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Beneficial Effects of Hypoxic Preconditioning on Human Umbilical Cord Mesenchymal Stem Cells

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Abstract

As human umbilical cord mesenchymal stem cells (hUC-MSCs) transplanation may be promising in heart failure treatment, it is important to know whether hypoxic preconditioning (HP) promote hUC-MSCs proliferation and differentiation and protect them against chemical hypoxic damages. This study aimed to investigate the effects of HP on proliferation and differentiation of human umbilical cord mesenchymal stem cells (hUC-MSCs). The study also aimed to confirm our hypothesis that HP could promote hUC-MSCs proliferation and differentiation to cardiomyocyte-like cells as well as effectively protecting hUC-MSCs and cardiomyocyte-like cells against chemical hypoxic damages. Isolated hUC-MSCs were cultured in hypoxia at 1%, 3% and 5% O_2 for 72 hours. 5-azacytidine (5-AZA) induced differentiation of hUC-MSCs to cardiomyocyte-like cells was determined by streptavidin-perosidase (SP) immunohistochemical staining and the content of troponin (TnI). Flow cytometry was used to measure cell cycle in hUC-MSCs and cardiomyocyte-like cells. The mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$), were measured in hUC-MSCs and cardiomyocyte-like cells during chemical hypoxia induced by cobalt chloride (100 μmol/L). HP optimally promoted the proliferation of hUC-MSCs at 3% O_2 and enhanced the differentiation of hUC-MSCs to cardiomyocyte-like cells by 5-AZA in a concentration-dependent manner. The cell cycle distribution of cardiomyocyte-like cells, but not hUC-MSCs, was clearly changed by HP. Chemical hypoxic damage, decreased $\Delta\Psi_m$ and increased $[Ca^{2+}]_m$, were alleviated significantly in HP-treated cells compared with the normaxia-treated cells. The results demonstrate that HP promoted hUC-MSCs proliferation and differentiation to cardiomyocyte-like cells, and protected both cell types against chemical hypoxic damage.

Key Words: chemical hypoxia, differentiation, human umbilical cord mesenchymal stem cells, hypoxic preconditioning, proliferation

Introduction

Along with improvements of living standard and changes of living style in the modern society, the incidence of cardiovascular diseases is increasing. Heart failure after myocardial infarction is a major factor for cardiac death. Traditional treatment for heart failure after infarction includes drug therapy, interventional therapy and surgery but none of these treatments can regenerate cardiomyocytes or prevent irreversible reduction of compensated cardiomyocytes after infarction (31). Myocardial regeneration is a clinical strategy

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to improve cardiac functions and prognosis of cardiovascular diseases for cardiovascular treatment in the future. Among the candidate approaches, stem cells may be promising in regenerative medicine for their self-renew and multipotency characteristics (13).

Human umbilical cord mesenchymal stem cells (hUC-MSCs) have been getting more attention for its convenience as a cell source, and there was little dispute in ethics. A large number of studies have demonstrated that mesenchymal stem cells (MSCs) can renovate myocardial injury, increase new capillaries and improve cardiac function and prognosis (12, 38, 41). In practical applications, however, the efficacy of stem cells transplantation is greatly limited because the survival rate of MSCs after transplantation is very low due to the hypoxic conditions in the pathological heart (15, 32, 33, 36). Therefore, improvement in proliferation and differentiation of MSCs, enhanced resistance against hypoxic injury of MSCs, and better MSC survival after transplantation will be pivotal factors for the success of stem cells transplantation.

It was reported that hypoxic preconditioning (HP) had positive effects on stem cells (8) such as increasing MSC longevity (11, 18), promoting MSC differentiation (1) and increasing MSC self-renew rates (27). But the optimal oxygen concentration and the duration of HP are uncertain. Furthermore, the effects of HP on proliferation of hUC-MSCs, differentiation of hUC-MSCs to cardiomyocyte-like cells, and damages of hUC-MSCs have not been elucidated.

Here we hypothesized that HP could promote proliferation and differentiation of hUC-MSCs to cardiomyocyte-like cells and could effectively protect hUC-MSCs and cardiomyocyte-like cells against chemical hypoxic damages. This study aimed to investigate the effects of HP on hUC-MSC proliferation and differentiation to cardiomyocyte-like cells, and the protection of HP on hUC-MSCs or cardiomyocyte-like cells against chemical hypoxic injury using cell culture, immunohistochemistry, flow cytometry and confocal microscope methods.

Materials and Methods

Isolation and Culture of hUC-MSCs

Fresh human umbilical cords were gathered from newborns normally delivered by healthy mothers. Written informed consents were obtained from the mothers, and the experimental procedures were approved by the Ethics Committee of Bethune International Peace Hospital. The umbilical cords were rinsed in sterile phosphate-buffered saline (PBS) until the cord blood was cleared, and the blood vessels were removed. The umbilical cords were cut into 2-3 cm length and opened up. The Wharton's jelly was scratched out with two tissue forceps and cut into small pieces of 1-3 mm. The fragments were then collected and cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37°C and 5% CO_2. The culture solution was first changed with the complete medium at 5th day of culture in which superficial cells were removed and the hUC-MSCs were further cultured. The medium was subsequently changed every 48-72 h. After the cells reached 80-90% confluency, they were detached with 0.25% trypsin/EDTA mixture (Sigma, St. Louis, MO, USA) for 1-2 min. When most adherent cell retracted and increased intercellular space were observed under microscope, the digestion was terminated with DMEM/F12 containing 10% FBS. After cells washed with DMEM/F12 and centrifuged at 1,000 rpm for 5 min, depositing cells were combined with DMEM/F12 medium containing 10% FBS to form single-cell suspension. Cells were inoculated into 75 cm^2 subculture bottles with 1×10^5 cell densities for passage culture, and the growth and morphological changes were observed using an inverted phase contrast microscope.

Identification of hUC-MSCs

The hUC-MSCs were identified according to hUC-surface antigen by flow cytometry and the multidirectional differentiation characteristics performed by *in vitro* induction test (17). To evaluate the expression of surface antigens, cultured hUC-MSCs were detached, washed, and resuspended in PBS at 1×10^6 cells/ml. Different antibodies were added to the cell suspension, and were incubated at room temperature in the dark for 30 min. The antibodies included sheep anti-mouse IgG1–FITC/IgG1–PE/IgG1-Percp isotype control (BD, Franklin Lakes, NJ, USA), human HLA-DR-Percp /CD34-PE/ CD45-FITC directly-labeled antibody (BD), sheep anti-mouse IgG1 k-FITC/ IgG1-PE isotype control (BD), anti-human CD90-FITC/CD73-PE directly-labeled antibody (BD), sheep anti-mouse IgG1 k-PE isotype control (ebioscience, USA), and anti-human CD105-PE directly-labeled antibody (ebioscience). Data were analyzed by flow cytometry with the EXPO-32 software (EPICS-XL4, BECKMAN, Pasadena, CA, USA).

To evaluate differentiation potentials, cultured MSCs at passage 3 were detached and replaced in twelve-well plates and six-well covered plates with a density of 2×10^4/ml. The confluent cells were cultured in differentiation media for induction of osteocyte- and adipocyte-like cells. Osteogenic differentiation medium consisted of 100 nM dexamethasone (DXM) (Sigma-Aldrich, USA), 10 nM β-phosphoglycerol (Sigma-Aldrich), 50 μM levorotation vitamin C (Sigma-Aldrich), and 50 nM FK506 (Sigma-Aldrich).

Chondrogenic differentiation medium consisted of 1 mM sodium pyruvate (Sigma-Aldrich), 0.1 nM DXM (Sigma-Aldrich), 0.1 mM levorotation vitamin C (Sigma-Aldrich), 10 ng/ml TGF-β 3 (R&D Systems, Minneapolis, MN, USA), 6.25 μg/ml TRF (AbCys, Paris, France), 6.25 μg/ml rh-insulin (Invitrogen, Carlsbad, CA, USA), 6.25 ng/ml seleninic acid (Sigma-Aldrich), 5.35 μg/ml linoleic acid (Sigma-Aldrich), and 1.25 ng/ml BBSA (Sigma-Aldrich). Adipogenic differentiation medium consisted of 1 μM DXM (Sigma-Aldrich), 0.5 mM IBMX (Sigma-Aldrich), 10 mg/l recombinant human insulin (Invitrogen) and 100 μM indometacin (Sigma-Aldrich). After 2 weeks of culture, osteogenic differentiation was confirmed according to calcium deposits by Alizarin red S staining (Sigma-Aldrich). Chondrogenic differentiation was confirmed according to specific cartilage matrix secretion by Alican (Sigma-Aldrich), and adipogenic differentiation was demonstrated according to lipid droplets by Oil red O (Sigma-Aldrich) staining.

HP and Growth Curve of hUC-MSCs

The hUC-MSCs were randomly divided into four groups: 1% hypoxia group (treated with 1% oxygen), 3% hypoxia group (3% oxygen), 5% hypoxia group (5% oxygen) and normoxia group (21% oxygen), all at a constant 5% CO_2 and variable N_2. For HP, the cultured hUC-MSCs at passage 3 were incubated in the corresponding oxygen concentration at 37°C for 72 h. After detachment with 0.25% trypsin/EDTA, hUC-MSCs were replaced in four 12-well plates. Three wells of hUC-MSCs were detached and the number of hUC-MSCs was counted. The growth curve of MSCs was constructed to compare the effects of HP on hUC-MSCs proliferation.

Induction of hUC-MSCs to Cardiomyocyte-Like Cells, Cell Cycle and Ultrastructure

After HP, the cultured hUC-MSCs at passage 3 were detached and inoculated in 6-well plates containing 5-nitrogen cytidine (5-AZA) (Sigma) for 24 h incubation at 37°C and 5% CO_2 in a saturated humidity incubator. The final concentration of 5-AZA for induction of cardiomyocyte-like cells from hUC-MSCs was 0, 50, 75, 100, 150 and 200 μM. The cells were then incubated with normal culture medium and the medium was refreshed every 1-2 days. Morphological changes of the cells were observed under microscope and photographed every 2 days.

The cell cycle of hUC-MSCs and differentiated cardiomyocyte-like cells was evaluated with flow cytometry. Ultrastructure of the hUC-MSCs and the differentiated cardiomyocyte-like cells was observed under an electronic microscope.

Conversion Rate of hUC-MSCs to Cardiomyocyte-Like Cells

Identification of cardiomyocyte-like cells is mainly dependent on intracellular troponin (cTnI). The streptavidin-perosidase (SP) immunohistochemical staining method and flow cytometry were used to determine cardiomyocyte-like cells after four weeks of induction, in which cTnI in the cardiomyocyte-like cells was stained and buffy-like granules that appeared in the cytoplasm were observed under microscope. After immunocytochemical staining and hematoxylin redye, five visual fields were selected randomly for each group and the cTnI-positive cardiomyocyte-like cells were counted under an inverted phase contrast microscope at 100× magnification. Conversion rate of hUC-MSCs to cardiomyocyte-like cells was calculated according to the following formula: the conversion rate = cTnI positive cell number/blue nucleus cell number (the total number of cells) × 100%.

Chemical Damage and Measurements of Mitochondrial Ca^{2+} and Membrane Potential in hUC-MSCs

The cultured hUC-MSCs in 6-well plates were treated with cobalt chloride ($CoCl_2$) at 100 μM to induce chemical hypoxia damage, or treated with the same volume of BPS as control for 24 h. Laser confocal microscopy was used to measure mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondria Ca^{2+} concentration ($[Ca^{2+}]_m$) in hUC-MSCs and cardiomyocyte-like cells. The $\Delta\Psi_m$ was monitored with a fluorescent probe containing Rhodamine123 (Rh-123) (Sigma), which is taken up into cells and localized into mitochondria. The diffusion and accumulation of Rh-123 in mitochondria is proportional to the degree of $\Delta\Psi_m$ (34). The hUC-MSCs were incubated with PBS containing 0.5 μM Rh-123 in the dark for 30 min at 37°C (9). Rh-123 fluorescence was excited at 490 nm, and the green emission fluorescence was detected with a long-pass filter of 530 nm to analyze $\Delta\Psi_m$. Five cells were selected randomly for measurement and were photographed, and the average fluorescence intensity was taken as $\Delta\Psi_m$. $[Ca^{2+}]_m$ was measured by loading cells with the Ca^{2+} fluorescent dye rhod-2-AM (Molecular Probes, USA). The cells were loaded with 10 μM rhod-2 acetoxymethyl ester for 120 min at 4°C, and then incubated for 30 min at 37°C in the culture medium. The two-step cold loading/warm incubation protocols led to exclusive loading of rhod-2 into the mitochondria (30). Rhod-2 fluorescence was excited at 530 nm and analyzed with emission monitor through a 510 and 570 nm band pass barrier filter. The fluorescence intensity was calculated from selected areas of

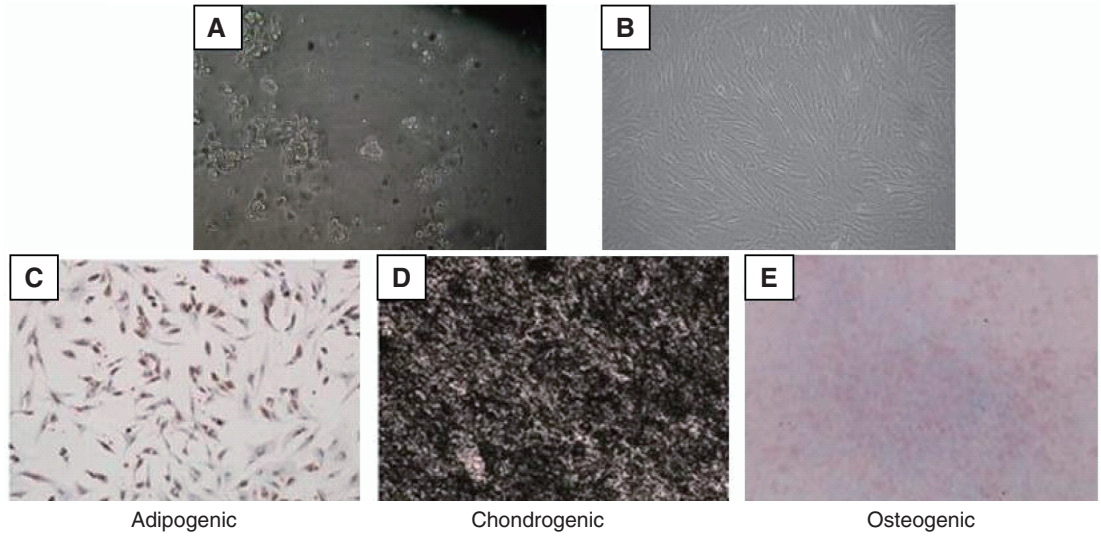

Fig. 1. hUC-MSCs under culture and after differentiation. A: hUC-MSCs cultured for 7 days (transformation); B: hUC-MSCs of third Passage. C-E: Multipotential differentiation of hUC-MSCs to adipogenic-like cells (C), chondrogenic-like cells (D) and osteogenic-like cells (E).

images and was taken as $[Ca^{2+}]_m$.

Data Statistics

All data were expressed as mean ± standard deviation (mean ± SD). Statistical analysis was performed with SPSS 13.0 software package. One-way analysis of variance (ANOVA) followed by a Dunnett's *post hoc* test was used to compare the data among multi groups and *T*-test was used to compare the data between two groups. Statistical P values < 0.05 were considered significant.

Results

Effects of HP on the Proliferation of hUC-MSCs

Adherent cells, mainly fibroblast-like and circular flat-like cells, were formed from cultured Wharton's jelly of the umbilical cord after 5-7 days of incubation. MSC colonies appeared after 10-14 days of culture. hUC-MSCs at passage 3 were used in this study. The fibroblast-like cells were long spindle in shape and displayed parallel arrangements, or spiral growth (Fig. 1, A and B).

The proliferation rate of hUC-MSCs was faster in HP conditions (1% to 5% O_2) than that in normoxic conditions, and the optimal hypoxic level for the promotion of hUC-MSCs proliferation was 3% O_2 (P < 0.01). There was no significant difference in the proliferation rate between 1% and 5% O_2 (P > 0.05). The difference of proliferation rate between 3% O_2 and normoxia appeared at 24 h of culture, and the difference between 1% O_2 or 5% O_2 and normoxia appeared at 48 h of culture. Those differences were more obvious when the culture time was extended (P < 0.01, Fig. 2). The results demonstrated that 3% O_2 was the optimal level for hUC-MSC HP treatment.

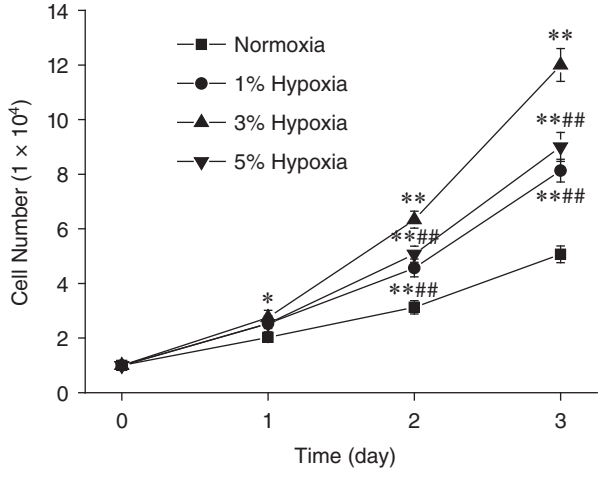

Fig. 2. Effects of hypoxic preconditioning at different oxygen concentrations on MSCs proliferation. Data are expressed as mean ± SD, n = 3 for each group, *P < 0.05, **P < 0.01 *vs.* normoxia, $^{\#\#}P$ < 0.01 *vs.* 3% hypoxia.

Effects of HP on hUC-MSCs Differentiation to Cardiomyocyte-Like Cells

The hUC-MSCs had a good multidirectional differentiation characteristic. The results showed that hUC-MSCs differentiated to adipogenic cells, osteogenic cells and chondrogenic cells in the corresponding induction medium (Fig. 1, C, D and E). The results

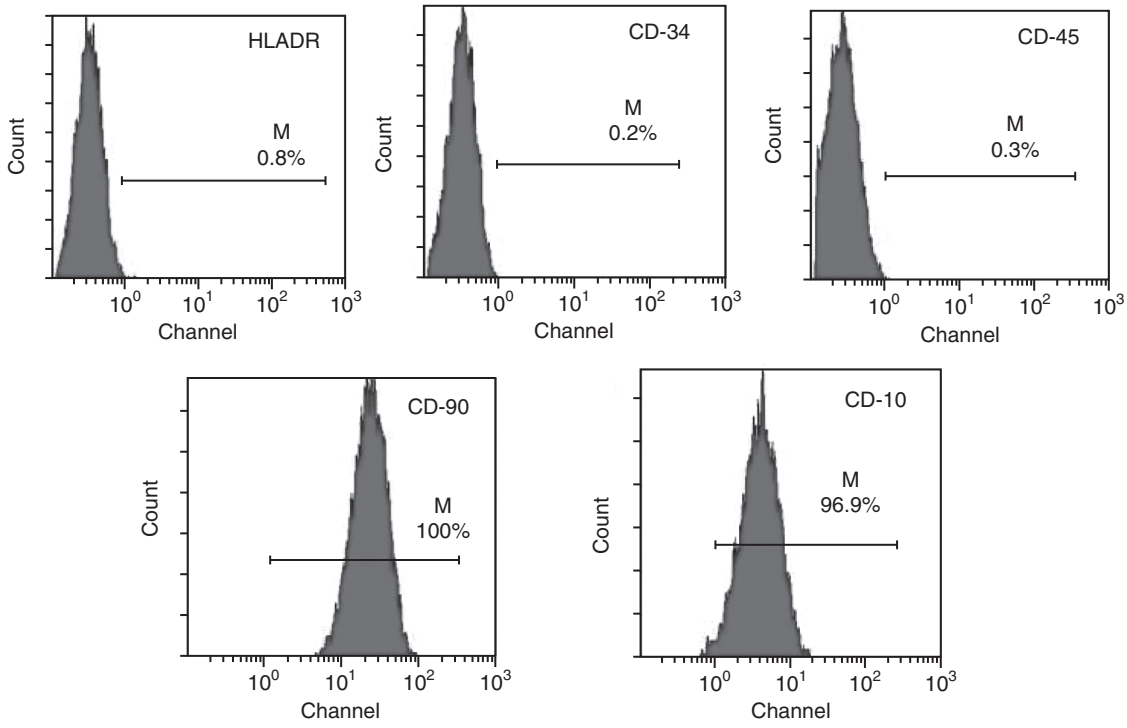

Fig. 3. Detection of surface antigens of hUC-MSCs by flow cytometry. Count, cell number; channel, relative strength of fluorescent signal; M, positive cells.

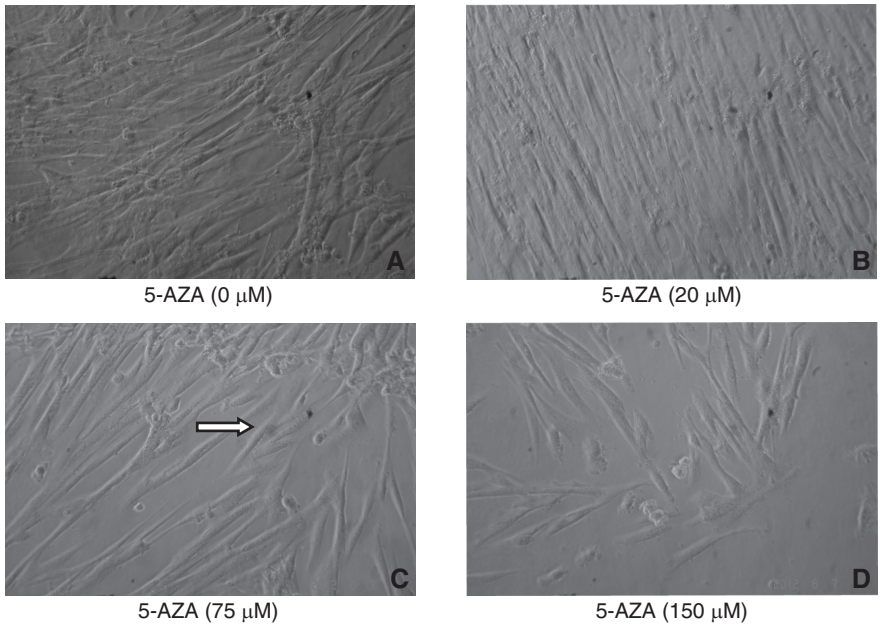

Fig. 4. Change of cell number and modality of MSCs induced by different concentrations of 5-AZA. Formation of bifurcate connection at 75 μM 5-AZA is indicated by arrow.

of flow cytometry showed that HALDR, CD34 and CD45 were not expressed on the MSC surface, and the positive rate was less than 2%. However, CD90 and CD105 were expressed strongly, and the positive rate was more than 95% (Fig. 3). The results demonstrated that all isolated and cultured hUC-MSCs in the study were highly purified mesenchymal stem cells.

After induction with 5-AZA for 24 h, the morphology of the hUC-MSCs changed significantly, the number of cells diminished, and cell proliferation was

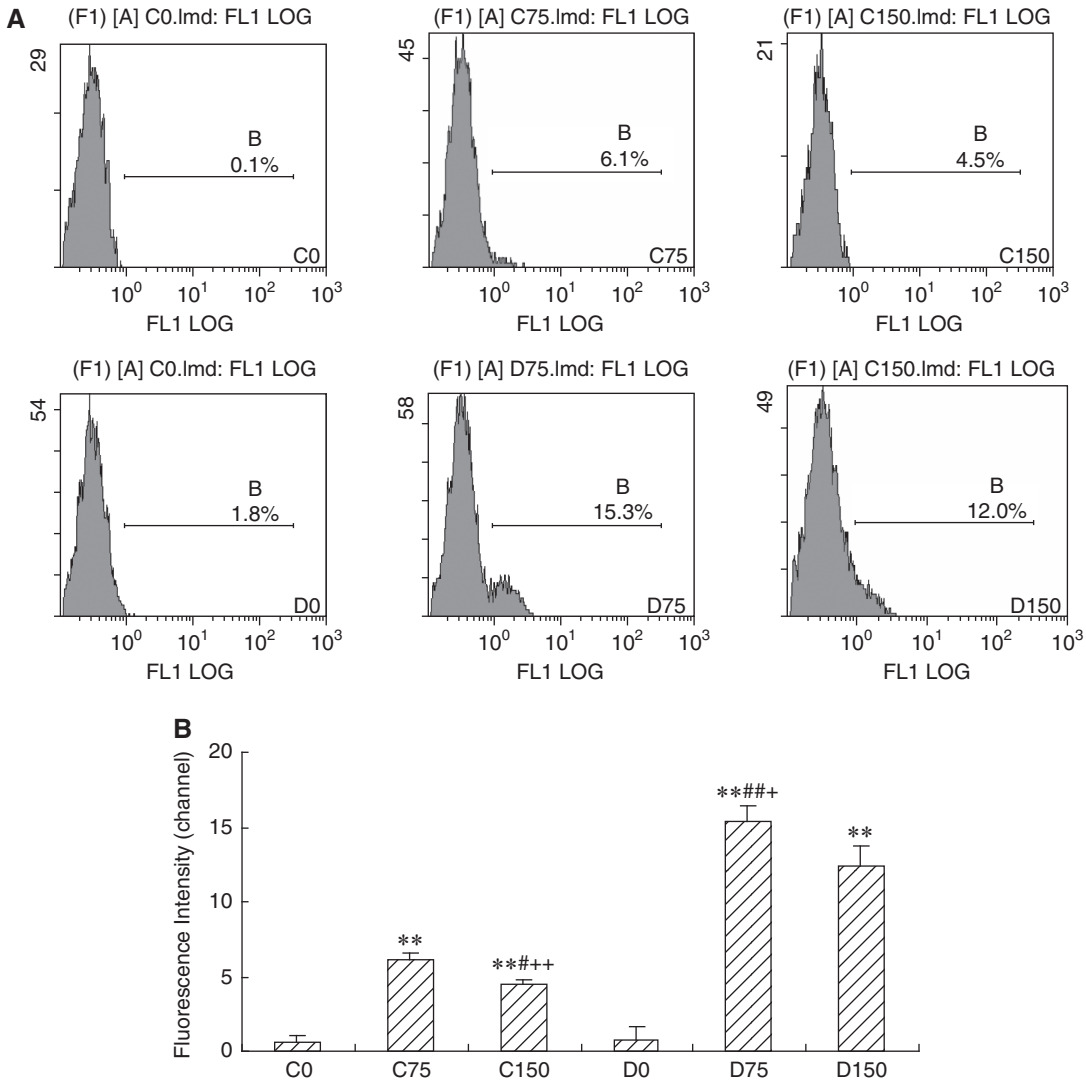

Fig. 5. Effects of hypoxia preconditioning on expression of TnI in cardiomyocyte-like cell induced by 5-AZA. A: Flow cytometry detection of TnI. B: quantification of data in A. C0, C75, C150: cells without 5-AZA or cells treated with 75 or 150 μM 5-AZA under normoxia, respectively; D0, D75, D150: cells without 5-AZA or cells treated with 75 or 150 μM 5-AZA under 3% hypoxia, respectively. Data are expressed as mean ± SD; n = 3 for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* corresponding C0 or D0, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ *vs.* C75; $^{+}P < 0.05$; $^{++}P < 0.01$ *vs.* D150.

arrested. The induced cells became rod-shape, short, thick and bifurcation on 5-AZA treatment compared with the control cells. And the cells displayed the characteristics of mutual connection and aggregative growth (Fig. 4). The morphological change was most obvious in cells treated with 75, 100 and 150 μM 5-AZA. The decrease of cell number was most obvious at 200 μM 5-AZA. The results suggest that 5-AZA at < 150 μM concentration could inhibit proliferation and promoted differentiation of hUC-MSCs. Higher 5-AZA (> 200 μM) could result in growth inhibition or cell death.

TnI is a subunit of Troponin which is the main regulatory protein of myocardium, which is in turn regulated by Nkx2.5 and GATA-4. Hence, TnI was selected as a marker of cardiomyocytes in this study (20). There was no cTnI expression in the control cells without 5-AZA treatment. The fraction of cTnI-positive cells was 15, 35, 32, 35 and 32% when treated with 50, 75, 100, 150 and 200 μM 5-AZA, respectively. Although the fraction of cTnI-positive cells on 200 μM 5-AZA treatment was comparable with that on 75-150 μM treatment, the total number of cells diminished.

The ratio of cTnI-positive cells was higher in HP-treated cells than that in the normoxia-treated cells (40.0 ± 3.0% *vs.* 34.2 ± 2.0%, $P < 0.05$) in 75 μM 5-AZA condition. Also, the ratio of cTnI-positive cells was higher in HP-treated cells than that in normoxia-treated cells (38.4 ± 2.6% *vs.* 33.2 ± 2.0%, $P < 0.05$) under 150 μM 5-AZA condition. In addition, the cTnI content induced by 5-AZA was significant higher in

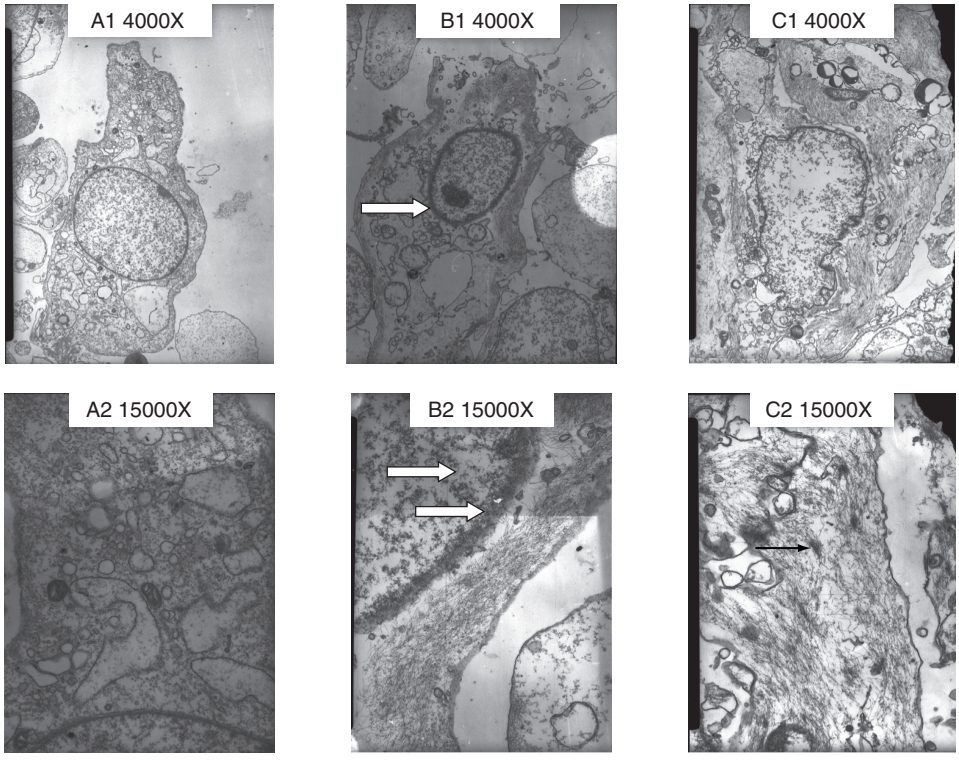

Fig. 6. Ultrastructure of stem celld and myocardial-like cells under electronic microscope. A: Stem cells: no myofilament, round, oval, clear, big nuclear and abundant organelles. B: cardiomyocyte-like cell induced by 5-AZA for 4 weeks: abundant myofilament under the cell membrane (indicated by open arrow). C: cardiomyocyte-like cell induced by 5-AZA for 4 weeks: abundant myofilament under the cell membrane, higher electron density in myofilament dense area and plaque-like structure, which looks like myofilament adhesion, Z or M belt (indicated by thin arrow).

HP-treated than that in normoxia-treated cadiomyocyte-like cells ($P < 0.05$, Fig. 5). The results suggest that the optimal concentration of 5-AZA for hUC-MSCs differentiation to cadiomyocyte-like cells was 75-150 μM, and HP promoted differentiation of hUC-MSCs induced by 5-AZA.

Effects of HP on the Cell Cycle and the Ultrastructure of hUC-MSCs and Cadiomyocyte-Like Cells

HP had no effects on the cell cycle of hUC-MSCs, but did change the cell cycle of cadiomyocyte-like cells induced by 5-AZA. In cadiomyocyte-like cells, G_1-phase cell population was decreased and the cells in the G_2 and S phase were increased ($P < 0.05$). The results showed that HP could block cell growth at the G_2 and S phase, and enhanced differentiation and proliferation to cadiomyocyte-like cells (Table 1).

Under transmission electron microscopy, hUC-MSCs without the 5-AZA treatment displayed large nuclei, clear cytoplasm, and abundant organelles but no filaments. After induction with 5-AZA, there were unequal amounts of muscle filaments in bundles, or vertical terraces arrangement appeared near the nucleus in the cells. The density of the muscle filaments was higher in the cardiomyocyte-like cells treated with hypoxia than that with normoxia. The sites of high-density myofilaments in some cells looked like plaque, which was likely the filament attachment site of Z or M band (Fig. 6).

Table 1. Effects of hypoxic preconditioning (3% O_2) on the cell cycle of hUC-MSCs and cardiomyocyte-like cells induced by different concentrations of 5-AZA

5-AZA (μM)	Stage	Normoxia	Hypoxia
0	G_1	94.13 ± 0.90	93.97 ± 1.59
	G_2	3.76 ± 0.71	2.04 ± 0.07
	S	2.84 ± 0.38	3.51 ± 0.11
75	G_1	93.93 ± 1.76	87.27 ± 0.91*
	G_2	3.07 ± 0.33	8.25 ± 0.06*
	S	2.98 ± 0.43	5.10 ± 0.30*
150	G_1	85.13 ± 2.15	75.70 ± 2.55*
	G_2	9.63 ± 0.61	14.63 ± 0.60*
	S	5.69 ± 0.32	9.82 ± 0.50*

Data were expressed as mean ± SD; n = 3 for each group. *$P < 0.05$ *vs*. corresponding normoxia.

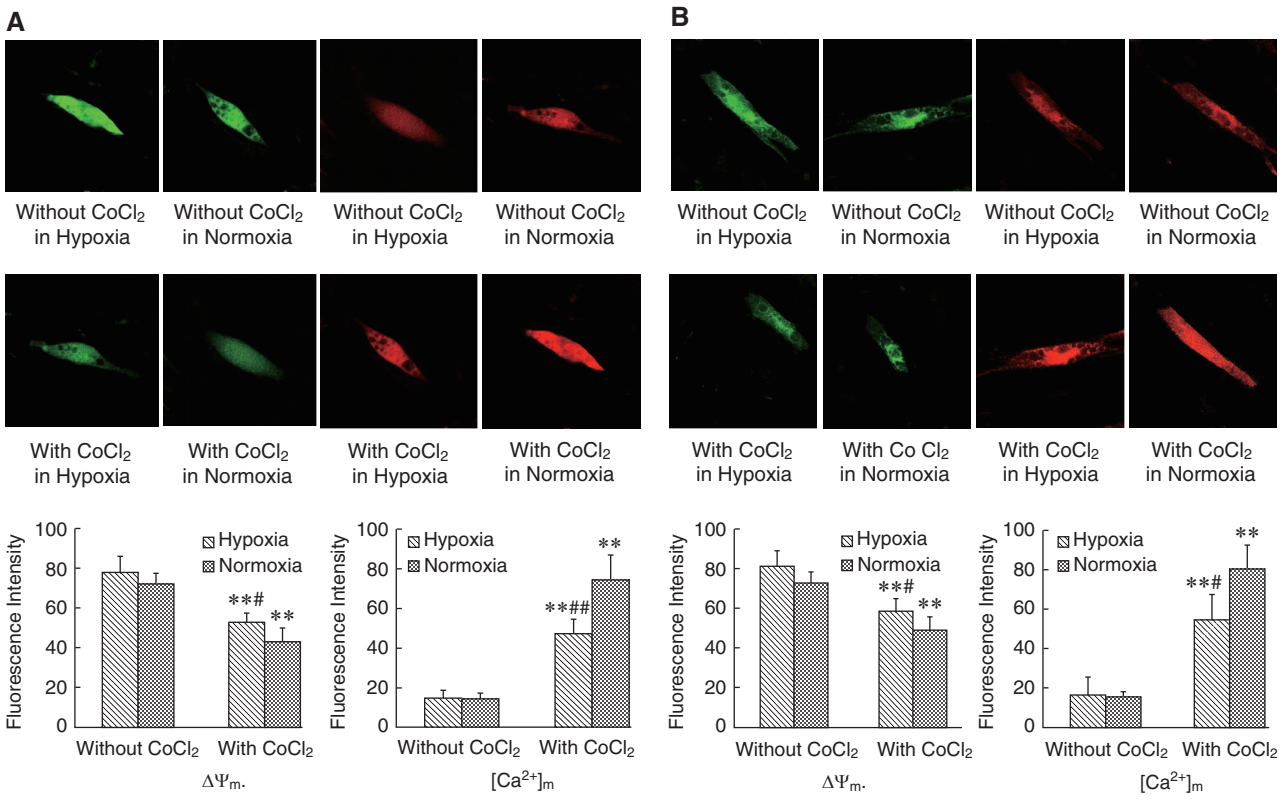

Fig. 7. Effects of hypoxia on mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial calcium ($[Ca^{2+}]_m$) in hUC-MSCs (A) and myocardium-like cell induced by 75 μM 5-AZA (B). Upper: Images of mitochondrial membrane potential and mitochondrial calcium under confocal microscope. Bottom: Quantitative comparison of mitochondrial membrane potential and mitochondrial calcium. Data are expressed as mean ± SD; n = 5 for each group. $^{**}P < 0.01$ *vs.* corresponding without $CoCl_2$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* corresponding normoxia.

Protective Effects of HP on hUC-MSCs against Mitochondria Damage Induced by Chemical Hypoxia

The $\Delta\Psi_m$ was decreased and $[Ca^{2+}]_m$ was increased significantly in hUC-MSCs and cadiomyocyte-like cells during chemical hypoxia ($P < 0.01$, Fig. 7, A and B), suggesting that the mitochondria of hUC-MSCs and cadiomyocyte-like cells were damaged. The $\Delta\Psi_m$ was increased and $[Ca^{2+}]_m$ was decreased significantly in HP-treated hUC-MSCs compared with the normaxia-treated hUC-MSCs ($P < 0.05$ or 0.01, Fig. 7A). The $\Delta\Psi_m$ was increased and $[Ca^{2+}]_m$ was decreased significantly in HP-treated cadiomyocyte-like cells induced by 75 μM 5-AZA compared with normaxia- treated cadiomyocyte-like cells ($P < 0.05$, Fig. 7B). Taken together, the results indicated that HP could effectively protect the mitochondria of hUC-MSCs and cadiomyocyte-like cells against chemical hypoxic damage induced by $CoCl_2$.

Discussion

In this study, immunohistochemistry and flow cytometry were used to investigate the effects of HP on the proliferation of hUC-MSCs in culture, the differentiation of hUC-MSCs to cardiomyocyte-like cells, and the effects of HP protection against chemical hypoxia injury on hUC-MSCs and cardiomyocyte-like cells. The result showed that 3% oxygen was an optimal hypoxia level for HP on proliferation and differentiation of hUC-MSCs. HP significantly accelerated the proliferation of hUC-MSCs, promoted the differentiation of hUC-MSCs to cardiomyocyte-like cells induced by 5-AZA, and effectively protected the mitochondria of hUC-MSCs and cardiomyocyte-like cells against the chemical hypoxic damage induced by $CoCl_2$.

Although many studies have been carried out for the effect of hypoxia on MSCs, disputes still exists on the effects of hypoxia on proliferation and differentiation of MSCs. For proliferation, some experiments found that certain level and duration of hypoxia could promote MSC proliferation (11, 25, 30), but others reported inhibition of proliferation if hypoxia was sustained more than 7 days (16). For differentiation, there were reports that hypoxia could promote stem cells differentiation to osteoblast and adipocyte (2, 35) and undergo chondrogenesis (4). But there were

conflicting reports that hypoxia inhibited the differentiation ability of stem cells (5, 40). Furthermore, there are no reports yet on the effects of HP on proliferation and differentiation of hUC-MSCs. This study confirmed the promotory effects of HP on proliferation and differentiation of hUC-MSCs.

The effects of hypoxia on the cells or body are known to be dependent on the degree and duration of hypoxia. Up to now, however, there was no consensus on the optimal hypoxia for the research of MSCs. Some researchers showed that hypoxia between 3% O_2 and 21% O_2 had no effects on differentiation ability of MSCs to osteoblast, while 1% O_2 had a significant inhibition on the differentiation of stem cells to osteoblast (16, 19, 37). But other studies demonstrated that only hypoxia below 5% O_2 could inhibit osteoblast differentiation (26). Also there was a report that hypoxia could maintain the differentiation characteristics of stem cells (24). Our results demonstrated that HP of 3% O_2 for 72 h showed the best efficiency in promoting hUC-MSCs proliferation and differentiation toward cardiomyocyte-like cells.

Numerous studies found that the therapeutic efficacy of MSCs transplantation on myocardium infarction was closely related to the survival rate of transplanted cells *in situ* (21, 32). In general, the transplantation survival rate of MSCs was extremely low at less than 1% (33, 36), suggesting a large number of transplanted cells was lost after transplantation. Only MSCs with long-term viability could differentiate into cardiomyocytes to execute myocardial repair (13). The severe loss of transplanted cells has greatly restricted the efficiency of stem cell transplantation (15). Researches showed that the loss of stem cells was mainly related to ischemia and hypoxia in the transplantation site (29). In the pathological myocardium, long-term ischemia and hypoxia often exist, resulting in anoxia for low blood reperfusion even if vessels were re-opened (7). Therefore, it is important to improve the survival rates of MSCs against hypoxia or ischemia of MSCs after transplantation. Generally, the mitochondrial membrane potential and mitochondrial calcium were often used to evaluate cellular functions. Many studies have shown that decreasing mitochondrial membrane potential is related to mitochondrial permeability transition (PT), which is related to opening of PT pore. At the same time, calcium can be overloaded through the opening PT pore. In this study, the mitochondrial membrane potential was found to decrease and the mitochondrial calcium was increased in hUC-MSCs and cardiomyocyt-like cells during chemic hypoxia induced by $CoCl_2$, reflecting damages to these cells. And the damages of hUC-MSCs and cardiomyocyt-like cells were effectively prevented by HP, demonstrating the protective effects of HP on hUC-MSCs and cardiomyocyt-like cells against chemical hypoxia injury.

Among different types of stem cells, MSCs or multipotent mesenchymal stromal cells were considered to be ideal tools for the treatment of many degenerative diseases (5, 10), with their characteristics of multi-lineage differentiation potentials (14, 28, 33), nutrition effects (6, 39), immunosuppressive effects (23) and angiogenic ability (22).

In conclusion, this study demonstrated for the first time that an optimal level of hypoxia preconditioning enhanced proliferation and differentiation of hUC-MSCs to cardiomyocyte-like cells, and protected hUC-MSCs against chemical hypoxia injury. These findings form the basis of our recent observation that cardiac transplantation with the HP-treated hUC-MSCs effectively alleviated the remodeling of left atrium and ventricle, diminished infarct area and myocardial fibration, and improved the pump function in post-infarction heart of rabbits (unpublished data). Hypoxic preconditioning might be a mechanism to provide beneficial effects to hUC-MSC-based cardiac transplantation.

Acknowledgments

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References

1. Akita, T., Murohara, T., Ikeda, H., Sasaki, K., Shimada, T., Egami, K. and Imaizumi, T. Hypoxic preconditioning augments efficacy of human endothelial progenitor cells for therapeutic neovascularization. *Lab. Invest*. 83: 65-73, 2003.
2. Basciano, L., Nemos, C., Foliguet, B., de Isla, N., de Carvalho, M., Tran, N. and Dalloul, A. Long term culture of mesenchymal stem cells in hypoxia promotes a genetic program maintaining their undifferentiated and multipotent status. *BMC Cell Biol*. 12: 12, 2011.
3. Bedada, F.B., Chan, S.S., Metzger, S.K., Zhang, L., Zhang, J., Garry, D.J., Kamp, T.J., Kyba, M. and Metzger, J.M. Acquisition of a quantitative, stoichiometrically conserved ratiometric marker of maturation status in stem cell-derived cardiac myocytes. *Stem Cell Reports* 3: 594-605, 2014.
4. Bertolo, A., Hafner, S., Taddei, A.R., Baur, M., Potzel, T., Steffen, F. and Stoyanov, J. Injectable microcarriers as human mesenchymal stem cell support and their application for cartilage and degenerated intervertebral disc repair. *Eur. Cell Mater*. 29: 70-81, 2015.
5. Boyette, L.B., Creasey, O.A., Guzik, L., Lozito, T. and Tuan, R.S. Human bone marrow-derived mesenchymal stem cells display enhanced clonogenicity but impaired differentiation with hypoxic preconditioning. *Stem Cells Transl. Med*. 3: 241-254, 2014.
6. Caplan, A.I. and Dennis, J.E. Mesenchymal stem cells as trophic mediators. *J. Cell. Biochem*. 98: 1076-1084, 2006.
7. Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P. and Gurtner, G.C. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat. Med*. 10: 858-864, 2004.

8. Delalat, B., Pourfathollah, A.A., Soleimani, M., Mozdarani, H., Ghaemi, S.R., Movassaghpour, A.A. and Kaviani, S. Isolation and *ex vivo* expansion of human umbilical cord blood-derived $CD34^+$ stem cells and their cotransplantation with or without mesenchymal stem cells. *Hematology* 14: 125-132, 2009.
9. Di Lisa, F., Blank, P.S., Colonna, R., Gambassi, G., Silverman, H.S., Stern, M.D. and Hansford, R.G. Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition. *J. Physiol*. 486: 1-13, 1995.
10. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D.J. and Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8: 315-317, 2006.
11. Estrada, J.C., Albo, C., Benguria, A., Dopazo, A., Lopez-Romero, P., Carrera-Quintanar, L., Roche, E., Clemente, E.P., Enriquez, J.A., Bernad, A. and Samper, E. Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis. *Cell Death Differ*. 19: 743-755, 2012.
12. Gavira, J.J., Perez-Ilzarbe, M., Abizanda, G., Garcia-Rodriguez, A., Orbe, J., Paramo, J.A., Belzunce, M., Rabago, G., Barba, J., Herreros, J., Panizo, A., de Jalon, J.A., Martinez-Caro, D. and Prosper, F. A comparison between percutaneous and surgical transplantation of autologous skeletal myoblasts in a swine model of chronic myocardial infarction. *Cardiovasc. Res*. 71: 744-753, 2006.
13. Geng, Y.J. Molecular mechanisms for cardiovascular stem cell apoptosis and growth in the hearts with atherosclerotic coronary disease and ischemic heart failure. *Ann. NY Acad. Sci*. 1010: 687-697, 2003.
14. Govindasamy, V., Ronald, V.S., Abdullah, A.N., Nathan, K.R., Ab Aziz, Z.A., Abdullah, M., Musa, S., Kasim, N.H. and Bhonde, R.R. Differentiation of dental pulp stem cells into islet-like aggregates. *J. Dent. Res*. 90: 646-652, 2011.
15. Herrmann, J.L., Abarbanell, A.M., Weil, B.R., Wang, Y., Wang, M., Tan, J. and Meldrum, D.R. Cell-based therapy for ischemic heart disease: a clinical update. *Ann. Thorac. Surg*. 88: 1714-1722, 2009.
16. Holzwarth, C., Vaegler, M., Gieseke, F., Pfister, S.M., Handgretinger, R., Kerst, G. and Muller, I. Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells. *BMC Cell Biol*. 11: 11, 2010.
17. Horwitz, E.M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Deans, R.J., Krause, D.S. and Keating, A. Clarification of the nomenclature for MSC: the International Society for Cellular Therapy position statement. *Cytotherapy* 7: 393-395, 2005.
18. Hu, X., Yu, S.P., Fraser, J.L., Lu, Z., Ogle, M.E., Wang, J.A. and Wei, L. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function *via* enhanced survival of implanted cells and angiogenesis. *J. Thorac. Cardiovasc. Surg.* 135: 799-808, 2008.
19. Hung, S.P., Ho, J.H., Shih, Y.R., Lo, T. and Lee, O.K. Hypoxia promotes proliferation and osteogenic differentiation potentials of human mesenchymal stem cells. *J. Orthop. Res*. 30: 260-266, 2012.
20. Malliaras, K., Makkar, R.R., Smith, R.R., Cheng, K., Wu, E., Bonow, R.O., Marbán, L., Mendizabal, A., Cingolani, E., Johnston, P.V., Gerstenblith, G., Schuleri, K.H., Lardo, A.C. and Marbán, E. Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (CArdiosphere-Derived aUtologous stem Cells to reverse ventricUlar dySfunction). *J. Am. Coll. Cardiol*. 63: 110-122, 2014.
21. Mangi, A.A., Noiseux, N., Kong, D., He, H., Rezvani, M., Ingwall, J.S. and Dzau, V.J. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat. Med*. 9: 1195-1201, 2003.
22. Martens, T.P., See, F., Schuster, M.D., Sondermeijer, H.P., Hefti, M.M., Zannettino, A., Gronthos, S., Seki, T. and Itescu, S. Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. *Nat. Clin. Pract. Cardiovasc. Med*. 3 (Suppl 1): S18-S22, 2006.
23. Nauta, A.J. and Fibbe, W.E. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 110: 3499-3506, 2007.
24. Nekanti, U., Dastidar, S., Venugopal, P., Totey, S. and Ta, M. Increased proliferation and analysis of differential gene expression in human Wharton's jelly-derived mesenchymal stromal cells under hypoxia. *Int. J. Biol. Sci*. 6: 499-512, 2010.
25. Panchision, D.M. The role of oxygen in regulating neural stem cells in development and disease. *J. Cell. Physiol*. 220: 562-568, 2009.
26. Raheja, L.F., Genetos, D.C. and Yellowley, C.E. The effect of oxygen tension on the long-term osteogenic differentiation and MMP/TIMP expression of human mesenchymal stem cells. *Cells Tissues Organs* 191: 175-184, 2010.
27. Saller, M.M., Prall, W.C., Docheva, D., Schonitzer, V., Popov, T., Anz, D., Clausen-Schaumann, H., Mutschler, W., Volkmer, E., Schieker, M. and Polzer, H. Increased stemness and migration of human mesenchymal stem cells in hypoxia is associated with altered integrin expression. *Biochem. Biophys. Res. Commun*. 423: 379-385, 2012.
28. Sasaki, M., Abe, R., Fujita, Y., Ando, S., Inokuma, D. and Shimizu, H. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J. Immunol*. 180: 2581-2587, 2008.
29. Shimizu, T., Sekine, H., Yamato, M. and Okano, T. Cell sheet-based myocardial tissue engineering: new hope for damaged heart rescue. *Curr. Pharm. Des*. 15: 2807-2814, 2009.
30. Simon, M.C. and Keith, B. The role of oxygen availability in embryonic development and stem cell function. *Nat. Rev. Mol. Cell Biol*. 9: 285-296, 2008.
31. Steinhauser, M.L. and Lee, R.T. Regeneration of the heart. *EMBO Mol. Med*. 3: 701-712, 2011.
32. Tang, Y.L., Tang, Y., Zhang, Y.C., Qian, K., Shen, L. and Phillips, M.I. Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. *J. Am. Coll. Cardiol*. 46: 1339-1350, 2005.
33. Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J. and Kessler, P.D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 105: 93-98, 2002.
34. Ubl, J.J., Chatton, J.Y., Chen, S. and Stucki, J.W. A critical evaluation of *in situ* measurement of mitochondrial electrical potentials in single hepatocytes. *Biochim. Biophys. Acta* 1276: 124-132, 1996.
35. Valorani, M.G., Montelatici, E., Germani, A., Biddle, A., D'Alessandro, D., Strollo, R., Patrizi, M.P., Lazzari, L., Nye, E., Otto, W.R., Pozzilli, P. and Alison, M.R. Pre-culturing human adipose tissue mesenchymal stem cells under hypoxia increases their adipogenic and osteogenic differentiation potentials. *Cell Prolif*. 45: 225-238, 2012.
36. Wang, X., Jameel, M.N., Li, Q., Mansoor, A., Qiang, X., Swingen, C., Panetta, C. and Zhang, J. Stem cells for myocardial repair with use of a transarterial catheter. *Circulation* 120: S238-S246, 2009.
37. Yang, D.C., Yang, M.H., Tsai, C.C., Huang, T.F., Chen, Y.H. and Hung, S.C. Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. *PLoS One* 6: e23965, 2011.
38. Yu, Y.S., Shen, Z.Y., Ye, W.X., Huang, H.Y., Hua, F., Chen, Y.H., Chen, K., Lao, W.J. and Tao, L. AKT-modified autologous intracoronary mesenchymal stem cells prevent remodeling and repair in swine infarcted myocardium. *Chinese Med. J. (Engl.)* 123: 1702-1708, 2010.

39. Zhang, M., Mal, N., Kiedrowski, M., Chacko, M., Askari, A.T., Popovic, Z.B., Koc, O.N. and Penn, M.S. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *FASEB J.* 21: 3197-3207, 2007.
40. Zhang, Y., Marsboom, G., Toth, P.T. and Rehman, J. Mitochondrial respiration regulates adipogenic differentiation of human mesenchymal stem cells. *PLoS One* 8: e77077, 2013.
41. Zhuang, Y., Chen, X., Xu, M., Zhang, L.Y. and Xiang, F. Chemokine stromal cell-derived factor 1/CXCL12 increases homing of mesenchymal stem cells to injured myocardium and neovascularization following myocardial infarction. *Chinese Med. J. (Engl.)* 122: 183-187, 2009.