Original Article
Overexpression of Hsp70 enhances MSCs survival and therapeutic efficiency in hindlimb ischemia

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Abstract: Background: Mesenchymal stem cells (MSCs) therapy has limitations in the therapeutic angiogenesis of ischemic limbs because of the low survival rates of MSCs after transplantation. Therapeutic angiogenesis using modified stem cells is a novel strategy for severe ischemic diseases. Objective: This study aimed to identify that overexpression of Hsp70 could enhance MSCs survival and therapeutic efficiency in the repair of hindlimb ischemia. Method: In this study, bone marrow-derived mesenchymal stem cells were obtained from C57BL/6J mice, and stably transduced with heat shock protein 70 (Hsp70, H-MSCs) by adenovirus vector. Hindlimb ischemia mice models were created and transplanted with H-MSCs, PBS, or mature MSCs into the ischemic muscles. Results: We observed that Hsp70 overexpression protected MSCs against cell death triggered by oxidative stress in vitro. Moreover, greater blood flow and vascular density increased in the H-MSCs group compared with control groups. The beneficial effects of Hsp70 overexpression were further found to be associated with enhanced Bcl-2/Bax activation and increased secretion of growth factors (FGF-2, HGF, VEGF, and IGF). Conclusion: These results showed that the genetic modification of MSCs using Hsp70 before transplantation could benefit the treatment of hindlimb ischemic diseases.

Keywords: Angiogenesis, heat shock protein 70, mesenchymal stem cells, hindlimb ischemia, transplantation

Introduction

Mesenchymal stem cells (MSCs) have multipotent differentiation properties, and therefore hold tremendous potential for regenerative therapies in cardiovascular and cerebrovascular ischemic diseases [1, 2]. Several clinical studies have reported that MSCs have been used for neovascularization of severe ischemic diseases [3, 4]. However, poor viability and differentiation ability of the transplanted cells are major limiting factors of stem-cell-based therapies [5]. Toma et al found extremely low survival rate (<1%) of MSCs after transplantation into injured mouse hearts at the fourth day [6]. The shortage of MSCs in the target organs, and their supposed functional withering in pathological conditions limit their therapeutic effects [7]. Recently, debate has arisen about the usefulness of stem-cell-based therapies after transplantation [8]. Enhancing the therapeutic angiogenesis efficiency poses a great challenge for therapeutic angiogenesis of ischemic tissues.

A series of approaches have been made to enhance the therapeutic effects of MSCs before transplantation, including pretreatment with transcription factors, growth factors and cytokines, preconditioning, and genetic modification, which showed salutary effects for the treatment of ischemic diseases [9-11]. The heat shock protein 70 (Hsp70) family are molecular chaperones that are over-active under various stimulates, such as hypoxia, heat shock, ischemia/reperfusion, glucose deprivation, and exposure to toxins [12]. As reported, hypoxia induced the increase of Hsp70 together with the protection of cardiomyocytes and endothelial cells under ischemia-reperfused conditions [13]. Over-expression of Hsp70 in cells through gene modification also greatly enhances the resistance of cardiomyocytes in vitro and in vivo in transgenic mice [14]. Moreover, deletion of

Hsp70 by siRNA results in the dysfunction of cardiomyocytes and impairment of the stress response of Hsp70-knockout hearts under ischemia/reperfusion conditions. Hsp70 protects cells from various damages such as hypoxia and environmental irritation, thereby protecting myocardial cells and exerting beneficial effects on endothelial cells [15, 16]. Although heat-shock modification has been tested to enhance cell survival under various stress conditions, the response of heat-shock to different cell types is variable and complex [17]. MSCs exhibit tremendous potential for regenerative medicine, therefore, it is important to identify the Hsp70-modified MSCs that can be the used for the repair of severe ischemic diseases.

In this study, we genetically engineered mouse MSCs with Hsp70 gene (H-MSCs) and examined cell survival, apoptosis, adhesion and migration. Hindlimb ischemic mice models were established and were transplanted with H-MSCs, MSCs, and PBS via intramuscle injection. Accordingly, we examined whether implantation of H-MSCs augments therapeutic angiogenesis and collateral vessel formation in the hindlimb ischemic mouse models.

Materials and methods

MSC culture

MSCs were isolated and expanded from the femurs and tibias of C57BL/6J mice (n=40) as previously described [18]. The MSCs were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-penicillin/streptomycin solution for three passages at 37°C in a humidified atmosphere containing 5% CO_2 in vitro.

P3 MSCs were incubated with phycoerythrin (PE)-conjugated anti-mouse antibodies against CD29, CD34, CD90, CD133, MHC-II, and fluorescein isothiocyanate (FITC)-conjugated CD31, and CD105 (eBioscience, San Diego, USA). The incubation was conducted for 30 minutes at 4°C in the dark and then washed three times in PBS. Normal rabbit IgG antibodies were used as negative control groups. Cells were then resuspended in 100 µl of PBS, and 0.5% Bull Serum Albumin (BSA). Resulting cells were tested using flow cytometry and analyzed with Cell Quest Pro Software (BD Biosciences, Rockville,

MD). The experiments were performed in triplicates.

Construction of Hsp70-Ad vectors and cell transfection

A high-capacity adenoviral vector (HC-Ad) was constructed as described previously. The HC-Ad-HSP70 was generated to overexpress the Hsp70 carrying a C-terminal 6-His tag. The vector HC-Ad-HSP70 produced enhanced GFP (EGFP). MSCs were cultured and infected with a stock AdV concentration of 1.5×10^6 infection units/L. Transduction efficiency was analyzed using flow cytometry and GFP expression analysis in the total cell population was conducted using Cell Quest Pro Software after 24 hours. Expression levels of Hsp70 were determined using Western blot analysis. Protein samples of H-MSCs and MSCs were extracted and subjected to SDS-PAGE/immunoblotting analysis using anti-Hsp70 antibody, and anti-β-actin antibody (1:500, Abcam, UK).

Cell viability and proliferation

For assessment of cell Viability and proliferation, the cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) was used on H-MSCs and MSCs following the manufacturers' instructions as previously described [19]. H-MSCs and MSCs growth were measured every day for six days, and each assay was performed in three times.

Multi-differentiation assays of H-MSCs

To evaluate the multi-lineage potential of MSCs differentiating into osteocytes and adipocytes, both the H-MSCs and control MSCs were subjected to stimulation for adipogenic and osteogenic induction media as previously described [20]. After three weeks, the induced cells were stained with alizarin red and Oil Red O. Accordingly, the expression of the osteogenic representative gene (Runx2), and the adipogenic representative gene (PPAR-r) were analyzed with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The cDNAs were analyzed using the following primers: Runx2, 5' GGA CTG TGG TTA CCG TCA T 3', 5' GGA GGA TTT GTG AAG ACT GTT 3'; and PPAR-r, 5' CAC TCG CAT TCC TTT GAC AT 3', 5' TTG ATC GCA CTT TGG TAT TCT 3'.
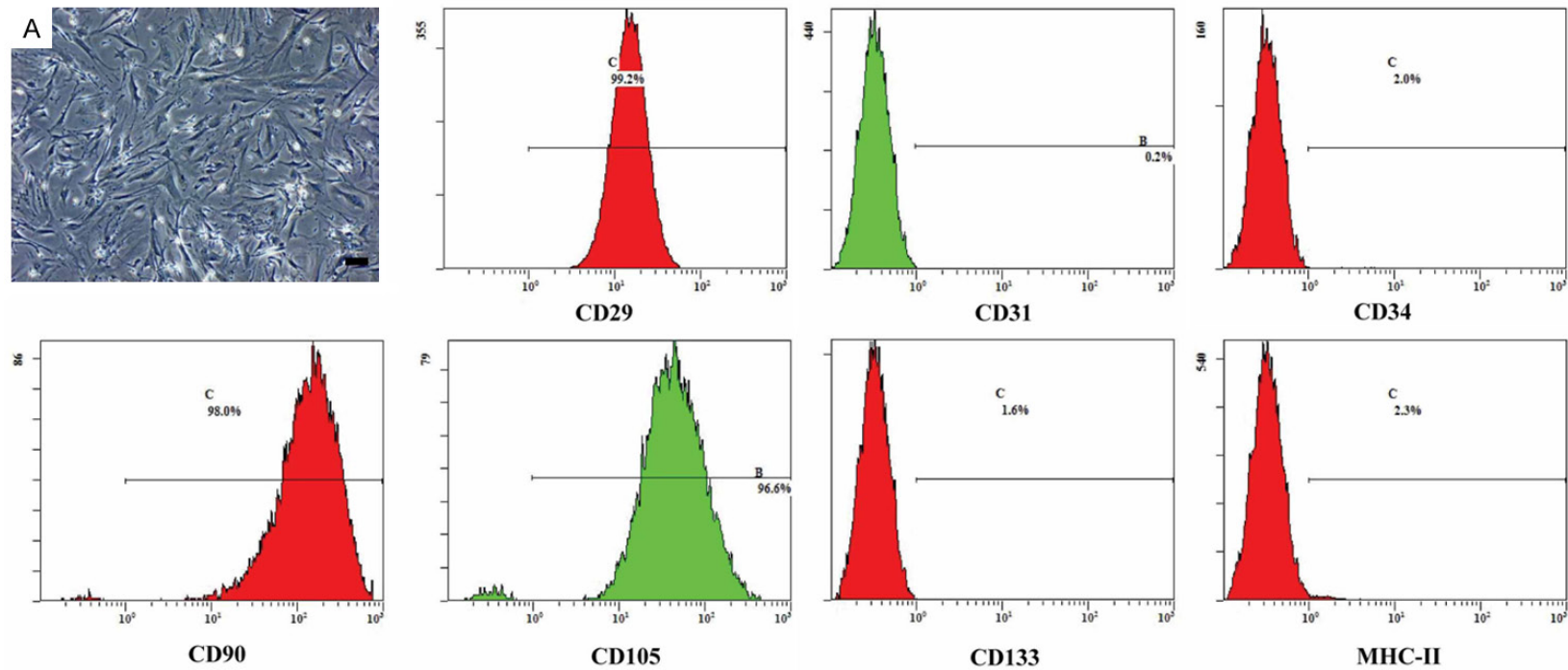
Figure 1. Characterization of MSCs. MSCs showed fibroblast-like attaching cells (A). Phenotypic analysis showed that P3 MSCs were strongly positive for CD29, CD90, and CD105, and negative for CD31, CD34, CD133, and MHC-II. Scale bar measures 100 μm.
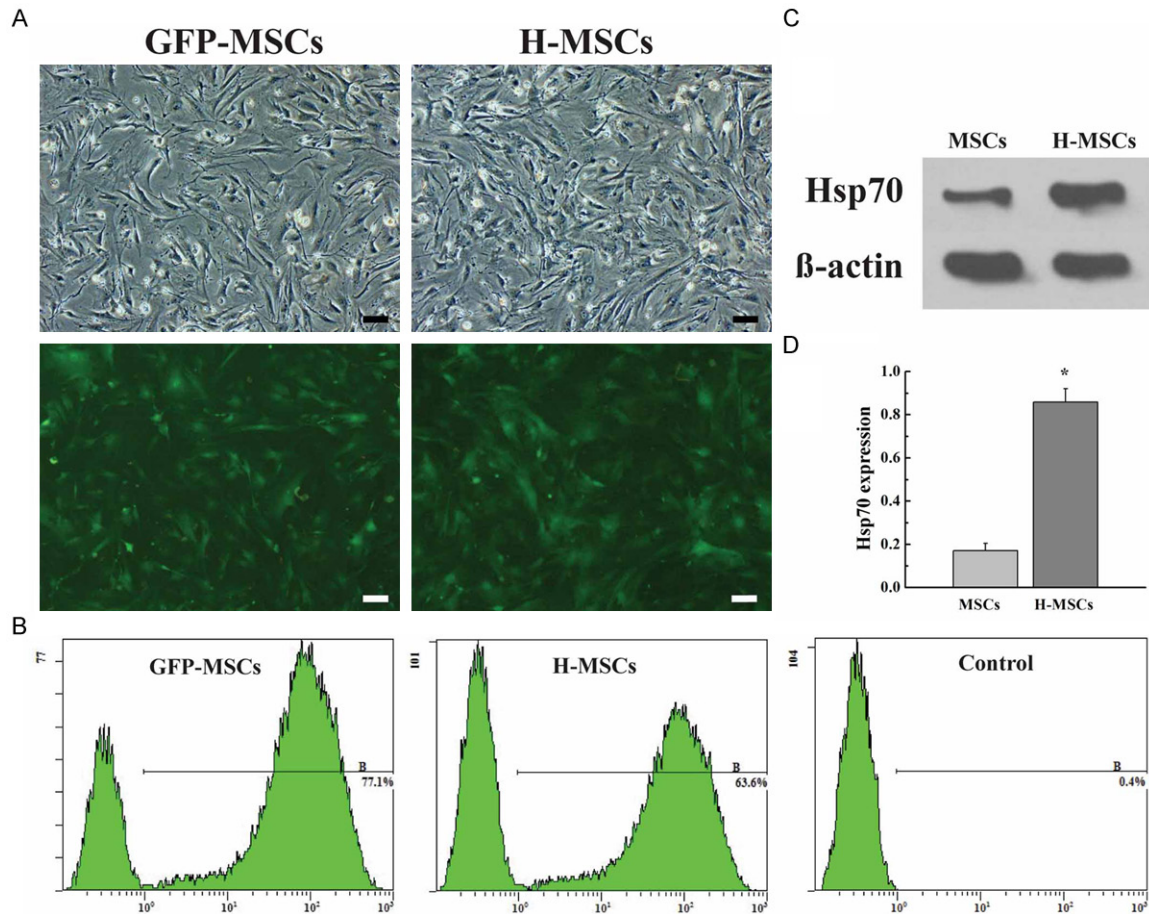
Figure 2. Overexpression of Hsp 70 in MSCs. A. Immunofluorescence staining showed that more than 75% of the MSCs expressed the Hsp70 protein. By contrast, the control MSCs lack the Hsp70 protein. B. Flow cytometer analysis showed that H-MSCs were strongly positive for the GFP. C. Western blot analysis showed Hsp70 was highly expressed in H-MSCs. D. Statistical analysis revealed that H-MSCs groups exhibited significant difference compared with MSCs groups (P<0.05). Scale bar measures 100 μm, *P<0.05.

Experimental hypoxia and Annexin V/PI staining

Both H-MSCs and control MSCs were cultured at 80% confluency and followed by incubation in hypoxic conditions (5% CO_2, 5% O_2, and 94% N_2) for 24 hours in an airtight and humidified hypoxic chamber (Anaerobic Environment, ThermoForma, Marietta, OH, USA) at 37°C. Apoptosis was tested using an Annexin V/PI apoptosis detection kit (Pharmingen, Annexin V-Pe Apoptosis Kit) and analyzed with flow cytometry. At least $5×10^4$ cells were analyzed in each sample. The levels of early apoptosis and late apoptosis/necrosis were measured as percentages of Annexin V^+/PI^- and Annexin V^+/PI^+ cells using Cell Quest software (BD Biosciences, Rockville, MD). Accordingly, the expression levels of the apoptotic Bcl-2/Bax genes were analyzed with qRT-PCR.

Adhesion and migration assay of H-MSCs

To explore the effect of Hsp70 on the adhesion and migration ability, MSCs were plated at a density of $1×10^3$ cm^2 in a 6-well plate and were incubated for 60 min at 37°C. The wells were washed with PBS, and the number of attached cells was determined by microscopic cell counting. Each experiment was repeated at least three times.

Migration was assayed by a transwell inserts (R&D Systems Inc., Minneapolis, USA). MSCs were trypsinized and suspended at a concentration of $1×10^5$ cells/100 μl in DMEM. The MSCs were placed in the upper wells, and 500 μL of DMEM containing 50 ng/mL of Hsp70 was placed in the lower wells. After six hours of incubation, the non-migrating cells were mechanically removed, and the remaining cells
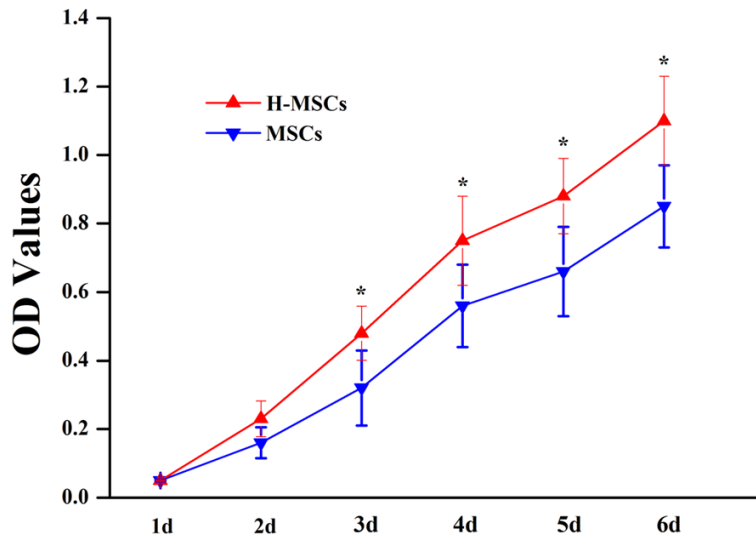
Figure 3. Cell viability of H-MSCs, *P<0.05.

were fixed in 4% paraformaldehyde and stained with 4', 6-diamidino-2-phenylindole (DAPI) (DAKO, USA). The cells were counted in six random microscopic fields.

Hindlimb ischemic mouse models and cell transplantation

All animal procedures were performed in accordance with the Guidelines of the Animal Experiment and Care Committee of Soochow University. The left hindlimb ischemia was induced in male C57BL/6J mice (six months old, n=32) as described previously [19]. The femoral artery was ligated and excised. No mice died during the experiment. Mice were randomly divided into four groups. The sham group (n=8), The control group (n=8) received phosphate-buffered saline (100 μl of PBS), the MSCs group (n=8) received MSCs (1×10⁶ cells/100 μl), and the H-MSCs group (n=8) received H-MSCs (1×10⁶ cells/100 μl). The ischemic muscles were implanted at three predetermined points after postoperative day one. At one, two, and three weeks after transplantation, a laser Doppler blood flow meter (Omegaflow floci, Omegawave) was used to measure the cutaneous blood flow.

After three weeks, angiogenesis and collateral vessel formation in the ischemic tissues were stained with anti-CD31 mAb (PECAM-1: Becton Dickinson). Five fields from the three different muscle samples of each animal were randomly

selected and analyzed for the capillary density as described previously.

ELISA of the cytokines

Conditioned media were obtained from cultured MSCs and H-MSCs after the 48 h incubation. The FGF-2, HGF, VEGF and IGF concentrations released in the media were measured using mouse ELISA kit (R&D Systems) following the manufacturer's instruction. All experiments were performed in triplicate wells and repeated at least three times.

Statistical analysis

Data are expressed as mean values ± standard deviation (SD). Statistical significance was evaluated using unpaired Student t test for comparison between 2 means and ANOVA for comparison among 3 groups, P<0.05 (*) was considered statistically significant.

Results

Isolation and characterization of MSCs

MSCs showed fibroblast-like attaching cells that were culture expandable (**Figure 1A**). Phenotypic analysis by flow cytometry demonstrated that P3 MSCs were strongly positive for the specific surface antigens CD29 (99.2% ± 3.5%), CD90 (98.0% ± 2.4%), and CD105 (96.6% ± 2.9%); and negative for the specific surface antigens CD31 (0.2% ± 0.05%), CD34 (2.0% ± 0.14%), CD133 (1.6% ± 0.19%), and MHC-II (2.3% ± 0.21%) (**Figure 1**). The control groups were also negative (0.2% ± 0.07%, data not shown).

Overexpression of Hsp70 in MSCs

Hsp70 gene was successfully transduced into MSCs by adenovirus vector after culturing for 24 h, and the transduction efficiency reached more than 75% (**Figure 2A**). No significant difference was observed in the transduced ratio between Hsp70 and GFP control. Fluorescence microscopy results showed that more than 70% of the MSCs expressed the Hsp70 markers
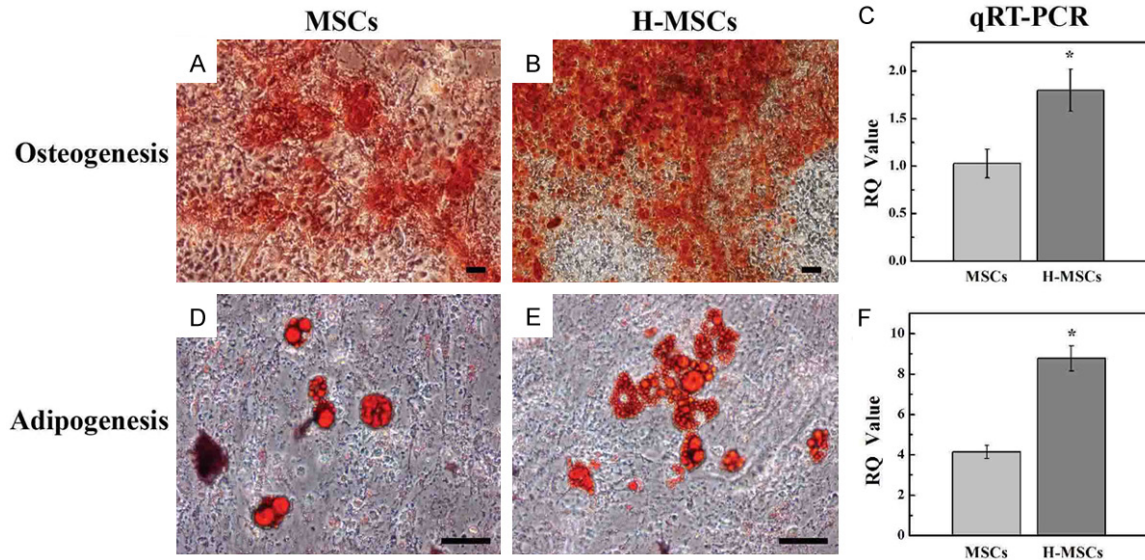
Figure 4. Multi-differentiation assays of H-MSCs. Osteocytes differentiation potential of the MSCs (A) and H-MSCs (B). qRT-PCR of Runx2 (C). Adipocytes differentiation potential of the MSCs (D) and H-MSCs (E). qRT-PCR of PPARr (F). Scale bar measures 100 µm, *P<0.05.

(**Figure 2A**). Flow cytometer analysis showed that H-MSCs were strongly positive for the GFP (63.6% ± 4.5%), and control GFP-MSCs (75.1% ± 5.2%), and negative for the control groups were (0.4% ± 0.02%) (**Figure 2B**). Western blot analysis demonstrated that Hsp70 was highly expressed in the transfected H-MSCs groups than that in the control MSCs (**Figure 2C**). Statistical analysis in **Figure 2D** revealed that H-MSCs groups were significantly different from untransfected MSCs groups (*P<0.05).

Hsp70 overexpression promotes cell viability

To further investigate the effects of Hsp70 over-expression on the viability and proliferation of MSCs, a cell viability test was performed using CCK-8 assay from day 1 to day 6 (**Figure 3**). The time line chart showed that MSCs and H-MSCs were initially in stationary phase at day 1. The cells underwent logarithmic growth period from day 2 to day 6. The results showed that the H-MSCs exhibited higher proliferation rate compared with the MSCs from day 3 to day 6 (*P<0.05). Our results indicate that Hsp70 enhanced the viability and proliferation of MSCs.
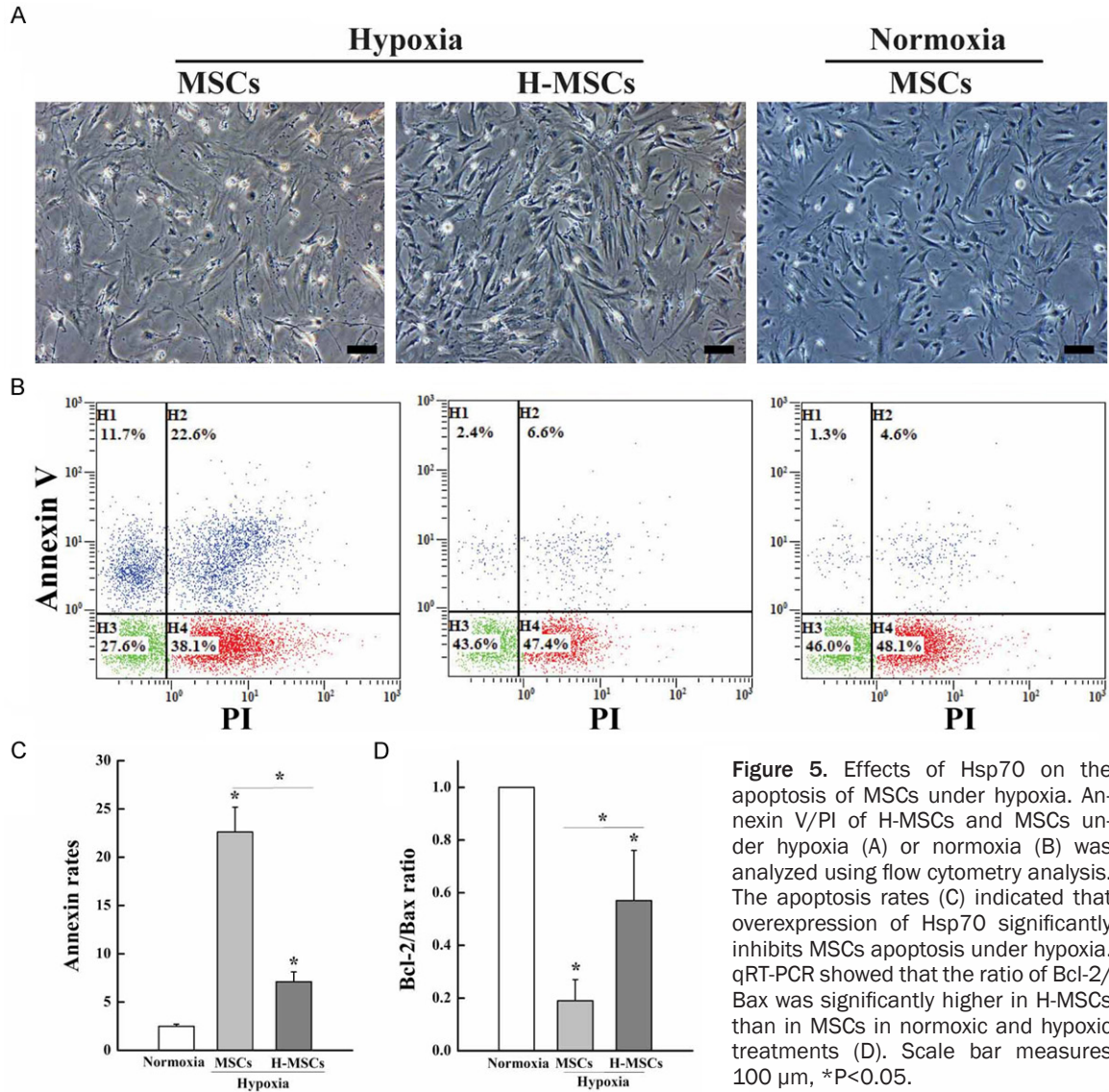
Multi-differentiation assays of H-MSCs

In this study, we compared the differentiation potential of the H-MSCs and MSCs in vitro. All the cells could differentiate into osteocytes

and adipocytes after three weeks induction, Alizarin red staining showed positive bone-forming capacity in H-MSCs groups (**Figure 4B**) and MSCs groups (**Figure 4A**). Differentiation of MSCs into adipocytes was confirmed by Oil Red-O staining and microscopic observation, in which red colored oil granules were observed in H-MSCs groups (**Figure 4E**) and MSCs groups (**Figure 4D**). The qRT-PCR of the relative gene expression levels in H-MSCs groups showed significant difference in the transcript levels of Runx2 (**Figure 4C**, *P<0.05) and PPAR-r (**Figure 4F**, *P<0.05) compared with the MSCs groups. Our results demonstrated that Hsp70 could enhance the multi-differentiation ability of MSCs.

Hsp70 overexpression inhibits MSCs apoptosis under hypoxia

In normoxic conditions, MSCs exhibited uniform fibroblast-like shapes (**Figure 5A**). However, P3 MSCs showed aging and dedifferentiation with irregular shapes under hypoxia (**Figure 5A**), which was quite different from the shapes of MSCs transfected with Hsp70. To explore the effect of Hsp70 on MSCs apoptosis under hypoxic conditions, the treated H-MSCs and MSCs were stained using Annexin V/PI apoptosis kit. Fow cytometry analysis demonstrated that the apoptotic percentage of H-MSCs groups under hypoxia was 6.6% ± 1.24% com-

Figure 5. Effects of Hsp70 on the apoptosis of MSCs under hypoxia. Annexin V/PI of H-MSCs and MSCs under hypoxia (A) or normoxia (B) was analyzed using flow cytometry analysis. The apoptosis rates (C) indicated that overexpression of Hsp70 significantly inhibits MSCs apoptosis under hypoxia. qRT-PCR showed that the ratio of Bcl-2/Bax was significantly higher in H-MSCs than in MSCs in normoxic and hypoxic treatments (D). Scale bar measures 100 µm, *P<0.05.

pared with MSCs control groups (22.6% ± 3.17%, **Figure 5B**). Quantitative analysis was conducted between the two groups (*P<0.05, **Figure 5C**) and compared with MSCs under normoxic conditions (4.6% ± 0.57%). RT-PCR also showed a significantly higher ratio of Bcl-2/Bax in Hsp70-MSCs than in MSCs in normoxic and hypoxic treatments (*P<0.05, **Figure 5D**). Compared with MSCs controls under hypoxic conditions in vitro, MSCs transfected with Hsp70 displayed higher viability and inhibited MSCs apoptosis under hypoxic conditions.

Adhesion and migration assay of H-MSCs

The effect of Hsp70 on the adhesion of MSCs was tested after incubating for 60 min. In

Figure 6A, a portion of round-shaped MSCs were attached to the plates of the six wells, however, more MSCs were in the H-MSCs groups than in that of the MSCs control groups as observed under a fluorescent microscope. Significant differences were observed between the H-MSCs groups and the MSCs control groups (*P<0.05, **Figure 6B**), which demonstrated that Hsp70 overexpression could effectively enhance the adhesion of MSCs.

The effect of Hsp70 on the migration of MSCs was assayed by a transwell inserts. **Figure 6C** demonstrated that more MSCs were observed in the H-MSCs groups than in that of the MSCs control groups. Quantitative data further indicated that there are significant differences
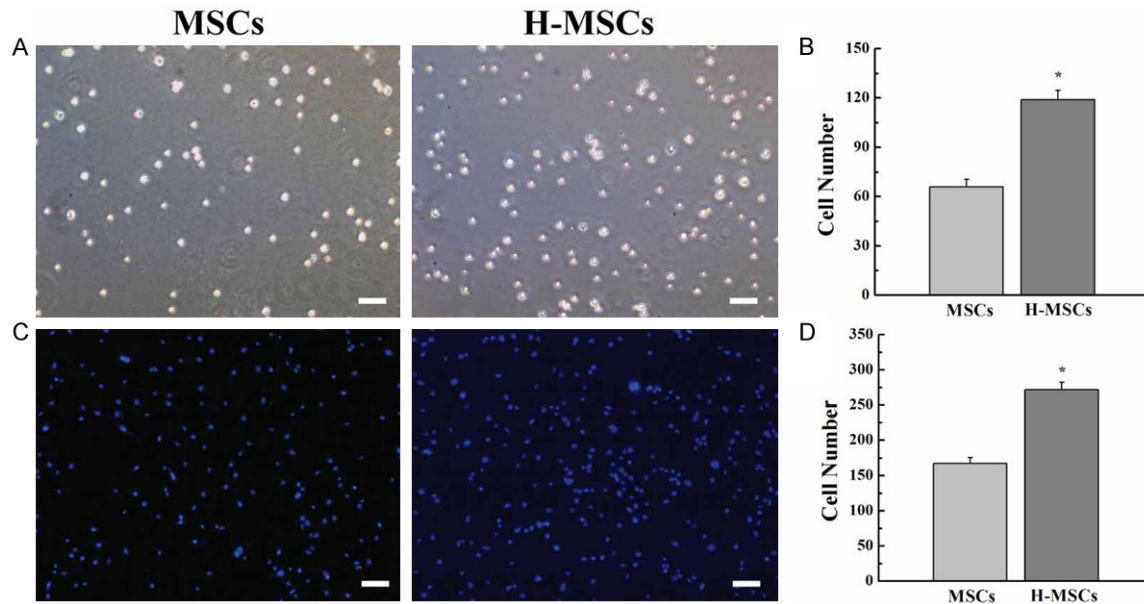
Figure 6. Adhesion and migration assay of H-MSCs. A. The effect of Hsp70 on the adhesion of MSCs and H-MSCs. B. Statistical analysis. C. The effect of Hsp70 on the migration of MSCs and H-MSCs. D. Statistical analysis. Scale bar measures 100 μm, *P<0.05.
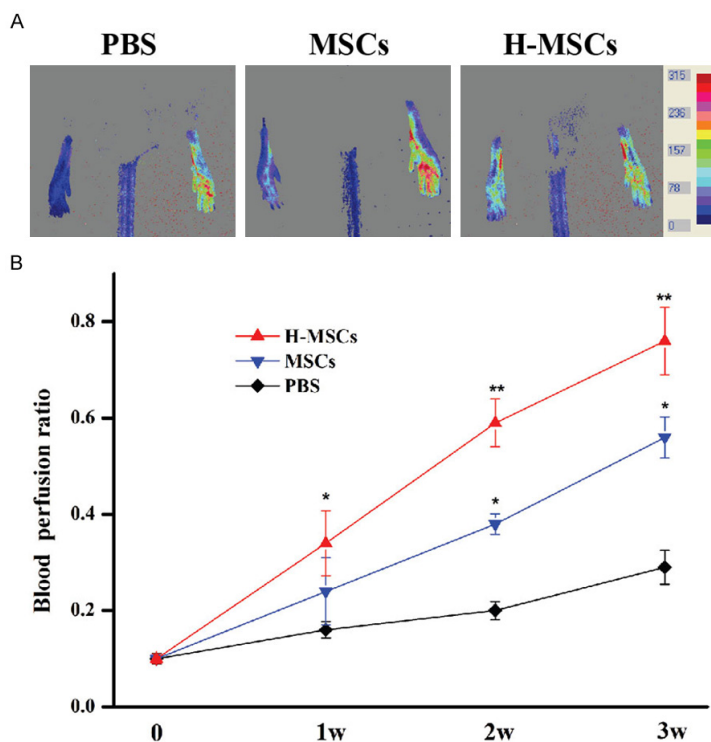

Figure 7. Laser Doppler images of blood flow, *P<0.05, **P<0.01. A. Representative color laser Doppler images of superficial blood flow in lower ischemic limbs. B. The time line chart indicated that the laser Doppler perfusion index. Scale bar measures 100 μm, *P<0.05.

Our results demonstrated that 50 ng/mL Hsp70 could enhance the migration of MSCs.

Effects of Hsp70 on therapeutic angiogenesis of hindlimb ischemia

In our study, no tumor and teratoma formation were observed in the ischemic muscles after H-MSCs and MSCs transplantation. At one, two and three weeks after cell transplantation, a laser Doppler blood flow meter was used to analyze the superficial blood perfusion in the lower left ischemic limbs (**Figure 7A**). The timeline chart indicated that the laser Doppler perfusion index was significantly higher in the Hsp70-MSCs group than in the MSCs group and the PBS control groups from the first week to the third week (*P<0.05, **Figure 7B**). Our results indicated that overexpression of Hsp70 enhanced the blood perfusion of MSCs-based therapy for ischemic muscles.

between the H-MSCs groups and the MSCs control groups (*P<0.05, **Figure 6D**) existed.

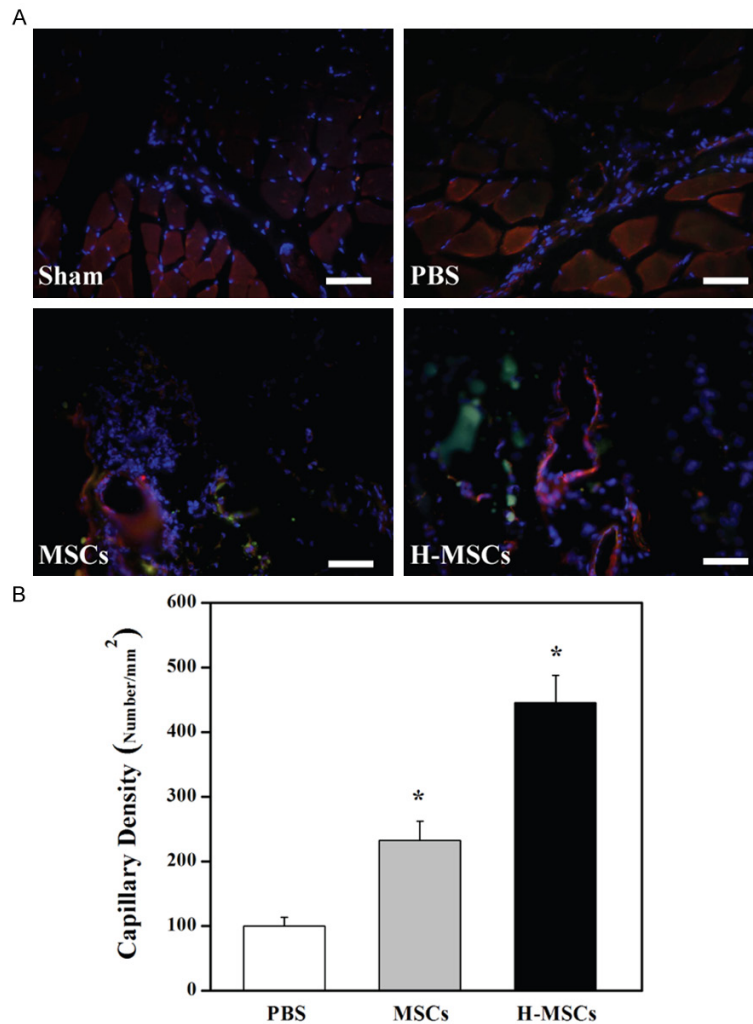To evaluate the effects of transplanted H-MSCs on the formation of new blood vessels, endo-

Figure 8. Histology staining of the ischemic muscles after cell transplantation. A. Representative immunohistochemistry staining for endothelial cells markers CD31. B. Quantitative analysis of the microvessel densities in H-MSCs and MSCs groups (P<0.05). Scale bar measures 100 μm, *P<0.05.

H-MSCs, we examined the secretion of major growth factors (FGF-2, HGF, VEGF, and IGF-1) of the H-MSCs and MSCs after being culture for 48 h. The cytokines and growth factors exhibited a significant increase in H-MSCs compared with MSCs (*P<0.05, **Figure 9**). Our results demonstrated that Hsp-70 may stimulate the paracrine mechanisms of MSCs to enhance the vascularization of ischemic muscles.

Discussion

In the present study, we demonstrated that overexpression of Hsp70 increased MSCs proliferation, multi-differentiation, adhesion, and migration and protected MSCs against cell apoptosis under hypoxia. Moreover, transplantation of Hsp70-MSCs enhanced blood flow and vascularization in the ischemic hindlimb compared with the MSCs and PBS groups. Meanwhile, overexpression of Hsp-70 was correlated with enhanced Bcl-2/Bax activation and increased secretion of cytokines (FGF-2, HGF, VEGF, and IGF) in MSCs. Taken together, our data suggest that genetic modification of MSCs with Hsp70 before transplantation could be salutary for treating ischemic hindlimbs.

Therapeutic use of MSCs has been suggested to be a promising clinical approach for the treatment of critical ischemic diseases [21, 22], however, it still has limitations due to the poor viability and differentiation ability of MSCs after cell transplantation. Many approaches have been applied to enhance the survival of transplanted MSCs [9, 10]. In our study, we modified MSCs with Hsp70 through adenovirus vector systems, which proved advantages in stabilizing the expressing of target genes without significant immune response. Cell death is influenced by an ischemic condition, characterized by lack of nutrients and oxygen [23]. Heat shock
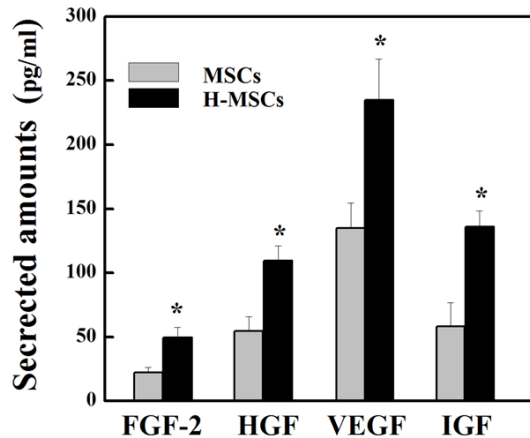
thelial cell marker CD31 was used to assay the capillary density of the ischemic limb muscles. Fluorescent microscopic examination detected that both the MSCs and H-MSCs groups exhibited a significant increase in the blood vessel densities, compared with the sham group and PBS control groups (**Figure 8A**). Meanwhile, H-MSCs groups showed higher capillary densities compared with that of PBS groups and MSCs control groups (*P<0.05) (**Figure 8B**). These results further suggest that Hsp70 could enhance the therapeutic angiogenesis in hindlimb ischemia.

Cytokines in H-MSCs

To assess the potential paracrine effects of H-MSCs on therapeutic angiogenesis effect of

Figure 9. Cytokines in H-MSCs. ELISA assay showed that the cytokines and growth factors exhibited a significant increase in H-MSCs compared with MSCs, *P<0.05.

protein (Hsp) is suggested as a tool to protect cellular survival under deleterious stimuli and irrigation [24]. In this study, H-MSCs showed a remarkable increase in viability and proliferation under hypoxic conditions compared with MSCs. When H-MSCs were transplanted into ischemic hindlimb muscles, the blood vessel densities and blood reperfusion were significantly improved compared with MSC-injected controls.

Although the mechanisms of Hsp70 on MSCs appear to be far more complex, it has been suggested that Hsp70 protects MSCs from apoptotic cell death by releasing angiogenic ligands, and inducing proliferation of stem cells [25]. Here, we observed an increase in the Bcl-2/Bax ratio. As reported by Yamagishi et al, HSP70 significantly prevents the arrest of DNA synthesis in cultured AGE-exposed pericytes by increasing the expression levels of BCL2, as well as inactivating BAX expression in retinal pericytes [26]. This mechanism aids pericyte survival, proving that Hsp70 directly prevents apoptosis of the MSCs. This is likely to involve Hsp70 inhibition of JNK, which induces apoptotic cell death in response to specific stimuli [17].

Another cytoprotective mechanism of Hsp70 may involve the paracrine effects by secretion of cytokines and growth factors [27, 28]. The present study clearly demonstrated the increased secretion of (FGF-2, HGF, VEGF, and IGF-1) from H-MSCs in response to the hypoxic conditions. This may be how the overexpression of Hsp70 enhances the proangiogenic

capacity of MSCs, therefore attenuating pathological muscle remodeling and promoting neovascularization. Taken together, HSP70 is emerging as a novel and suitable candidate for new therapeutic approaches in modifying MSCs for treating critical ischemic diseases.

Disclosure of conflict of interest

None.

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