

Original Article

Hypoxia-inducible factor 1 α protects mesenchymal stem cells against oxygen-glucose deprivation-induced injury via autophagy induction and PI3K/AKT/mTOR signaling pathway

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Abstract: Mesenchymal stem cell (MSC) transplantation is a promising therapeutic strategy for myocardial infarction. The survival rate of the grafted MSCs is limited by the conditions of hypoxia and low nutrient levels. In this study, we investigated the role of hypoxia-inducible factor 1 alpha (Hif-1 α) in oxygen-glucose deprivation (OGD)-induced injury in MSCs. Hif-1 α was overexpressed or suppressed in MSCs by transfection with a Hif-1 α expressing vector or Hif-1 α -specific siRNA, respectively. Then MSCs were exposed to OGD, and the changes in cell viability, cell cycle distribution and apoptosis were respectively monitored by MTT assay and flow cytometry. Additionally, expression levels of Beclin1, LC3 I and LC3 II, as well phosphorylation of PI3K, AKT and mTOR were detected by RT-PCR and Western blotting. The results indicated that Hif-1 α overexpression improved cell viability, reduced G1 phase cells accumulation, and suppressed apoptosis under OGD condition ($P < 0.05$). Beclin1 expression and the LC3 II/LC3 I ratio were increased by Hif-1 α overexpression, and were decreased by Hif-1 α knock-down ($P < 0.05$). In addition, PI3K, AKT and mTOR were inactivated by Hif-1 α overexpression, and phosphorylated by Hif-1 α knock-down ($P < 0.05$). In conclusion, these data suggest that Hif-1 α overexpression protects MSCs from OGD-induced injury via a mechanism in which autophagy and PI3K/AKT/mTOR pathway are implicated.

Keywords: Hif-1 α , MSC, OGD, autophagy, PI3K/AKT/mTOR pathway

Introduction

Mesenchymal stem cells (MSCs) are a class of multipotent adult stromal cells found in various tissues, such as bone marrow, umbilical cord blood, adipose-derived stromal cells, peripheral blood, fetal liver, lung, amniotic fluid, and placenta [1]. MSCs can differentiate into various connective tissues as well as other cell types, including myocytes and neurons [2]. Additionally, MSCs have been shown to provide cytokines and growth factors support for maintaining the functions of hematopoietic and embryonic stem cells [3]. Based on their low immunogenicity as well as the ease of harvesting and high cell yield, MSCs are believed to be a promising stem cell population for cell-based strategies [4]. For instance, MSC transplantation is

considered to be a potential therapeutic strategy for myocardial infarction [5]. However, the survival rate of the grafted MSCs is unsatisfactory, since they need to survive under conditions of hypoxia and low nutrient levels [6].

Hypoxia-inducible factor 1 (Hif-1) is one of the main components of the cellular response to lack of oxygen, and is a major transcription factor involved in cell adaptation to hypoxia [7]. Hif-1 α , a subunit of Hif-1, undergoes rapid degradation by an ubiquitin-proteasome system under normoxic conditions [8]. Hif-1 α has been shown to modulate the expression of multiple target genes involved in angiogenesis, energy metabolism, apoptosis, autophagy, proliferation, and other adaptive responses to hypoxia [9, 10]. Studies in MSCs have shown that Hif-1 α

The role of Hif-1 α in OGD-injured MSC

induces osteogenesis and angiogenesis, and promotes osteogenic differentiation [11]. However, the role for Hif-1 α in improving the survival rate of MSCs under hypoxic conditions remains to be established.

Oxygen-glucose deprivation (OGD) is a commonly used model to mimic an ischemic milieu *in vitro*. In the current study, OGD model was adopted in MSCs, and the expression of Hif-1 α was upregulated or suppressed by transfection with a Hif-1 α expressing vector or Hif-1 α -specific siRNA. The role of Hif-1 α in OGD-induced injury was investigated by monitoring cell viability, cell cycle distribution and apoptosis. Furthermore, the effects of Hif-1 α on MSCs were evaluated by measuring the changes in the expression of autophagy-related factors and PI3K/AKT/mTOR pathway proteins. This study may provide evidence to indicate the feasibility of using Hif-1 α modification as an effective strategy to improve MSCs survival following OGD exposure.

Materials and methods

Animals and MSC isolation

Specific pathogen-free grade male Sprague-Dawley (SD) rats (weight 200-225 g) were purchased from Vital River Laboratories (Beijing, China). All the animal experiments conducted in this study were approved by the Animal Ethics Committee of Southern Medical University and were performed according to the instructions of our institute. The rats were anesthetized by intraperitoneal (IP) injection of chloral hydrate (350 mg/kg) and then euthanized by cervical dislocation for sampling. MSCs obtained from the femurs and tibias were cultured with Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and maintained in a humidified incubator at 37°C under 5% CO₂ [12].

Plasmids and siRNA transfection

Full-length rat Hif-1 α cDNA was amplified by PCR using the following primer sequences: forward (5'-CTA GCT AGC TAG ACC ATG GAG GGC GGC-3') and reverse (5'-CGG GAT CCT TAT CAG TTA ACT TGA TCC-3'). The pC-Hif-1 α plasmid was generated by introducing the amplified cDNA sequence into pcDNA3.1 (Sangon Biotech, Shanghai, China); pcNC was used as a negative

control. The primer sequences of for generation of the Hif-1 α -specific siRNA were as follows: forward (5'-TCG AGG AAG GAA CCT GAT GCT TTA TTC AAG AGA TAA AGC ATC AGG TTC CTT CTT A-3') and reverse (5'-CTA GTA AGA AGG AAC CTG ATG CTT TAT CTC TTG AAT AAA GCA TCA GGT TCC TTC C-3'). siNC was used as a negative control.

Cells were transfected using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol. Stable Hif-1 α transfectants were generated under G418 (Gibco, Paisley, UK) selection.

OGD induction

MSCs were incubated with glucose-free medium and then exposed to hypoxic conditions (5% CO₂ and 95% N₂). After 8 h, MSCs were harvested for use in subsequent analyses [13, 14].

Cell viability assay

Cell viability was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to standard method [15]. The transfected MSCs were seeded in 96-well plates (5 × 10³ cells/well) and cultured for 48 h under OGD condition before the addition of 20 μ L MTT reagent (5 mg/mL, Sigma-Aldrich, St Louis, MO, USA). After incubation for 4 h at 37°C, 150 μ L dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added and the absorbance at 570 nm was measured using an iMark microplate reader (Bio-rod, Hercules, CA, USA).

Apoptosis assay

Cell apoptosis was analyzed using an Annexin V: FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's recommendations. Following exposure to OGD condition, 1 × 10⁵ MSCs were washed in PBS twice and fixed in 200 μ L Annexin-binding buffer containing 10 μ L Annexin V-FITC and 5 μ L PI. After incubation at room temperature for 30 min in the dark, 300 μ L PBS was added for flow cytometric analysis of apoptotic cells using a FACScan flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Cell cycle analysis

Cell cycle distribution was detected using the CycleTestPlus DNA Reagent Kit (BD Biosciences,

The role of Hif-1 α in OGD-injured MSC

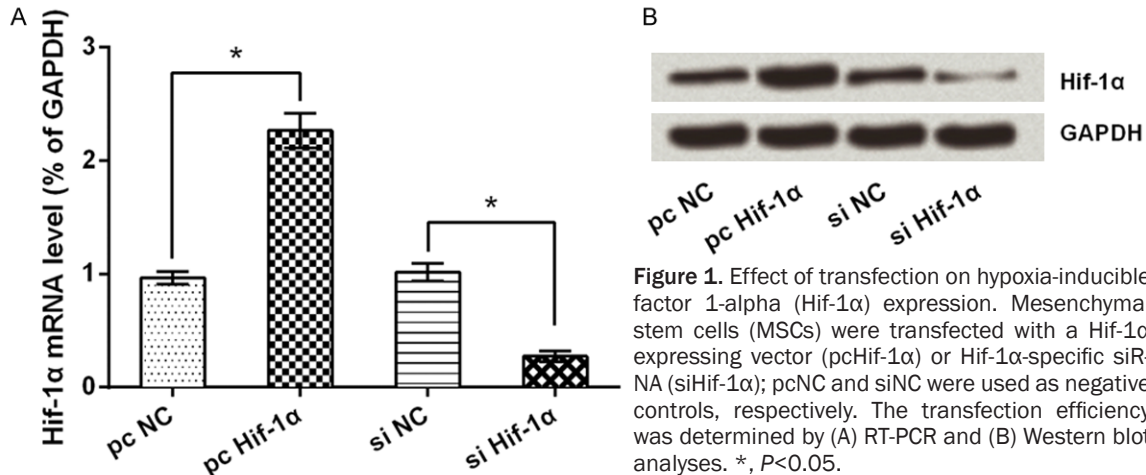


Figure 1. Effect of transfection on hypoxia-inducible factor 1-alpha (Hif-1 α) expression. Mesenchymal stem cells (MSCs) were transfected with a Hif-1 α expressing vector (pcHif-1 α) or Hif-1 α -specific siRNA (siHif-1 α); pcNC and siNC were used as negative controls, respectively. The transfection efficiency was determined by (A) RT-PCR and (B) Western blot analyses. *, $P < 0.05$.

es) according to the manufacturer's recommendations. Following exposure of OGD condition, the transfected MSCs were harvested and fixed overnight at 4°C in 300 μ L PBS and 700 μ L chilled ethanol. The fixed cells were then resuspended gently in 50 μ g/mL PI containing 0.1% Triton X-100 and 20 μ g/mL RNase A for 30 min at 37°C in the dark. Three thousand events per sample were recorded using the FACScan flow cytometer (Beckman Coulter, Fullerton, CA, USA).

RT-PCR

Following exposure of OGD condition, total RNA was isolated from the transfected MSCs using TRIzol reagent (Invitrogen) and DNaseI (Promega). cDNA was prepared by reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). RT-PCR was carried out using FastSTART Universal SYBR Green Master (ROX) (Roche) with the following primers: Hif-1 α forward (5'-CAT CAG CTA TTT GCG TGT GAG GA-3') and reverse (5'-AGC AAT TCA TCT GTG CTT TCA TGT C-3'); GAPDH forward (5'-GCA CCG TCA AGG CTG AGA AC-3') and reverse (5'-TGG TGA AGA CGC CAG TGG A-3').

Western blot analysis

Following exposure of OGD condition, total cellular proteins were extracted from the transfected MSCs using a Radio Immunoprecipitation Assay Kit (Beyotime, Shanghai, China) supplemented with protease inhibitors (Roche) according to the manufacturer's instructions. The protein concentration was quantified using the

BCATM Protein Assay Kit (Pierce, Appleton, WI, USA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were then incubated with 5% non-fat dried milk for 1 h at room temperature prior to incubation overnight at 4°C with primary antibodies for the detection of Hif-1 α , Beclin1, LC3 I, LC3 II, p-PI3K, PI3K, p-AKT, AKT, p-mTOR, mTOR (1:1,000; all from Cell Signaling Technology), and GAPDH (1:1,000; Chemicon International). After washing in TBST buffer, the membranes were incubated with the appropriate secondary detection antibodies (1:5,000; Odyssey, LICOR) for 1 h at room temperature. The signals were acquired with the Odyssey infrared imaging system and analyzed as specified in the Odyssey software manual [13]. GAPDH was used as an internal reference.

Statistical analysis

All data represent the mean \pm standard deviation (SD) of three independent experiments. Statistical analyses were performed using the SPSS version 13.0 program (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using Student's t-test and $P < 0.05$ was considered to indicate statistical significance.

Results

Effect of transfection on Hif-1 α expression

MSCs were divided into four groups and respectively transfected with pcNC, pcHif-1 α , siNC and siHif-1 α . Transfection efficiency was evalu-

The role of Hif-1 α in OGD-injured MSC

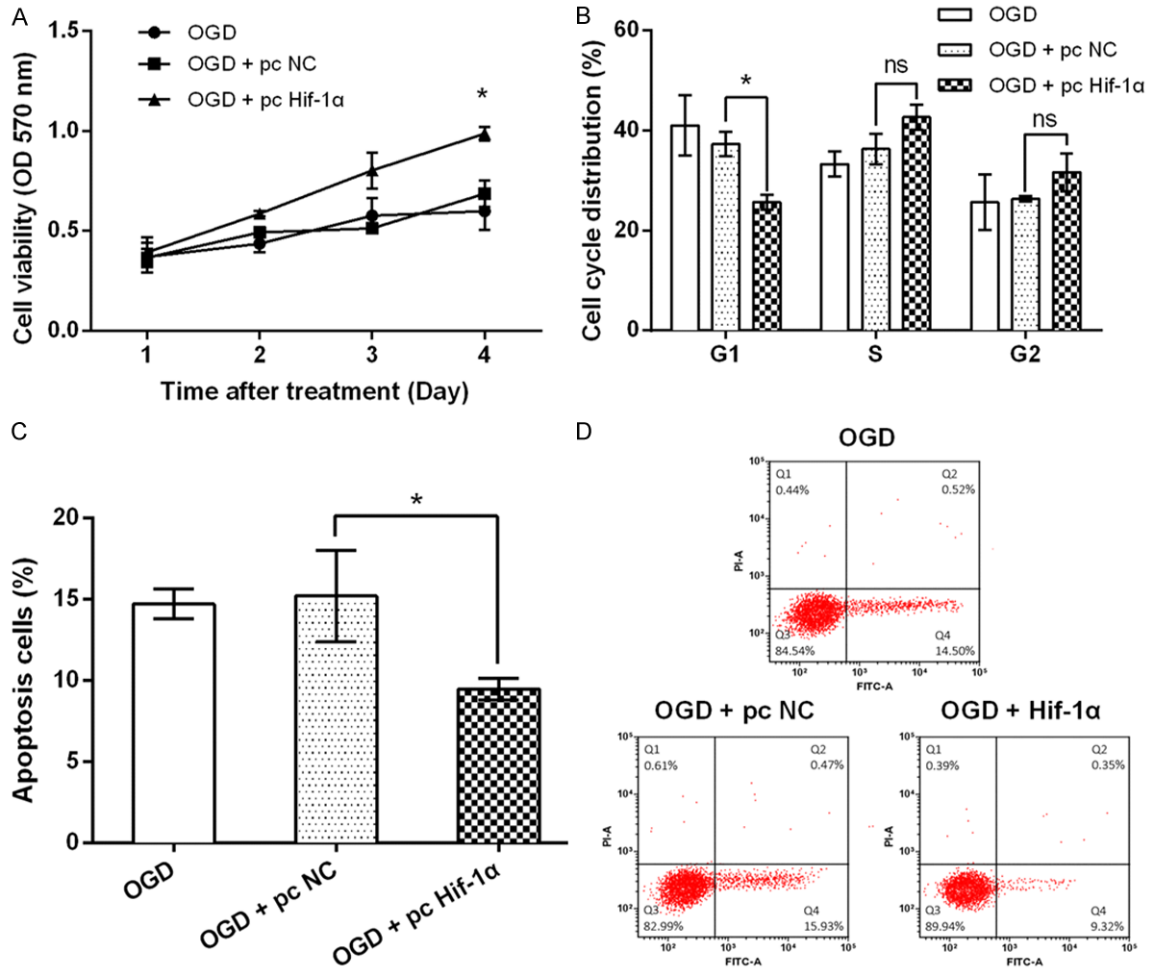


Figure 2. Overexpression of hypoxia-inducible factor 1 alpha (Hif-1 α) protected mesenchymal stem cells (MSCs) against oxygen-glucose deprivation (OGD)-induced injury. MSCs were transfected with a Hif-1 α expressing vector (pcHif-1 α) or its negative control (pcNC), and then exposed to OGD condition for 8 h. MTT assay and flow cytometry were performed to detect the changes in (A) cell viability, (B) cell cycle distribution, and (C and D) apoptosis. *, $P < 0.05$; ns, no significance.

ated by RT-PCR and Western blotting analyses. As expected, both the mRNA and protein levels of Hif-1 α were upregulated by transfection with the Hif-1 α expressing vector (pcHif-1 α) and were downregulated by transfection with Hif-1 α -specific siRNA (siHif-1 α), when compared with their correspondingly negative controls ($P < 0.05$; **Figure 1A** and **1B**).

Overexpression of Hif-1 α protected MSCs against OGD-induced injury

Next, the functional effects of Hif-1 α on OGD-injured MSCs were assessed by evaluating the changes in cell viability, cell cycle distribution and apoptosis. MTT analysis revealed that cell viability was significantly promoted by Hif-1 α

overexpression ($P < 0.05$; **Figure 2A**). Flow cytometry analytical results showed that Hif-1 α overexpression arrested much less cells in G1 phase and suppressed apoptotic cells rate ($P < 0.05$; **Figure 2B-D**). These data suggested that Hif-1 α overexpression protected MSCs from OGD-induced damage by controlling cell viability, cell cycle distribution and apoptosis.

Overexpression of Hif-1 α modulated expression of autophagy-related factors and PI3K/AKT/mTOR pathway proteins

It is well-known that Beclin1 is an autophagy-related gene, and the conversion of LC3 I to LC3 II is a hallmark of autophagy induction [16, 17]. Impaired autophagy is reflected by sup-

The role of Hif-1 α in OGD-injured MSC

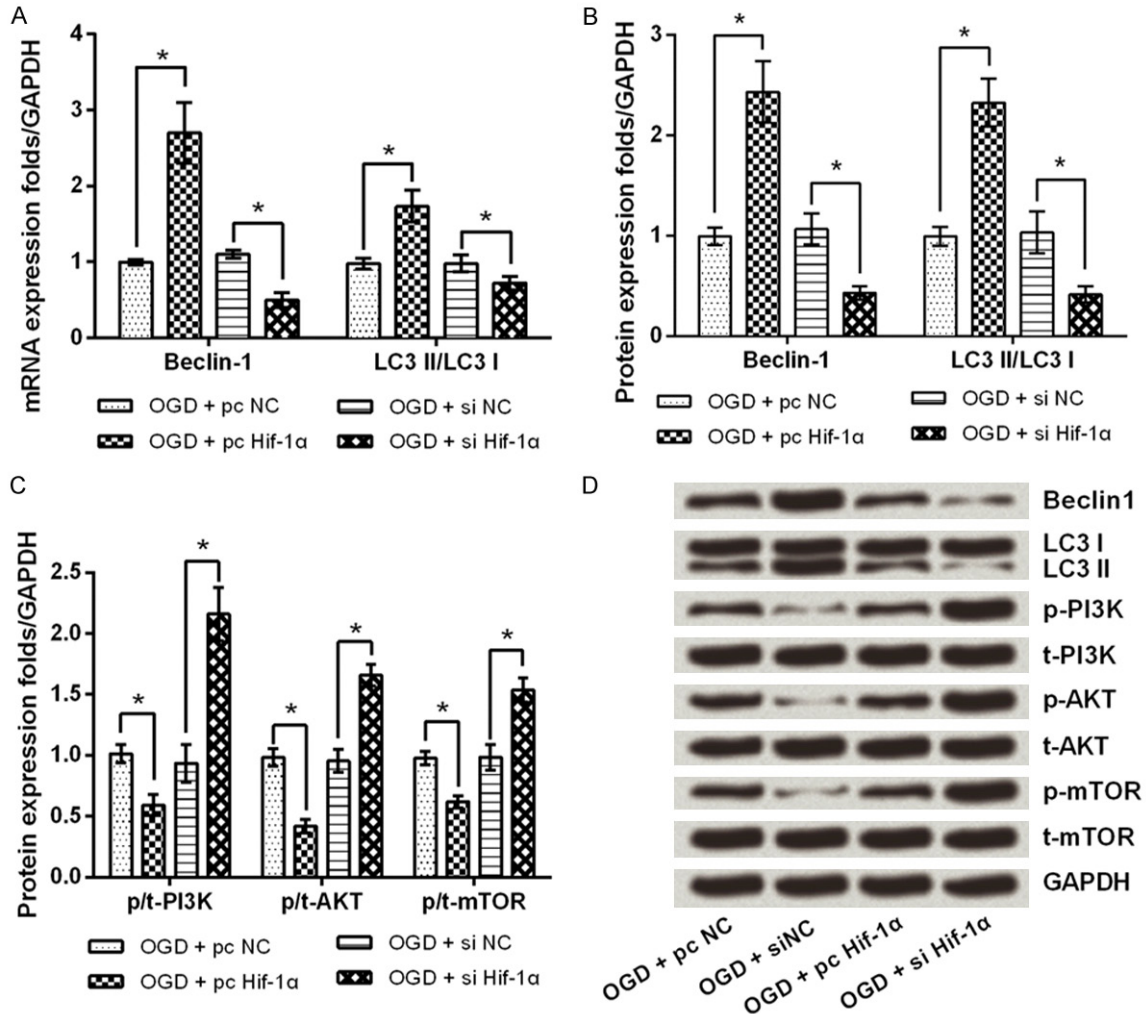


Figure 3. Overexpression of hypoxia-inducible factor 1 alpha (Hif-1 α) modulated the expression of autophagy-related factors and PI3K/AKT/mTOR pathway proteins. Mesenchymal stem cells (MSCs) were transfected with a Hif-1 α expressing vector (pcHif-1 α) or Hif-1 α -specific siRNA (siHif-1 α); pcNC and siNC were used as negative controls, respectively. The transfected cells were then exposed to oxygen-glucose deprivation (OGD) condition for 8 h. A: mRNA levels of Beclin1, LC3 I and LC3 II were measured by RT-PCR. B-D: Protein levels of Beclin1, LC3 I, LC3 II, p-PI3K, t-PI3K, p-AKT, t-AKT, p-mTOR and t-mTOR were detected by Western blotting. *, $P < 0.05$.

pression of the LC3 II/LC3 I ratio and Beclin1 expression [17, 18]. In the current study, RT-PCR and Western blotting analyses were performed to monitor the levels of LC3 I, LC3 II and Beclin1 in MSCs. Increases in Beclin1 expression and the LC3 II/LC3 I ratio were observed in Hif-1 α overexpressing-MSCs, while were decreased in Hif-1 α silencing-MSCs ($P < 0.05$; **Figure 3A, 3B and 3D**). These data provide support for the hypothesis that autophagy is induced by Hif-1 α overexpression after OGD induction.

Extensive research suggests that autophagy is induced by inhibition of the PI3K/AKT/mTOR signaling pathway [19, 20]. Since Hif-1 α increased the level of autophagy, we examined the

ability of Hif-1 α to modulate the PI3K/AKT/mTOR signaling pathway. The results of Western blot analysis shown in **Figure 3C and 3D** revealed that Hif-1 α overexpression notably suppressed the activation of PI3K, AKT and mTOR, while Hif-1 α knock-down exhibited the completely opposite impacts ($P < 0.05$). These findings indicated that Hif-1 α overexpression protected MSCs from OGD-induced damage through the suppression of the PI3K/AKT/mTOR signaling pathway.

Discussion

Recent studies have indicated that MSC death after transplantation is a negative factor impa-

cting on the outcome of cell-based therapies for myocardial infarction [21]. High sensitivity to the hypoxic and glucose-deficient environment have been implicated as major determinants of MSC fate in these procedures [22, 23]. OGD induces apoptosis in approximately 40% of transplanted MSCs within 4 h [23]. Thus, strategies that improve the survival rate of MSCs under OGD condition are urgently required.

In this study, overexpression and suppression of Hif-1 α in MSCs was achieved by transfection with a Hif-1 α expressing vector and Hif-1 α -specific siRNA, respectively. Hif-1 α overexpression resulted in an increased MSCs viability, a reduction of G1 phase cells population, and a decrease of apoptotic cells rate. Furthermore, increases in Beclin1 expression and the LC3 II/LC3 I ratio, as well as inactivation of the PI3K, AKT and mTOR proteins were observed in Hif-1 α overexpressing-MSCs.

Currently, regulation of the Hif-1 pathway is of great interest due to its capacity to direct MSC behavior, while the role of Hif-1 α in MSC survival is controversial. Some studies suggest that Hif-1 α is responsible for the proliferation of MSCs under hypoxic conditions [11] and has a limited, but pivotal role in enhancing MSCs lifespan [24]. In contrast, some evidences indicate that Hif-1 α expression is increased in hypoxic MSCs and is associated with a decreased proliferation rate and accumulation of cells in the G1 phase [25]. The supply of glucose as an indispensable energy source for cells is limited under ischemic conditions. Glucose depletion is responsible for the impairment of MSC survival and function [26]. Hypoxic conditions result in massive MSC death due to complete exhaustion of glucose; however, MSCs are able to withstand exposure to severe, continuous hypoxia provided that a glucose supply is available [26]. Furthermore a high-glucose concentration attenuated hypoxia-induced expression of Hif-1 α in mouse MSCs [27].

In the current study, Hif-1 α -modified MSCs were exposed to hypoxia and glucose-free conditions, *i.e.*, OGD, to investigate the protective effects of Hif-1 α under such conditions. Hif-1 α overexpression improved the resistance of MSCs to OGD-induced injury, indicating Hif-1 α played a protective role in OGD-injured MSCs.

Autophagy is a double-edged sword that can serve as a protection mechanism but can also

contribute to cell damage [28]. Autophagy can be induced by changes in environmental conditions, such as hypoxia and nutrient depletion [28, 29]. More importantly, autophagy is rapidly induced by hypoxia via a Hif-1-dependent pathway [29]. In cardiomyocytes, autophagy triggered by hypoxia is enhanced by Hif-1 α overexpression; moreover, Hif-1 α -mediated autophagy ameliorates the reduction in cell viability induced by hypoxia [30]. The findings of the present study demonstrated that Hif-1 α regulated the induction of autophagy-related factors, such as Beclin1 and LC3 II/LC3 I ratio, which might be associated with the functional impact of Hif-1 α on OGD-damaged MSCs.

To further explore the hypothesis that Hif-1 α -induced autophagy protected MSCs against OGD-induced injury, we monitored the expression of PI3K/AKT/mTOR signaling pathway proteins. PI3K is a crucial upstream autophagy regulator, and PI3K/AKT signaling is a well-characterized pathway that contributes to mTOR activation [31]. During early hypoxia, PI3K/AKT/mTOR seems to be involved in the action of insulin on Hif-1 α activity by positive regulation of Hif-1 α translation [32]. The data obtained in the current study indicated that Hif-1 α accumulation promoted autophagy, at least in part, by blocking the PI3K/AKT/mTOR signaling pathway. However, further studies are required to confirm this hypothesis.

In conclusion, this study shows that Hif-1 α overexpression protects MSCs against OGD-induced injury by promoting viability, reducing the G1 phase cell population, and suppressing apoptosis. Autophagy and PI3K/AKT/mTOR signaling pathway are implicated in the mechanism by which Hif-1 α protects MSCs against OGD-induced injury in MSCs.

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Disclosure of conflict of interest

None.

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The role of Hif-1 α in OGD-injured MSC

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