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ORIGINAL PAPER

Effect of low oxygen tension on the biological characteristics of human bone marrow mesenchymal stem cells

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Abstract Culture of mesenchymal stem cells (MSCs) under ambient conditions does not replicate the low oxygen environment of normal physiological or pathological states and can result in cellular impairment during culture. To overcome these limitations, we explored the effect of hypoxia (1 % O_2) on the biological characteristics of MSCs over the course of different culture periods. The following biological characteristics were examined in human bone marrow-derived MSCs cultured under hypoxia for 8 weeks: proliferation rate, morphology, cell size, senescence, immunophenotypic characteristics, and the expression levels of stemness-associated factors and cytokine and chemokine genes. MSCs cultured under hypoxia for approximately 2 weeks showed increased proliferation and viability. During long-term culture, hypoxia delayed phenotypic changes in MSCs, such as increased cell volume, altered morphology, and the expression of senescence-associated-β-gal, without altering their characteristic immunophenotypic characteristics. Furthermore, hypoxia increased the expression of stemness and chemokine-related genes, including *OCT4* and *CXCR7*, and did not decrease the expression of *KLF4*, *C-MYC*, *CCL2*, *CXCL9*, *CXCL10*, and *CXCR4* compared with levels in cells cultured under normoxia. In conclusion, low oxygen tension improved the biological characteristics of MSCs during ex vivo expansion. These data suggest that hypoxic culture could be a useful method for increasing the efficacy of MSC cell therapies.

Keywords Mesenchymal stem cell · Hypoxia · Biological characteristics · Cell therapy

Introduction

Human mesenchymal stem cells (MSCs) possess enormous potential for tissue repair and renewal of damaged cells, which is attributed to their multipotent differentiative capacity (Sasaki et al. 2008; Toma et al. 2002), trophic activity (Caplan and Dennis 2006; Zhang et al. 2007), potent immunosuppressive effects (Aggarwal and Pittenger 2005; Nauta and Fibbe 2007), and ability to induce vascularization (Martens et al. 2006). These characteristics of MSCs make them promising therapeutic tools for use in the clinic. Clinical applications require a large number of cells to ensure the survival of a sufficient number of MSCs after transplantation. This requirement still poses a major obstacle for the achievement of successful clinical applications. MSCs are present at very low numbers in tissues despite the fact that they can be isolated from a wide variety of sources (Aust et al. 2004; Pittenger et al. 1999), and it is not possible to isolate the large numbers of MSCs required for clinical trials from a single donor for each therapy (Trachtenberg et al. 2011;

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Hare et al. 2012; Tan et al. 2012). Thus, ex vivo expansion of MSCs is necessary for developing and maintaining MSCs for cell therapy. Conventional expansion procedures for MSCs are performed under ambient oxygen levels (21 % O_2). However, in vivo MSCs are not usually exposed to such a high concentration of oxygen, which is approximately 4–10-fold greater than the concentration of oxygen in their natural niches (Antoniou et al. 2004; Chow et al. 2001). The ambient oxygen concentration might cause environmental stress to the in vitro cultured MSCs. Numerous studies have presented data correlating the ambient oxygen concentration with negative effects on MSCs, including early senescence, longer population doubling time, DNA damage (Estrada et al. 2012; Fehrer et al. 2007), and poor engraftment following transplantation (Mohamadnejad et al. 2010; Schächinger et al. 2006). These data have raised serious concerns regarding the therapeutic efficacy and safety of MSCs.

The low oxygen concentration in the stem cell niche or physiological microenvironment is an important component in the maintenance of MSC properties such as self-renewal, multipotency, and extended survival (Cipolleschi et al. 1993; Packer and Fuehr 1977). Thus, oxygen tension during in vitro expansion of MSCs might play a crucial regulatory role in the maintenance of stem cell properties. Recent studies show that the proliferation, differentiation, and survival of MSCs are affected by culture under low oxygen tension (Martin-Rendon et al. 2007; Grayson et al. 2007; Holzwarth et al. 2010; Annabi et al. 2003). However, the degree and duration of hypoxia described in previous studies vary greatly and may account, in part, for the conflicting effects of hypoxia on the proliferation and differentiation capacities of MSCs (Holzwarth et al. 2010; Salim et al. 2004; Lennon et al. 2001; Malladi et al. 2006).

In this study, we investigated the biological characteristics, such as the proliferation rate, cell size, morphology, senescence, immunophenotypic characteristics, and the expression of several genes, of bone marrow (BM)-MSCs under hypoxia over several cell passages. We observed that culture of MSCs under hypoxia improved their biological characteristics during ex vivo expansion. These results suggest that culture under hypoxia could be a useful method for increasing the efficacy of MSC-based cell therapies.

Materials and methods

Isolation and culture of human BM-MSCs

The institutional Review Board of the Samsung Medical Center approved the protocols used in this study (IRB No.2012–11-003). Iliac crest BM aspirates were obtained from five normal adult volunteers. Mononuclear cells were isolated from normal BM aspirates using Ficoll-Hypaque (Histopaque-1077; Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. Cells were plated at 3×10^5 cells/cm^2 in low glucose Dulbecco's Modified Eagle's Medium (LG-DMEM; Biowest, Nuaille, France) containing 10 % fetal bovine serum (FBS; Biowest) and 100 U/mL penicillin/streptomycin (Life Technologies-Gibco, Rockville, MD, USA). After 24 h, nonadherent cells were removed. Adherent cells were cultured for an additional 5–10 days, until they were ~70 % confluent. At this stage, the primary culture cells were trypsinized using 0.05 % Trypsin/EDTA (Life Technologies-Gibco) and designated as passage #1 (P1). MSCs were subcultured once per week by plating at a density of 1000 cells/cm^2 prior to dividing MSCs into two groups (normoxia and hypoxia groups).

Immunophenotypic analysis of BM-MSCs

Antibodies against the human antigens CD14, CD34, CD45, CD73, CD90, CD105, and CD166 and the histocompatibility antigen DR alpha chain (HLA-DR) were purchased from Becton Dickinson Bioscience (BD Bioscience, San Jose, CA, USA). A total of 5×10^5 cells were resuspended in 0.1 mL phosphate-buffered saline (PBS, Biowest) and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for 30 min at room temperature. FITC- or PE-conjugated human IgGs were used as isotype controls at the same concentrations as those of the specific antibodies. The fluorescence intensity of the cells was evaluated by flow cytometry (BD FACSVerse™; BD Bioscience), and the data were analyzed using the BD FACSuite™ software (BD Bioscience).

Differentiation of BM-MSCs

Adipogenic differentiation After reaching confluence, cells were cultured for 14–21 days in LG-DMEM containing 10 % FBS, 1 μM dexamethasone, 500 μM isobutyl methylxanthine (Sigma-Aldrich), 100 μM indomethacin (Sigma-Aldrich), and 10 μg/mL insulin (Sigma-Aldrich). Adipogenic differentiation was evaluated by detecting cellular accumulation of neutral lipid vacuoles via staining with Oil-red O (Sigma-Aldrich) solution.

Osteogenic differentiation MSCs were plated at 5×10^5 cells/well in 6-well plates in LG-DMEM containing 10 % FBS, allowed to adhere overnight, and the medium was then replaced with LG-DMEM containing 10 % FBS supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 100 μM ascorbate-2-phosphate (Sigma-Aldrich). The medium was changed every third day. After 14–21 days, osteoblast differentiation was determined by measuring alkaline phosphatase expression.

Chondrogenic differentiation After reaching confluence, 1×10^6 cells were pelleted in a 15-mL tube by centrifugation at 300×*g* for 5 min. Pelleted cells were resuspended and cultured for 14–21 days in LG-DMEM containing 1× insulin-transferrin-selenium (ITS; Life technologies-Gibco), 1 mM sodium pyruvate (Life Technologies-Gibco), 0.1 μM dexamethasone, 397 μg/mL ascorbate-2-phosphate, and 10 ng/mL transforming growth factor-β1 (R&D Systems, Minneapolis, MN, USA). Chondrogenic induction was evaluated at 80 % confluence by staining with toluidine blue to detect extracellular accumulation of chondrocyte matrix (Sigma-Aldrich).

Culture of BM-MSCs under hypoxic and normoxia

BM-MSCs derived from five donors (P5 D1 MSC, P5 D2 MSC, P5 D3 MSC, P2 D4 MSC, and P5 D5 MSC) were maintained under normoxia (37 °C, 5 % CO_2, 95 % air) for 7 days and then divided into two groups, a normoxia group and a hypoxia group (37 °C, 1 % O_2, 5 % CO_2, and 94 % N_2). Cells were plated at a density of 1000 cells/cm^2 and placed in a normoxia or a hypoxia chamber. Cells were observed on day 7 of culture using a phase contrast microscope (Olympus CK40, Melville, NY, USA). Cells were harvested using 0.05 % trypsin/EDTA, incubated with 4 % trypan blue solution, and counted using a hemocytometer (Marienfeld, German). Cells in each group were counted and subcultured once per week for 2 weeks. Among MSCs derived from different donors, donor 1 (D1) MSCs were counted and passaged under normoxia or hypoxia once per week for 8 weeks. Cell growth was assessed by counting cumulative cell numbers each week following initial plating at a density of 1000 cells/cm^2. Cumulative cell numbers were counted for 8 weeks in four independent experiments. At each passage, the number of cell divisions was calculated using the following formula: number of cell divisions = $\mathrm{Log}_2(N/N0)$, where $N0$ is the initial number of cells seeded and N is the final number of cells after 7 days of incubation.

Apoptosis assay by flow cytometry

Apoptosis assays were performed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis antibody (BD Bioscience) according to the manufacturer's instructions. Briefly, BM-MSCs initially plated at 1000 cells/cm^2 were maintained for 7 days under normoxia or hypoxia and then subcultured once per week. After 2 weeks, cells were collected and resuspended in binding buffer. Annexin V-FITC and propidium iodide (PI) were added, and the reaction was incubated in the dark for 15 min. The fluorescence intensity of the cells was evaluated by flow cytometry (BD FACSVerse™), and the data were analyzed using the BD FACSuite™ software.

RNA extraction and RT-PCR analysis

Total RNA was isolated from BM-MSCs cultured under hypoxic or normoxia using an RNeasy kit (Lifethechnology-Ambion, Carlsbad, CA, USA) and was used as a substrate for the QuantiTect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The cDNAs were amplified by PCR using the primers shown in Table 1. The band intensity of each PCR product was measured using NIH image/ImageJ and normalized against that of GAPDH mRNA.

Cell size measurements

BM-MSCs initially plated at a density of 1000 cells/cm^2 were maintained for 7 days under normoxia or hypoxia and then subcultured once per week. After 6 weeks, cells were collected and resuspended in FACS buffer (BD Bioscience). Cell size was measured by flow cytometry (BD FACSVerse™), and the data were analyzed using BD FACSuite™ software. FSC-A parameters of the software were used for cell size measurements, as recommended by BD (see BD FACService TECHNOTES, Customer Focused Solutions, Vol. 9 No. 4 October, 2004; Shapiro 2003).

Quantitative SA-β-galactosidase assay

The cells were cultured at a density of 4×10^3 cells/cm^2 in 6-well plates containing media. The cells were fixed with 4 % paraformaldehyde in PBS, washed with PBS, and then stained using an senescence-associated (SA) β-gal staining kit (Cell BioLabs, San Diego, CA, USA) for 10 h in an incubator chamber at 37 °C in the dark. Positive cells were counted and results were expressed as the mean percentage of SA-β-gal-positive cells among total cells.

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical significance of $P < 0.05$ was determined using independent Student's t test.

Results

Characteristics of BM-MSCs

MSCs derived from five donors were used in this study. MSCs isolated from adult human BM were morphologically heterogeneous and fibroblastic in shape (Fig. 1a). At the second passage, the presence of mesenchymal lineage-associated cell surface markers on the MSCs was confirmed by flow cytometry analysis. MSCs were uniformly positive for CD73,

Table 1 Primer sequences used for RT-PCR

Target		Primer sequence[a]	Cycle number	Product size (bp)
OCT4	F	5′-AAGCGATCAAGCAGCGACTA-3′	31	230
	R	5′-CCTCAGTTTGAATGCATGGG-3′		
KLF4	F	5′-CCCAATTACCCATCCTTCCT-3′	29	210
	R	5′-TGCCTTGAGATGGGAACTCT-3′		
C-MYC	F	5′-GGAAGAAATTCGAGCTGCTG-3′	34	229
	R	5′-TTGATGAAGGTCTCGTCGTC-3′		
CCL2	F	5′-GCAGCAAGTGTCCCAAAGAA-3′	30	203
	R	5′-AACAGGGTGTCTGGGGAAAG-3′		
IL6	F	5′-TACCCCCAGGAGAAGATTCC-3′	26	199
	R	5′-GCCATCTTTGGAAGGTTCAG-3′		
CXCL9	F	5′-TGTTCTTCTACCACCCACCAG-3′	30	203
	R	5′-CAGCATGCTAACAGGCTTAGG-3′		
CXCL10	F	5′-CTGTACGCTGTACCTGCATCA-3′	35	172
	R	5′-TTCTTGATGGCCTTCGATTC-3′		
CXCR4	F	5′-CTGGCCTTCATCAGTCTGGA-3′	35	167
	R	5′-TCATCTGCCTCACTGACGTT-3′		
CXCR7	F	5′-ACCAAATGATCTGCCCTGGA-3′	27	182
	R	5′-GACAGCTGCGTCATCAAGAG-3′		
GAPDH	F	5′-TCAACGGATTTGGTCGTATTGGG-3′	25	234
	R	5′-TGATTTTGGAGGGATCTCGC-3′		

OCT4 octamer-binding transcription factor 4, *KLF4* Kruppel-like factor 4, *C-MYC* v-myc avian myelocytomatosis viral oncogene homolog, *CCL2* C-C motif chemokine ligand 2, *IL6* interleukin 6, *CXCL9* C-X-C motif chemokine 9, *CXCL10* C-X-C motif chemokine 10, *CXCR4* C-X-C motif chemokine receptor 4, *CXCR7* C-X-C motif chemokine receptor 7, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

[a] Forward (F) and reverse (R) primers used to detect mRNA expression of the indicated targets

CD90, CD105, and CD166 and negative for CD14, CD34, CD45, and HLA-DR (Fig. 1b). The MSC differentiation potential was then assessed using osteogenic, adipogenic, and chondrogenic induction media. The cells differentiated into all three lineages (Fig. 1c).

Hypoxia increased the proliferation rate and viability of MSCs

MSCs derived from five different donors were cultured under hypoxia (1 % O_2) or normoxia for 2 weeks (Fig. 2a), and the influence of oxygen tension on proliferation and viability was investigated. The proliferation rate of MSCs cultured under hypoxia was higher than that of MSCs cultured under normoxia (Fig. 2b). The number of cell divisions of D1 MSC, D2 MSC, D3 MSC, D4 MSC, and D5 MSC under hypoxia and normoxia was 2.26 ± 0.14 vs. 1.64 ± 0.15, 2.74 ± 0.23 vs. 1.92 ± 0.18, 1.92 ± 0.13 vs. 1.66 ± 0.09, 2.80 ± 0.25 vs. 2.05 ± 0.20, and 3.14 ± 0.19 vs. 1.80 ± 0.13, respectively. Flow cytometry analysis using annexin V staining showed that there were more annexin V-positive cells among MSCs cultured under normoxia than among MSCs cultured under hypoxia (Fig. 3), indicating that hypoxia was more efficient in reducing cell death than normoxia.

Hypoxia delayed the decrease in the proliferation rate and prevented changes in phenotype over time in culture

To investigate whether long-term exposure of cultures to hypoxia affects the biological characteristics of MSCs, D1 MSCs were cultured under hypoxia or normoxia for 8 weeks (Fig. 4a) and proliferation rate, morphology, cell size, senescence, and immunophenotypes were examined over the course of several culture periods. Over time, growth was markedly higher for MSCs cultured under hypoxia than for those cultured under normoxia (Fig. 4b). The number of cell divisions was higher for MSCs cultured under hypoxia than for those cultured under normoxia (Fig. 4c). P6 MSC cell numbers under hypoxia and normoxia over a period of 1 week were 2.57 ± 0.18 and 2.19 ± 0.09, respectively, and P7 MSC cell numbers under hypoxia and normoxia over a period of 2 weeks were 2.53 ± 0.12 and 1.65 ± 0.02, respectively. The number of cell divisions of P11 MSCs cultured under hypoxia and normoxia for 7 weeks was 1.86 ± 0.19 and 1.18 ± 0.06, respectively, and that of P12 MSCs under hypoxia and normoxia for 8 weeks was 1.31 ± 0.19 and 0.90 ± 0.04, respectively. Over time in culture, a greater number of large and flat cells were detected in MSCs cultured under normoxia than in those

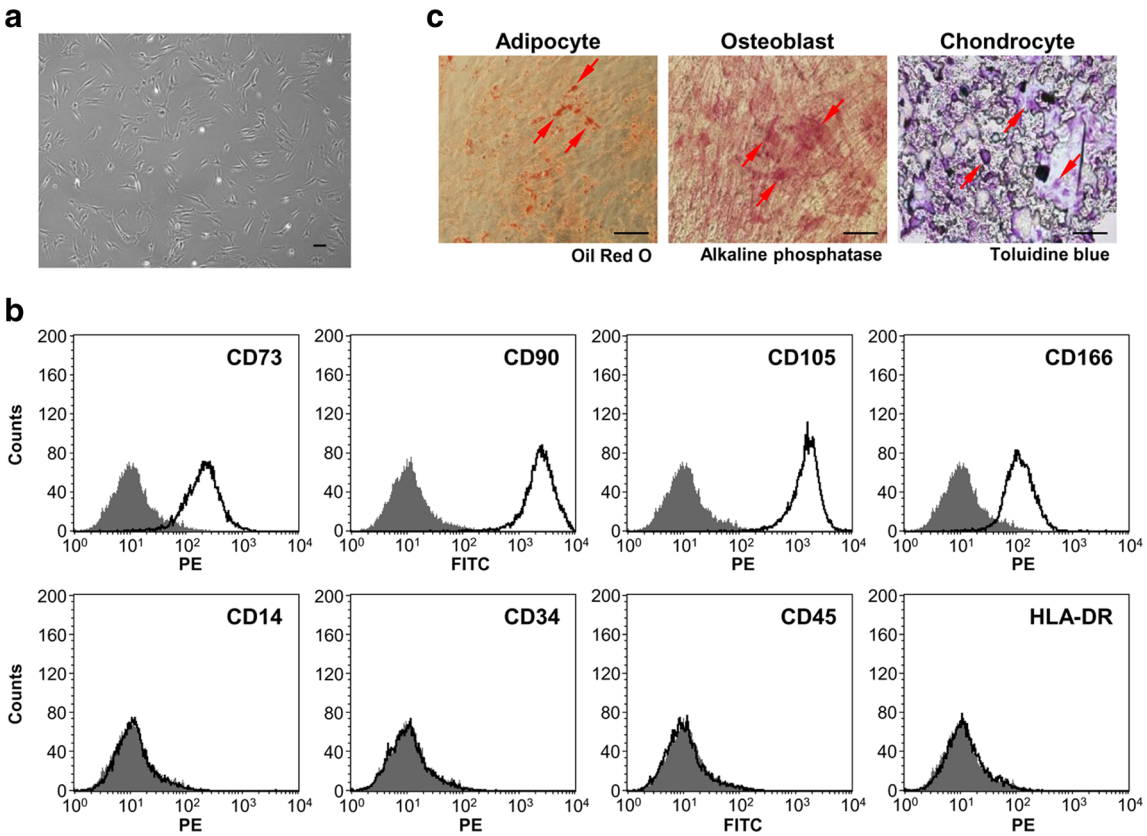

Fig. 1 Characteristics of MSCs derived from human BM. **a** Representative morphological appearance of BM-MSCs. The cells exhibited a spindle-shaped or fibroblastic morphology. *Scale bar* at 100 μm. **b** Flow cytometric analysis of MSC surface markers. The expression of surface antigens was plotted against appropriate human IgG isotype controls (*gray histograms*). MSCs used in this study were positive for CD73, CD90, CD105, and CD166 and negative for CD14, CD34, CD45, and HLA-DR (*clear histograms*). **c** Differentiation of BM-MSCs. The cells were incubated for 14–21 days in the presence of specific differentiation agents for osteoblasts, chondrocytes, or adipocytes. Differentiation into the adipocyte lineage was demonstrated by staining with Oil-red O (*red arrow*), indicating intracellular lipid accumulation. Differentiation into osteoblast was demonstrated by staining with alkaline phosphatase (*red arrow*), indicating mineralization of the extracellular matrix. Differentiation into chondrocyte was demonstrated by staining with Toluidine Blue (*red arrow*), indicating the deposition of proteoglycans and lacunae formation. *Scale bar* at 100 μm

cultured under hypoxia. MSCs cultured under hypoxia were smaller in size than those cultured under normoxia (Fig. 5a), and the cells retained their spindle-shaped morphology (Fig. 5b), implying that hypoxia delayed the increase in cell volume over time in culture. In addition, senescent phenotypes were confirmed by the expression of SA-β-gal. More SA-β-gal-positive cells were detected in MSCs cultured under normoxia than in those cultured under hypoxia (Fig. 5b). SA-β-gal-positive cells were approximately 4 % of total P5 MSCs (0 week) cultured

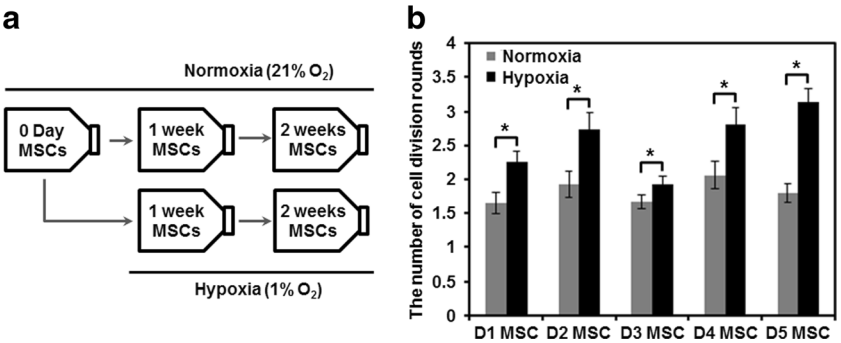

Fig. 2 Proliferation rates of MSCs cultured under hypoxia or normoxia for 2 weeks. **a** Schematic drawing of MSC culture under different oxygen tensions. BM-MSCs derived from five different donors were cultured and maintained under hypoxia or normoxia for 2 weeks. **b** The number of cell divisions of MSCs cultured under hypoxia or normoxia for 2 weeks. The number of cell divisions was calculated with respect to cell number. *$P < 0.05$

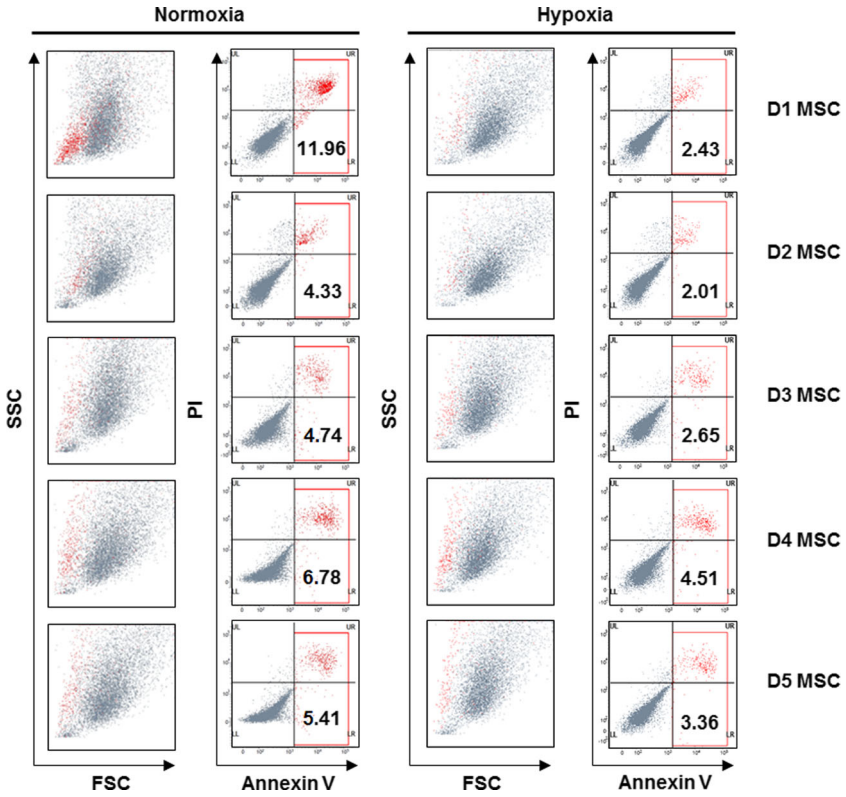

Fig. 3 Flow cytometric apoptosis analysis of MSCs cultured under hypoxia or normoxia for 2 weeks. BM-MSCs derived from five different donors were cultured and maintained under hypoxia or normoxia for 2 weeks. Cell apoptosis was assessed using flow cytometry-based annexin V-FITC and PI. Annexin V staining is indicative of apoptosis; PI staining is indicative of apoptosis and necrosis

under normoxia and 79 % and 30 % of P13 MSCs (8 weeks) cultured under normoxia and hypoxia, respectively (Fig. 5c). MSCs cultured under hypoxia for 8 weeks were positive for typical MSC antigens (CD90 and CD105) but negative for the typical hematopoietic antigen, CD45 (Fig. 5D), implying that long-term exposure to hypoxia did not alter the typical immunophenotypic characteristics of MSCs.

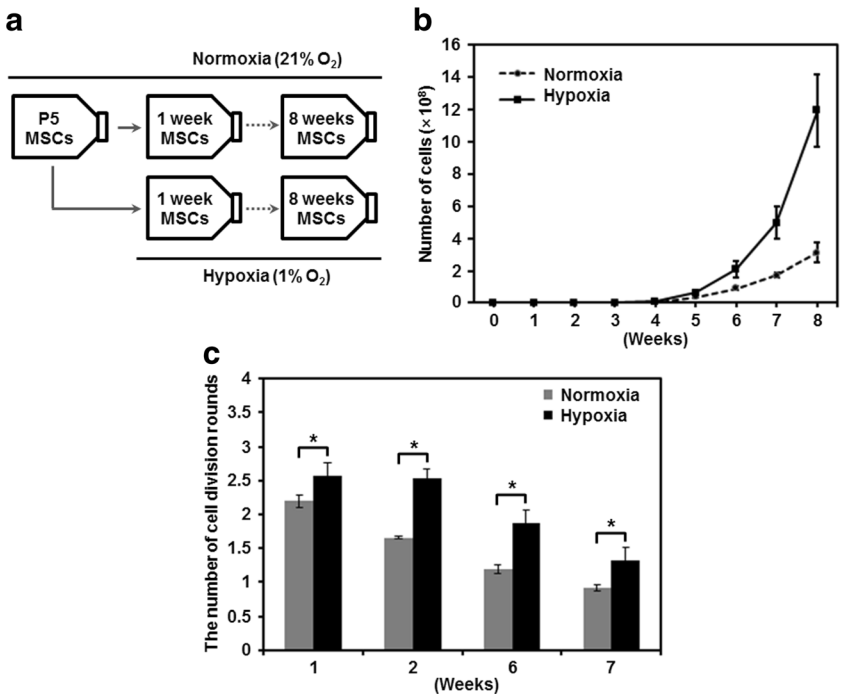

Fig. 4 Proliferation rates of MSCs under hypoxia or normoxia during long-term cultivation. **a** Schematic drawing of a long-term MSC culture under different oxygen tensions. D1 MSCs at passage 5 were cultured and maintained under hypoxia or normoxia for 8 weeks (passage 13). **b** Long-term growth curve of MSCs cultured under hypoxia or normoxia. Cells were counted and passaged once per week, and cumulative cell numbers were determined for 8 weeks. Long-term growth curves represent four independent experiments performed at each oxygen concentration. **c** The number of cell divisions of MSCs cultured under hypoxia or normoxia. The number of cell divisions was calculated with respect to cell number. $*P < 0.01$

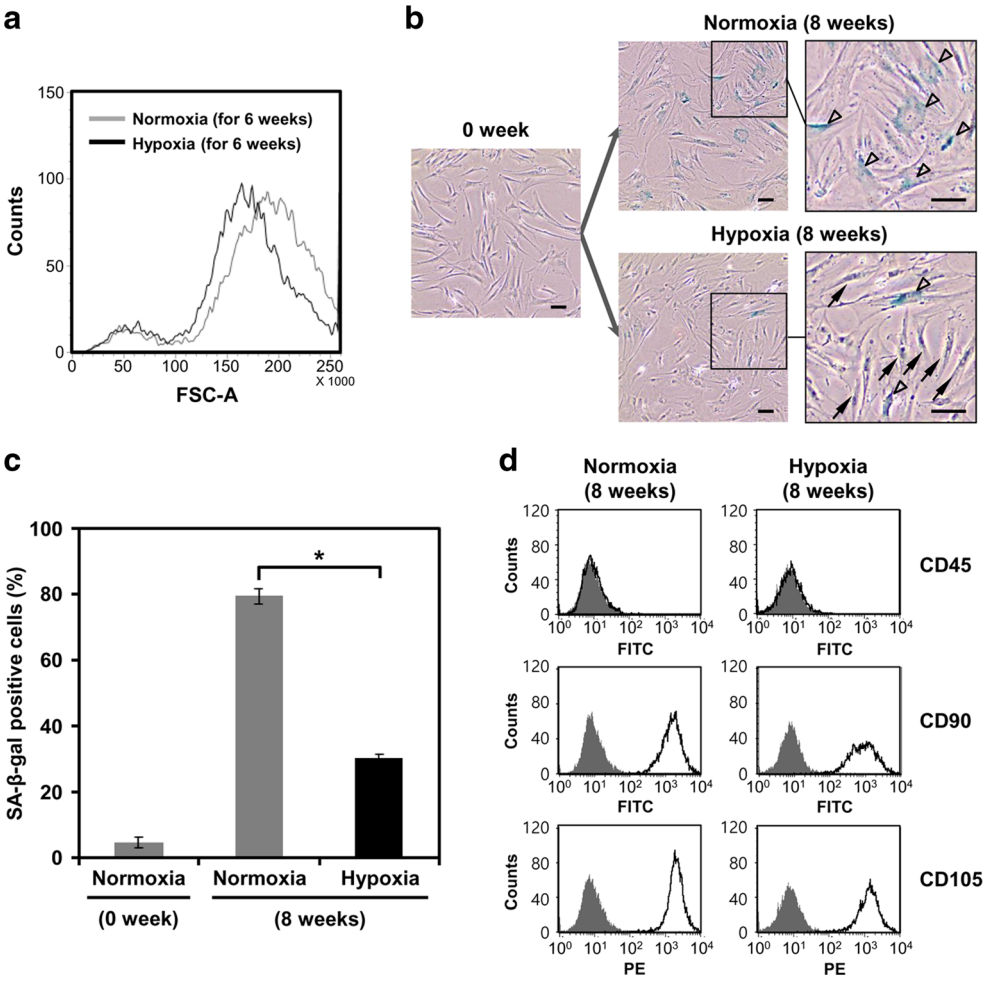

Fig. 5 Phenotypic characteristics of MSCs under hypoxia or normoxia during long-term cultivation. **a** Change in cell size of MSCs under hypoxia or normoxia over time in culture. D1 MSCs at passage 5 were cultured under hypoxia or normoxia for 6 weeks. The size of MSCs was measured using flow cytometry and is reflected by the forward-scatter signal. **b** Morphology and senescence-associated β-galactosidase (SA-β-gal) staining of MSCs cultured under hypoxia or normoxia for 8 weeks. *Blank arrow head* indicates SA-β-gal-positive cell and *black arrow* indicates SA-β-gal-negative cell that grows in culture with a spindle-shaped morphology. *Scale bar* at 100 μm. **c** Percentage of SA-β-gal-positive cells was counted in three independent experiments. $*P < 0.01$. **d** Flow cytometric analysis of surface markers of MSCs cultured under hypoxia or normoxia for 8 weeks. All MSCs cultured under hypoxia or normoxia were negative for FITC-conjugated CD45 and positive for FITC-conjugated CD90 and PE-conjugated CD105

Hypoxia increased the expression of stemness and chemokine-related genes

The expression of functional genes, such as stemness, cytokine, and chemokine genes, and their receptors, was investigated using MSCs derived from five different donors and cultured under hypoxia or normoxia for 1 or 2 weeks. RT-PCR analysis showed that hypoxia increased the expression of octamer-binding transcription factor 4 (*OCT4*) and C-X-C motif chemokine receptor 7 (*CXCR7*) in MSCs (Fig. 6). The expression of other cytokine and chemokine genes, including Kruppel-like factor 4 (*KLF4*), v-myc avian myelocytomatosis viral oncogene homolog (*C-MYC*), and C-C motif chemokine ligand 2 (*CCL2*), was not lower under hypoxia than under normoxia for cultured MSCs from any donor (Fig. 6). The expression of interleukin 6 (*IL6*) in D1, D3, and D5 MSCs cultured under hypoxia was similar to that in MSCs cultured under normoxia, while it was downregulated in D2 and D4 MSCs cultured under hypoxia (Fig. 6). The expression of C-X-C motif chemokine ligand 9 (*CXCL9*) and *CXCR4* was hardly detected in MSCs cultured under hypoxia and normoxia. RT-PCR analysis with a high cycle number showed that hypoxia slightly upregulated the expression of *CXCR10*, although it was low under each culture condition (Fig. 6).

Discussion

The microenvironment of MSCs in tissues is characterized by limited oxygen availability (hypoxia), which activates many stress and survival pathways in stem cells (Buravkova et al. 2014). Indeed, O_2 levels in the bone marrow, from which MSCs were obtained in our study, remain as low as 1–6 % (Eliasson et al. 2010; Chow et al. 2001). Thus, culturing MSCs under hypoxia can mimic the natural microenvironment of stem cells and allow investigations of the proliferation, differentiation, senescence, metabolic balance, and other physiological aspects of these cells, which have potentially important clinical applications (Rosová et al. 2008). Previous studies mainly reported the positive effects of hypoxia on the biological characteristics of human MSCs (Saller et al. 2012; Lennon et al. 2001; Hung et al. 2007; Grayson

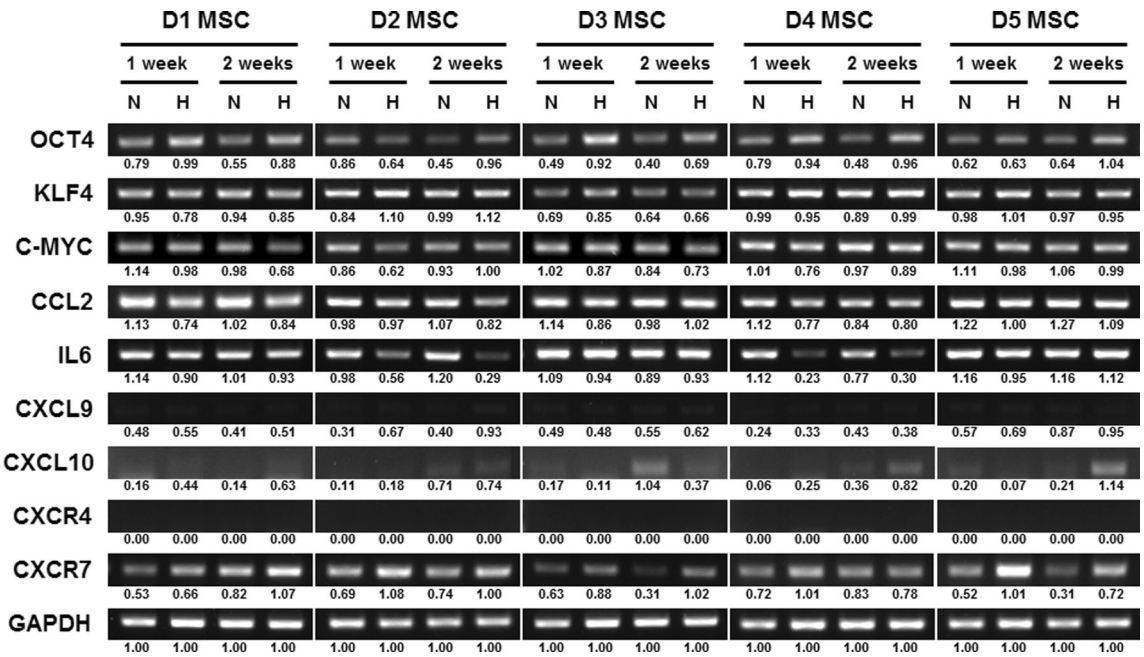

Fig. 6 RT-PCR analysis of stemness, cytokine, and chemokine gene expression in MSCs cultured under hypoxia or normoxia. The expression of stemness, cytokine, chemokine genes, and their receptors was examined by semiquantitative RT-PCR in MSCs derived from five different donors and cultured under different oxygen tensions for 1 or 2 weeks. The values below each panel show the quantitative gene expression data in terms of the change in the mRNA level relative to that of GAPDH mRNA. *H* hypoxia, *N* normoxia

et al. 2007). On the other hand, several studies showed negative or no effects of hypoxia on MSCs (Holzwarth et al. 2010; Roemeling-van Rhijn et al. 2013; Raheja et al. 2011; Salim et al. 2004; Zhu et al. 2006; Malladi et al. 2006). These discrepancies may be due to the variation in the oxygen tension, the duration of hypoxic culture, comparison of a limited range of biological characteristics employing a low number of MSCs, and the variation in the system that was used in each study to control the oxygen level. In particular, 1 % O_2 had conflicting effects on the biological characteristics, such as proliferation, survival, and migration, of MSCs (Rosová et al. 2008; Holzwarth et al. 2010; Roemeling-van Rhijn et al. 2013; Tsai et al. 2011; Hung et al. 2007; Raheja et al. 2011). Therefore, to clarify the effect of hypoxia on the biological characteristics of MSCs, additional data are necessary to support these findings. Our study shows the effect of hypoxia (1 % O_2) on various biological characteristics, including proliferation, morphology, viability, cell size, expression of various genes, and a senescence marker, of human MSCs derived from different donors over time in culture. Hypoxia increased cell proliferation and viability, inhibited senescence, and regulated the expression of several stemness- and chemokine-related genes. These results suggest that culture under hypoxia could help overcome the limitations of culturing MSCs under ambient oxygen conditions.

Oxygen concentration is an important component of the stem cell niche and plays a prominent role in stem cell fate determination. Low oxygen tension is important for maintaining the plasticity and proliferation of stem cells. We demonstrated here that BM-MSCs cultured under hypoxia had higher proliferation rates than BM-MSCs cultured under normoxia. This finding is consistent with previous reports that demonstrated increased proliferation of BM-MSCs cultured under hypoxia (Ren et al. 2006; Grayson et al. 2007). In stem cells, maintenance of proliferation is affected by molecular mechanisms mediated by stemness genes, such as *KLF4*, *OCT4*, and *C-MYC*. KLF4 prevents embryonic stem cell (ESC) differentiation (Zhang et al. 2010) and maintains MSCs in an undifferentiated state (Saulnier et al. 2011). Oct4 is a transcription factor essential for self-renewal and survival of MSCs (Tsai et al. 2012), and C-MYC plays a vital role in cell proliferation and differentiation of adult stem cells (Bhandari et al. 2011). In this study, we showed that MSCs cultured under hypoxia for approximately 2 weeks had higher rates of cell proliferation and *OCT4* expression than cells cultured under normoxia and maintained the expression of *KLF4*, *C-MYC* of MSC at levels similar to those of cells cultured under normoxia. The higher proliferation rates correlated with increased rates of cell division and cell survival under hypoxia.

We observed a senescent phenotype characterized by enlarged and flattened cell morphology, increased cell volume, and the expression of SA-β-gal during long-term culture of MSCs under normoxia. By contrast, MSCs cultured under hypoxia maintained the phenotypic characteristics of early passage MSCs to a greater degree than in MSCs cultured under normoxia in terms of morphology, cell size, proliferation, and senescence. Aging or replicative senescence

negatively affects proliferation of MSCs as the cells lose their stemness and undergo cell cycle arrest (Boyette and Tuan 2014; Yu and Kang 2013). The delay in development of the senescent phenotype in MSCs cultured under hypoxia may be because they tend to maintain a higher rate of cell proliferation than MSCs cultured under normoxia. This opens the prospect of obtaining large amounts of cells with the desired biological characteristics during long-term culture.

The success of cell-based therapies is critically dependent on the engraftment of the transplanted cells. The objective of stem cell regenerative therapy is to treat damaged organ tissues in a manner that avoids cell death and/or inappropriate tissue remodeling (Burdon et al. 2011). Recently, the novel mechanisms underlying the therapeutic effects of MSCs were shown to include the paracrine actions by cytokines, chemokines, growth factors, and their receptors (Caplan and Dennis 2006; Zhang et al. 2007; Gnecchi et al. 2008; Figueroa et al. 2012; Liu et al. 2012; Hung et al. 2007). In this study, we found that the expression of *CXCR7* and *CXCL10* was increased in MSCs cultured under hypoxia. In addition, the expression of chemokines and their receptor, including *CCL2*, *CXCL9*, and *CXCR4* by MSCs cultured under hypoxia, was similar to that of MSCs cultured under normoxia. Recent studies show that a hypoxic environment increases the expression of vascular endothelial growth factor (VEGF), a factor important for angiogenesis (Crisostomo et al. 2008), and of chemokine receptors, such as CXCR4, CXCR7, and CX3CR1 (Liu et al. 2012; Hung et al. 2007), which facilitate the tissue-specific trafficking of MSCs. Our data and other reports show that MSCs cultured under hypoxia could be more effective for engraftment than MSCs cultured under normoxia. Furthermore, MSC receptors and secreted factors are thought to be important for the therapeutic action of MSCs. Interestingly, the expression of *IL6* was maintained in MSCs from some donors cultured under hypoxia at levels similar to those in MSCs cultured under normoxia, but it was downregulated in MSCs from other donors. IL6 is involved in the inhibition of monocyte differentiation toward DCs, decreasing their stimulatory effect on T cells (Djouad et al. 2007; Jiang et al. 2005), while the secretion of IL6 by MSCs delays apoptosis of lymphocytes and neutrophils (Raffaghello et al. 2008; Xu et al. 2007). Through further investigation, it will be important to determine whether the relative differences in the levels of gene expression between different donors under hypoxia are a critical factor for the selection of potent MSCs.

For MSCs to adapt to low oxygen, they have to sense and respond to changes in oxygen levels. Under hypoxia, MSCs may undergo changes in their biological characteristics via several molecular mechanisms. For instance, in a hypoxic environment, hypoxia-inducible factor 1α (HIF-1α) prevents TCA cycle activity and results in lower reactive oxygen species (ROS), slowing the rate of telomere shortening (Bodnar et al. 1998; Richter and Zglinicki 2007); as a consequence, replicative senescence may be delayed. Moreover, a hypoxic environment induces higher proliferation rates (Estrada et al. 2012; Fehrer et al. 2007; Nekanti et al. 2010) by lowering ROS levels and upregulating the expression of Notch target genes (e.g., Hes and Hey genes), resulting in the upregulation of several stem cell markers. For therapeutic applications, it will be important to improve the biological characteristics of stem cells under hypoxia to generate MSCs that can adapt to and function in the in vivo environment. Recently, murine MSCs cultured under hypoxia showed improved skeletal muscle regeneration, blood flow, and vascular formation compared with MSCs cultured under normoxia (Leroux et al. 2010). Furthermore, culture under hypoxia causes MSCs to grow faster and increases the rapidly self-renewing cell population, and also increases the levels of secreted factors, as shown in this study. These effects of hypoxic culture conditions on MSCs may lead to the development of better cell therapy strategies.

In conclusion, hypoxia had a dramatic effect on the biological characteristics of BM-MSCs. Hypoxia increased MSC proliferation, upregulated the expression of *OCT4* and *CXCR7*, and delayed alterations in phenotypic characteristics such as cell size, morphology, and senescence. Our findings should help formulate guidelines for the collection of optimal MSCs for cell therapy.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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