Effects of hypoxia-inducible factor-1α on the proliferation and apoptosis of human synovial mesenchymal stem cells

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Abstract. Hypoxia is a constant feature of the synovial microenvironment. How synovial mesenchymal stem cells (SMSCs) proliferate and differentiate in a hypoxic environment over a long period of time has aroused the interest of researchers. The aim of the present study was to explore the effects of hypoxia-inducible factor-1α (HIF-1α) on the proliferation and apoptosis of human SMSCs. SMSCs were harvested and cultured under different concentration of oxygen, normoxia (21% O_2), hypoxia (5% O_2) and severe hypoxia (0.5% O_2) to determine its effect on the expression of HIF-1α. Then, the cells were collected and cell proliferation and apoptosis were detected at severe hypoxia (0.5% O_2) and hypoxia (5% O_2) conditions following HIF-1α siRNA transfection. There were no significant changes in cellular proliferation or apoptosis when cultured in normoxia (21% O_2), hypoxia (5% O_2) or severe hypoxia (0.5% O_2). However, the mRNA and protein expression of HIF-1α were markedly upregulated in the hypoxic conditions. Further experiments suggested that the proliferation of SMSCs was obviously suppressed and apoptosis was markedly increased under severe hypoxic (0.5%) and hypoxic (5% O_2) conditions following HIF-1α siRNA transfection. In conclusion, HIF-1α effectively improved the tolerance of SMSCs to hypoxia, which may promote cellular proliferation and prevent the apoptosis of SMSCs under hypoxic conditions.

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Introduction

The study of mesenchymal stem cells (MSCs) has advanced considerably throughout the past few decades, and rapid progress has been made in utilizing MSCs to study disease pathogenesis, to discover biomarkers and novel targets, and to validate cell-based tissue engineering therapies (1-4). In the past few years, synovial mesenchymal stem cells (SMSCs) have attracted increasing attention as a promising cell source for tissue engineering as SMSCs have an intriguing multi-lineage developmental plasticity *in vitro* and *in vivo* (5-7). Synovial tissue can be obtained by minimally invasive surgery such as arthroscopy (8), and similar to adult MSCs from other sources, SMSCs can be isolated and expanded more efficiently *in vitro* (8,9). In addition to the ability to self-replicate and differentiate into multiple lineages, the immune-privileged nature of SMSCs implies their potential utilization in allogeneic cell-based settings (10,11). Articular synovium is avascular and exists in a low oxygen microenvironment. The oxygen tension ranges from 1 to 7% in the knee joints of different age groups (12,13). As oxygen tension seems to be a nonnegligible regulator of cell proliferation and differentiation of SMSCs, it is therefore essential to study the survival mechanism of SMSCs in a hypoxic environment.

Hypoxia-inducible factor-1α (HIF-1α) is a heterodimeric transcription factor that can be induced under hypoxic condition (14,15). The stimulation and activity of HIF-1α have been demonstrated to be mediated at different levels throughout the cell cycle (16). Some regulatory pathways, including Wnt, Notch, PTEN, JAK/STAT, LOX and FBW7 are involved in regulating the metabolism, angiogenesis, metastasis and invasion of MSCs via HIF-1α mediation (17). HIF-1α undergoes rapid degradation under normoxic conditions with a half-life of only approximately 5 min (18). In comparison, in hypoxic conditions, intermediate metabolites stabilize the expression of HIF-1α by inhibiting the activity of proliferol hydroxylase, and promotes its migration into the nucleus to combine with HIF-1β to form HIF-1 heterodimers (19-21). Numerous studies have demonstrated that HIF-1α participates in the regulation of angiogenesis, cell growth and glucose metabolism (22-24).

HIF-1α has been reported to be abundantly expressed in synovial tissues of patients with rheumatoid arthritis, osteoarthritis and temporomandibular joint disorders, which are stimulated by a series of immune factors such as inflammation

and oxidative stress response (25-27). However, the expression of HIF-1α in normal human synovial membrane and the role of HIF-1α in synovial membrane adapting to different oxygen environments have not been reported. In particular, the effect of low oxygen tension on HIF-1α expression in SMSCs has not been characterized. Therefore, in order to determine the initial effects of HIF-1α in SMSCs, we investigated the expression of HIF-1α in SMSCs under different oxygen conditions and observed the effect of HIF-1α on the proliferation and apoptosis of human SMSCs *in vitro*.

Materials and methods

Tissue harvest and cell culture. Synovial tissue from six patients with a spectrum of knee conditions including ligament, meniscal, and cartilage injury were collected by arthroscopy. Ethical approval for this study was granted by the Institution Review Board of the Affiliated Hospital of Nanjing Medical University, and all study participants were recruited after providing informed written consent. SMSCs were isolated using an enzyme digestion procedure according to a previously described method (28). The culture medium (DMEM/Nutrient Mixture F-12 Ham supplemented with 20% FBS) was changed every 3 days. According to the manufacturer's protocol, $1.0x10^6$ SMSCs were cultured for 21 days in adipogenic medium (Adipogenic Base Media, StemXVivo; R&D Systems) for adipogenesis detected by Oil red-O staining. The same method was used for osteogenesis with osteogenic medium (Osteogenic Base Media, StemXVivo; R&D Systems) and Alizarin red staining was conducted.

Hypoxia exposure. The 2nd passage of $1.0x10^5$ SMSCs was cultured for 7 days under different ambient oxygen tension, including normoxia (21% O_2), hypoxia (5% O_2) or severe hypoxia (0.5% O_2) at 37˚C in a humidified incubator with 5% CO_2.

Cell transfection. The 2nd passage of $1.0x10^5$ cells were transfected with small interfering RNA (siRNA) as previously described (29). SMSCs were placed into 6-well plates for 24 h. Cells were transfected with specific siRNA (Ambion; Thermo Fisher Scientific, Inc.) targeting HIF-1α using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, SMSCs were collected for further analysis.

Proliferation assay. A Cell Counting Kit-8 (CCK-8) assay was used to evaluate cell viability. Briefly, the SMSCs were collected and seeded into 96-well plates at a dose of $5.0x10^3$/ml. Then, after cell culture for 1 to 7 days, 10 µl of CCK-8 solution (Nanjing Jiancheng Biotechnology Institute) was added into each well. Cells were cultured at room temperature for 4 h in the dark. The absorbance was measured at 450 nm by a microplate reader (Bio-Rad, Inc.).

Flow cytometry. To determine the phenotypes of the SMSCs, flow cytometric analysis was used. The 2nd passage of $1.0x10^6$ SMSCs was collected and the cells were suspended in PBS before being incubated with the following antibodies (Agilent Technologies, Inc.) for 90 min at 37˚C: FITC-conjugated anti-human CD147 (cat. no. SZB10284; 1:500 dilution), CD90 (cat. no. bs-10430R; 1:200 dilution), CD105 (cat. no. bs-0579R; 1:10,000 dilution), CD44 (cat. no. K001677P; 1:500 dilution), CD117 (cat. no. 130-098-570; 1:500 dilution), CD34 (cat. no. bs-0765R-2; 1:200 dilution), CD14 (cat. no. K101533P; 1:200 dilution) and CD45 (cat. no. bs-10600R; 1:200 dilution). The cell phenotypes were analyzed using an FC 500 flow cytometer (BD Biosciences).

RT-qPCR. TRIzol reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA of the SMSCs. The target gene and an endogenous control β-actin were amplified by qPCR using the SYBR Green PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). GAPDH was used as an internal reference. The primers for PCR were as follows: GLUT3 forward, 5'-CGGCTTCCTCATTACCTTC-3' and reverse, 5'-GGCACG ACTTAGACATTGG-3'; HIF-1α forward, 5'-TAAAGGAAT TTCAATATTTGATGGG-3' and reverse, 5'-AAAGGGTAA AGAACAAACACACAG-3'; GAPDH forward, 5'-GGA GCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGT TGTCATACTTCTCATGG-3'. The thermocycling conditions were 25˚C for 5 min, 42˚C for 60 min and at 95˚C for 15 sec. Fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (30) normalized to GAPDH.

Western blot analysis. SMSCs were harvested on ice in PBS and centrifuged at $1.3x10^4$ g for 10 min. Total protein of the SMSCs was isolated using lysis buffer (Sigma-Aldrich; Merck-Millipore) and quantified using a bicinchoninic acid (BCA) assay (Beyotime Biotechnology, Inc., China). Then, 20 µg protein was electrophoresed on 10% gel with SDS-PAGE and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was then blocked with 5% nonfat milk for 2 h at 4˚C and was then incubated with GLUT3 (cat. no. bs-20225R; 1:500 dilution; BIOSS), HIF-1α (cat. no. K000487P; 1:500 dilution; Beijing Solarbio Science & Technology Co., Ltd.), cleaved caspase3 (cat. no. 1083-10; 1:500 dilution; BioVision, Inc.), Bax (cat. no. K002397P; 1:500 dilution; Beijing Solarbio Science & Technology Co., Ltd.) and Bcl-2 (cat. no. K003505P; 1:500 dilution; Beijing Solarbio Science & Technology Co., Ltd.) antibodies and GAPDH antibody (cat. no. G5262-1VL; 1:3,000 dilution; Sigma-Aldrich; Merck KGaA) overnight at 4˚C. Then, the membranes were re-incubated with secondary antibodies (Cell Signaling Technology, Inc., USA). The signal was visualized using a photographic developer (Invitrogen Life Technologies, Inc., USA) and densitometry was performed using ImageJ (version 1.25; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are reported as the means ± standard errors. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Parameters of cells under normoxic (21% O_2), hypoxic (5% O_2) and severe hypoxic (0.5% O_2) conditions were determined by one-way ANOVA. Parameters of cells between groups following siRNA-induced HIF-1α knockdown were analyzed using a t-test. $P<0.05$ was considered statistically significant.

Results

Characterization of the SMSCs. Multilateral form fibre cells and a few spindle-shaped cells were observed among the primary

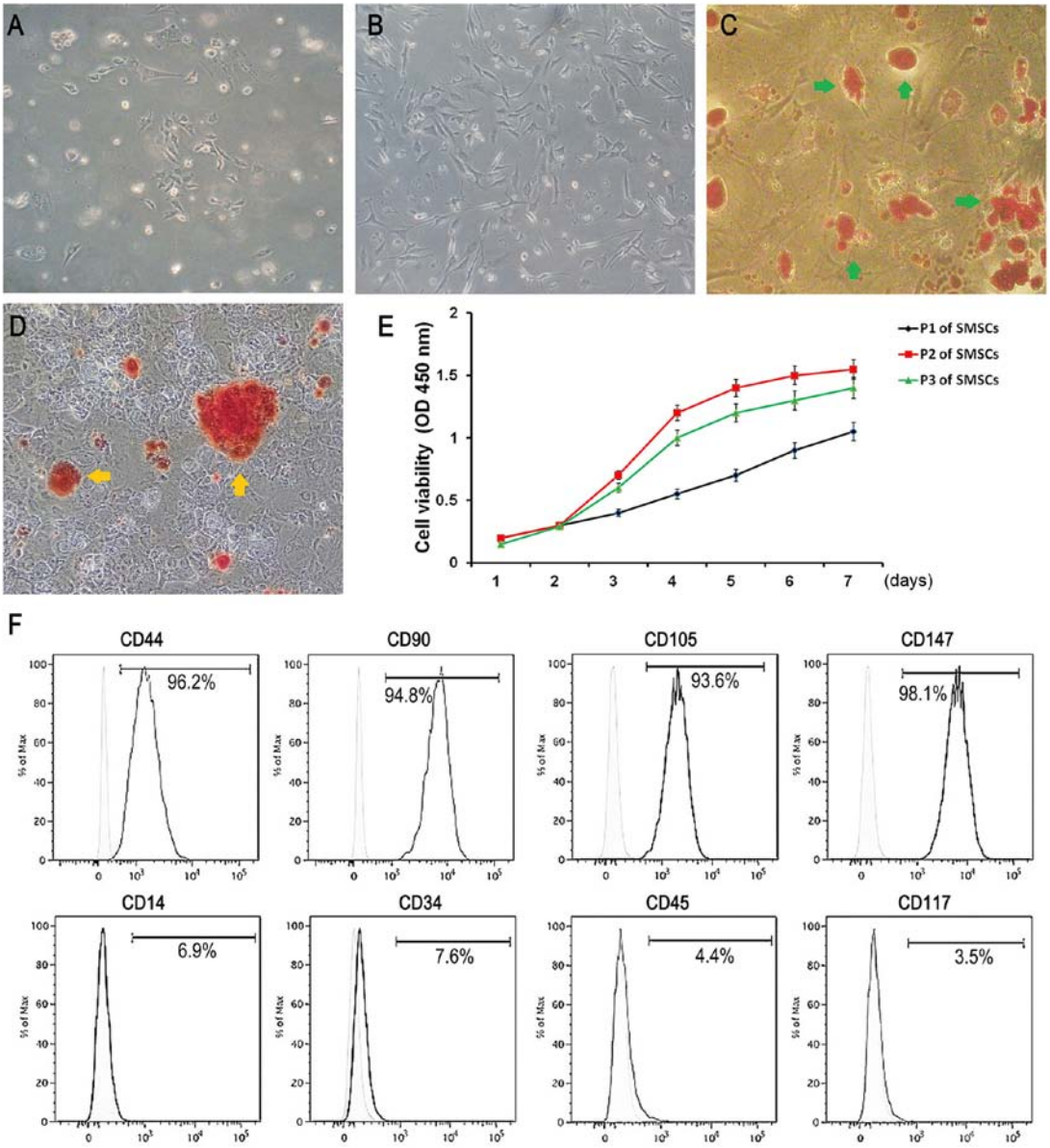

Figure 1. Characteristics of SMSCs. (A and B) Observation of primary SMSCs and P2 SMSCs, respectively (magnification, x40). (C) Oil red staining indicates lipid droplet formation (green arrows) after adipogenic differentiation for 14 days (magnification, 40). (D) Alizarin red staining demonstrates the formation of mineralized nodules (yellow arrows) after osteogenic induction for 21 days (magnification, x40). (E) Cell proliferation of P1 to P3 SMSCs was determined by CCK-8 assay. P2 SMSCs revealed the highest rate of proliferation. (F) Flow cytometric analysis showed that the surface markers CD44, CD90, CD105, CD147, CD14, CD34, CD45 and CD117 were expressed on average on 96.2, 94.8, 93.6, 98.1, 6.9, 7.6, 4.4 and 3.5% of the SMSCs, respectively. All data are averages ± standard deviations (SD) (error bars) from 3 to 5 independent experiments. SMSCs, synovial mesenchymal stem cells.

cells and the 2nd passage of the SMSCs (Fig. 1A and B). Oil red O-positive lipid droplets could be obviously observed in the SMSCs after adipogenic induction for 3 weeks (Fig. 1C). Similarly, Alizarin red S staining showed that some mineralized nodules were effectively formed in the isolated SMSCs after three weeks of osteoinduction (Fig. 1D). These results demonstrated that the SMSCs exhibited the potential of multidirectional differentiation. CCK-8 assays demonstrated that the SMSCs increased over time in exponential growth, and the 2nd passage of the SMSCs exhibited the highest proliferation (Fig. 1E). The immunophenotypes were determined via flow cytometry. We found that the surface markers of CD44, CD90, CD105, CD147, CD14, CD34, CD45 and CD117 were expressed on average on 96.2, 94.8, 93.6, 98.1, 6.9, 7.6, 4.4 and 3.5% of the SMSCs, respectively (Fig. 1F), which fits the criteria that we previously reported (28).

Characteristics of the SMSCs under hypoxic conditions. To observe the effects of different oxygen concentration microenvironments on the proliferation and apoptosis of SMSCs, cells were cultured under oxygen environment of normoxia (21% O_2), hypoxia (5% O_2) and severe hypoxia (0.5% O_2). Our findings suggested that there were no significant changes in cell viability or GLUT3 mRNA and protein expression following incubation under different oxygen conditions (Fig. 2A-C). Interestingly, the mRNA and protein expression of HIF-1α was significantly upregulated under hypoxic (5% O_2) and severe hypoxic (0.5% O_2) conditions (Fig. 2D and E). Additionally, no

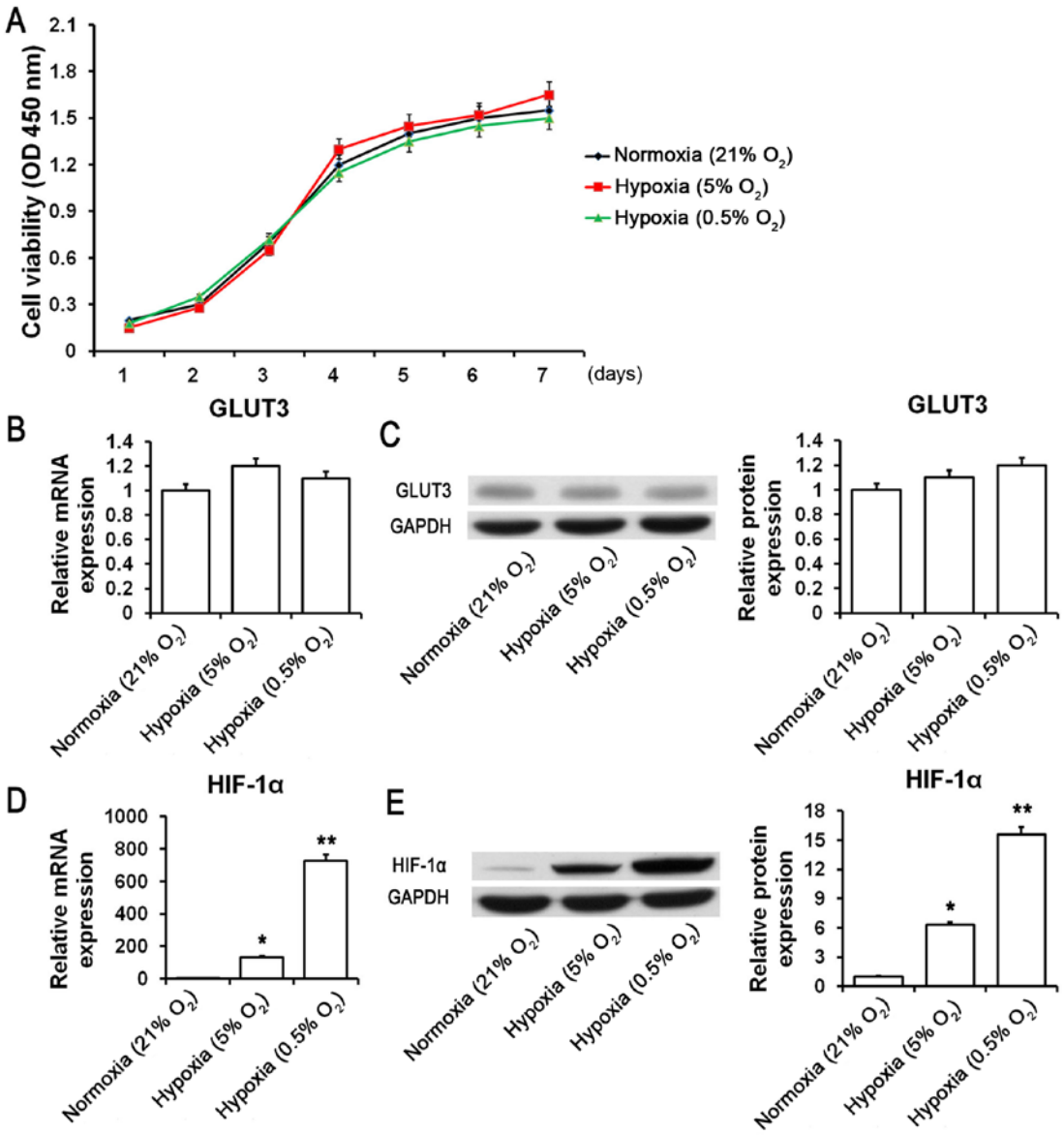

Figure 2. Changes in cell proliferation of SMSCs under normoxic (21% O_2), hypoxic (5% O_2) and severe hypoxic (0.5% O_2) conditions. (A) Cell proliferation of SMSCs was determined by CCK-8 assay, and no obvious differences were found under the different oxygen conditions. (B and C) No significant changes were found for mRNA and protein expression of GLUT3 as detected by RT-PCR and western blot analysis, respectively. (D and E) The mRNA and protein expression of HIF-1α were both significantly upregulated under hypoxic (5% O_2) and severe hypoxic (0.5% O_2) conditions following RT-PCR and western blot detection. All data are averages ± SD (error bars) of at least 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, as compared with the normoxia (21% O_2) group. SMSCs, synovial mesenchymal stem cells; GLUT3, glucose transporter 3 (also known as SLC2A3); HIF-1α, hypoxia-inducible factor-1α.

obvious changes were found in the protein expression levels of cleaved caspase 3, Bax and Bcl-2 (Fig. 3A). The level of apoptosis determined by flow cytometry also revealed no significant changes (Fig. 3B). These results indicated that HIF-1α may be engaged in regulating the proliferation and apoptosis of SMSCs.

Effect of HIF-1α on SMSCs under hypoxic condition. To characterize the specific mechanism of HIF-1α on SMSCs, siRNA-induced HIF-1α knockdown was conducted under severe hypoxic (0.5% O_2) and hypoxic (5% O_2) conditions. Our findings suggested that the cell viability and GLUT3 mRNA and protein expression were markedly suppressed following siRNA-induced HIF-1α knockdown (Fig. 4A-C). The mRNA and protein expression of HIF-1α were significantly suppressed following si-HIF-1α knockdown under severe hypoxic (0.5% O_2) and hypoxic (5% O_2) conditions (Fig. 4D and E). In addition, the protein expression of cleaved caspase 3 and Bax was significantly increased and the Bcl-2 expression was significantly decreased following HIF-1α knockdown (Fig. 5A). Flow cytometric analysis revealed that the apoptosis ratio was significantly increased following siRNA-induced HIF-1α knockdown (Fig. 5B). These results suggested that HIF-1α could effectively improve the tolerance to hypoxia of SMSCs, which might promote cellular proliferation and prevent the apoptosis of SMSCs under hypoxic conditions.

Discussion

Hypoxia occurs commonly in many types of mammalian tissues, such as synovium, cartilage and intervertebral

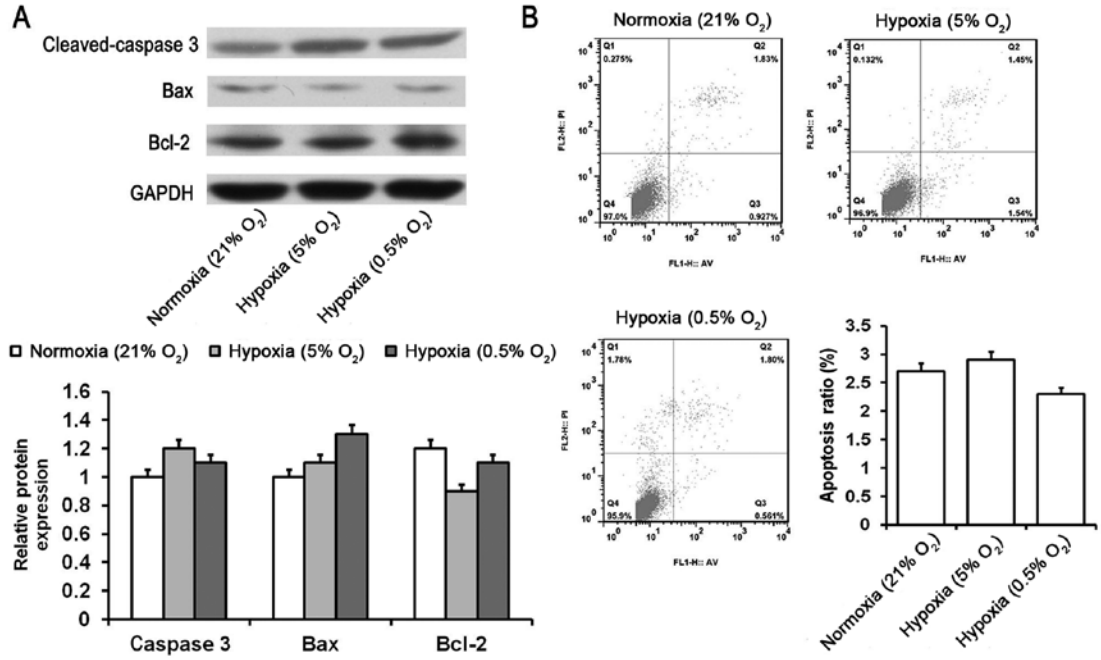

Figure 3. Changes in cell apoptosis of SMSCs under normoxic (21% O_2), hypoxic (5% O_2) and severe hypoxic (0.5% O_2) conditions. (A) The protein expression of cleaved-caspase 3, Bax and Bcl-2 revealed no significant changes by western blot analysis. (B) The apoptosis ratio revealed no significant changes following flow cytometry detection. All data are averages ± SD (error bars) of at least 3 independent experiments. SMSCs, synovial mesenchymal stem cells.

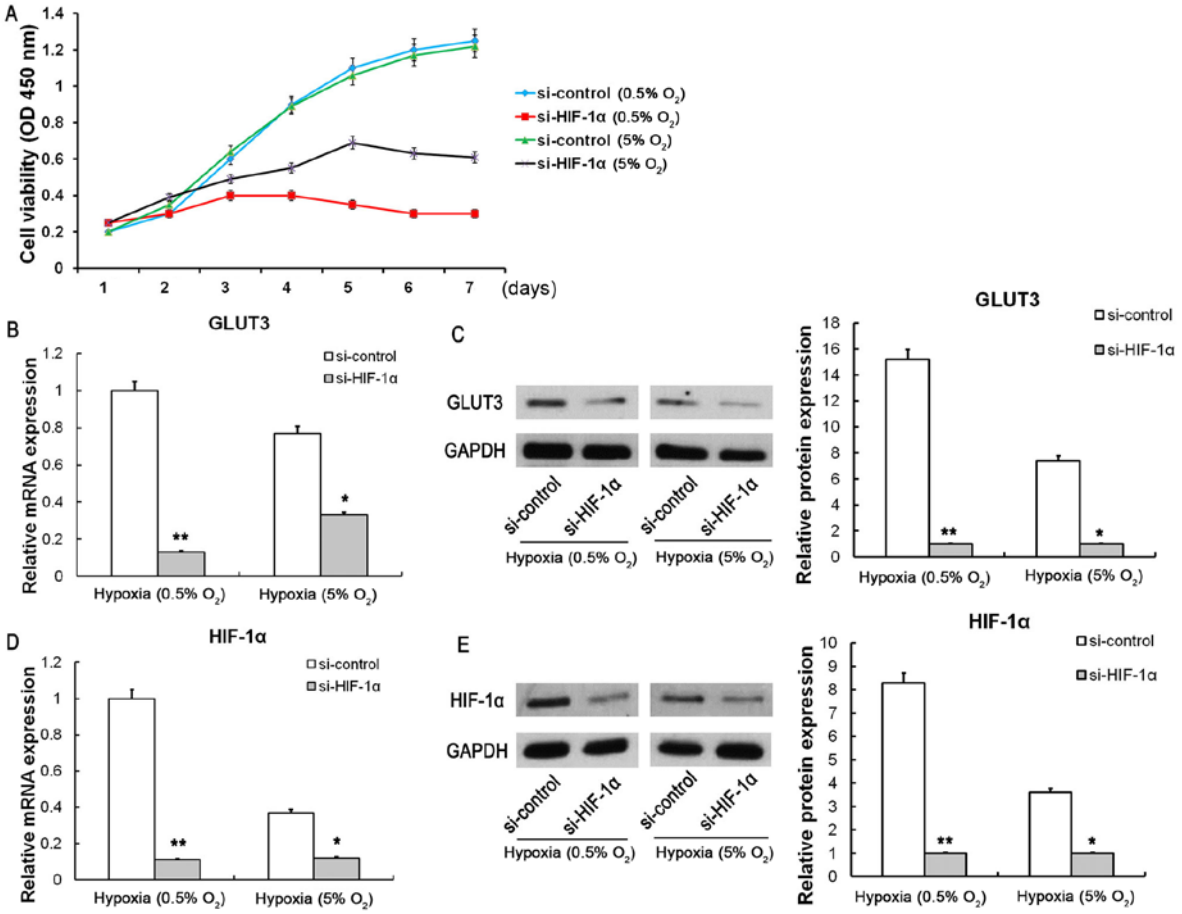

Figure 4. Changes in cellular proliferation and apoptosis of SMSCs followed si-HIF-1α knockdown under severe hypoxic (0.5% O_2) and hypoxic (5% O_2) conditions. (A) Cell proliferation of SMSCs detected by CCK-8 assay was effectively suppressed followed si-HIF-1α knockdown. (B and C) The mRNA and protein expression of GLUT3 as detected by RT-PCR and western blot analysis were significantly downregulated followed si-HIF-1α knockdown. (D and E) The mRNA and protein expression of HIF-1α as detected by RT-PCR and western blot analysis were significantly suppressed followed si-HIF-1α knockdown. All data are averages ± SD (error bars) of at least 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, compared with the si-control group. SMSCs, synovial mesenchymal stem cells; HIF-1α, hypoxia-inducible factor-1α.

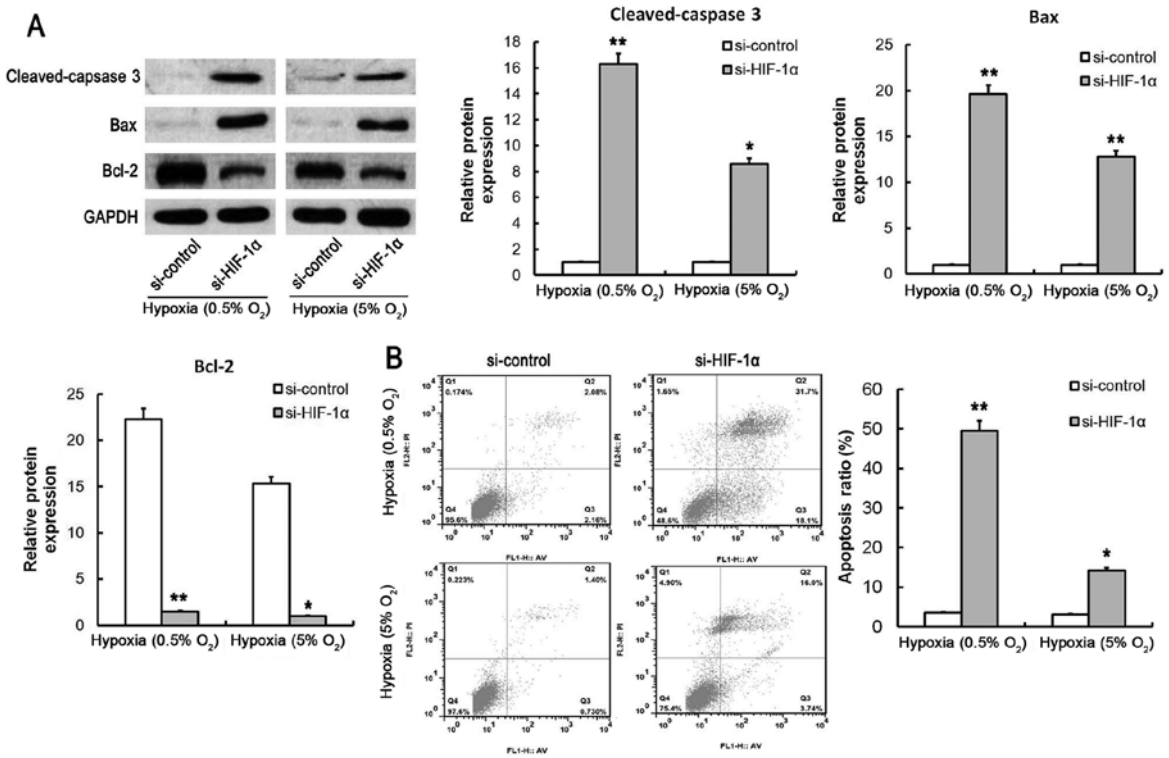

Figure 5. Changes in the cell apoptosis of SMSCs followed si-HIF-1α knockdown under severe hypoxic (0.5% O_2) and hypoxic (5% O_2) conditions. (A) The protein expression of cleaved caspase 3 and Bax detected by western blot analysis was significantly increased and Bcl-2 expression was significantly decreased followed si-HIF-1α knockdown. (B) The apoptosis level as detected by flow cytometry was obviously increased following si-HIF-1α knockdown. All data are averages ± SD (error bars) of at least 3 independent experiments. *P<0.05, **P<0.01, compared with the si-control group. SMSCs, synovial mesenchymal stem cells; HIF-1α, hypoxia-inducible factor-1α.

discs, where cells with a poor O_2 supply can adapt to these low oxygen tension conditions by activating survival pathways (31-33). Synovium is a type of connective tissue covering most of the inner joint structure, including the inner surface of the joint fibrous capsule, intra-articular ligaments, tendons, and bone surface (34). Synovial mesenchymal stem cells (SMSCs) are the principal cell type of synovial tissue, which are related to the synthesis of hyaluronate and the secretion of synovial fluid (35,36). In the present study, to explore the effects of hypoxia on SMSCs, SMSCs were cultured under normoxic and hypoxic conditions. We found that there were no significant changes in the cellular proliferation and apoptosis of SMSCs under normal and hypoxic conditions. Apoptotic proteins, such as cleaved caspase 3, Bax and Bcl-2 also exhibited no significant change under normal and hypoxic conditions. However, the levels of HIF-1α mRNA and protein were significantly increased under hypoxic condition. These results demonstrated that HIF-1α may be expressed in large quantities under hypoxic conditions, which could improve the hypoxic tolerance and inhibit the spontaneous apoptosis of SMSCs.

To verify the specific role of HIF-1α in SMSCs under hypoxic conditions and to further explore the relationship between HIF-1α and the apoptosis of SMSCs, we conducted a selective inhibition of HIF-1α under 0.5% O_2 and 5% O_2 hypoxic conditions by siRNA interference. Our findings revealed that the cellular proliferation was significantly inhibited and the apoptosis ratio was significantly increased after HIF-1α knockdown. Western blot assays demonstrated that the protein expression levels of cleaved caspase 3 and Bax were obviously increased, and Bcl-2 expression was significantly inhibited. These results suggest that the HIF-1α gene plays a protective role in cellular proliferation and apoptosis of SMSCs in a hypoxic environment.

The particular mechanisms may be summarized as follows. In the adaptive response of cells to changes in oxygen, the activated HIF-1α induced by hypoxia was found to stimulate more than 100 downstream genes for mediating the process of cell proliferation and survival (37,38). These metabolic processes are involved in cell proliferation, migration, glucose metabolism and angiogenesis (39,40). Prior studies have noted that HIF-1α mediates adaptive metabolic responses to hypoxia by decreasing flux via the tricarboxylic acid cycle and increasing flux via the glycolytic pathway, in order to meet the energy demands of rapidly growing tissue (23,41). Therefore, cells under hypoxic conditions tend to burn more glucose in order to achieve adequate energy requirements for cell survival. The evidence suggests that HIF-1α mediates this metabolic transformation by inducing the overexpression of glucose transporters (GLUTs) and enzymes that are involved in the glycolysis pathway, thereby increasing glucose entry into cells (42,43).

In the present study, mRNA and protein expression of GLUT3 were markedly suppressed following HIF-1α knockdown, suggesting that HIF-1α may promote the glucose metabolic conversion of SMSCs under hypoxic condition. As mentioned in a literature review, the HIF-1α transcriptional induction of a variety of angiogenic factors, such as vascular

endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) promotes the synthesis of endogenous angiogenesis, which in turn promotes neovascularization to enrich cells for growth (44). Moreover, HIF-1α was found to promote cell migration into oxygen-rich regions through inducing secretion via transcriptional activation growth factors such as fibroblast growth factor 11 (FGF11), transforming growth factor β3 (TGF-β3), insulin-like growth factor (IGF) and epidermal growth factor (EGF) (45-47).

In conclusion, we preliminarily explored the effects of HIF-1α on the proliferation and apoptosis of human SMSCs, and the results suggest that HIF-1α activation in SMSCs is probably one of the key mechanisms mediating the ability of SMSCs to adapt to hypoxic environments.

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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

DH and XS conceived and designed the study. DH, XS, WZ and XG performed the experiments and analyzed the data. WZ and XG wrote the paper. DH and WZ reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval for this study was granted by the Institution Review Board of the Affiliated Hospital of Nanjing Medical University, and all study participants were recruited after providing informed written consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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