduction pathway plays an important in the pathogenesis of mental disorders[26]. Moreover, DIXDC1 has been reported to promote the proliferation of Schwann cells after sciatic nerve crush[15]. Of note, a study[14] uncovered that DIXDC1 expression is increased in the brain after traumatic brain injury, and DIXDC1 is involved in regulating astrocyte proliferation. High levels of DIXDC1 are found in gliomas, and knockdown of DIXDC1 inhibits the proliferation and migration of glioma cells[27]. These studies suggest that DIXDC1 plays a pivotal role in neuronal development and neurological disease by affecting differentiation, proliferation, survival, and migration. However, whether DIXDC1 plays a role in cerebral ischemia/reperfusion injury remains unknown. In this study, we observed that DIXDC1 expression was significantly upregulated in response to OGD/R treatment. Functional experiments showed that overexpression of DIXDC1 improved neuronal survival and attenuated neuronal apoptosis induced by OGD/R treatment, indicating a neuroprotective role of DIXDC1. Therefore, upregulation of DIXDC1 may be an adaptive response of neurons to promote their survivability against OGD/R-induced insults.

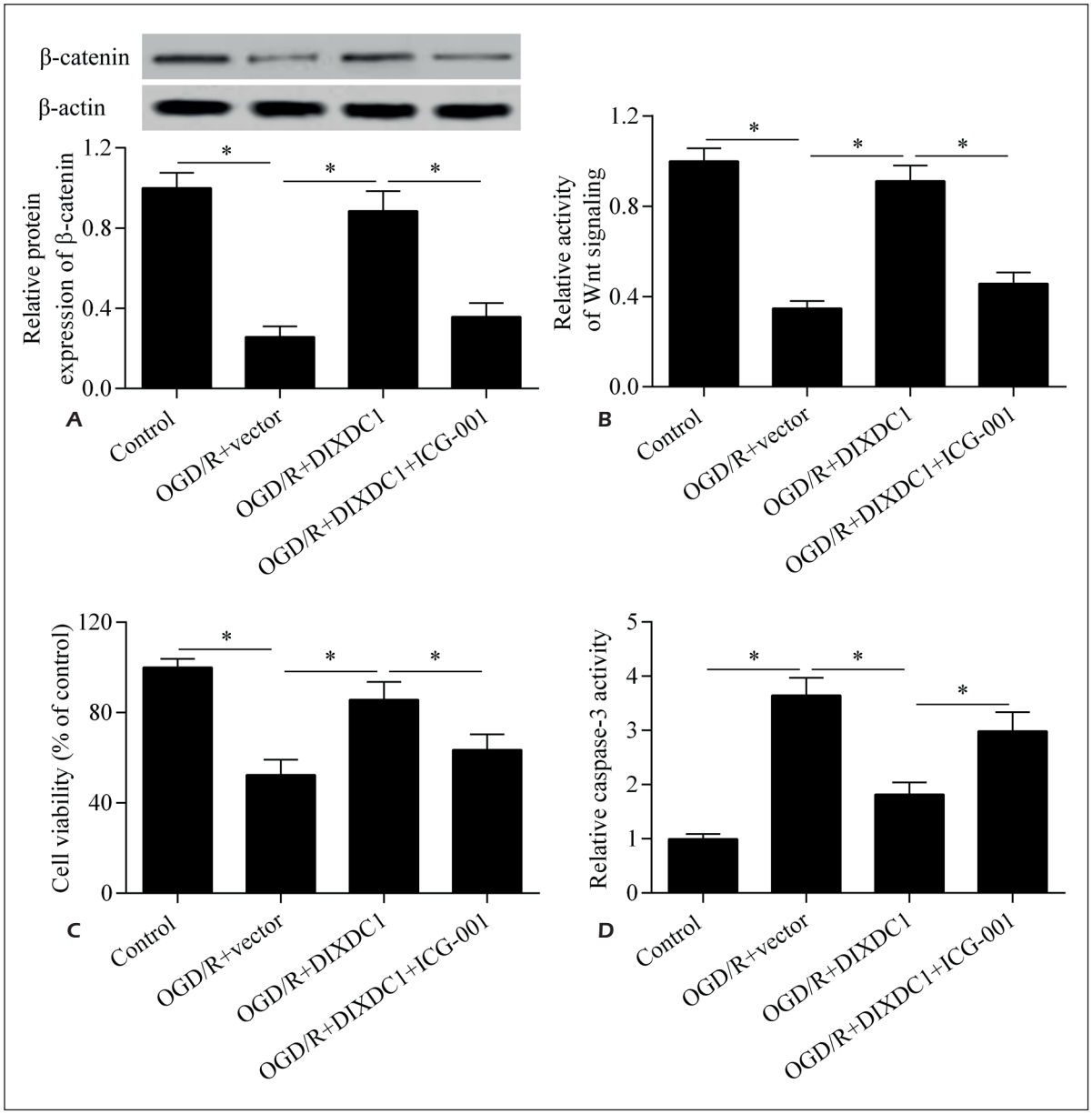

Figure 5. Inhibition of Wnt/β-catenin signaling reverses the protective effect of DIXDC1. HT22 cells were transfected with the pcDNA3.0-DIXDC1 vector and treated with or without ICG-001 (10 μM) for 24 h before being subjected to OGD/R treatment. **A**, Relative protein expression of β-catenin was detected by Western blot. **B**, Wnt signaling activity was determined by TOPflash luciferase reporter assay. **C**, Cell viability was assessed by MTT assay. **D**, Cell apoptosis was evaluated by caspase-3 activity assay.

DIXDC1 has been suggested as a positive regulator of Wnt/β-catenin signaling. DIXDC1 can form homomeric and heteromeric complexes with Dvl and Axin, two major Wnt downstream mediators, to activate the Wnt/β-catenin signaling pathway[28,29]. Multiple evidence[6,8,30,31] has reported that DIXDC1 regulates cell proliferation and the cell cycle via activation of the Wnt/β-catenin signaling pathway. These findings propose that the biological function of DIXDC1 is mainly associated with its regulatory effect on Wnt/β-catenin signaling. Consistently, our results demonstrated that overexpression of DIXDC1 prevented OGD/R-induced injury accompanied by increased β-catenin expression and high activity of Wnt signaling. Notably, inhibition of Wnt/β-catenin significantly reversed the DIXDC1-mediated neuroprotective effect. These results indicate that DIXDC1 protects neurons against OGD/R-induced injury through activation of Wnt/β-catenin

signaling. Furthermore, activation of Wnt/β-catenin signaling is involved in protecting neurons against OGD/R or cerebral ischemia-induced apoptosis[32,33]. Therefore, targeting DIXDC1 to modulate the Wnt/β-catenin signaling pathway may have a potential application for preventing neuronal injury during cerebral ischemia/reperfusion injury.

Conclusions

We demonstrated that overexpression of DIXDC1 could protect neurons against OGD/R-induced injury *in vitro* through activation of the Wnt/β-catenin signaling pathway. These results indicate that DIXDC1 may play an important role in regulating neuronal survival during cerebral ischemia and reperfusion injury *in vivo*. Activation of the Wnt/β-catenin signaling pathway by DIXDC1 may represent a potential and promising strategy for preventing neuronal damage. However, the precise role of DIXDC1 in cerebral ischemia and reperfusion injury requires further investigation using animal models *in vivo*.

Conflict of Interest

The authors declare that they have no conflict of interest.

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