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Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions

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Abstract

Low oxygen tension is a potent differentiation inducer of numerous cell types and an effective stimulus of many gene expressions. Here, we described that under 8% O_2, bone marrow stromal cells (MSCs) exhibited proliferative and morphologic changes. The level of differentiated antigen H-2Dd and the number of G_2/S/M phase cells increased evidently under 8% O_2 condition. Also, the proportion of wide, flattened, and epithelial-like cells (which were alkaline phosphatase staining positive) in MSCs increased significantly. When cultured in adipogenic medium, there was a 5- to 6-fold increase in the number of lipid droplets under hypoxic conditions compared with that in normoxic culture. We also demonstrated the existence of MSC differentiation under hypoxic conditions by electron microscopy. Expression of Oct4 was inhibited under 8% O_2 condition, but after adipocyte differentiation in normoxic culture and hypoxia-mimicking agents cobalt chloride ($CoCl_2$) and deferoxamine mesylate (DFX) treatments, Oct4 was still expressed in MSCs. These results indicate hypoxia accelerates MSC differentiation and hypoxia and hypoxia-mimicking agents exert different effects on MSC differentiation.
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Bone marrow contains cells that meet the criteria for stem cells of nonhematopoietic tissue are currently referred to either as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as marrow stromal cells (MSCs), because they appear to arise from the complex array of supporting structure found in marrow [1]. MSCs are a mixed cell population that generates bone, cartilage, fat, and fibrous connective tissue. Recently several investigators showed that stromal cells from many tissues contain stem cells with multipotential [2–6]. Thus, classical MSCs seem to contain undefined subfractions of stem cell population with great differentiation potential. At present, MSCs were considered a new resource to develop replacement tissues for congenital or degenerative disorders [7–9].

Low oxygen tension is a potent differentiation inducer of numerous cell types and an effective stimulus of gene expression [10–12]. Recent studies showed that hypoxia and hypoxia-mimicking agent $CoCl_2$ could trigger the differentiation of APL (acute promyelocytic leukemia) cells and variations of oxygen concentration modified self-renewal and cycling of hematopoietic stem cells [13]. It has

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been reported that 1% hypoxia modified proliferation and differentiation of $CD34^+$ CML cells [14]. Furthermore, it has been reported that the proportional of osteochondrogenesis in IMDM with 10% FBS culture under hypoxic conditions (5% O_2 concentration) was higher than that under normoxic conditions in vitro and in vivo [15].

In the present study, we showed hypoxia could accelerate the proliferation and differentiation of MSCs. At the same time, effects of hypoxia-mimicking agent $CoCl_2$ on MSCs were also addressed, which suggested that hypoxia and $CoCl_2$ had different effects on MSCs.

Materials and methods

Animals. Eight- to 12-week-old male and female Balb/c mice were used as bone marrow (BM) donors. All mice were purchased from Laboratory Animal Center (Beijing University School of Medicine). All animal handle and experimental procedures were approved by the Animal Care and Use Committee of the Chinese Academy of Medical Sciences.

Isolation of MSCs from bone marrow and culture conditions. BM was collected from the tibial and femoral diaphysis. The ends of bones were cut, and the marrow was flushed with 5 ml of Iscove's modified Dulbecco's medium (IMDM; Gibco) by using a needle and syringe. After being gently resuspended in medium by passing through a series of 19, 21, and 23 G needles, the whole BM cells were plated at a constant density of 10^5 cells/cm^2 in IMDM with 10% fetal bovine serum (FBS, Hyclone) in T25 culture flasks (Corning). Whole BM cells were first cultured under normoxic conditions, then nonadherent cells were removed thoroughly 24 h later, and the culture medium was replaced. Remaining adherent cells were incubated at 37 °C, in an atmosphere of hypoxic conditions (8% O_2, 5% CO_2, blended with 86% N_2) or normoxic conditions (5% CO_2, 95% room air), respectively. The medium was replaced every 2–3 days. After the culture reached confluence, the cells were detached by 0.25% trypsin and 1 mM EDTA, and replated at 1:2.

Hypoxia was induced by placing cells in a modular airtight humidified chamber flushed with a gas mixture which consisted of 8% O_2/5% CO_2 balanced with N_2 at 37 °C, and O_2 concentrations were measured by a miniOX1 oxygen meter as described [16]. The medium was saturated with 87% N_2/5% CO_2/8% O_2 in advance. For exposure to nonhypoxic conditions, cells were maintained in standard culture conditions of 37 °C in humidified 5% CO_2/95% room air as previously described. Cobalt chloride ($CoCl_2$) and deferoxamine mesylate (DFX) (Sigma) were dissolved in PBS and used at a final concentration of 100 μM and 120 μM respectively under normoxic conditions. The adherent cells were cultured in the presence or absence of $CoCl_2$ untill the cells reached 70–80% confluence.

Western blot. The expression levels of HIF-1α in MSCs under normoxic conditions, hypoxic conditions, and normoxic conditions with 100 μM $CoCl_2$ were analyzed by Western blot. An amount of 40 μg total protein from whole cell lysates was separated by SDS–PAGE and immunoblotted with an antibody against HIF-1α. Expression levels of HIF-1α were normalized to those of β-actin for loading differences.

Proliferation assay. Cells were incubated under different oxygen concentrations. Cell viability and number were determined by trypan blue staining.

Cell cycle analysis. Cell cycle analysis was performed by DNA content analysis of PI-stained cells after 7–8 days in culture. 10^6 cells were fixed and permeabilized in 70% cold ethanol at 4 °C for 2 h, washed twice with PBS, and treated with 200 μg/ml RNase A for 30 min at 37 °C. Cells were then incubated in 40 μg/ml propidium iodide for 5 min at room temperature and placed on ice until flow cytometric analysis.

Cellular immunophenotype analysis. Antibodies against CD13, CD44, I-Ad, and H-2Dd were used to analyze cells under different oxygen concentrations. Cells were removed from the culture after 7–8 days and analyzed for cell surface antigen expression. For indirect immunofluorescent analyses, cells were suspended at a concentration of 1×10^6 cells/ml in fluorescent-activated cell sorter (FACS) buffer (1% bovine serum albumin and 0.1% sodium azide in PBS, pH 7.2), and incubated with primary antibody (4 μg/ml monoclonal rat anti-mouse antibodies) (all from BD Pharmingen) for 30 min at 4 °C. Same-species, same-isotype irrelevant antibody was used as negative control. After two washes with FACS buffer, cells were incubated with fluorochrome-conjuncted secondary antibodies (fluorescein isothiocyanate [FITC]-conjugated goat anti-rat IgG) for 15 min at room temperature. After staining, all samples were kept on ice until analyzed on the FACSCalibur flow cytometer (Becton–Dickinson).

Alkaline phosphatase staining. For alkaline phosphatase staining, cells were fixed with cold methanol for 2 min and washed in 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 10 mM $MgCl_2$ buffer for 10 min, then stained with fast 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium alkaline phosphatase substrate (Sigma) for 5–10 min and rinsed in water.

Electron microscopy. Transmission electron microscopy was performed to assess the morphological characteristics of the cells. Cells under different oxygen concentrations were collected by a cellular scraper, and immediately fixed at 4 °C with 4% paraformaldehyde and 0.1% glutaraldehyde. All samples were post-fixed in osmium and routinely processed for transmission electron microscopy. A JEOL 100CXII (JEOL, Tokyo, Japan) transmission electron microscope was used.

Adipocyte differentiation. MSCs were plated in 60% DMEM-LG, 40% MCDB-201, supplemented with 2% FBS and 2×10^{-9} M dexamethansone (DEX) for 7–8 days. For Sudan-black staining of cytoplasmic triglyceride lipid droplets, cells were fixed with 10% formalin for 10 min, rinsed in water, and then stained with 0.5% Sudan-black (Sigma) in 60% ethanol for 10 min and rinsed in water.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using the acid–guanidinium–phenol–chloroform (AGPC) method, and cDNA was synthesized from 5 μg of total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies) in 50 μL of reaction solution according to the manufacturer's instructions. After cDNA synthesis, products were amplified using sequence-specific primers under the following condition: 93 °C for 3 min/93 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s (35 cycles)/72 °C for 7 min. Primers for Oct4 amplification were 5′-TGG CAT ACT GTG GAC CTC AGG TT-3′ and 5′-TTT CCA AAG AGA ACG CCC AGG G-3′ (319 bp); Primers for Flt-1 5′-TGT GGA GAA ACT TGG TGA CCT-3′ and 5′-TGG AGA ACA GCA GGA CTC CTT-3′ (504 bp); Primers for flk-1 5′-AGA ACA CCA AAA GAG AGG AAC G-3′ and 5′-GCA CAC AGG CAG AAA CCA GTA G-3′ (383 bp); Primers for VEGF 5′-GCA GGC TGC TGT AAC GAT GAA G-3′ and 5′-CAA GGC TCA CAG TGA TTT TCT GGC-3′ (185 bp); Primers for peroxisome proliferator-activated receptor-γ2 (PPARγ2) 5′-GGT GAA ACT CTG GGA GAT TC-3′ and 5′-CAA CCA TTG GGT CAG CTC TTG-3′ (267 bp); Primers for osteopontin 5′-CCA GGT TTC TGA TGA ACA GTA TCC-3′ and 5′-ACT TGA CTC ATG GCT GCC CTT T-3′ (162 bp). Primers for β-actin were 5′-GAG ACC TTC AAC ACC CCA GCC-3′ and 5′-GGG AAA TCG TGC GTG ACA TT-3′ (265 bp). PCR products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide.

Statistical analysis. Student's t test, 2-tailed, was used to assess the differences of proliferation between data from hypoxic and normoxic culture conditions. Data were presented as means ± standard deviation. P values <0.05 were considered significant.

Results

Proliferative response of MSCs to low oxygen and $CoCl_2$

It has been reported that hypoxia could drive proliferation of human pulmonary artery fibroblasts and the mechanism was also discussed [17]. However, the effect of low oxygen on MSC differentiation and proliferation has not been evaluated. We examined the protein levels of

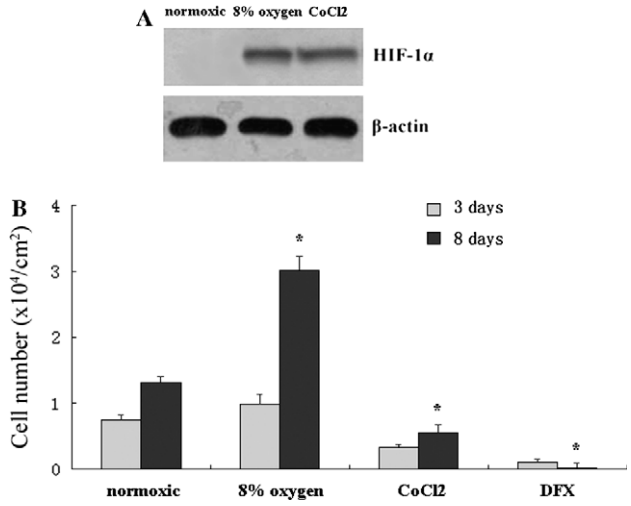

Fig. 1. (A) Western blot of HIF-1α expression of MSCs. 1, Under normoxic conditions; 2, under 8% low oxygen conditions; 3, treated with $CoCl_2$ (100 μM). (B) Number of MSCs after different treatments for 3 and 8 days observed after initial plating of cells. Three groups represented, respectively: 21%, cultured under normoxic conditions; 8%, cultured under 8% low oxygen conditions; $CoCl_2$, treated with $CoCl_2$ (100 μM). 4, Treated with DFX (120 μM). the number of cells initially plated was $1 \times 10^4/cm^2$. The results represent the mean cell number ± standard deviation ($n = 3$). $^*P < 0.05$ relative to normoxic control at the same day. *Abbreviations:* $CoCl_2$, cobalt chloride; DFX; deferoxamine mesylate.

HIF-1α under normoxic conditions, 8% low oxygen conditions and normoxic conditions with 100 μM $CoCl_2$. The protein level of HIF-1α was undetectable under normoxic condition, but significantly increased when MSCs were cultured in 8% low oxygen condition and normoxic condition with 100 μM $CoCl_2$ (Fig. 1A). So, we chose 8% oxygen as hypoxic conditions and $CoCl_2$ at 100 μM was used in the following studies. Cells were exposed to 21% O_2 (normoxia), 8% O_2, and $CoCl_2$ (100 μM), DFX (120 μM), respectively, in the expansion medium (IMDM supplemented with 10% FBS). After 7–8 days' treatment, adherent cells were trypsinized and counted using hemocytometer. Cell viability was quantified with trypan-blue exclusion assay. As shown in Fig. 1B, an approximate 2.8-fold increase in cell number was observed under 8% O_2 compared normoxic culture, while $CoCl_2$ inhibited the proliferation of MSCs. Furthermore DFX (120 μM) almost resulted in all cells' death.

Morphology changes of MSCs after different treatments

During the logarithmic growth phase from about day 6 to day 12, the morphology in each group was investigated. The normoxic culture was used as control. All the results were compared with the morphology of MSCs under normoxic conditions (Fig. 2A and B). As Colter et al. showed that the marrow stromal cells in our culture contain at least two morphologically distinct kinds of cells: spindle-shaped cells (RS) and large cuboidal or flattened cells [18]. And after different treatments, the proportion of two kinds of cells appeared to be inconsistent. Following the treatment of $CoCl_2$, MSCs exhibited the volume of survival cells to be low and small, and the wide-flattened cells in stromal cells almost disappeared. These small-size cells were fibroblast-like and had an increased nuclei-cytoplasm ratio (Fig. 2E). However after exposure to 8% O_2, the proportion of spindle-shaped cells decreased, and the proportion of flattened cells greatly increased. The increase in large flat cells was accompanied by an increase in alkaline phosphatase (Fig. 2G–J). Afterwards, we demonstrated that the ALP positive cells in Fig. 2G–J were not osteoblasts. When total RNA was isolated and analyzed by RT-PCR, expression of β-actin, used as an internal control, was the same in normoxic and hypoxic cells, whereas in neither case was osteopontin marker expressed (Fig. 2K). Almost all cells treated with DFX were dead at 6 days (Fig. 2F).

To further explore the influence of hypoxia on MSCs, we observed the ultra-structural change under 8% O_2 using transmission electron microscopy. Hypoxic cells contained a large number of unidentified vacuoles (Fig. 3), which were the markers of differentiation cells [18].

The status of cell cycle and the analysis of cellular immunophenotype under low oxygen culture.

As the number of MSCs cells significantly increased under hypoxic conditions, we hypothesized that hypoxia might drive MSCs into cell cycle. Fig. 4A shows that 19.42 ± 3.9% of MSCs entered into G_2-M/S phase after 8 days' hypoxic culture. Hypoxic culture caused an approximate 0.52-fold increase for cell in G_2-M/S phase when compared to normoxic culture. Given that cell division increased under hypoxic conditions, we next measured the expression of differentiated antigen H-2Dd and IA-d. Low expression of MHC antigen is important in cell transplantation therapy and MSCs have been reported to express low level of MHC [19]. The level of IA-d and CD13 under two different conditions was almost unchanged. However, the percentages of CD44 and H-2Dd in MSCs under 8% oxygen were significantly higher than under normoxic condition. Fig. 4B shows the phenotype of MSCs from a representative experiment under normoxic and hypoxic conditions.

Because the cell number was too limited after $CoCl_2$ treatment, the FACS analysis was not introduced.

Adipocyte differentiation

To induce adipocytes differentiation, MSCs were cultured at $2–3 \times 10^4/cm^2$ in 60% DMEM-LG, 40% MCDB-201 with 2×10^{-9} M DEX and 2% FBS. Adipocytes-like cells containing lipid droplets appeared (Fig. 5). Compared with normoxic culture, hypoxic culture showed an approximate 5.6-fold (5.6 ± 1.8, $n = 10$) increase in the number of lipid droplets after 8 days and a 3.2-fold (3.2 ± 1.6, $n = 10$) for 4 days (lipid droplets per field were counted, and the average of 10 fields was evaluated). But MSCs were cultured only in IMDM supplemented with 10% FBS without

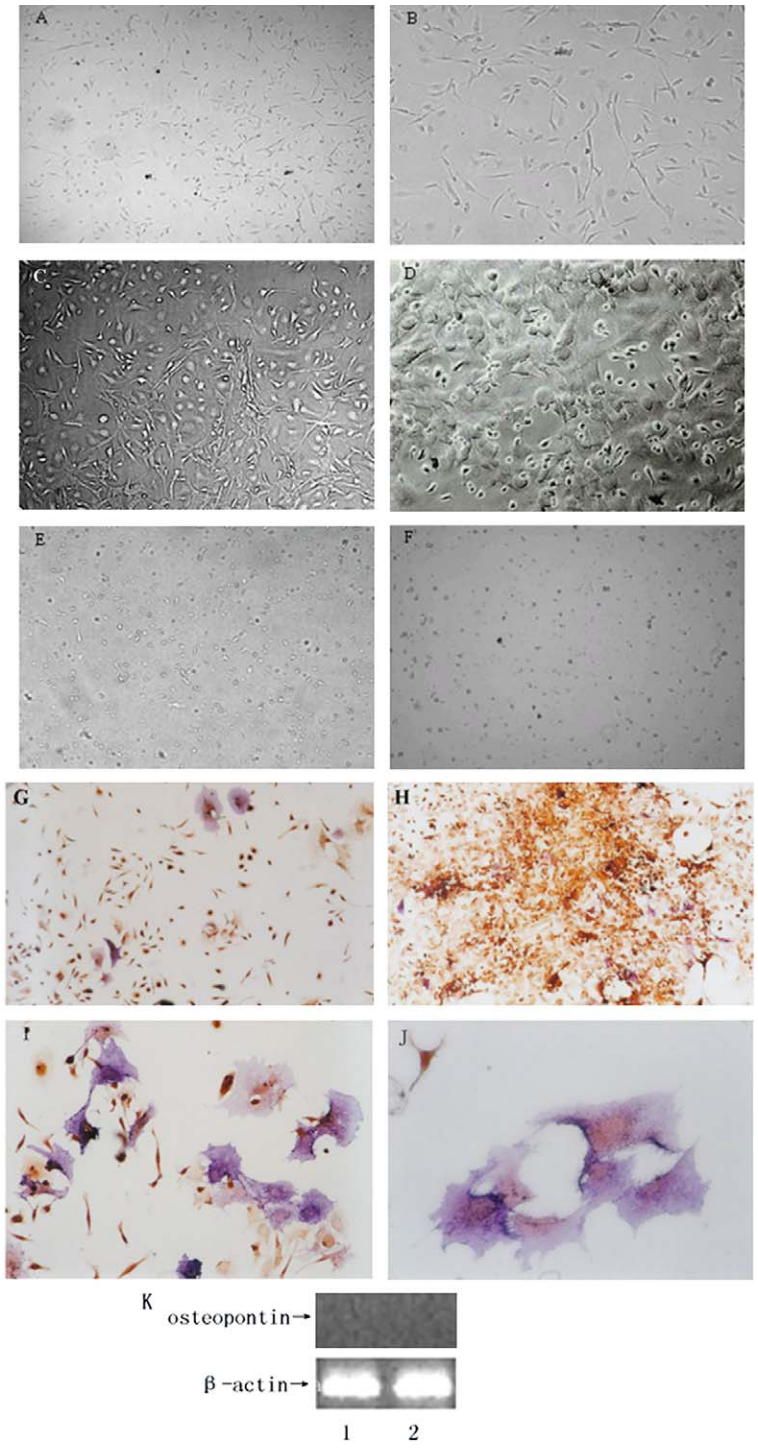

Fig. 2. Time course of MSC morphology and proliferation assay under different culture conditions and the number of ALP positive cells in MSCs increased under hypoxic conditions compared to normoxic conditions. MSCs were cultured under normoxic (21% O_2) conditions for 7 days (A, original magnification 100×; B, original magnification 200×); under 8% O_2 conditions for 7 days (C, original magnification 100×; D, original magnification 200×); and MSCs were treated with $CoCl_2$ for 7 days (E, original magnification 100×), with DFX for 10 days (F, original magnification 100×). The morphologic changes were analyzed by invert microscopy. The results are representative of three independent experiments. (G) MSCs were cultured at 8 days under normoxic conditions. The number of ALP positive cells was short (magnification 100×) (ALP positive and negative cells were stained purple and orange, respectively). (H) MSCs were cultured under 8% O_2 at 10 days. Fibroblast-like cells and cells grew mixedly, the ALP positive cells (wide-flattened endothelial-like cells) increased (magnification 100×). (I) Identical with (B) (magnification 200×). (J) The morphology of ALP positive cells (magnification 400×). (K) The osteopontin gene expression analysis of marrow stromal cells by RT-PCR lanes: 1, MSCs were cultured under 21% O_2; 2, under 8% O_2. Total RNA was analyzed by RT-PCR for osteopontin (162 bp) and β-actin (263 bp) mRNA. *Abbreviations:* ALP, alkaline phosphatase staining; RT-PCR, reverse transcription-polymerase chain reaction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

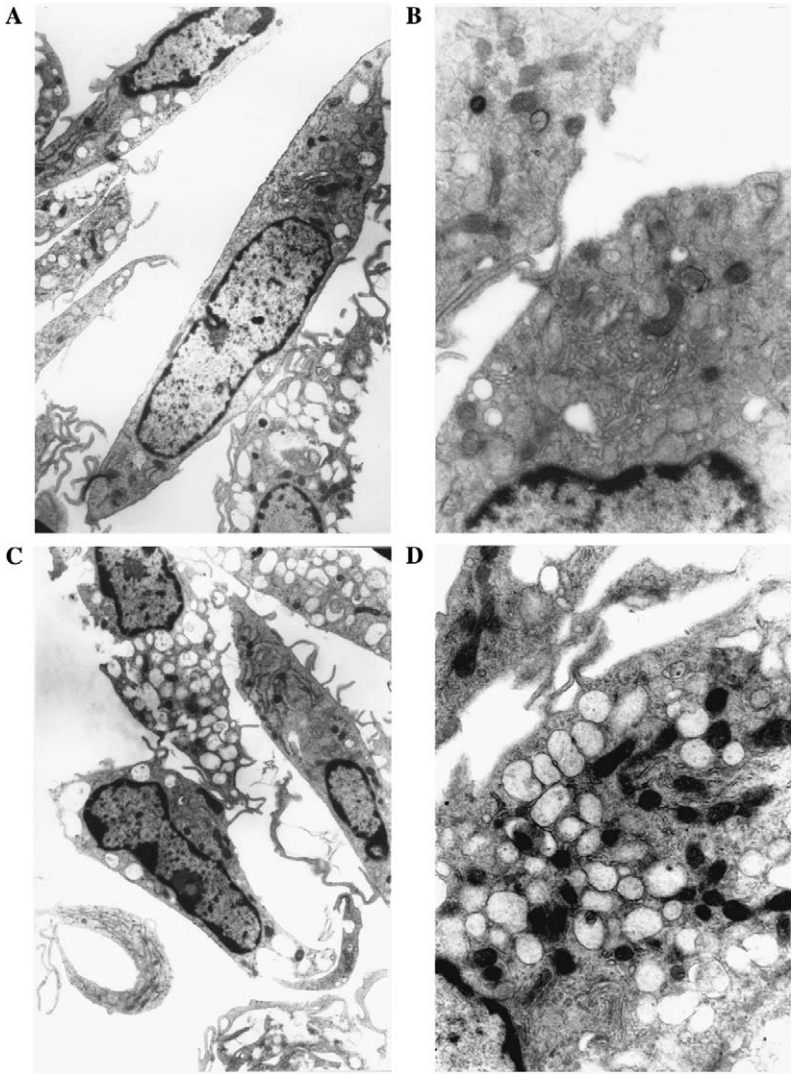

Fig. 3. The ultrastructural morphological evaluation of MSCs under different oxygen conditions. (A) The ultrastructure in MSCs under normoxic culture (4000×) for 9 days. (B) The ultrastructure of MSCs under normoxic culture (16,000×) for 9 days. (C) The ultrastructure in MSCs under 8% hypoxic culture (4000×) for 9 days. (D) The ultrastructure in MSCs under 8% hypoxic culture (16,000×) for 9 days. Hypoxic cells contained a large number of unidentified vacuoles under 8% hypoxic conditions.

DEX; there was no lipid formation. When total RNA was isolated and analyzed by RT-PCR, expression of β-actin, used as an internal control, was the same in normoxic and hypoxic cells, whereas the adipocyte marker PPAR2 was not expressed in untreated cells but was expressed in DEX-treated cells (Fig. 5E). In the study, to show the distinct number difference of adipocytes between normoxic and hypoxic conditions more apparently, we used a very low DEX strength to induce adipocytes comparing with the reported dosage (10^{-8} M DEX).

Expression of Oct4 under different conditions

Changes of gene expression at early period of hypoxia were evaluated. As hypoxia can trigger the activation of several signal pathways in cells [20,21], it may lead to the changes of MSCs. We found MSCs expressed Oct4, which is one of the important markers of pluripotential stem cells. By RT-PCR, we showed that 8% hypoxia markedly inhibited the expression of Oct4 (Fig. 6A). Under hypoxic culture, the expression of Oct4 could not be detected on days 1 and 6, while other treatments, including $CoCl_2$, DFX, VEGF, and adipocyte differentiation, did not affect Oct4 expression. Thus, $CoCl_2$ and DFX, hypoxia-mimcking agents, have different influence on Oct4 compared with 8% low oxygen.

In the next set of experiments, we analyzed the expression status of other genes, such as VEGF, flk-1, and Flt-1 after 24 h of different treatments (Fig. 6B). VEGF could be activated through intrinsic pathways, which were induced by HIF-1α accumulation. The figure shows that all the treatments could not affect the flk-1 gene expression. VEGF was expressed either in $CoCl_2$ or DFX groups. Eight percent of low oxygen had no evident effects on VEGF expression. None of the groups expressed Flt-1 gene.

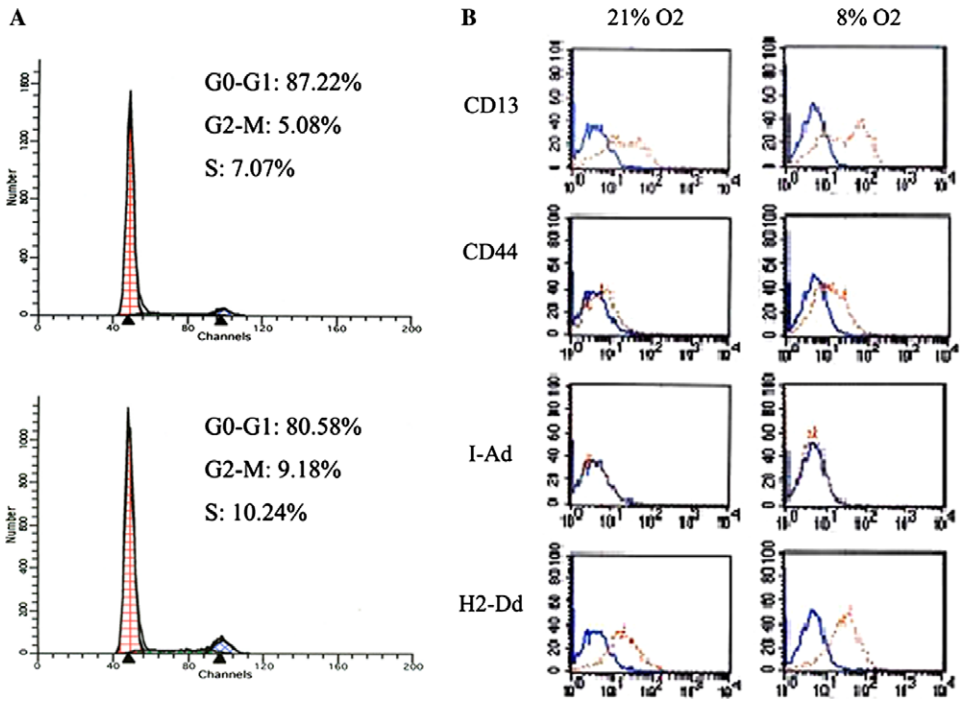

Fig. 4. Progression of hypoxia-stimulated MSCs through G_0/G_1 and into G_2-M/S phases of cell cycle. Histogramic DNA content-related cell cycle distribution and the changes of cell phenotypes. (A) MSCs were cultured under 21% O_2 (upper) and 8% O_2 (lower). (B) Effect of hypoxia on the cell surface antigen expression by hypoxia. MSCs under different oxygen concentrations' culture were harvested and labeled with antibodies against CD13, CD44, H-2Dd, and IA-d or control IgGs as indicated and analyzed by FACS. Plots show isotype control IgG-staining profiles (blue lines) versus specific antibody staining profile (red lines). The results are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

So O_2 concentration may be one of the important factors in maintaining pluripotential stem cells in MSCs population. Moreover, the existence of Oct4-positive cells relies on an appropriate density of stromal cells. The expression of Oct4 disappeared when cells were seeded at a low density ($<2 \times 10^3/cm^2$).

Discussion

MSCs-based therapy is of potential value in tissue replacement and regeneration, and has been studied in clinical trials in autologous and allogeneic settings [22]. Recent studies have shown that the proportion of osteogenesis cells under hypoxic conditions was greater than under normoxic conditions in vitro and in vivo [16]. Our results in the study showed that the marrow stromal cells under our culture contained very primitive cells which were Oct4-positive and have adipocyte differentiation ability and adipocyte differentiation was also accelerated under 8% O_2 in adipose differentiation condition. Therefore, we thought that perhaps hypoxia could trigger the proliferation and differentiation of adult mice marrow-derived MSCs and change their biologic characteristics at the same time. Subsequently, we observed the changes of proliferation and differentiation morphology under light or electronic microscopy and the increased number of G_2/S/M period cells.

Because marrow stromal cells contained two morphologically distinct cell types: spindle-shaped cells and large flat cells (more mature cells). Early colonies also contain a third kind of cell: very small round cells that rapidly self-renew. Moreover, samples enriched for the small cells had a greater potential for multipotential differentiation than samples enriched for the large cells. Whereas the cultured cells under 8% O_2 exhibited a larger proportion of large flat cells than 21% O_2. So low oxygen tension caused the stromal cells' differentiation and the higher number of large mature cells. In detail, the morphology changes under 8% O_2 also showed that the number of large flat and epithelioid cells increased which had a larger subpopulation of giant fat cells (being alkaline phosphatase positive) compared with 21% O_2. As ZX Song in 1983 has reported, our results were similar to the radioresistant murine marrow stromal cells isolated by her in appearance. Song and Quesenberry demonstrated the cell type could produce hematopoietic factor and induce pre-B cell formation [23–25]. In a word, the ALP positive cells would be significant in the research of early hemopoiesis regulation. According to our study, the population could resist low oxygen stimulation and proliferate fast under this condition. Although we have not succeeded, we observed that the subpopulation number under 5% O_2 culture was more than 21% O_2 (not shown) and reasonably hypoxic culture would finally be an effective way to isolate and purify the ALP positive cell pop-

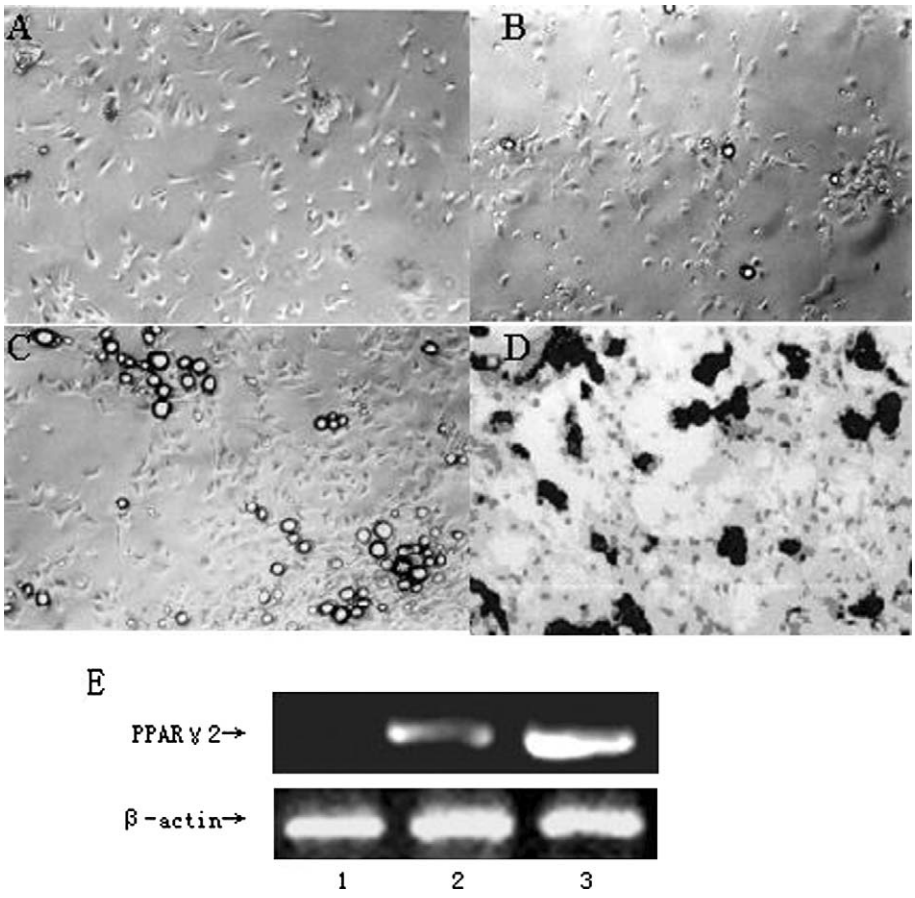

Fig. 5. Adipocyte differentiation and Sudan-black staining of MSCs. (A) MSCs were cultured in IMDM supplemented with 10% FBS under normoxic conditions, no lipid droplets formed. (B) MSCs were cultured in adipogenic medium under normoxic conditions, as evidenced by a few cytoplasmic lipid droplets. (C) MSCs were cultured in adipogenic medium under hypoxic conditions (8% O_2), as evidenced by more cytoplasmic lipid droplets. (D) Sudan-black staining of MSCs cytoplasmic lipid droplets (the black granules). (E) the PPARγ 2 gene expression analysis of marrow stromal cells by RT-PCR lanes: 1, MSCs were cultured under 21% O_2 without DEX; 2, under 21% O_2 with DEX; 3, under 8% O_2 with DEX. Total RNA was analyzed by RT-PCR for PPARγ-2 (267 bp) and β-actin (263 bp) mRNA. *Abbreviations:* ALP, alkaline phosphatase staining; IMDM, Iscove's modified Dulbecco's medium; DEX, dexamethasone; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction.

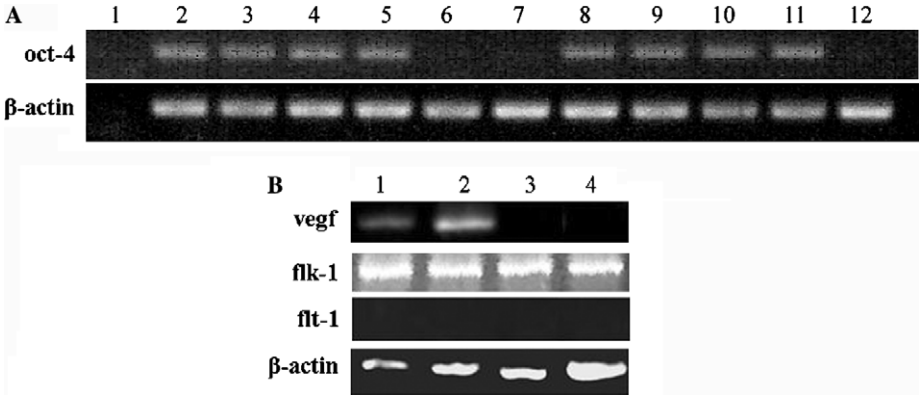

Fig. 6. The expression analysis of Oct4 and VEGF, flk-1, and Flt-1 in MSCs under different culture conditions by RT-PCR. (A) Lanes: 1, negative control, -RT; 2–4, MSCs were cultured under normoxic conditions plated at $1 \times 10^5/cm^2$. 2, Untreated cells; 3, passaged cells; 4, high confluent cells; 5–7, MSCs were cultured under 8% O_2. 5, At 6th hour; 6, at 24th hour; 7, at 6th day; 8 and 9, MSCs were treated with $CoCl_2$ (100 μM). 8, For 24 h; 9, for 6 days; 10 and 11, MSCs were treated with adipogenic medium and VEGF; 12, MSCs were passaged at a low density ($2 \times 10^3/cm^2$). (B) Lanes: 1, MSCs cultured under normoxic condition; 2, MSCs cultured under 8% O_2; 3, MSCs treated with $CoCl_2$; 4, MSCs treated with DFX. *Abbreviations:* RT-PCR, reverse transcription-polymerase chain reaction; $CoCl_2$, cobalt chloride.

ulation. In addition, we showed that the cell cycle, differentiated phenotype, and Oct4 expression changed evidently under 8% O_2. We also found that the higher proliferation of MSCs was accompanied by an increased cell number in G2-M/S phase, and the higher expression of differentiated antigen H-2Dd was also observed. The phenotype of MSCs in normoxic culture is the major histocompatibiliy complex (MHC) class II(I-Ad) negative, and it expresses low levels of matrix markers CD44 and middle levels of MHC class I(H-2Dd) and granule marker CD13, but after low oxygen tension treatment, the expression of H-2Dd and CD44 increased. H-2Dd class I molecule is very important in allograft rejection because Ly49A, the prototype receptor, inhibits NK cell function upon the interaction with it [26,27]. The increased expression of MHC I class molecule H-2Dd antigen would indicate that the cells were more aging and had more chance to immune-rejection. Therefore, we concluded that MSC proliferation and differentiation increased simultaneously under hypoxic conditions.

At the same time, Oct4, which is a marker of primitive stem cells, was downregulated. Oct4 represents the most important marker of pluripotent stem cells, such as ES cells and MAPC [2,28]. MSCs in culture expressed Oct4 indicated that at least a subpopulation of cells in MSCs may be pluripotent like embryonic stem cells. Therefore, it is important to find and identify Oct4-positive cells from bone marrow and other tissues in the research of their plasticity. The opening and closure of this gene may represent the differentiation potential of MSCs. We found that 8% hypoxia could trigger differentiation of Oct4-positive cells within short time. Other treatments (such as adipogenic differentiation or $CoCl_2$) did not change Oct4 expression. We also found that at low seeding density, transcription of Oct4 stopped. According to the known knowledge and understanding of stem cells [29], we hypothesized that Oct4-positive cells maintaining in MSCs population may rely on a ''niche''. When the important microenvironment components in the niche were disturbed under low cell concentration, the Oct4-positive cells differentiated into mature cells. Simultaneously, expression of H-2Dd antigen and the cells in G_2/S/M phase increased under hypoxic conditions when Oct4 gene was closed. Even at early stage of embryonic differentiation, the expression of Oct4 gene would continue for a comparatively long time [28]. So not all treatments would lead to the closure of Oct4 in MSCs. Oct4-positive cells may be the primitive stem cells, which were not easily mobilized by general inducers such as DEX $CoCl_2$ except hypoxic conditions. And once this gene is closed, the proportional of differentiated cells increased evidently. We found that 8% O_2 and hypoxia-mimiking agent have different effects on MSCs. The difference was represented mainly in two aspects. One aspect was the morphology and growth rate, the other was the gene expression pattern. Eight percent oxygen accelerated MSC proliferation, while $CoCl_2$ inhibited cell growth. At the same time, $CoCl_2$ could stimulate the expression of vasculogenesis relative genes VEGF, which were increased by oxygen deprivation [30,31], 8% hypoxia could not. Not $CoCl_2$ but 8% O_2 could trigger MSCs differentiation in vitro. The cause of these differences may be related to the difference in the activated signal pathways. Though both hypoxia and $CoCl_2$ could induce HIF-1α accumulation and activate some other signal pathways (such as MAPK, ERK1/2 MAPK, etc) [32,33], the levels of HIF-1α were different under low O_2 and $CoCl_2$ treatments. Cobalt has been widely used in the treatment of anemia and as a hypoxia mimic in cell culture and it is known to activate hypoxic signaling by stabilizing the hypoxia inducible transcription factor 1α (HIF-1α) [34]. However, cobalt exposure can lead to tissue and cellular toxicity. Vengellur A results showed that HIF-1α was playing a major role in mediating cobalt-induced toxicity in mouse embryonic fibroblasts and may offer a possible mechanism for the underlying pathology of injuries seen in workers exposed to environmental contaminants that can influence the hypoxia signaling system, such as cobalt [35]. As our data indicated that proliferation of MSCs was reduced in the presence of $CoCl_2$ compared to normoxic. Our findings in the paper showed that after adding $CoCl_2$ and DFX, protein of hypoxia-inducible factor 1α, VEGF gene was expressed.

In our experiments, the cells died after DFX (120 μM) treatment at initially plated. The reason for this might be that the used intensity was higher and the cell toxicity of DFX could not be neglected as Kim et al. have demonstrated. Although when the cells were cultured at the fifth day, the state of cells had no evident change after 24 h of DFX (120 μM) [36].

It has been reported that $CoCl_2$ and DFX activate hypoxia-inducible factor 1α, which stimulates the transcription of several genes that are associated with hypoxia. HIF-1 stimulates the transcription of genes, such as EPO and VEGF whose protein products function to either increase oxygen availability by promoting erythropoiesis and angiogenesis [37,38] and HIF-1α plays an essential role in cellular and systematic oxygen homeostasis [39].

Taken together, our results indicate that hypoxia accelerates MSC proliferation and differentiation, leading to the stop of Oct4 transcription. Future research on the mechanism underlying these effects may lead to a better understanding between hypoxia and stem cell maintenance.

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