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*Original Article*

# Silenced Notch1 attenuates autophagy in PC12 cells following oxygen-glucose deprivation-induced injury

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**Abstracts:** Ischemic cerebrovascular disease (ICD) or ischemic stroke is one of the most severe and common neurological diseases in the elderly population. Autophagy is a highly conserved process in eukaryotes and a key decision-maker contributing to neuronal fate. Until now, reports on the role of the Notch pathway in autophagy of ICD are limited. In this study, PC12 cells were screened for neuron-like cells that were positive for MAP2. The silenced Notch1 transfected neuron-like cells were used for the establishment of an oxygen glucose deprivation (OGD) model which was verified with cell viability by MTT assay and apoptosis examination by flow cytometry. After establishing the OGD model, the mRNA and protein expression of Notch1, Beclin1, and LC3-I/LC3-II were analyzed by RT-qPCR and Western blot. In the established OGD model, the cell viability was decreased significantly with 72.98% of apoptosis. Following the introduction of silenced Notch1, the mRNA and protein expression of Notch1, Beclin1 and LC3 were upregulated remarkably in neuron-like PC12 cells exposed to OGD compared with the control, increased markedly in Notch1 siRNA transfected neuron-like cells post-exposure to OGD compared with Notch1 siRNA transfected neuron-like cells without the treatment of OGD, and down-regulated distinctly in Notch1 siRNA transfected neuron-like cells post-exposure of OGD compared with those without transfection. We also observed that the levels of LC3-II rose greatly in OGD cells without Notch1 siRNA transfection compared with the control, but declined in OGD cells with Notch1 siRNA transfection compared with those without transfection. The Notch pathway might be partly associated with autophagy in neurons post-exposure to OGD highlighting it as a potential target for ICD treatment.

**Keywords:** Notch1, ischemic cerebrovascular disease, oxygen-glucose deprivation, autophagy, siRNA

## Introduction

Ischemic cerebrovascular disease (ICD) or ischemic stroke is one of the most serious and common neurological diseases in the elderly with high mortality and is becoming a heavy burden to patients [1]. In China, more than 200 million people suffer from stroke each year, most of whom (70-80%) become disabled, while ischemic stroke accounts for about 80% of strokes [2]. ICD can be attributed to several factors including oxidative stress, activation of inflammatory processes, excitotoxicity, $Ca^{2+}$ overload and apoptosis [3]. The characteristic pathological features of ICD include destruction and/or dysfunction of neuron cells and glia over-activation in the ischemic brain which ultimately lead to neurological deficits [4].

Autophagy is a highly conserved process in eukaryotes by which organelles and biological molecules are engulfed to form autophagosomes and delivered to the lysosome for degradation and recycle [5]. Neuronal autophagy has reportedly two unique features. One feature is that autophagy does not appear in the brain of mice without food for 48 h and brain nutrients are assumed to be compensated from other organs under starvation [6]. The other feature is that autophagosomes which exist in the whole cytoplasm and lysosomes residing usually in the juxtanuclear cytoplasm of the cell body in the neuron are localized in different places. If dendrites or axons are damaged, autophagosomes will not fuse with lysosomes to degrade sequestrated materials [7]. Increasing evidence supports that autophagy is a double-edged sword in cell survival, which

means that active autophagy may promote cell survival by digesting the misfolded protein or exacerbate cerebral cell apoptosis [8]. Due to cellular housekeeping functions, death-prevention as well as cell death [9], autophagy is also called as Type II Programmed Cell Death (PCD) to distinguish it from apoptosis which is often defined as Type I PCD [10]. Traditionally, cerebral ischemia induced cell death is considered to be exclusively necrotic, but mounting research indicates that neuronal cell death in ischemic penumbra is due to apoptosis [11]. Importantly, many data suggest that the process of autophagy could be a key decision-maker that determines neuronal fate [12].

Cerebral ischemia can activate multiple signaling pathways including PI3K/Akt/mTOR [13], AMPK/mTOR [14], NF-κB/mTOR [15], MAPK/mTOR [16] and HIF-1α/BNIP3 [17] that subsequently activate autophagy. Notch signaling is an evolutionarily conserved pathway which is fundamental for neuronal development and specification. For decades, the Notch pathway has been revealed to be important not only in embryonic development but also in brain maturity of vertebrates and invertebrates, neural progenitor regulation, neuronal connectivity, synaptic plasticity as well as learning/memory. In addition, Notch is aberrantly upregulated in ischemic injury [18]. However, the role of the Notch pathway in autophagy of ICD has not been fully investigated.

In this study, Notch1 was silenced and introduced into neuron-like cells derived from PC-12 to survey mRNA and protein expression of Notch1 and autophagy-related Beclin and LC3 I/LC3 II in an oxygen glucose deprivation (OGD) model. Taken together, we found that the Notch pathway was associated with autophagy of ICD when neuron cells were pre-exposed to OGD.

## Material and methods

### *Cell culture and induction*

PC12 cells obtained from Innovat (Wuxi, Jiangsu, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin (Invitrogen, Carlsbad, CA, USA), and 100 mg/ml streptomycin (Invitrogen Corp., Carlsbad, CA, USA) in an atmosphere of 5% $CO_2$ and 95% air at 37°C. Cells were differentiated by the addition of nerve growth factor (150 ng/mL) for 48 h prior to experiments.

### *Oxygen-glucose deprivation (OGD)*

PC12 cells were deprived of oxygen and glucose for 2 h to simulate ischemic injury in vitro as previously described [19]. PC12 cells were washed with sterile phosphate buffer solution (PBS) and incubated in Earle's balanced salt solution. The cells were then incubated in a hypoxia chamber (HF100, Heal Force, China) with a compact gas oxygen controller to maintain oxygen concentration at 1% by injecting a gas mixture of 94% $N_2$ and 5% $CO_2$ for 2 h. Normal control cells were incubated in a regular cell culture incubator (HF90, Heal Force, China) under normoxic conditions.

### *Cell transfections*

Three Notch1 interference sequences were synthesized by GenePharma (Shanghai, China) as follows: Notch1-siRNA1, 5'-UUAACAUCUUGUCUGCAGGTT-3', Notch1-siRNA2, 5'-ACAAACCAUAUGGACAGGGTT-3', Notch1-siRNA3, 5'-ACAAACCAUAUGGACAGGGTT-3'. A random negative interference sequence was set as control: NC-siRNA, 5'-ACGUGACACGUUCGGAGAATT-3'. The siRNA were transfected into the OGD cells using Lipofectamine® 2000 Transfection Reagent (Invitrogen Life Technologies) following the manufacturer's instructions. The transfection efficiencies were evaluated by qRT-PCR.

### *MTT assay*

PC12 cells ($1\times10^5$ cells/mL, 0.1 mL) transfected with or without Notch siRNA were seeded into a 96-well plastic plate for 48 h of growth and then the cells were treated with OGD as described above. After OGD, MTT (10 mg/mL, Sigma, CA, USA) was added to each well and incubated at 37°C in normoxia for 4 h. Dimethyl sulfoxide (DMSO, Sangon Biotech, Shanghai, China) was added to each well for 10 min to dissolve the dark blue crystals and the absorbance was read at 570 nm on a microplate reader (Multiskan Mk3, Thermo Scientific, MA, USA).

### *Fluorescent identification*

PC12 cells suffering from OGD were fixed with 4% paraformaldehyde for 15 min. After washing with sterile PBS, Triton X-100 (0.1%) was

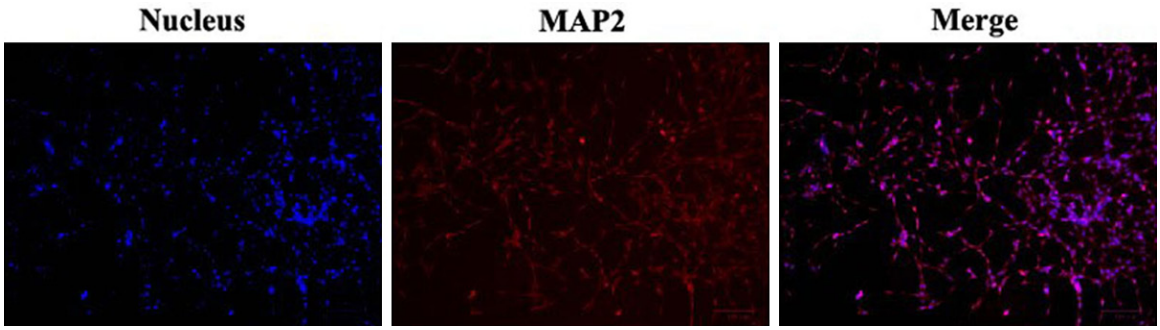


**Figure 1.** Induction of PC12 cells into neuron-like cells by 150 ng/mL NGF for 6 days. (Left) The cell nucleus were stained with DAPI showing mazarine. (Middle) MAP2 were coated with primary and secondary antibody and emitted red fluorescence [33]. Merge of DAPI and MAP2 fluorescence.

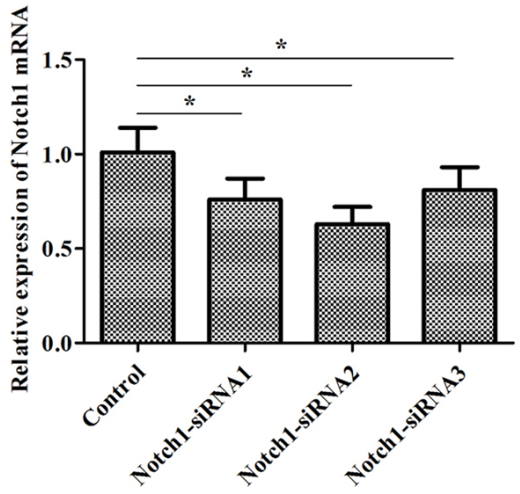


**Figure 2.** Relative mRNA expression of three Notch1 siRNAs in PC12 cells transfected with Notch1 siRNA. A random negative interference sequence was set as control. *, $p < 0.05$.

added and incubated with cells for 10 min. After PBS washing, the suspension was sealed by addition of 10% goat serum for 30 min, then incubated separately with one drop of rabbit-anti-rat primary antibody MAP2 overnight at 4°C and FITC-labeled secondary antibody for 20 min. Finally, the cells were mixed with DAPI for 5 min at room temperature and photographed by a fluorescent microscope.

*Apoptosis assay by flow cytometry*

Following OGD, PC12 cells was stained with Annexin V-FITC staining solution at 4°C for 15 min, and then with propidium iodide solution at 4°C for 5 min in the dark. The samples were then immediately analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using CellQuest Pro software.

*Gene analysis*

The expression of Notch1, Beclin1 and LC3-I/LC3-II mRNA were analyzed by RT-qPCR on total RNA isolated from PC12 cells using the Trizol reagent according to the supplier's protocols. Reverse transcription was performed with 200 ng of RNA in a total volume of 10 μL using One-Step RT-PCR kit according to manufacturer's recommendations. RT-qPCR was performed on the 7900HT Fast Real-Time PCR system using TaqMan gene expression assays probes (Applied Biosystems). The primers used for Notch1 were 5'-TGCCTGACGCCTACCAAC-3' (forward) and 5'-TGTCTTCCCTGACCATCC-3' (reverse), for Beclin1 were 5'-GAAGACAGAGCGATGGTAG-3' (forward) and 5'-ATGGTGCTGTTGTTGGAC-3' (reverse) and for LC3-II/LC3-I were 5'-TGCCAATACAATGGTCAA-3' and 5'-ATGTCTGCTTCGCCTCTT-3' (reverse). GAPDH was as an internal control with primers as 5'-GCAAGTTCAACGGCACAG-3' (forward) and 5'-CGCCAGTAGACTCCACGAC-3' (reverse). The PCR was carried out in a total volume of 10 μL containing 1.5 μL of diluted and preamplified cDNA, 10 μL of TaqMan Gene Expression Master Mix, and 1 μL of each fluorescence TaqMan probe. The cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles, each one consisting of 45 sec at 95°C and 45 sec at 59°C with final extension at 72°C for 60 sec. Samples were run in triplicate and the mean value was calculated for each case. The data were managed using the

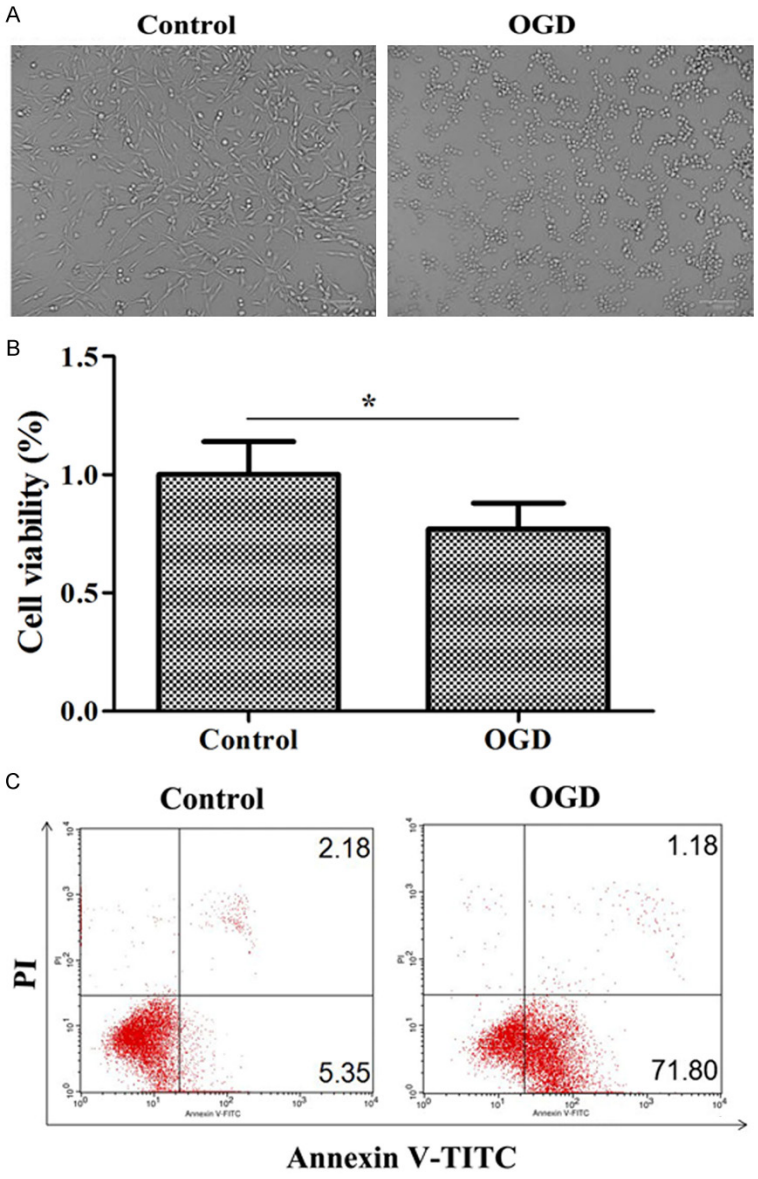


**Figure 3.** Establishment and identification of the OGD model. A. Representative images of PC12 cells exposed to OGD. B. Cell viability evaluated by the MTT assay method. C. Apoptosis examination by flow cytometry after Annexin V-FITC staining. The cells without OGD were set as control. *, $p < 0.05$.

Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated by using the comparative $C_t$ method and obtaining the fold change value ($2^{-\Delta\Delta Ct}$) according to previously described protocol [20].

*Western blot analysis*

Total protein concentrations were determined by a BCA Protein assay reagent kit (TransGen, Beijing, China). Tissue total protein (50 μg) was separated by 12% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was blocked with PBS-T containing 5% non-fat dry milk for 1 h, and then incubated overnight at 4°C with the corresponding primary antibodies (1:1000, Abcam). After washing 3 times with PBS-T, the membrane was incubated with secondary antibody (1:4000, Solarbio). An anti-β-actin antibody (1:4000, Abcam) served as the control. The protein band was visualized using an enhanced chemiluminescence (ECL) kit and the density of each band was quantified using a Western blotting detection system (Quantity-One software; Bio-Rad Laboratories, Hercules, CA, USA).

*Statistical analysis*

The SPSS 19.0 software was used for the statistical analyses. Results were expressed as mean ± SD. Multi-group comparisons were made with one-way analyses of variance (one-way ANOVAs), and two-group comparisons were made with Bonferroni tests. The values were considered to be significant when P was less than 0.05.

## Results

*Cell induction, Notch1 siRNA construction, and OGD model establishment*

The neuron-like cells were induced from PC-12 cell by NGF (150 ng/mL) for consecutive six days. Immunofluorescent staining showed that the induced cells were MAP2-positive which emitted strong red fluorescence (**Figure 1**). The results indicated the neuron-like PC12 cells were induced successfully for subsequent experiments. Three Notch1 siRNA sequences were introduced into the neuron-like PC-12 cells. The transfection efficiencies were evaluated by qRT-PCR analyses of the expres-

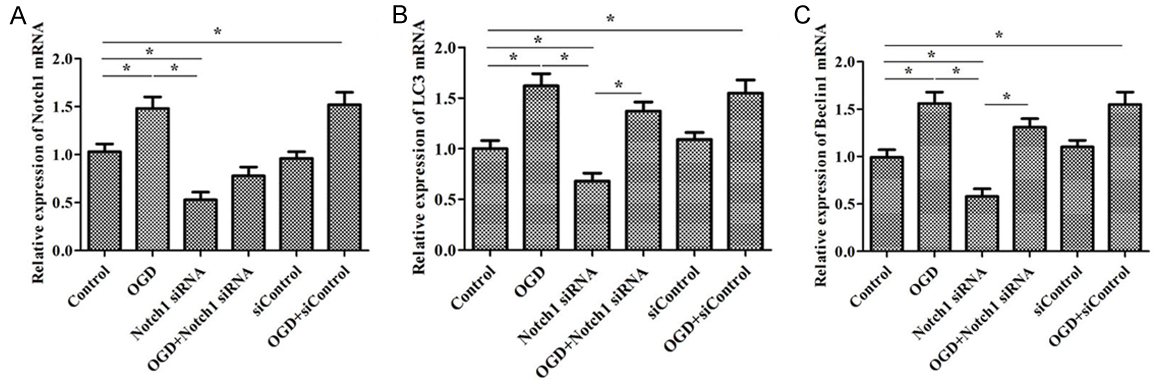


**Figure 4.** Relative mRNA expression of (A) Notch1, (B) LC3 and (C) Beclin1 in PC12 cells. *, $p < 0.05$.

sion of Notch1 mRNA. We observed that the three siRNAs were all able to inhibit Notch1 mRNA expression significantly ($p < 0.05$). The Notch1-siRNA2 was selected for further studies due to the best inhibition efficacy (**Figure 2**). In the established OGD model, we found disappearance of the cell synapse, ground, and apoptotic cells (**Figure 3A**). MTT assay showed that cell survival decreased prominently in the OGD model (**Figure 3B**). Followed by Annexin V-FITC staining, most of the cells (72.98%) exposed to OGD commenced apoptosis (**Figure 3C**). These results reflected the establishment of the OGD model in the neuron-like PC12 cells.

*Effects of Notch1 siRNA on expressions of autophagy-related genes*

To investigate the involvement of Notch1 in autophagy of OGD cells, the mRNA and protein expression of Notch1, Beclin1, and LC3-II/LC3-I were analyzed after the transfection of Notch1 siRNA. As shown, the mRNA expression of Notch1, Beclin1 and LC3 were upregulated significantly in the OGD model compared with the control ($p < 0.05$). Following transfection of the Notch1 siRNA, the expression of the three genes decreased notably in the control compared with that without transfection ($p < 0.05$). In the OGD model, the three genes were inhibited in the cells transfected with Notch1 siRNA compared with those without transfection ($p < 0.05$), but still increased remarkably compared with the control transfected with Notch1 siRNA ($p < 0.05$, **Figure 4A-C**). These results could also be observed in the analyses of protein levels (**Figure 5A** and **5B**). Further, the levels of LC3-II rose greatly in OGD cells without Notch1 siRNA transfection compared with the control, but declined in OGD cells with Notch1 siRNA transfection compared with those without transfection. ($p < 0.05$, **Figure 5A** and **5B**).

## Discussion

Two major approaches are currently being employed in the treatment of ICD. One approach aims to restore blood flow (reperfusion) in the injured region with thrombolytic, antithrombotic, and anti-aggregation drugs, such as recombinant tissue plasminogen activator (rt-PA), but the use is limited by the short therapeutic window (3 h) and high risk of hemorrhagic complications [12]. The other approach is described as neuroprotection focusing on retard neuronal cell death by regulating endogenous cytoprotective response and/or metabolic signaling pathways related with cell injury [21]. The neuroprotective approach in ICD is based on the existence of a special area embracing the ischemic core and separating the core from healthy tissue, namely penumbra which is thought to be recoverable [22]. In penumbra, the cells will also die and effective neuroprotection approach is dedicated to reducing cell death by extending penumbra survival, reducing ischemic inflammation and reperfusion injury [23].

Notch1 is mainly expressed in pyramidal neurons of the cortex and hippocampus [24], and behaves as a “plasticity molecule” to regulate spine morphology, synaptic plasticity, and memory processing [25]. Other Notch proteins were either not associated with ICD (such as Notch 3) or expressed too limited (such as

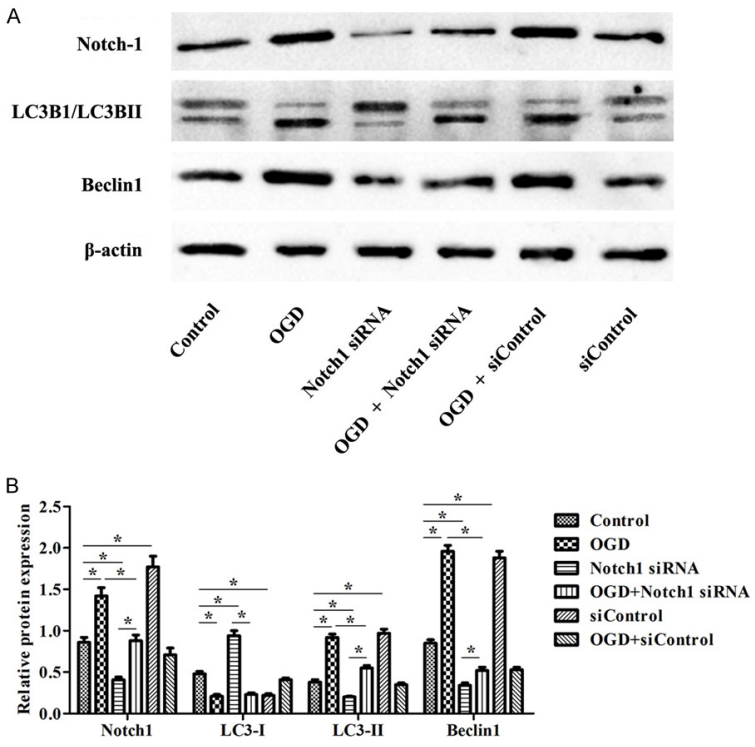


**Figure 5.** Evaluations of autophagy-related proteins. (A) Representative images of Western blot and (B) relative protein expression of Notch1, LC3-I, LC3-II and Beclin1 in PC12 cells. *, $p < 0.05$.

Notch 2) to be detected in brain (such as Notch 4) [18, 24]. Following ischemic injury, increasing evidence demonstrates that Notch signaling is aberrantly up-regulated resulting in a series of effects [26, 27]. In our study, Notch1 level increased significantly post-OGD, while silenced Notch1 level decreased accordingly consistent with most reports. Nevertheless, we noticed that Notch pathway could play a role in damage as well as regeneration in OGD [18, 28, 29]. Herein, Notch1 seems to induce autophagy in PC12 cells and play a role in damage as it could increase the exprression of autophagy-related proteins including LC3-II and Beclin1. LC3-II is localized at the membrane of the autophagosome, and LC3-phospholipid conjugates have faster electrophoretic mobility than unconjugated LC3-I [30]. Thus, LC3-II was defined as the marker of autophagosome and the ratio of LC3-II/LC3-I reflected autophagy induction [31]. We also found that the level of LC3-II increased notably in our OGD model even after the treatment of si-Notch1 compared with the control, indicating Notch1 might be partly implicated in the regulation of autophagy.

Although the consolidated agreement on the underlying mechanism of autophagy has not been reached in ICD, there are at least five viewpoints proposed: 1) Active autophagy in ICD protects neurons from death; 2) Active autophagy in ICD has a destructive role; 3) The degree of autophagy determines the fate of cells in ICD; 4) The role of autophagy is determined by the time at which it is induced after ICD; 5) Autophagy can be interfered in ICD [32]. From the obtained results, it could be revealed readily that Notch1-mediated autophagy exerted a harmful effect in our experiment. Meanwhile, we notice that the correlation among apoptosis, autophagy, and necrosis in ICD remains unclear, and the three pathologic processes can occur concurrently within damaged neurons in ICD. However, autophagy is presumed to act as an important modifier of cell death in neurological disorders [30].

In conclusion, an OGD model was established and Notch1 siRNA was constructed. It could be found that silenced Notch1 inhibited the expression of autophagy-related proteins LC3-II and Beclin1 in OGD cells. Based on the fact that Notch1 siRNA transfected OGD cells still had evident upregulation of LC3-II and Beclin1 compared with Notch1 siRNA transfected control, we propose that the Notch pathway might be partly associated with autophagy in ICD. In subsequent studies, more efforts will be needed to investigate the correlation among apoptosis, autophagy, and necrosis.

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## Disclosure of conflict of interest

None.

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