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Original Article

Hypoxia inducible factor 1α promotes survival of mesenchymal stem cells under hypoxia

Bingke Lv*, Feng Li*, Jie Fang, Limin Xu, Chengmei Sun, Jianbang Han, Tian Hua, Zhongfei Zhang, Zhiming Feng, Xiaodan Jiang

*Department of Neurosurgery, Zhujiang Hospital, Southern Medical University; The National Key Clinical Specialty; The Engineering Technology Research Center of Education Ministry of China; Guangdong Provincial Key Laboratory on Brain Function Repair and Regeneration, Guangzhou 510282, China. *Equal contributors.*

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Abstract: Mesenchymal stem cells (MSCs) are ideal materials for cell therapy. Research has indicated that hypoxia benefits MSC survival, but little is known about the underlying mechanism. This study aims to uncover potential mechanisms involving hypoxia inducible factor 1α (HIF1A) to explain the promoted MSC survival under hypoxia. MSCs were obtained from Sprague-Dawley rats and cultured under normoxia or hypoxia condition. The overexpression vector or small interfering RNA of *Hif1a* gene was transfected to MSCs, after which cell viability, apoptosis and expression of HIF1A were analyzed by MTT assay, flow cytometry, qRT-PCR and Western blot. Factors in p53 pathway were detected to reveal the related mechanisms. Results showed that hypoxia elevated MSCs viability and up-regulated HIF1A ($P < 0.05$) as previously reported. HIF1A overexpression promoted viability ($P < 0.01$) and suppressed apoptosis ($P < 0.001$) under normoxia. Correspondingly, HIF1A knockdown inhibited viability ($P < 0.05$) and promoted apoptosis ($P < 0.01$) of MSCs under hypoxia. Expression analysis suggested that p53, phosphate-p53 and p21 were repressed by HIF1A overexpression and promoted by HIF1A knockdown, and B-cell CLL/lymphoma 2 (BCL2) expression had the opposite pattern ($P < 0.05$). These results suggest that HIF1A may improve viability and suppress apoptosis of MSCs, implying the protective effect of HIF1A on MSC survival under hypoxia. The underlying mechanisms may involve the HIF1A-suppressed p53 pathway. This study helps to explain the mechanism of MSC survival under hypoxia, and facilitates the application of MSCs in cell therapy.

Keywords: Hypoxia inducible factor 1α, mesenchymal stem cell, hypoxia, apoptosis, p53

Introduction

Mesenchymal stem cells (MSCs) have developed rapidly to be a research focus during the past decades for their enormous potentiality in the clinical treatment of various diseases. Derived from bone marrow, adipose tissue, umbilical cord and other tissue and organs of both the newborn and the adults [1], MSCs are distinguished by the great capacity of self-renewal and differentiation, by which they are able to differentiate into multiple cell types including osteoblasts, chondrocytes, neuron-like cells and muscle cells under different induction conditions [2-4]. Besides, the immunoregulatory role of MSCs has also been reported, which burnish the potential application of MSCs in cell and gene therapy for various diseases and tissue regeneration [5, 6]. Although promising results have been achieved, more efforts are still urgently needed to delineate the mechanism of MSCs and to improve the efficacy of MSC therapy.

Hypoxia is a crucial physiological and pathological phenomenon, in which the capacities of many cells, including MSCs, are altered. Studies have found that moderate hypoxia conditions *in vitro* facilitate the survival and growth of MSCs [7, 8]. Actually, MSCs grow in a relative low oxygen concentration *in vivo*, lower than 5% in mouse bone marrow for instance [9]. These evidences imply the possibility of using proper hypoxic niche to improve the culture method for MSCs before transplantation or further clinical application. Moreover, the underlying mechanism regarding MSCs under hypoxia needs to be addressed, which will facilitate the research on MSCs with gene modification [10].

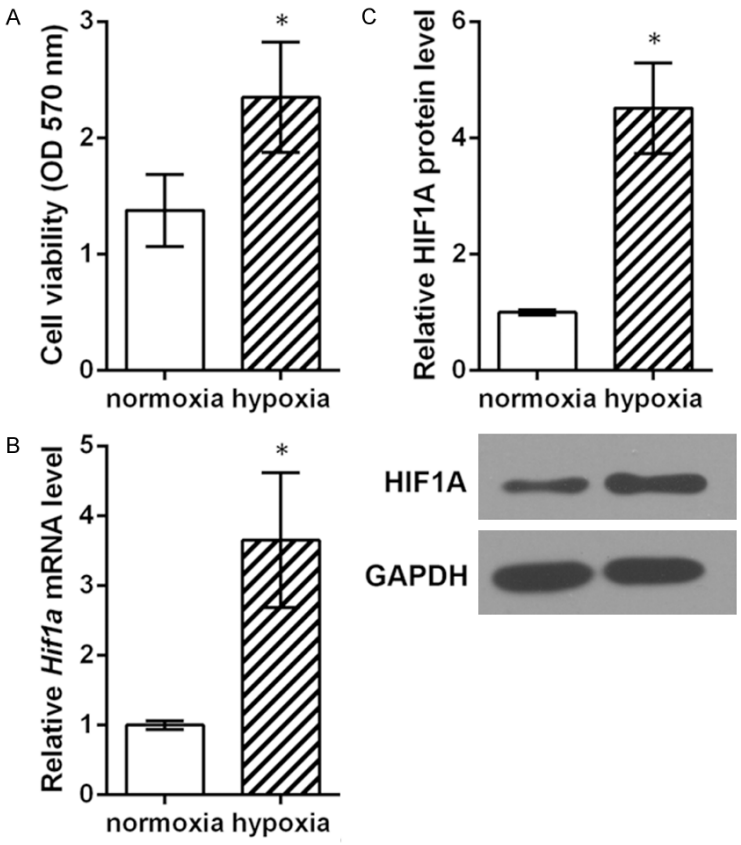

Figure 1. Hypoxia induces viability of rat mesenchymal stem cells (MSCs). Rat MSCs were cultured under normoxia or hypoxia conditions for 48 h, after which MTT assay, qPCR and Western blot were performed. MSCs under the hypoxia condition possessed higher cell viability and HIF1A expression levels. A: Cell viability detected by MTT assay at optical density (OD) of 570 nm. B: mRNA level of hypoxia inducible factor 1α (*Hif1a*) quantified by qPCR. C: Protein level of HIF1A detected by Western blot and normalized with GAPDH. *P < 0.05.

Increasing research has suggested that hypoxia inducible factor 1α (HIF1A) expression is elevated during hypoxia [11]. Cellular HIF1A is in a dynamic equilibrium of expression and degradation: when the oxygen concentration increases, HIF1A is hydroxylated and interacts with von Hippel-Lindau tumor suppressor (VHL), which leads to the ubiquitination and degradation of HIF1A. The hydroxylation is suppressed by hypoxia, elevating HIF1A protein level [12]. Since hypoxia is a common feature of solid tumors, HIF1A elevation plays pivotal roles in the pathogenesis of tumors [13]. Furthermore, HIF1A is capable of promoting the self-renewal and proliferation of MSCs, as well as regulating MSC differentiation [14, 15]. However, evidence is limited to decipher the mechanism of HIF1A in regulating MSC survival under hypoxia.

This study aims to investigate the effects of HIF1A on MSC survival under hypoxia and to elucidate possible mechanisms. MSCs of Sprague-Dawley (SD) rats were isolated and cultured under normoxia or hypoxia conditions. HIF1A was overexpressed or knocked down by cell transfection, after which cell viability and apoptosis were assessed. The expression of p53 pathway was analyzed by Western blot. These results were expected to elucidate the role and mechanism of HIF1A in regulating MSC survival under hypoxia, thus facilitating research on MSC clinical application.

Materials and methods

Animals and cells

SD rats (5 females and 5 males, 1 month old, 180 ± 20 g) were purchased from SLAC (Shanghai, China) and used in this study for MSC isolation. The rats were kept in laboratory environment at 24°C and 50% humidity for acclimatization, anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g) and sacrificed by injecting about 2 mL air into the caudal vein. All the animal experiments were approved by a local ethics committee and performed in accordance to the instructions of our institute.

The isolation of MSCs was performed based on a previous study [16] with minor changes. The femur and tibia of the rats were dislocated and immersed in 75% ethyl alcohol for 15 min. The epiphyses at both sides was cut off and the bone marrow was flushed with Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). Then cells were seeded in 6-well plates at a density of 5×10^6 cells/well and incubated in humid air containing 5% CO_2 at 37°C. MSCs were purified by changing the medium to remove non-adher-

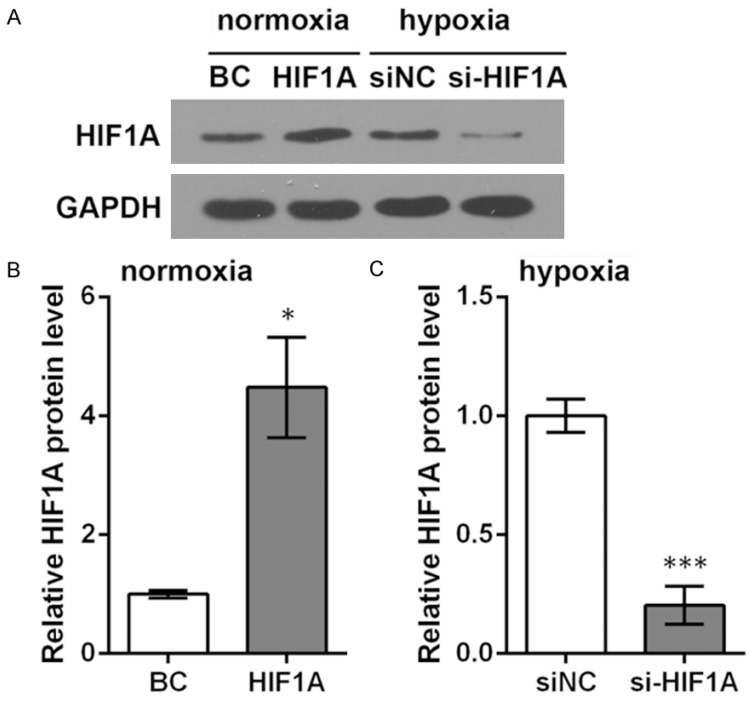

Figure 2. Verification of hypoxia inducible factor 1α (HIF1A) overexpression and knockdown. Rat mesenchymal stem cells (MSCs) were cultured under normoxia or hypoxia conditions. Cell transfection was performed to overexpress or knock down HIF1A. The overexpression vector of HIF1A was transfected for overexpression and the small interfering RNA of HIF1A (si-HIF1A) was transfected for knockdown. Blank vector (BC) and siRNA negative control (siNC) were used as the corresponding control. Western blot was performed at 48 h post transfection. HIF1A was effectively overexpressed or knocked down. A: HIF1A protein level detected by Western blot. GAPDH was used as an internal control. B: Quantification of HIF1A protein level according to Western blot results after cell transfection in MSCs cultured under normoxia. C: Quantification of HIF1A protein level according to Western blot results after cell transfection in MSCs cultured under hypoxia. *P < 0.05. ***P < 0.001.

ent cells. Briefly, the medium was half changed at 48 h after seeding the cells, and completely changed every 3 d thereafter. The cells were passaged when the confluency reached 80%. The cells were analyzed by flow cytometry using EasyCyte 6HT2L (Merck Millipore, Boston, MA, USA), and collected for further experiments when the percent of CD44- or CD29-positive cells was over 97% and simultaneously the percent of CD45- or CD34-positive cells was less than 3% [17].

MSCs were divided into two groups: normoxia and hypoxia, which were culture under normoxia and hypoxia conditions, respectively. The normoxia group was kept in the abovementioned conditions, while the hypoxia group was shifted to a tri-gas incubator HERA cell 150i (Thermo Scientific) containing 92% N_2, 5% CO_2 and 3% O_2 at 37°C.

Transfection

The overexpression vector containing the complete coding sequence of rat *Hif1a* (GenBank Accession NM_024359) was constructed using pcDNA3.1 vector (Thermo Scientific, Carlsbad, CA, USA) and screened by Ampicillin (100 μg/mL, Solarbio, Beijing, China). The correct sequence was further verified by sequencing. The blank vector was also prepared as a negative control for transfection (BC). The specific small interfering RNA (siRNA) for *Hif1a* and the negative siRNA control (siNC) were synthesized by Genscript (Nanjing, China). Cell transfection was performed at 48 h after hypoxia treatment was started using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. MSCs (1×10^5) were seeded in each well of 24-well plates 1 d before transfection. In each well, 1 μg of the overexpression vector or 10 pmol of siRNA were added, and the plates were incubated at 37°C for 48 h, after which MTT assay, flow cytometry, qPCR and Western blot were performed on these transfected MSCs.

MTT assay

Cell viability was measured by the MTT method using MTT Cell Proliferation Assay (ATCC, Manassas, VA, USA). Briefly, MSCs were seeded at 1×10^5 cells/well, and 10 μL of MTT Reagent was added to the well for 4 h incubation. Then 100 μL of Detergent Reagent was added and the plates were incubated in the dart at room temperature for 2 h to dissolve the purple precipitates. Optical density (OD) was measured at 570 nm.

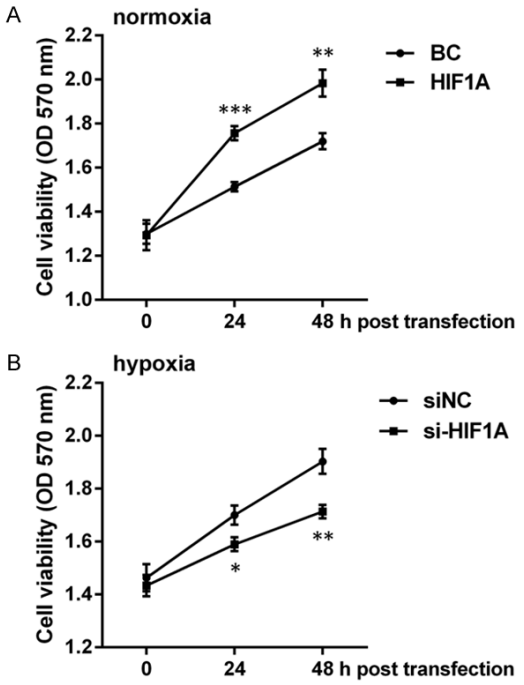

Figure 3. Hypoxia inducible factor 1α (HIF1A) regulates rat mesenchymal stem cell (MSC) viability. MSCs were cultured under normoxia or hypoxia conditions. Cell transfection was performed to overexpress or knock down HIF1A. Cell viability was detected by MTT assay at 0, 24 and 48 h post transfection. A: HIF1A overexpression by vector transfection induces MSC viability under normoxia compared to blank vector (BC). B: HIF1A knockdown by small interfering RNA (si-HIF1A) suppresses MSC viability under hypoxia compared to siRNA negative control (siNC). *P < 0.05. **P < 0.01. ***P < 0.001.

Flow cytometry

Flow cytometry was performed to assess cell apoptosis after MSCs were stained by fluorescein isothiocyanate (FITC) and propodium iodide (PI) using Annexin V-FITC Apoptosis Kit (BioVision, Milpitas, CA, USA). Cells (1×10^5) were collected and resuspended in Binding Buffer, and then 5 μL of Annexin V-FITC and 5 μL of PI were added. After incubated in the dark at room temperature for 5 min, the cells were detected by flow cytometry. FITC-positive and PI-negative cells were considered as apoptotic cells.

qPCR

Total RNA was extracted from MSCs using Trizol (Invitrogen) and purified by RNA Cleanup Kit (CWbio, Beijing, China) according to the manufacturer's instructions. The quality of RNA was examined by 1% agarose gel electrophoresis. First-strand complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR was performed to quantify the level of *Hif1a* mRNA on QuantStudio 6 Flex Realtime PCR system (Thermo Scientific) with the specific primer for *Hif1a*: forward 5'-GGCTA CAGTA CTGCA CCAAC T-3' and reverse 5'-TGGCA TTCGA AGGAC ATGGT-3'. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method normalized to *Gapdh* (forward 5'-CGCAT TGCCA GACAT ATCAG C-3' and reverse 5'-AGGTG AAGCA GGCTC AATCA A-3').

Western blot

Protein of MSCs was extracted using Cell Protein Extraction Reagent (Boster, Wuhan, China) according to the manufacturer's instructions. The protein was migrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk in phosphate buffered saline (PBS) for 4 h at 4°C and then incubated in the specific antibodies against HIF1A (ab1, Abcam, Cambridge, UK), p53 (ab28), phospho-p53 (p-p53, ab38-497), p21 (ab80633) and B-cell CLL/lymphoma 2 (BCL2, ab196495) overnight at 4°C. Anti-GAPDH (ab8245) was used as an internal control. Membranes were washed in PBS for 5 times and then incubated in goat anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase for 2 h at room temperature. ECL Plus Western Blotting Substrate (Thermo Scientific) was used to visualize the signals. The signal intensity was quantified by ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Experiments were performed in triplicate. Results were expressed as mean ± standard deviation. Comparison between two groups was performed by Student's *t* test in SPSS 20 (IBM, New York, NY, USA). $P < 0.05$ was considered statistically significant.

Results

Hypoxia induces MSC viability and HIF1A expression

Existed research has found that MSCs benefit from the hypoxia condition. In order to eluci-

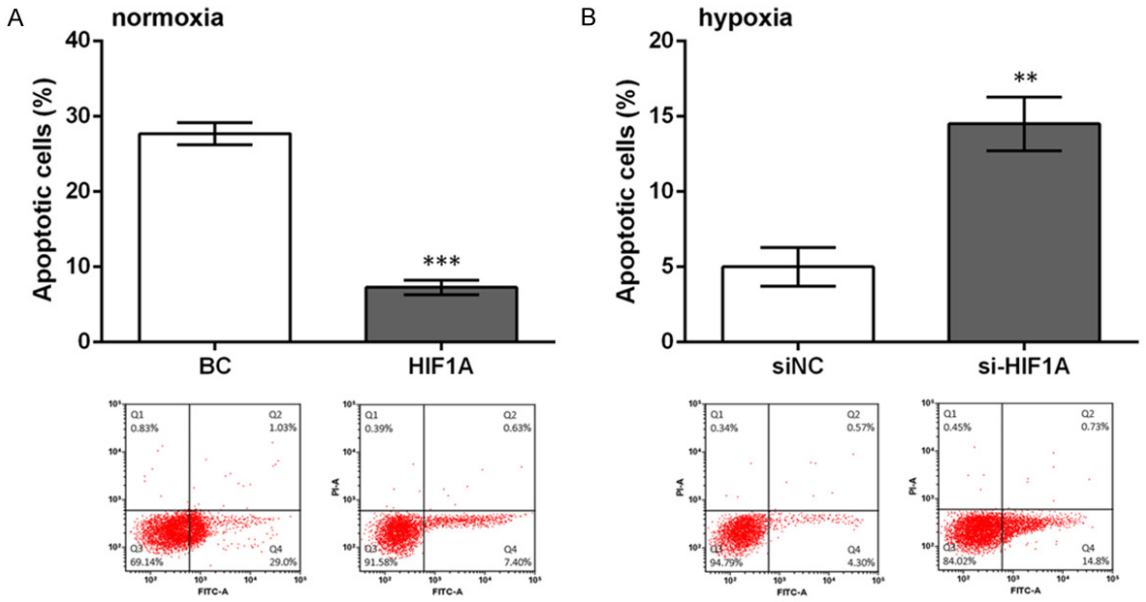

Figure 4. Hypoxia inducible factor 1α (HIF1A) regulates rat mesenchymal stem cell (MSC) apoptosis. MSCs were cultured under normoxia or hypoxia conditions. Cell transfection was performed to overexpress or knock down HIF1A. Percent of apoptotic cells were detected by flow cytometry at 48 h post transfection. A: HIF1A overexpression by vector transfection reduces MSC apoptosis under normoxia compared to blank vector (BC). B: HIF1A knockdown by small interfering RNA (si-HIF1A) increases MSC apoptosis under hypoxia compared to siRNA negative control (siNC). **P < 0.01. ***P < 0.001.

date the underlying mechanism, we first tested *in vitro* cell viability changes after MSCs were culture under normoxia or hypoxia for 48 h. MTT assay showed that hypoxia induced MSC viability ($P < 0.05$, **Figure 1A**). Meanwhile, as a factor closely related to hypoxia changes, HIF1A mRNA and protein levels were both promoted after hypoxia treatment ($P < 0.05$, **Figure 1B** and **1C**), suggesting the possible involvement of HIF1A in the hypoxia-induced MSC viability. Thus in the following experiment, we tried to reveal the role of HIF1A in regulating MSCs during hypoxia.

HIF1A regulates MSC viability and apoptosis

Since HIF1A had a higher level under the hypoxia condition, it was overexpressed in MSCs cultured under normoxia and knocked down in MSCs cultured under hypoxia to reflect its potential roles. But before further detection, we verified whether cell transfection successfully induced HIF1A level change by Western blot. Results showed that HIF1A overexpression vector in the normoxia groups significantly elevated HIF1A protein level ($P < 0.05$, **Figure 2A** and **2B**). si-HIF1A markedly reduced HIF1A protein level in the hypoxia-treated MSCs ($P < 0.001$, **Figure 2A** and **2C**). Thus these transfected MSCs were valid for further experiments.

MSC viability was detected after transfection. HIF1A overexpression significantly promoted the viability of MSCs cultured under normoxia at 24 and 48 h post transfection ($P < 0.001$ and $P < 0.01$, **Figure 3A**). In MSCs cultured under hypoxia, HIF1A knockdown had considerable effects suppressing MSC viability at 24 and 48 h post transfection ($P < 0.05$ and $P < 0.01$, **Figure 3B**). Cell apoptosis change was also detected. The percent of apoptotic MSCs under normoxia was obviously reduced by HIF1A overexpression ($P < 0.001$, **Figure 4A**), and that under hypoxia was increased by HIF1A knockdown ($P < 0.01$, **Figure 4B**). Taken together, HIF1A might induce viability and suppress apoptosis in rat MSCs.

HIF1A regulates p53 pathway

To explore the underlying mechanism of HIF1A in regulating MSCs, factors in p53 pathway pivotal to cell apoptosis were detected by Western blot after cell transfection. In MSCs under normoxia, HIF1A overexpression significantly suppressed the level of p53, p-p53 and p21 and

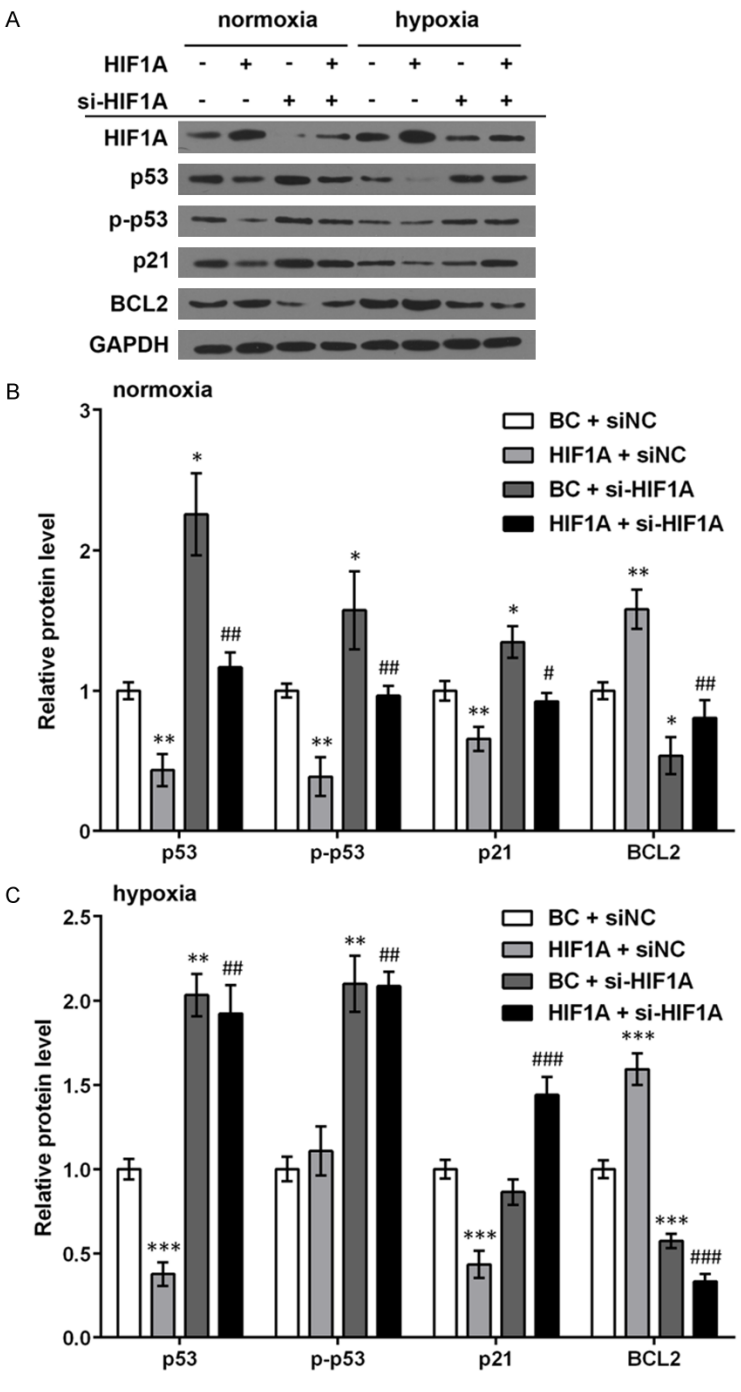

Figure 5. Hypoxia inducible factor 1α (HIF1A) regulates p53 pathway in rat mesenchymal stem cells (MSCs). MSCs were cultured under normoxia or hypoxia conditions. Cell transfection was performed to overexpress or knock down HIF1A. Blank vector (BC) and siRNA negative control (siNC) were used as the corresponding control. Western blot was performed at 48 h post transfection and GAPDH was used as an internal control. Protein levels of p53, phospho-p53 (p-p53), p21 and B-cell CLL/lymphoma 2 (BCL2) were significantly regulated by HIF1A overexpression or knockdown. A: Protein levels of p53 pathway factors detected by Western blot. B: Quantification of Western blot results for MSCs under normoxia. C: Quantification of Western blot results for MSCs under hypoxia. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the BC + siNC group. #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to the HIF1A + siNC group.

increased the level of BCL2 ($P < 0.01$, **Figure 5A** and **5B**). HIF1A knockdown had opposite effects ($P < 0.05$). Furthermore, HIF1A knockdown could impede the function of HIF1A overexpression on these factors ($P < 0.05$ or $P < 0.01$). In MSCs under hypoxia, HIF1A overexpression suppressed p53 and p21, and increased BCL2 ($P < 0.001$, **Figure 5A** and **5C**), while p-p53 did not show significant changes ($P > 0.05$). HIF1A knockdown induced opposite changes ($P < 0.01$ or $P < 0.001$), although p21 were not changed greatly ($P > 0.05$). Also, HIF1A knockdown could attenuate the effect of HIF1A overexpression. Based on these results, it was possible that HIF1A could suppress factors in p53 pathway, which might be associated with its role in rat MSCs.

Discussion

MSCs are mostly growing in a hypoxia niche *in vivo*, and moreover, MSCs cultured under hypoxia *in vitro* also exhibits better growth and survival status. This study detected a higher MSC viability and elevated HIF1A expression under hypoxia. In order to explain the related mechanisms, HIF1A overexpression and knockdown was performed. It was found that overexpression of HIF1A under normoxia promoted viability and inhibited apoptosis of MSCs. Consistently, HIF1A knockdown in MSCs cultured under hypoxia suppressed viability and elevated apoptosis. The altered HIF1A expression was accompanied by the modulated p53, p21 and BCL2 levels, which are all factors associated with p53 pathway.

Based on the former studies that MSC viability and proliferation can be elevated by both short-term and continuous exposure to hypoxia [18, 19], this study was started from the viability of MSCs continuously cultured under hypoxia compared to normoxia, and found promoted viability by hypoxia, which was consistent to those existed studies. Furthermore, along with the elevated cell viability, both mRNA and protein levels of HIF1A in MSCs were also up-regulated, which was in line with the HIF1A protein processing that has been well-delineated before [12]. Thus it was tempting to speculate the regulatory role of HIF1A in the hypoxia-induced MSC viability.

The regulatory effects of HIF1A on cell viability and apoptosis have reported in various cell types. HIF1A induces proliferation of disease cells such as non-small cell lung cancer cells [20] and stem cells like human embryonic stem cells [21] and adipose-derived stem cells [22]. Besides, the suppressive effect of HIF1A on cell apoptosis has also been revealed. It inhibits retinoblastoma (Rb) cell apoptosis, thus contributing to poor Rb cell chemosensitivity [23], while its down-regulation is associated with elevated neuronal apoptosis [24]. While several reports have addressed that HIF1A promotes apoptosis in some diseases [25], it is likely that the role of HIF1A depends on specific cell types and conditions. With respect to MSCs under normoxia and hypoxia condition, HIF1A is capable of enhancing cell viability and reducing cell apoptosis, thus promoting MSC survival.

We further investigated the underlying mechanism of HIF1A during hypoxia by detecting factors in p53 pathway, since p53 has been intensively studied in the regulation of cell survival [26]. MSCs with p53 knockdown exhibit higher proliferative capacity [27] and a lower apoptosis rate [28]. Our results showed that HIF1A overexpression suppressed p53, p-p53 and downstream factors p21 but elevated BCL2, while HIF1A knockdown had the opposite effects. BCL2 is an anti-apoptotic factor whose transcription can be suppressed by p53 [29]. Besides, p21 can be transcriptionally activated by p53 [30] and participates in apoptotic regulation through modulating cell cycle [31]. Hence the results of this study suggested that HIF1A was capable of regulating p53 and its downstream apoptotic factor p21 and BCL2 in MSCs. Furthermore, p53 is a key modulator during the regulated apoptosis by HIF1A [32], which raises the possibility that HIF1A reducing MSC apoptosis may be associated with its suppression on p53 pathway. The regulation of p53, p-p53, p21 and BCL2 by HIF1A was detected in both normoxia and hypoxia conditions, although no significant changes was found in p-p53 by HIF1A overexpression or p21 by si-HIF1A, which may generate from influences from other regulators. Further validation is needed to decipher the sophisticated mechanism centering HIF1A.

Recent research has raised concerns about the advantage of gene-modified MSCs in improving outcome of transplantation. For example, the specific gene-modified MSCs aggrandize arteriogenesis in a porcine model of myocardial ischemia [33], attenuate cerebral infarction in a rat model [34] and expedite MSC invasion into tumors [35]. In view of these findings, this study uncovered several factors like HIF1A and p53 that may be modified in MSCs for transplantation, while the causal relationship between these factors still requires further investigation. Our study also emphasizes the advantages of the hypoxia condition in improving MSC survival, which agrees with a former study that hypoxic pretreatment improves effects of MSC sheets [36], emphasizing the optimization of culture condition for MSC therapy.

Collectively, this study revealed one possible mechanism of HIF1A regulating p53 pathway to explain the improved MSC survival under hypoxia condition. Up-regulation of HIF1A is associated with the enhanced viability and suppressed apoptosis of rat MSCs *in vitro*, which indicates the improved survival of cultured MSCs. These results provide alternatives to support the application of MSCs in cell therapy and tissue engineering.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Xiaodan Jiang, Department of Neurosurgery, Zhujiang Hospital, Southern Medical University; The National Key Clinical Specialty; The Engineering Technology Research Center of Education Ministry of China; Guangdong Provincial Key Laboratory on Brain Function Repair and Regeneration, Guangzhou 510282, China. E-mail: jiangxd@smu.edu.cn

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