Original Article
The protective effect of leukemia inhibitory factor on apoptosis of BMSCs induced by hypoxia and serum-deprivation

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Abstract: Objectives: Bone marrow-derived mesenchymal stem cells (BMSCs) - based tissue engineering is an important strategy for treatment of bone defects. However, the ischemia environment limits the survival and biological functions of BMSCs. The present study aimed to investigate the effect of leukemia inhibitory factor (LIF) on the apoptosis of BMSCs induced by hypoxia and serum-deprivation (H&SD) as well as the underlying pathway mechanism. Methods: Mitochondrial membrane potential (MMP) was determined by flow cytometry. The apoptotic phenomenon of nuclear morphology was detected by fluorescence microscope. The ratio of apoptotic BMSCs was investigated by Annexin V/propidium iodide (PI) double staining and flow cytometric analysis. The expression of apoptosis-related molecules was detected by quantitative polymerase chain reaction (qPCR) and western blotting. Results: H&SD treatment induced a series of apoptotic phenotypes, including the downregulation of MMP, the apoptotic phenomenon of nuclear morphology, the increased rate of BMSCs at early and late apoptotic stage, and the reduced B-cell lymphoma-2 (Bcl-2)/Bcl-2-associated X (Bax) ratio. Administration of recombinant LIF alleviated the apoptosis of BMSCs induced by H&SD, which was reflected in recovery of MMP, morphology of nuclei, rate of apoptotic cells and inhibition of cleaved Caspase-3. The results of western blot demonstrated that phosphorylation of janus kinase (JAK) 1 and signal transducer and activator of transcription (STAT) 3 was inhibited by H&SD treatment, which was upregulated by LIF administration. JAK1-specific inhibitor GLPG0634 or STAT3-specific inhibitor S3I-201 eliminated the protective effects of LIF on the apoptosis of BMSCs. Conclusion: These data indicated that LIF played a protective role in apoptosis of BMSCs induced by ischemia via activating JAK1/STAT3 signaling pathway.

Keywords: BMSCs, LIF, hypoxia, serum-deprivation, apoptosis, JAK1/STAT3

Introduction

Bone defects caused by various reasons are a big risk for quality of life of patients and a heavy burden on society [1]. Cell-based bone tissue engineering is an important approach to treat bone defects. Various adult stem cells, especially bone marrow-derived mesenchymal stem cells (BMSCs) are the common seed cells, due to the sufficient sources and convenience of harvest [2]. BMSCs have many biological ad-

vantages for regeneration, including multipotent differentiation potentials, supportive capacity, anti-inflammatory and immune-modulatory properties [3]. The application of BMSCs in various tissue injuries has been translated from laboratory research into clinical practice. For example, endogenous or exogenous BMSCs were utilized to achieve the repair of long bone and vertebrae fractures caused by trauma or tumor [3]. Also, the potential of BMSCs therapy in live cirrhosis has been explored in different

clinical trials, which showed extremely promising outcomes in hepatic regeneration [4]. Intracerebral BMSCs transplantation has been showed to be a promising therapeutic approach to improve neurogenesis in pre-clinical stroke models [5].

However, BMSCs-based bone tissue engineering still faces many challenges like uncertain efficiency. One of the reasons is that bone defects are always accompanied with insufficiency of blood supply and nutrient-deficiency. Whether by direct injection or implantation using scaffold, BMSCs will face a harsh living microenvironment. It is proved that hypoxia, nutrient-deficiency, accumulation of inflammatory cytokines will induce cell apoptosis [6, 7]. These factors will disrupt the normal physiological functions and activities of cells and negatively affect the efficacy of BMSC-based bone tissue engineering. Existing studies have observed that human mesenchymal stem cells can survive and maintain their activity for at least six weeks after being implanted into nude mice [8]. However, other studies have found that within 4 weeks after implantation of the BMSCs-scaffold complex, the cell number dropped dramatically [9]. Mass death of BMSCs occurred after three days in a hypoxic environment [10]. When BMSCs were implanted into the ectopic osteogenic site, they started to die within three days and were barely detectable after 14 days [11]. Similarly, when sheep mesenchymal stem cells with composite scaffolds were implanted in vivo, the cells almost disappear after 14 days [12]. Despite the different scaffold materials used in these studies, the survival and activity of BMSCs in hypoxic environment still need to be maintained or enhanced. It can be seen that how to maintain the survival of stem cells and truly exert the functions of implanted BMSCs is an unavoidable basic problem in bone tissue engineering. Accordingly, treatment of seed BMSCs to help them function better in harsh environment, such as administration of helpful cytokines.

Leukemia inhibitory factor (LIF) belongs to the interleukin-6 family and is a cytokine which plays multiple biological functions. Various kinds of tissues and cells can spontaneously secret or induce via stimulation LIF secretion and its receptors [13]. BMSCs, hematopoietic stem cells, embryonic stem cells and osteo-

blasts are target cells of LIF. The current study found that the main functions of LIF include maintaining the self-renewal of stem cells [14, 15], participating in the regulation of bone metabolism [16, 17] and so on. The mature LIF protein is a secreted protein consisting of at least 179 amino acids. The molecular weight of LIF core protein is 20 KD, and the molecular weight can vary from 32 KD to 67 KD according to the degree of glycosylation. At the amino acid sequence level, mouse, rat and human LIF proteins share a high degree of homology [18]. In terms of secondary structure, LIF protein has 4 α-helices. The tertiary structure of LIF has four helices A, B, C and D from the N-terminus to the C-terminus, which are linked together by two long loops of AB and CD and a short loop of BC [19]. There are three functional sites on the epitope of LIF, which can interact with LIFR and the gp130 receptor subunit respectively [20]. LIF first binds to LIF receptor (LIFR) on the target cell membrane in low-affinity form, and then binds to gp130 to form a high-affinity heterodimeric complex, which initiates a series of signaling molecules and transduction pathways in the cell to play its biological role. Gp130 is a common signal transduction subunit owned by members of the IL-6 cytokine family. Current studies have found that the activation of gp130 can activate signaling pathways such as janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling [21].

LIF plays an important role in the biological functions of various kinds of stem cells. For embryonic stem cells (ESCs), LIF is able to maintain the self-renewal and participate in the regulation of differentiation through activating STAT3 and following cascade molecules [22]. As for hematopoietic stem cells (HSCs), LIF can promote the proliferation of primitive multipotential progenitor cells and stimulate the quiescent cells to enter the cell cycle [23] and protect HSCs after retroviral transfection to maintain their survival [24]. In mesenchymal stem cells (MSCs), LIF plays a vital role in maintaining the multidirectional differentiation ability. The secretion level of is an important indicator of the early undifferentiated stat of MSCs [25]. Additionally, previous study of our group found that a sustained hypoxic microenvironment induced upregulation of LIF expression in a rat model of periodontal loading [26]. Another study showed that injection of LIF cDNA plas-

mid promoted ossification of bone defect [27]. These studies suggested that LIF can protect osteoblast-related cells and promote bone tissue repair in local hypoxia microenvironment.

Given the above, the aim of the present study is to explore the potential protective effect of LIF on the BMSCs in ischemia environment and the involved pathway mechanism.

Materials and methods

Isolation and culture of BMSCs

Based on the classical method as previously reported [28], rat bone marrow mesenchymal stem cells (BMSCs) were isolated from male wistar rats, age 35-38 days and weighing 90±10 g. Samples of bone marrow were harvested in accordance with the Animal Care and Use Committee approval of School and Hospital of Stomatology, Wuhan University. The femora were excised aseptically, cleaned of soft tissues, and passed through 3 washes with Phosphate Buffered Saline (PBS). The ends of the bones were removed, and the marrow flushed out. The released cells were collected in two 75 cm² flasks (Corning, USA) containing 10 ml of 10% fetal bovine serum (FBS, Hyclone, USA) in α-MEM. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2, at 37°C. After 72 h, all medium was aspirated and replaced to remove the non-adherent cells. The medium was replaced every 2-3 days.

Induced ischemia and treatment of BMSCs

Hypoxia and serum deprivation (H&SD) condition was designed to mimic the in vivo conditions of ischemia. Briefly, cells were washed with serum-free α-MEM twice and placed in serum-free medium containing the required treatment, then were cultured in a hypoxic incubator under 1% O_2 and 5% CO_2 at 37°C. For normal control, cells were incubated in complete medium and in a humidified atmosphere of 20% O_2 and 5% CO_2 at 37°C. Cells exposed to hypoxia & SD alone were used as the apoptotic controls. In preliminary experiments, a dose-dependent study involving culture of cells with recombinant rat leukemia inhibitory factor (LIF) (Millipore, LIF 3010, USA) of 0, 10, 20, 40 and 80 ng/ml was carried out and 40 ng/ml was determined as the optimum concentration of LIF for modulating typical apoptosis. The cells

were then exposed to hypoxia & SD in the presence of LIF with/without 100 µM S3I-201 (STAT3 inhibitor, Selleck) or 1 µM GLPG0634 (JAK1 inhibitor, Selleck).

Assessment of morphological changes

Chromosomal condensation and nuclear fragmentation were evaluated using Hoechst 33258 (Beyotime, China). BMSCs were fixed in 4% paraformaldehyde for 30 min at room temperature. After fixing, the cells were carefully washed twice with PBS and then exposed to 5 µg/ml Hoechst 33258 for 10 minutes in the dark. All samples were observed and photographed using a fluorescence microscope. Apoptotic cells were characterized by morphological changes such as cell shrinkage, nuclear condensation, and fragmentation.

Detection of apoptotic cells with flow cytometry

Apoptosis of BMSCs with different treatment was assessed by Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (C1062, beyotime) according to the manufacturer's protocols. In brief, cells were collected, washed with ice-cold PBS, and then resuspended in 500 µl 1 × binding buffer solution with 5 µl Annexin V-FITC and 10 µl PI. After the incubation of 5 min at room temperature in the dark, the samples were immediately analyzed by bivariate flow cytometry. 1×10^4 cells were analyzed in each of the samples.

Measurement of mitochondrial membrane potential

Rhodamine123 has been commonly used for monitoring the mitochondrial transmembrane potential. Briefly, MSCs with different treatment were collected and stained in cell culture medium containing 0.1 µM Rhodamine123 (Selleck) for 30 minutes at 37°C in the dark. After washing twice with pure culture medium, the staining of samples was quantified by flow cytometry. 1×10^4 cells were analyzed in each of the samples.

Quantitative polymerase chain reaction (qPCR) of B-cell lymphoma-2 (Bcl-2)/Bcl-2-associated X (Bax) and Bcl-2 interactingmediator of cell death (Bim) expression

Total RNA was isolated from cultured MSCs using TRIzol reagent (Invitrogen, Carlsbad, CA,

Table 1. Primer sequences for qPCR (5'-3')

Genes	Forward	Reverse
Bcl-2	ACTTCTCTCGTCGCTACCGTCGC	AGAGCGATGTTGTCCACCAGGG
Bax	CCAGGACGCATCCACCAAGAAG	CCCAGTTGAAGTTGCCGTCTGC
LIF	TTTGCCGTCTGTGCAACAAG	TGGACCACCGCACTAATGAC
LIFR	CCAGATTCCCCAAGGTCCAC	AAGGACCACCCTCCTCCATT
GAPDH	GACAACTTTGGCATCGTGGA	ATGCAGGGATGATGTTCTGG

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; LIF, leukemia inhibitory factor; LIFR, Leukemia inhibitory factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

USA) according to the manufacturer's instructions. 1 μg of RNA was reverse transcribed to cDNA (20 μl) with PrimeScript RT reagent Kit With gDNA Eraser (Takara Bio Inc., Tokyo, Japan). Gene sense and antisense primers were listed in **Table 1**. Quantitative real-time PCRs were performed with the SYBR Premix Ex Taq II (Takara Bio Inc., Tokyo, Japan) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The standard PCR conditions were as follows: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, 60°C for 34 seconds. All quantitations were normalized to an internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples of cells cultured in normal conditions were set as the control group. The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression.

Protein extraction and western blot analysis

For analysis of protein levels, cells were placed on ice and rinsed three times with cold PBS. Then RIPA lysis buffer (Beyotime, China) was used to lyse the cells to isolate total proteins according to its protocol. Western blots were performed as described previously. Stat3, phosphorylated Stat3 (p-Stat3) (Abcam, Cambridge, UK) and caspase-3 (Cell Signaling Technology, Danvers, MA, USA) monoclonal antibodies were used in conjunction with a horseradish peroxidase-conjugated secondary anti-body (Pierce). Equal loading was ensured using a GAPDH monoclonal primary antibody (Sigma).

Statistical analysis

Data was expressed as mean ± SD. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were evalu-

ated using Student's t-test. *P*-value < 0.05 was required for statistical significance.

Results

Identification of rat bone marrow mesenchymal stem cells

The BMSCs were isolated from 4-week-old male wistar rats via whole bone marrow adherent method. At passage 3, the cells showed a uniform fusiform morphology and adherent growth (**Figure 1A**). After osteogenic differentiation for 14 days, the calcium depositions were observed by alizarin red staining (**Figure 1B**). After adipogenic induction for 14 days, the lipid droplets were detected by oil red O staining (**Figure 1C**). Also, the cell markers of third-passage cells were determined by flow cytometry. The results showed that the cells strongly expressed the cell markers of mesenchymal stem cells CD90, CD29, CD105 and CD44 (**Figure 1D-G**), and the hematopoietic cell marker CD45 was negatively expressed (**Figure 1H**). The above data confirmed that the isolated cells were mainly BMSCs.

Hypoxia and serum-deprivation treatment induced apoptosis of BMSCs

Ischemia model of BMSCs was mimicked by hypoxia and serum-deprivation combination treatment (H&SD) in vitro. The apoptotic features of BMSCs were detected from various aspects, including morphology, mitochondrial membrane potential (MMP), flow cytometric analysis of Annexin V/PI double staining and expression alterations of apoptosis-related molecules.

As the earliest event in the apoptosis cascade, the decrease of MMP is an important characteristic of cell apoptosis. Once the MMP collapses, cell apoptosis will be irreversible. As shown in **Figure 2A**, **2B**, hypoxia and serum-free treatment led to a time - dependent down-regulation of MMP in BMSCs.

In the process of apoptosis, the change of nuclear morphology is the most obvious evidence. Chromatin in the nucleus of cells at early and middle stage of apoptosis aggregated and distributed at the edge of nuclear mem-
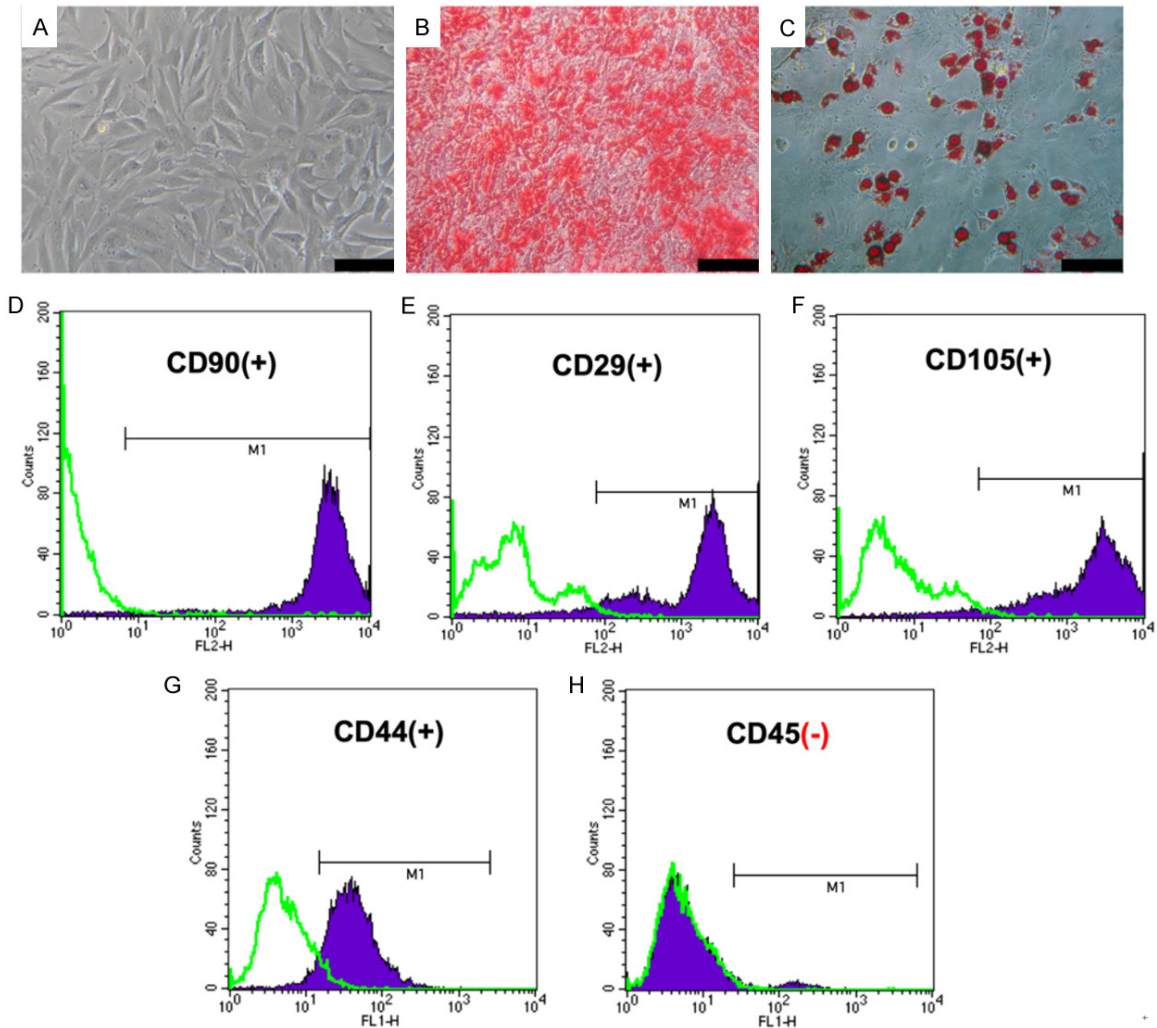
Figure 1. Identification of rat bone marrow-derived mesenchymal stem cells (BMSCs). A. The general view of BMSCs at passage 3 under phase-contrast microscopy (bar = 100 μm; magnification = 200×). B. The alizarin red staining of BMSCs after 14 days of osteogenic induction. C. The oil red O staining of BMSCs after 14 days of adipogenic induction. D-H. The flow cytometry showed the BMSCs were positive for cell markers of mesenchymal stromal cells CD90, CD29, CD105 and CD44, and negative for hematopoietic cell marker CD45.
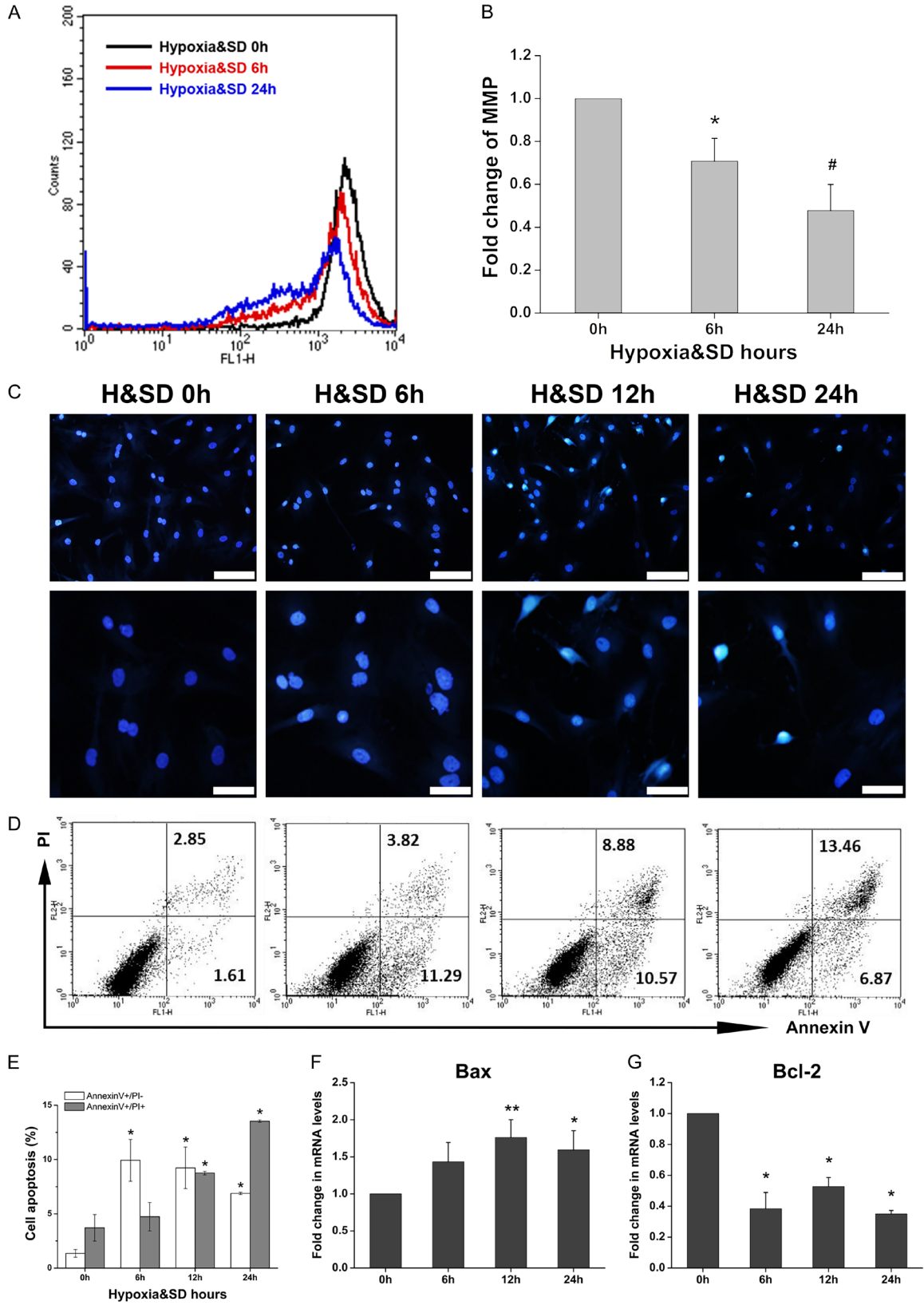
brane. At the late stage of apoptosis, nuclear fragmentation occurred, and highly aggregated apoptotic bodies were observed. As shown in **Figure 2C**, the nuclei of BMSCs in normal culture conditions exhibited uniform blue fluorescence. After hypoxia and serum-deprivation treatment for 6 hours, chromatin aggregated in some cells and the nuclear membrane budded. After 12 hours, highly agglutinated chromatin with high fluorescence was detected, located at the edge of the nuclei. After 24 hours, the proportion of apoptotic nuclei further increased.

Annexin V/PI double staining and flow cytometry were used to detect the rate of apoptotic BMSCs. The population of Annexin V+/PI- was

the cells at early stage of apoptosis and that of Annexin V+/PI+ was at the late stage of apoptosis. As shown in **Figure 2D**, **2E**, the rates (%) of cells at early apoptotic stage of H&SD for 0, 6, 12, 24 h were 1.36±0.35, 10.13±1.92, 9.24±1.91, 6.89±0.10 respectively, while the rates (%) of cells at late apoptotic stage were 3.72±1.22, 4.75±1.31, 8,76±0.17, 13.54± 0.11. The data demonstrated that the early apoptosis rate of the treated BMSCs was significantly upregulated after 6, 12 and 24 hours, while the late apoptosis rate was significantly increased after 12 and 24 hours.

At the molecular level, qPCR was applied to observe the expression alteration of apoptosis-
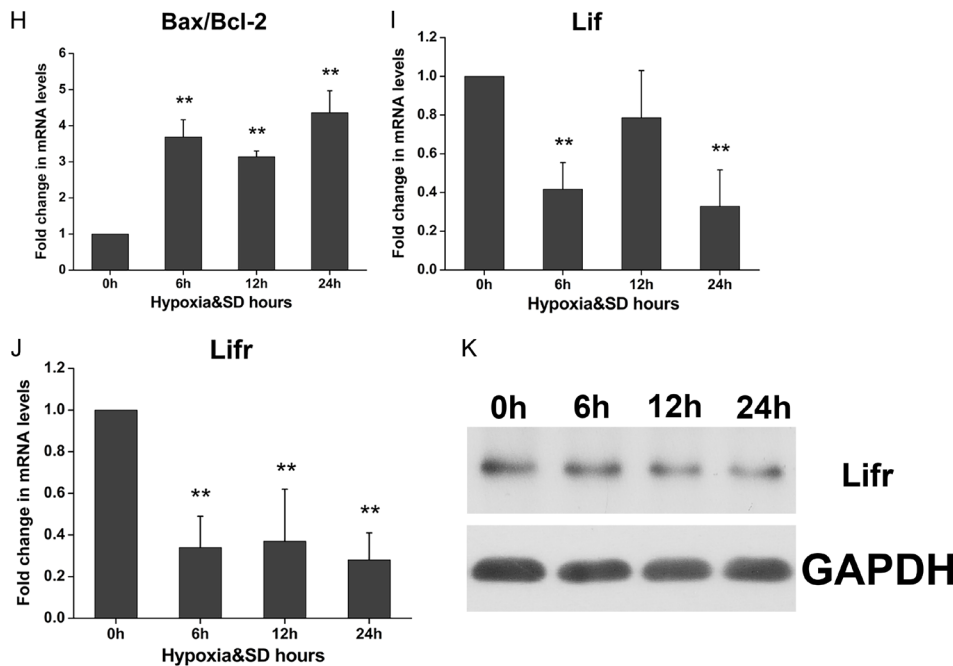
Figure 2. Hypoxia and serum-deprivation (H&SD) treatment induced apoptosis of BMSCs. (A) Typical histogram of flow cytometric analysis of Rh123 staining. (B) Quantitative analysis of mean fluorescence intensity. (C) Nuclear morphology of BMSCs at different timepoints of H&SD treatment after Hoechst 33258 staining (upper, bar = 100 μm, magnification = 200×; lower, bar = 50 μm, magnification = 400×). (D) Typical four-quadrant diagrams of flow cytometric analysis of Annexin V/PI double staining. (E) Quantitative analysis of the rate of cell apoptosis. The qPCR results showed the mRNA expression alterations of Bcl-2-associated X (Bax) (F), B-cell lymphoma-2 (Bcl-2) (G), Bax/Bcl-2 (H), Leukemia inhibitory factor (LIF) (I) and Leukemia inhibitory factor receptor (LIFR) (J). (K) Western blot showed the protein level of LIFR was downregulated after H&SD treatment ($^*P < 0.05$, $^{**}P < 0.01$).

related genes. The results revealed that Bax mRNA expression was upregulated, and Bcl-2 mRNA expression was downregulated after H&SD treatment (**Figure 2F-H**). At last, the LIF and its receptor LIFR were decreased (**Figure 2I-K**).

Above all, hypoxia and serum-deprivation treatment resulted in typical apoptotic phenomenon in BMSCs. More importantly, LIF and LIFR were decreased in the apoptosis process.

LIF rescued the apoptosis of BMSCs induced by hypoxia and serum-deprivation

According to the previous experimental data, H&SD treatment for 6 hours when the early apoptosis was the most obvious was chosen as the observation time in this part. Based on the exploration of experimental conditions (**Figure 3A**), the administrative concentration of recombinant LIF was set as 40 ng/ml.

As shown in **Figure 3B**, after stained with Rh123, BMSCs in normal culture condition showed bright green fluorescence, while the

BMSCs with H&SD treatment showed dim green fluorescence, LIF administration enhanced the fluorescence of apoptosis model. Flow cytometry was used to analyze the MMP of BMSCs, consistent with the observation of fluorescence microscope, the results showed that MMP was downregulated by H&SD treatment and rescued by LIF administration (**Figure 3C**, **3D**).

As to the morphology, which was shown in **Figure 3E**, the nuclei of the control group were complete and uniform, while the apoptotic group showed apoptotic characteristics. LIF administration played a therapeutic role, while LIF neutralizing antibody eliminated it.

The flow cytometric analysis of Annexin V/PI double staining (**Figure 3F**) demonstrated that LIF attenuated the rates of early, late (**Figure 3G**), and general (**Figure 3H**) apoptotic BMSCs, while LIF neutralizing antibody counteracted the effect.

At last, western blot was applied to detect the cleaved Caspase-3 in apoptosis process of BMSCs. As shown in **Figure 3I**, no cleaved
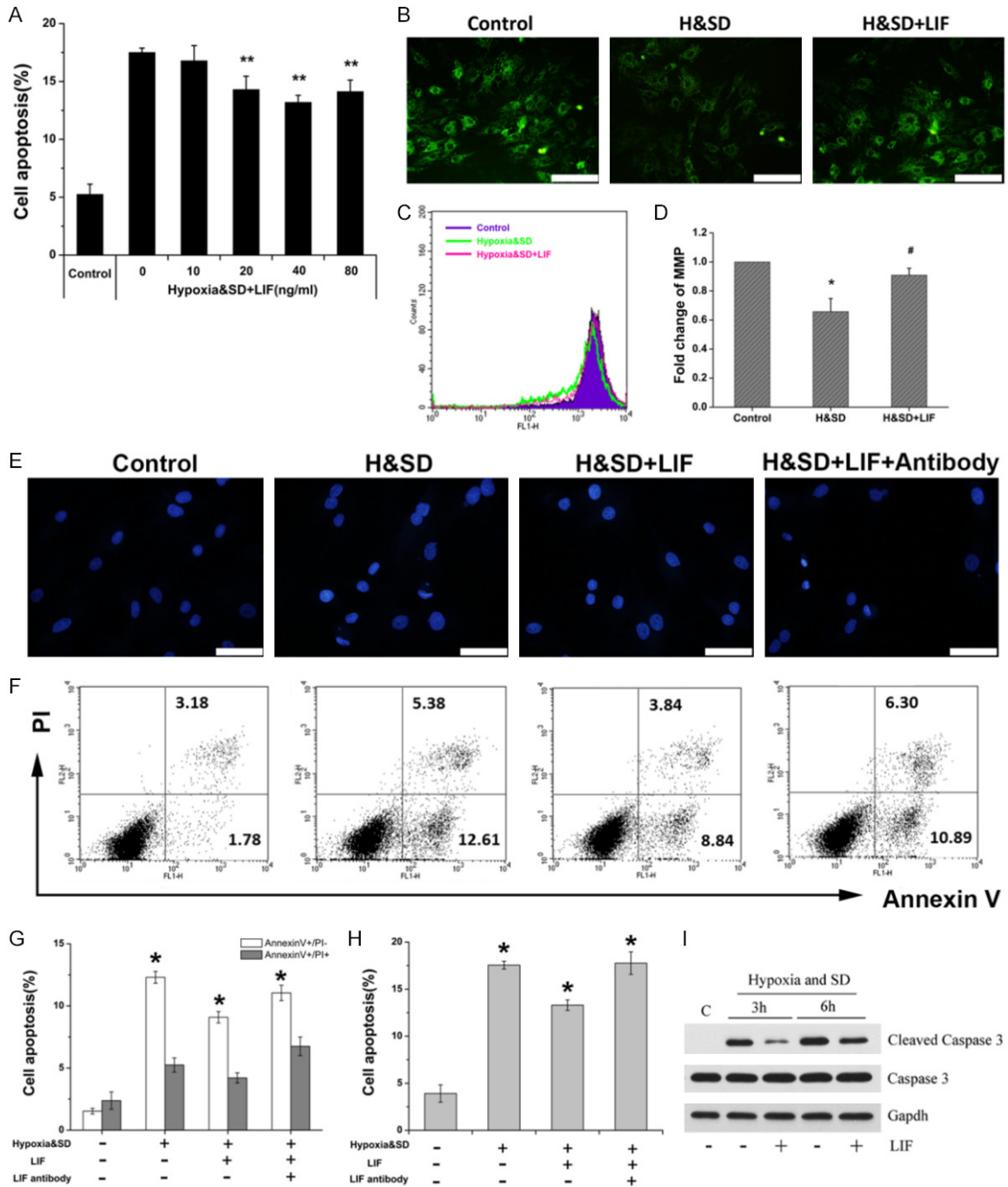
Figure 3. Leukemia inhibitory factor (LIF) rescued the apoptosis of BMSCs induced by hypoxia and serum-deprivation. A. The effect of different LIF concentration on the apoptosis rate. B. Typical views of Rh123-stained BMSCs under fluorescence microscope (bar = 50 μm, magnification = 400×). C. Typical histogram of flow cytometric analysis of Rh123 staining. D. Quantitative analysis of mean fluorescence intensity. E. Nuclear morphology of BMSCs at different timepoints of H&SD treatment after Hoechst 33258 staining (bar = 50 μm, magnification = 400×). F. Typical four-quadrant diagrams of flow cytometric analysis of Annexin V/PI double staining. G. Quantitative analysis of the rate of early (Annexin V+/PI-) and late (Annexin V+/PI+) cell apoptosis. H. Quantitative analysis of the rate of general cell apoptosis. I. Western blot of cleaved and general Caspase 3 ($^*P < 0.05$, $^{**}P < 0.01$).

Caspase-3 was detected in control group of BMSCs. Cleaved Caspase-3 increased after H&SD treatment and LIF administration significantly inhibited it.
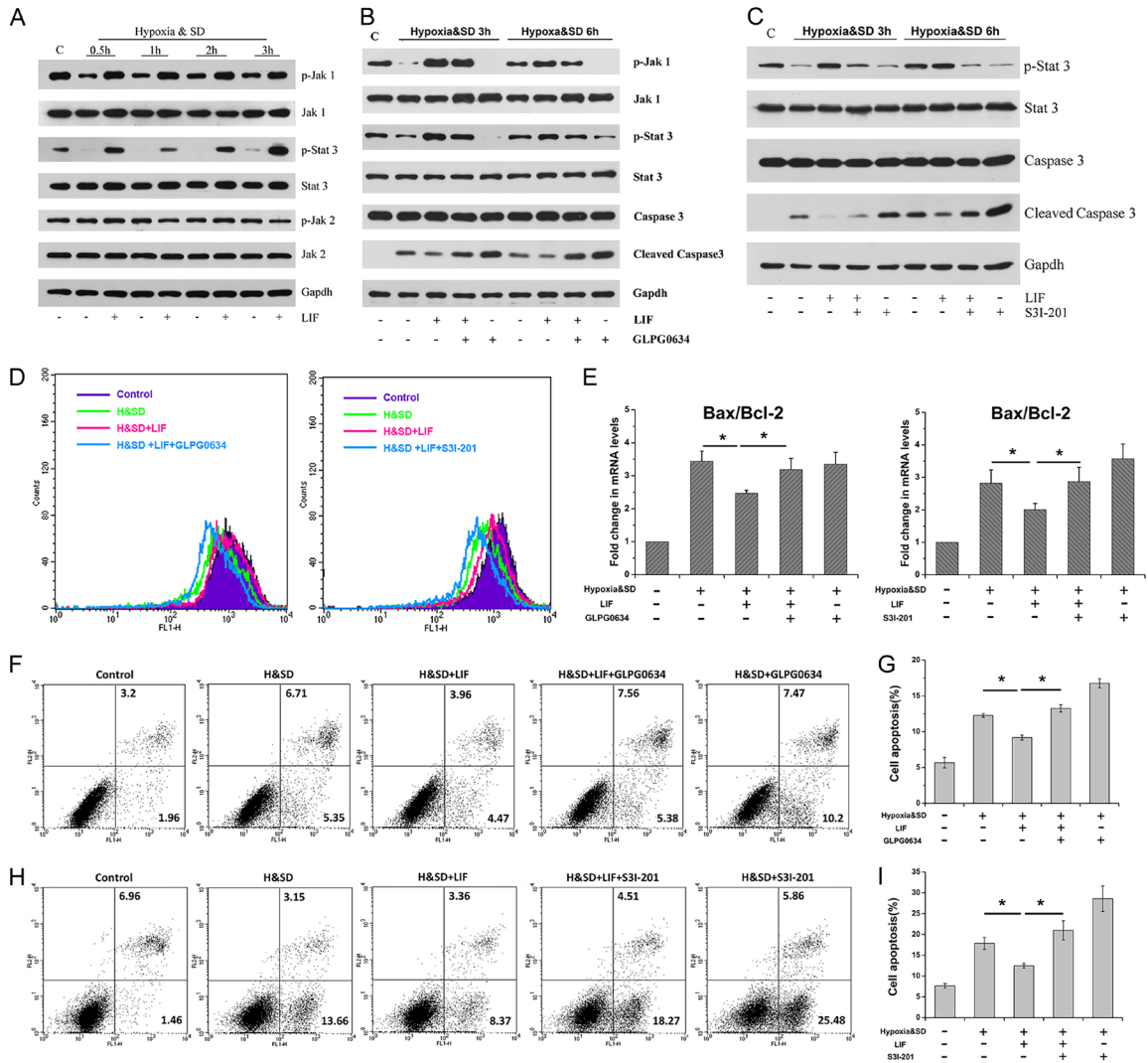
Figure 4. The signaling pathway mechanism of LIF-mediated anti-apoptosis effect. (A) Phosphorylation of janus kinase (JAK) 1, JAK2 and signal transducer and activator of transcription (STAT) 3 in H&SD-treated BMSCs with or without LIF. (B) Effect of LIF and/or JAK1-specific inhibitor GLPG0634 on activation of JAK1, STAT3 and Caspase 3. (C) Effect of LIF and/or STAT3-specific inhibitor S3I-201 on activation of STAT3 and Caspase 3. (D) Typical histogram of flow cytometric analysis of Rh123 staining in different groups of BMSCs. (E) Effect of LIF and/or GLPG0634 or S3I-201 on the Bax/Bcl-2 ratio. (F) Typical four-quadrant diagrams of flow cytometric analysis of Annexin V/PI double staining in H&SD-treated BMSCs with LIF and/or GLPG0634. (G) Quantitative analysis of the rate of general cell apoptosis in (F). (H) Typical four-quadrant diagrams of flow cytometric analysis of Annexin V/PI double staining in H&SD-treated BMSCs with LIF and/or S3I-201. (I) Quantitative analysis of the rate of general cell apoptosis in (H) ($^{*}P < 0.05$).

Above all, it could be concluded that LIF played a protective role in apoptosis of BMSCs from various aspects including MMP, morphology and expressions of apoptosis-related molecules.

The mechanisms of LIF-mediated anti-apoptotic effect

LIF plays biological functions via binding to its receptor LIFR on the cell membrane and then

activating a series of signal molecules and transduction pathway, like JAK/STAT signaling pathway and so on.

First of all, to determine whether the JAK/STAT signaling pathway was involved in H&SD-induced apoptosis of BMSCs and the following therapeutic effect of LIF, western blot was conducted to detect the phosphorylation of JAK1, JAK2 and STAT3. As shown in **Figure 4A**, H&SD treatment significantly decreased the phos-
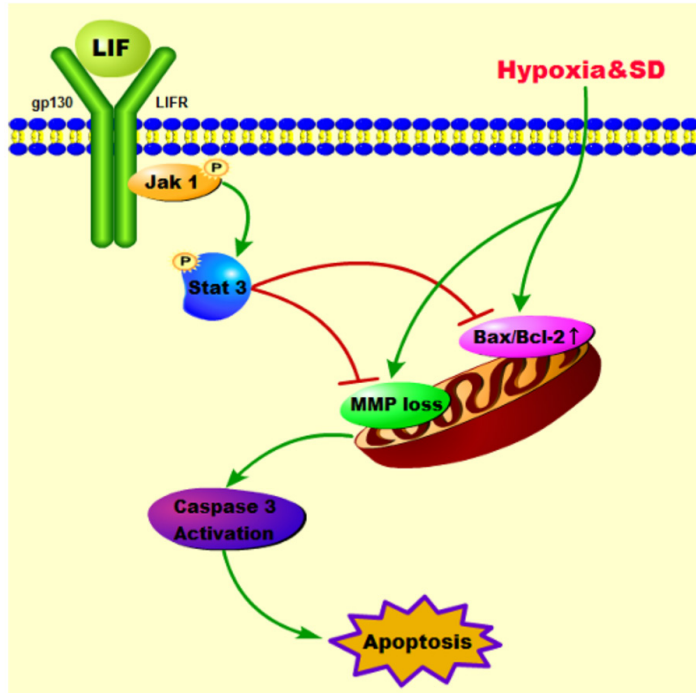
Figure 5. Diagram of the present study. In vitro ischemia model via hypoxia and serum-deprivation induced apoptosis of BMSCs, reflecting in loss of MMP, elevation of Bax/Bcl-2 ration and activation of Caspase-3. After bonding to LIFR, LIF promoted phosphorylation of Jak1 and Stat3, which led to the protection of mitochondrial function and inhibition of apoptosis.

phorylation of JAK1 and STAT3 in BMSCs, which was significantly rescued by LIF application. The phosphorylation of JAK2 did not change among all groups.

Accordingly, the JAK1-specific inhibitor GLP-G0634 and STAT3-specific inhibitor S3I-201 were introduced into the following experiments. As shown in **Figure 4B**, single GLPG0634 was proven to completely inhibit JAK1 phosphorylation and downstream STAT3 phosphorylation. LIF elevated the H&SD-induced downregulation of JAK1 and STAT3 phosphorylation and suppressed the H&SD-induced upregulation of cleaved Caspase-3, and the therapeutic effect of LIF was eliminated by GLPG0634. Similarly, as shown in **Figure 4C**, S3I-201 completely suppressed the activation of STAT3 and eliminated the therapeutic effect of LIF in apoptosis of BMSCs.

Furthermore, MMP detection was conducted to determine the effect of GLPG0634 and S3I-201 on protective role of LIF. The results demonstrated that GLPG0634 or S3I-201 signifi-

cantly decreased the LIF-elevated MMP in apoptosis (**Figure 4D**). Consistently, GLPG0634 or S3I-201 significantly upregulated the LIF-inhibited Bax/Bcl-2 ratio in apoptosis (**Figure 4E**). At last, Annexin V/PI double staining was used to analyze the apoptosis rate of BMSCs. The data demonstrated that GLPG0634 (**Figure 4F**, **4G**) or S3I-201 (**Figure 4H**, **4I**) raised the LIF-downregulated rate of apoptotic BMSCs after H&SD treatment.

The above results indicated that LIF played the protective role in H&SD-induced apoptosis of BMSCs via JAK1/STAT3 signaling pathway.

Discussion

For the first time, the present study demonstrated that LIF played a protective role in the apoptosis of BMSCs induced by hypoxia and serum-deprivation via activating JAK1/STAT3 signaling pathway (**Figure 5**).

Ensuring the survival and normal function of the implanted cells like BMSCs in the site of bone defect is the premise of bone tissue engineering. However, the poor survival status of seed cells after implantation caused by insufficient local blood supply limited the clinical application of BMSCs-based bone tissue engineering [11]. Due to the local lack of blood supply in the bone defect site, an environment of hypoxia and nutrient deficiency is formed. Both hypoxia and nutrient deficiency are risk factors that can induce apoptosis [29]. What's more, current studies have demonstrated that nutrient deprivation was a greater risk factor affecting cell survival than hypoxia. BMSCs were highly tolerant to simple hypoxia, and it has been reported that the growth of cells was not significantly affected at 2% oxygen concentration [30]. When human BMSCs were exposed to less than 1% oxygen, no obvious cell death was observed after 48 hours, but the death rate was significantly upregulated after complete serum deprivation [10]. Based on the two main characteristics of tissue ischemia in vivo,

the present study used hypoxic culture to simulate the hypoxic environment in vivo, and serum deprivation to simulate the nutrient deficient environment in vivo. An in vitro cell culture model with hypoxia and serum-deprivation for BMSCs was established to mimic tissue ischemia in vivo. Consistent with published literature [31], hypoxia and serum-deprivation induced apoptosis of MSC, as indicated by morphological changes of nuclei, dysfunction of MMP, accumulation of Bax, and Annexin V/PI double staining. Also, in accordance with another study [32], as to the treatment of apoptosis via additive, the timepoint of six hours was selected because hypoxia and serum-deprivation culture for six hours had induced obvious apoptotic phenomenon in BMSCs.

Current studies believed that there are two main pathways of apoptosis: the exogenous pathway mediated by death receptors and the endogenous pathway mediated by mitochondria. The mitochondrial pathway is the main approach for cells to undergo apoptosis after exogenous stimulation. The alteration of mitochondrial membrane potential is considered to be the specific early event of the mitochondrial apoptosis pathway [33]. Once stimulated by apoptotic signals, mitochondria release pro-apoptotic factors such as cytochrome C (Cyt C) to form apoptotic bodies, which further activate Caspase-3 to clew cellular structural proteins and cause DNA degradation, leading to cell apoptosis [34, 35]. The reduction of MMP and Cyt C release are regulated by Bcl-2 family members [36]. Bax and Bcl-2 are the most representative apoptosis-promoting and apoptosis-inhibiting proteins in the Bcl-2 family respectively, and Bax is the dominant inhibitor of Bcl-2 [37]. Bax and Bcl-2 form positive and negative regulation of cell apoptosis, and the ratio of Bax/Bcl-2 determines whether cells tend to be apoptotic [38]. In this study, the ratio of Bax/Bcl-2 in BMSCs increased significantly after hypoxia and serum-deprivation treatment, suggesting that the cells tended to undergo apoptosis. Additionally, hypoxia and serum-deprivation resulted in a time-dependent decrease in MMP, indicating that dysfunction of mitochondrial occurred and mitochondrial apoptosis pathway was initiated. Consistent with reported study, the in vitro ischemia model of BMSCs was successfully established, which laid a foundation and preliminary basis for following research.

In the present study, LIF administration showed a protective role in apoptosis of BMSCs induced by hypoxia and serum-deprivation, including improving the MMP, nuclei morphology, cell apoptosis rate and inhibiting the activation of Caspase-3. Caspase signaling pathway is a vital cellular pathway of cell apoptosis, and Caspase-3 is a key molecule in the execution of apoptosis. Caspase-3 activation happens at the end of the cascades of both death-receptor-mediated exogenous apoptosis and mitochondria-mediated endogenous apoptosis [33]. After enzymatic hydrolysis of Caspase-3 precursor, a small C-terminal peptide fragment is released to generate the active cleaved Caspase-3 fragment, which cleaves the cytosolic and cytonuclear substrate, leading to apoptosis [39]. Studies have shown that release of Cyt C and activation of Caspase-3 increased in BMSCs treated hypoxia and serum-deprivation [40, 41]. In the present study, Caspase-3 was significantly activated in BMSCs after 6 hours of hypoxia and serum-deprivation treatment, while LIF played an inhibitory role in the process. Consistent with the present study, a lot of reports in the literature have reported the inhibitory effect of LIF on cell apoptosis. Negoro found that LIF inhibited cell apoptosis by blocking doxorubicin-induced reduction of Bcl-xl and activation of Caspase-3 in cardiomyocytes [42]. Hunt observed that LIF suppressed staurosporine-induced Caspase-3 activation and DNA fragmentation in myoblasts [43]. Kerr found that injection of exogenous LIF inhibited oligodendrocyte apoptosis in a mouse model of spinal cord injury [44]. Ruan confirmed that transforming growth factor β (TGF-β) upregulated the expression of anti-apoptotic gene Bcl-xl and downregulated the expression of pro-apoptotic gene Bid by stimulating the secretion of LIF, thus inhibiting the apoptosis of osteoclasts [45]. These results suggested that LIF is involved in the regulation of apoptosis in a variety of cells under different physiological or pathological environments.

The JAK/STAT signaling pathway can mediate a wide range of biological processes such as inflammatory responses, oxidative stress, cell damage and apoptosis [46]. Under the stimulation of a variety of different cytokines or growth factors, JAKs can activate the expression of STATs protein. After binding to the cytoplasmic JAKs binding site of recipient cells, JAKs phos-

phorylation occurs. Activated JAKs further recruit and activate the corresponding STATs in the cytoplasm, and then bind to the promoter of the target gene in the signaling process [47]. STAT3 is an important signal for cell survival protection. Activation of STAT3 can promote cell survival and inhibit cell apoptosis [48]. Reports in the literature have demonstrated that JAK2/STAT3 signaling plays an essential protective role in ischemia injury of myocardial and brain tissues [49, 50]. However, the present study found that exogenous LIF stimulation significantly increased the activation levels of JAK1 and STAT3 without changing the activation level of JAK2. The study innovatively found that LIF maintained the survival of BMSCs through JAK1/STAT3 signaling. In BMSCs-based bone tissue engineering, using LIF to amplify JAK1/STAT3 signaling pathway in seed cells or host to enhance the survival of BMSCs may be a new therapeutic strategy to improve the bone regeneration.

Based on the results, there are several clinical implications from the present study. First, H&SD could induce the apoptosis of BMSCs, so oxygen-releasing and nutrient-loaded scaffolds should be fabricated and utilized in the bone tissue engineering to improve the local microenvironment [51]. Second, the present study demonstrated that JAK1/STAT3 signal axis is an essential signal for the survival of BMSCs in the ischemic environment. Therefore, amplification of JAK1/STAT3 signal to maintain the vitality of BMSCs may be a therapeutic strategy to achieve optimal bone regeneration. Activated JAK1/STAT3 by regenerative cytokine, like interleukin (IL)-10, was reported to participate in cell survival and anti-inflammatory response [52, 53]. Moreover, LIF supplement could counteract ischemia-induced apoptosis of BMSCs and enhance the survival rate, which is the innovative finding of the study and has certain positive significance for clinical practice. It offered a new strategy of LIF application to treat the seed cells before implantation to improve the survival status of BMSCs.

Disclosure of conflict of interest

None.

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