# Continuous hypoxia regulates the osteogenic potential of mesenchymal stem cells in a time-dependent manner

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**Abstract.** The effects of hypoxia on the osteogenic potential of mesenchymal stem cells (MSCs) have been previously reported. From these studies, possible factors affecting the association between hypoxia and the osteogenic differentiation of MSCs have been suggested, including hypoxia severity, cell origin and methods of induction. The effect of the duration of hypoxia, however, remains poorly understood. The aim of the present study was to investigate the effect of continuous hypoxia on the induced osteogenesis of MSCs. Rat MSCs were isolated and cultured *in vitro*. Once the cells had been cultured to passage three, they were switched to 1% oxygen and cultured either with or without osteogenic medium, while cells in the control groups were cultured under normoxia in corresponding conditions. Four osteogenic differentiation biomarkers, runt-related transcription factor 2, osteopontin, osteocalcin and alkaline phosphatase, were analyzed by quantitative polymerase chain reaction and western blotting at defined intervals throughout the culture period. In addition, Alizarin Red staining was used to assess changes in mineralization. The results showed that 1% hypoxia was able to enhance and accelerate the osteogenic ability of the MSCs during the initial phases of differentiation, and the protein expression of certain associated biomarkers was upregulated. However, continuous hypoxia was shown to impair osteogenesis in the latter stages of differentiation. These findings suggest that hypoxia can regulate the osteogenesis of MSCs in a time-dependent manner.

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## Introduction

Mesenchymal stem cells (MSCs) are progenitors of mesoderm tissue, and can be induced to differentiate into adipocytes, osteocytes, chondrocytes and myocytes under the appropriate conditions (1,2). Studies investigating *in vitro* tissue engineering have broadened the application of, and basic research into, MSCs and their regulators (3-5). Oxygen has been shown to act not only as a cellular substrate, but also as a communication signal that can regulate stem cell properties, including survival, multiplication, differentiation and 'stemness' (6-10). It is unknown whether the native microenvironment of MSCs should be defined as hypoxic or primary normoxic (11); however, it has been established that oxygen concentration is a critical regulator of MSCs, and may therefore control their differentiation fate.

MSCs have been considered the most important seed cells, and are widely used in the experimental and clinical treatment of musculoskeletal disorders and degenerative diseases (12,13). A central issue in the treatment of bone defects and necrosis, is how to promote the multiplication and ensure the effective osteogenesis of MSCs at the delivery site. Furthermore, the ultimate results of transplanting MSCs into a hypoxic environment *in vivo* are poorly understood, and the underlying mechanisms are unclear. Controversy remains regarding the effects of hypoxia on the osteogenic differentiation of MSCs (14,15). Salim *et al* (9) found that a short period of anoxia exposure downregulated runt-related transcription factor 2 (Runx2) and bone morphogenetic protein 2 expression. This resulted in the inhibition of key steps in the differentiation of pluripotent mesenchymal precursors and committed osteoblasts into the osteogenic lineage. Through microarray profiling, the downregulation of Runx2 was indicated to be a potential mechanism underlying the anoxia-induced inhibition of differentiation (9). Similar results were reported by Holzwarth *et al* (16), who showed that human MSCs (hMSCs) exhibited a reduced rate of proliferation under reduced oxygen tension compared with that under 21% oxygen; the cells instead accumulated in the $G_1$ phase. Adipogenic and osteogenic differentiation were also shown to be severely impaired, although the increase in oxygen levels from 1 to 3% restored the capacity of the cells for osteogenic differentiation (16). Potier *et al* (17) indicated that temporary exposure (48 h) of hMSCs to hypoxia led to

the limited stimulation of angiogenic factor secretion but to the persistent (up to 14 days) downregulation of several osteoblastic markers. This suggested that the exposure of MSCs *in vivo* to hypoxic conditions following transplantation could affect their osteogenic potential. By contrast, Grayson *et al* (18) demonstrated that pretreatment with hypoxia could enhance the proliferation and tissue formation of hMSCs. A 30-fold increase in hMSC expansion was obtained over six weeks without loss of multi-lineage differentiation capabilities when hMSCs were maintained in an atmosphere of 2% hypoxia (18). It is considered that the effect of hypoxia on MSCs depends upon the severity of hypoxia, the origin of the cells and the method of osteogenic induction (6,19). However, the effect of the duration of hypoxia on the osteogenic potential of MSCs is currently unknown.

In the present study, the effects of continuous hypoxia (1% oxygen concentration) on the osteogenic potential of MSCs in long-term culture were investigated. Four osteogenic biomarkers, Runx2, osteopontin (OPN), osteocalcin (OCN) and alkaline phosphatase (ALP), were detected at mRNA and protein levels during the course of differentiation.

Table I. Primers used for the quantitative polymerase chain reaction.

| Gene | Orientation | Sequence (5'-3') |
|---|---|---|
| β-actin | Forward | TTCTTTGCAGCTCCTTCGTTGCCG |
| | Reverse | TGGATGGCTACGTACATGGCTGGG |
| ALP | Forward | TATGTCTGGAACCGCACTGAAC |
| | Reverse | CACTAGCAAGAAGAAGCCTTTGG |
| OCN | Forward | GCCCTGACTGCATTCTGCCTCT |
| | Reverse | TCACCACCTTACTGCCCTCCTG |
| OPN | Forward | CCAAGCGTGGAAACACACAGCC |
| | Reverse | GGCTTTGGAACTCGCCTGACTG |
| Runx2 | Forward | ATCCAGCCACCTTCACTTACACC |
| | Reverse | GGGACCATTGGGAACTGATAGG |

ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; Runx2, runt-related transcription factor 2.

## Materials and methods

*MSC isolation and culture*. The study protocol was approved by the Ethics Committee of the Shanghai Sixth People's Hospital (Shanghai, China). Rat mesenchymal stromal cells (rMSCs) were isolated from femoral marrow specimens and maintained according to local regulations. Bone marrow samples were obtained from three rats and numbered accordingly. rMSCs were harvested by gently flushing bone marrow from the femora with α Minimum Essential Medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and 1% antibiotic solution. When rMSCs reached 60-70% confluence, they were detached and cryopreserved at passage 1 ($P_1$) (90% FBS and 10% dimethylsulfoxide). For each experiment, a new batch of rMSCs was thawed and cultured to $P_3$ under normoxia, for further use.

*Multipotent differentiation assay*. The potential of rMSCs for multipotent differentiation into osteogenic and adipogenic lineages was assessed. For the evaluation of induced osteogenesis, rMSCs were seeded at a density of $5x10^4$ cells/well and cultured in osteogenic differentiation medium containing dexamethasone, ascorbate and β-glycerophosphate (Sigma) for two weeks. Alizarin Red staining was performed to assess cellular mineralization in the differentiation medium. This was performed by fixing the cells with 100% ethanol for 15 min and then staining with 0.2% Alizarin Red S solution, with a pH of 6.36-6.4, at room temperature for 1 h. Adipogenic differentiation was performed according to conventional methodology, using culture medium containing 1 $\mu$M dexamethasone and 5 $\mu$g/ml insulin. The cultured cells were stained with Oil Red (Sigma) to detect fat droplets.

*Conditions of hypoxia and osteogenic induction*. Methodology for the generation of hypoxic conditions and osteogenic induction was based on that outlined in our previous study (20). Normoxic (21% $O_2$) and hypoxic (1% $O_2$) conditions were used continuously for culturing the cells in a humidified atmosphere. The proliferation and differentiation conditions of the cells were equal for both levels of $O_2$. When the rMSCs reached $P_3$ under normoxia, the cells were then either further cultured under normoxia or were transferred to a hypoxia incubator (Forma™ Series II 3110 Water Jacketed $CO_2$ Incubator; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained in 1% $O_2$. Osteogenic differentiation was induced in 50% confluent rMSC cultures by switching to osteogenic induction medium. Control cells were cultured in parallel under hypoxia and normoxia without osteogenic induction medium. After 3, 7, 14 and 21 days of culture, cells were harvested and cryopreserved for subsequent analysis.

*Quantitative polymerase chain reaction (qPCR)*. The expression level of the mRNA transcripts was assessed by two-step qPCR. In brief, total RNA was prepared with TRIzol® Plus (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. A TURBO DNA-free™ kit (Applied Biosystems, Foster City, CA, USA) was employed for the removal of possible contaminated genomic DNA and the subsequent removal of DNase itself from the total RNA preparation. cDNA was then synthesized from total RNA using oligo (dT) and ReverTra Ace® reverse transcriptase (Toyobo, Osaka, Japan). qPCR was performed and analyzed by kinetic qPCR using the ABI PRISM 7900 system (Applied Biosystems) with SYBR Green Realtime PCR Master Mix Plus (Toyobo) for relative quantification of the indicated genes. The transcript of β-actin was used as an endogenous control for normalization.

The primers used for Runx2, OPN, OCN and ALP are listed in Table I. All the PCR primers were designed to span introns to discriminate the cDNA from genomic amplicons, according to the manufacturer's instructions. The amplification efficacy of each PCR reaction was assessed initially with a serial dilution of control samples, which fell into the range of 95-105%. The comparative cycle threshold method was utilized to assess the levels of each mRNA transcript relative to the level of β-actin mRNA transcript in the same sample.

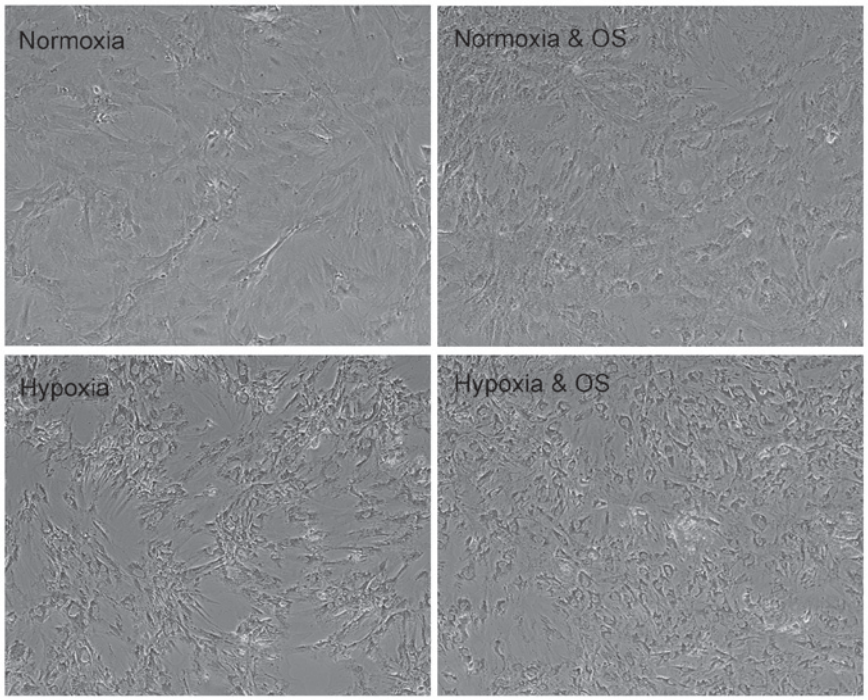


Figure 1. Photomicrographs of rMSCs. rMSCs lost their spindle shape upon culturing under hypoxic conditions and following OS induction. OS, osteogenic; rMSCs, rat mesenchymal stromal cells.

*Western blotting*. Nuclear proteins were isolated from the rMSCs, following cellular centrifugation at 4˚C, using a nuclear protein extraction kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions. The protein contents of the cell lysates were determined using a micro bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc., Rockford, IL, USA). Protein from the cell lysates was mixed with 4X loading buffer containing 10 mM dithiothreitol and boiled for 10 min, prior to electrophoresis by 10% SDS-PAGE. Following transfer onto polyvinylidene fluoride membranes and blocking, the membranes were incubated overnight at 4˚C with monoclonal antibodies for hypoxia-inducible factor 1α (HIF-1α; 1:3,000; Abcam, Cambridge, MA, USA), Runx2 (1:1,000; Abcam), OPN (1:2,000; R&D Systems, Minneapolis, MN, USA), OCN (1:3,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or ALP (1:1,000; Santa Cruz Biotechnology, Inc.). Following several washes in Tris-buffered saline with Tween®20 (TBST), the membranes were subsequently incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Sigma) diluted 1:3,000 in blocking buffer. The membranes were then washed three times with TBST, and signals were detected by enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.) and exposure to X-ray film. The density of the bands was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). The expression of HIF-1α was normalized to β-actin expression in the same lane. Data from each experiment are presented as the fold increase relative to the control group.

*Alizarin Red staining*. Alizarin Red staining was performed to assess the mineralization of the cultured cells in the differentiation media at the different stages. The methodology utilized was in accordance with that described previously in the text.

*Statistical analysis*. All numerical data are expressed as the mean ± standard error. A Student's t-test was employed to compare differences between groups with SPSS 17.0 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significantly difference. All experiments were performed in triplicate.

## Results

*Morphology and surface antigen expression of rMSCs*. Cell morphology under 1% oxygen or atmospheric conditions was observed by light microscopy, with photomicrographs taken at 3, 7, 14 and 21 days. This showed that rMSCs cultured in 21% $O_2$ without osteogenic induction were morphologically indistinguishable up to passage $P_3$. However, in comparison with normoxic $P_1$-$P_3$ cells, rMSCs under hypoxic conditions lost their spindle shape when treated with osteogenic induction medium (Fig. 1).

*Potency of induced multi-lineage differentiation*. Alizarin Red S staining revealed that rMSCs cultured in osteogenic medium for two weeks had significant calcium deposition (Fig. 2A). In the case of adipogenic differentiation, the accumulation of multiple droplets, identified by Oil Red staining, occurred after three weeks of culture (Fig. 2B). The data indicated that the collected rMSCs had acquired the potency of induced multi-lineage differentiation.

*qPCR analysis of osteogenesis-related biomarkers*. A high Runx2 mRNA expression level was maintained in

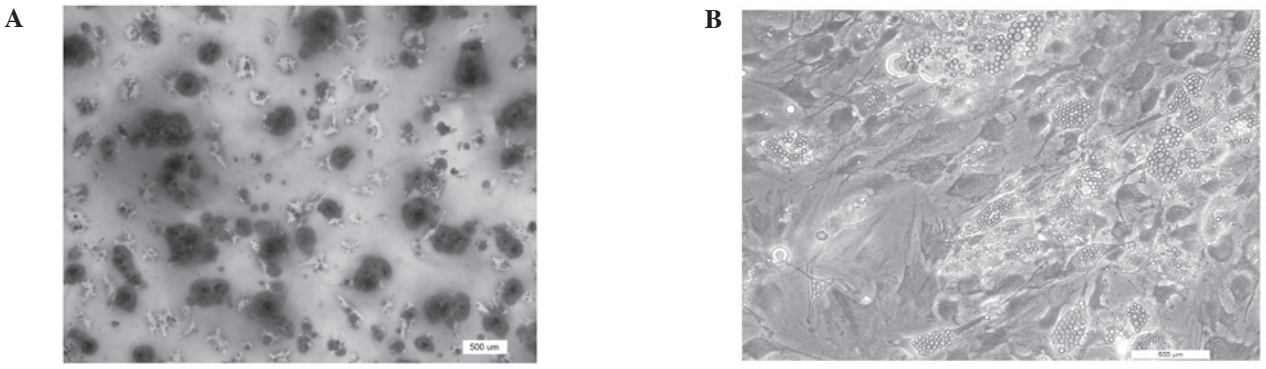


Figure 2. Multipotency of induced differentiation. (A) Induced osteogenic differentiation was confirmed by Alizarin Red S staining. (B) Accumulation of multiple fat droplets indicated by Oil Red staining in cells cultured in adipogenic medium for three weeks.

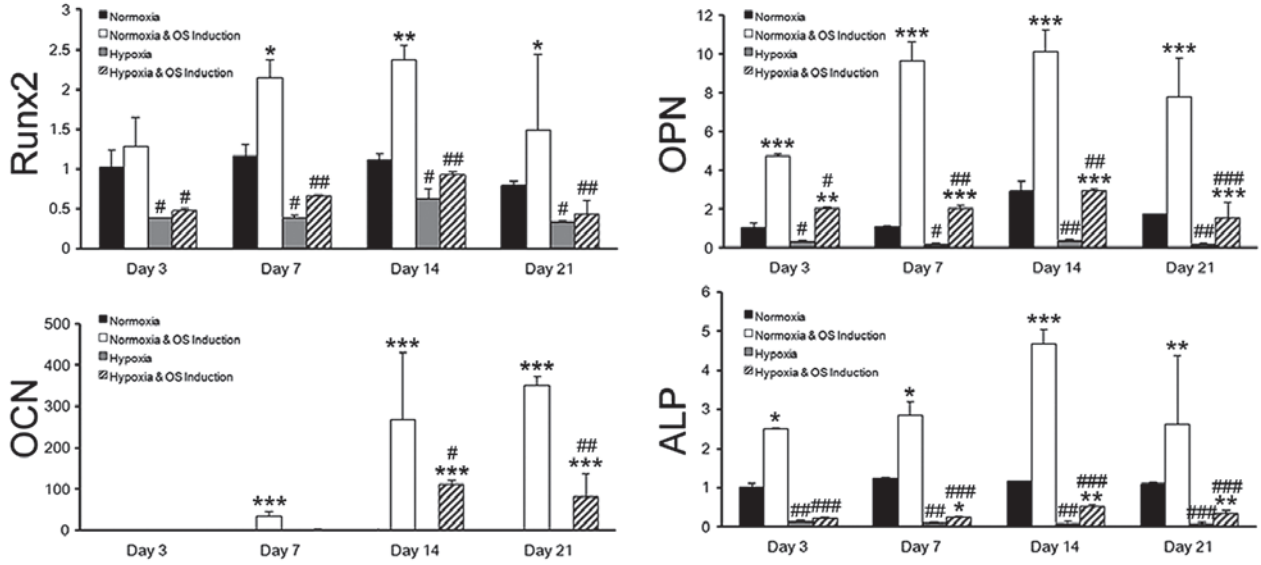


Figure 3. Quantitative polymerase chain reaction of osteogenesis-related biomarkers. Runx2 expression remained higher in normoxia-treated rat mesenchymal stromal cells, irrespective of whether they were cultured in OS induction medium. Runx2 expression remained at a low level under hypoxic conditions. The pattern of expression of OPN, OCN and ALP was similar to that of Runx2, with the levels of OPN, OCN and ALP all significantly lower in hypoxia-treated cells. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 versus the non-OS induction group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 versus the corresponding normoxia group. OS, osteogenic; Runx2, runt-related transcription factor 2; OPN, osteopontin; OCN, osteocalcin; ALP, alkaline phosphatase.

normoxia-treated rMSCs, irrespective of whether they were cultured in osteogenic induction medium. However, Runx2 mRNA expression was low under hypoxic conditions. As an early biomarker of osteogenesis, the suppression of Runx2 indicated that the osteogenic capacity of the rMSCs was suppressed by hypoxia. The expression pattern of OPN, OCN and ALP was similar to that of Runx2, with significantly lower mRNA levels observed in hypoxia-treated cells than in normoxia-treated cells (Fig. 3). Overall, early-, medium- and mature-stage osteogenic biomarkers were all downregulated at the mRNA level in a hypoxic environment.

*Western blot analysis of osteogenesis-related biomarkers*. On the third day of culture, the expression of Runx2 was similar between cells cultured under normoxia and hypoxia, irrespective of osteogenic induction; however, by days 7, 14 and 21, the protein expression of Runx2 was reduced in hypoxia-treated cells. The protein expression levels of OCN and ALP peaked on day 7 in the hypoxia-treated cells. On day 14, the expression of ALP was maintained at a similar level in the hypoxia-treated rMSCs, whereas OCN expression was significantly downregulated in the hypoxia plus OS induction group. OPN expression remained repressed in cells cultured under hypoxia with osteogenic induction. On day 14, no significant differences were observed in the expression levels of OCN and ALP between the hypoxia- and normoxia-treated groups. under the same conditions. The levels of OCN and ALP were higher in hypoxia-treated cells than those in cells treated solely with normoxia on day 21 (Fig. 4). It was additionally observed that, in hypoxia-treated rMSCs, the duration of Runx2 expression was relatively short and the expression of OCN and ALP was advanced, indicating that hypoxia may accelerate the osteogenic differentiation of rMSCs and osteocyte maturation.

*Alizarin Red staining*. The results of the Alizarin Red staining directly indicated that osteogenic ability was enhanced in hypoxia-treated rMSCs as compared with controls on days 3, 7 and 14. Comparison between the groups showed that osteo-

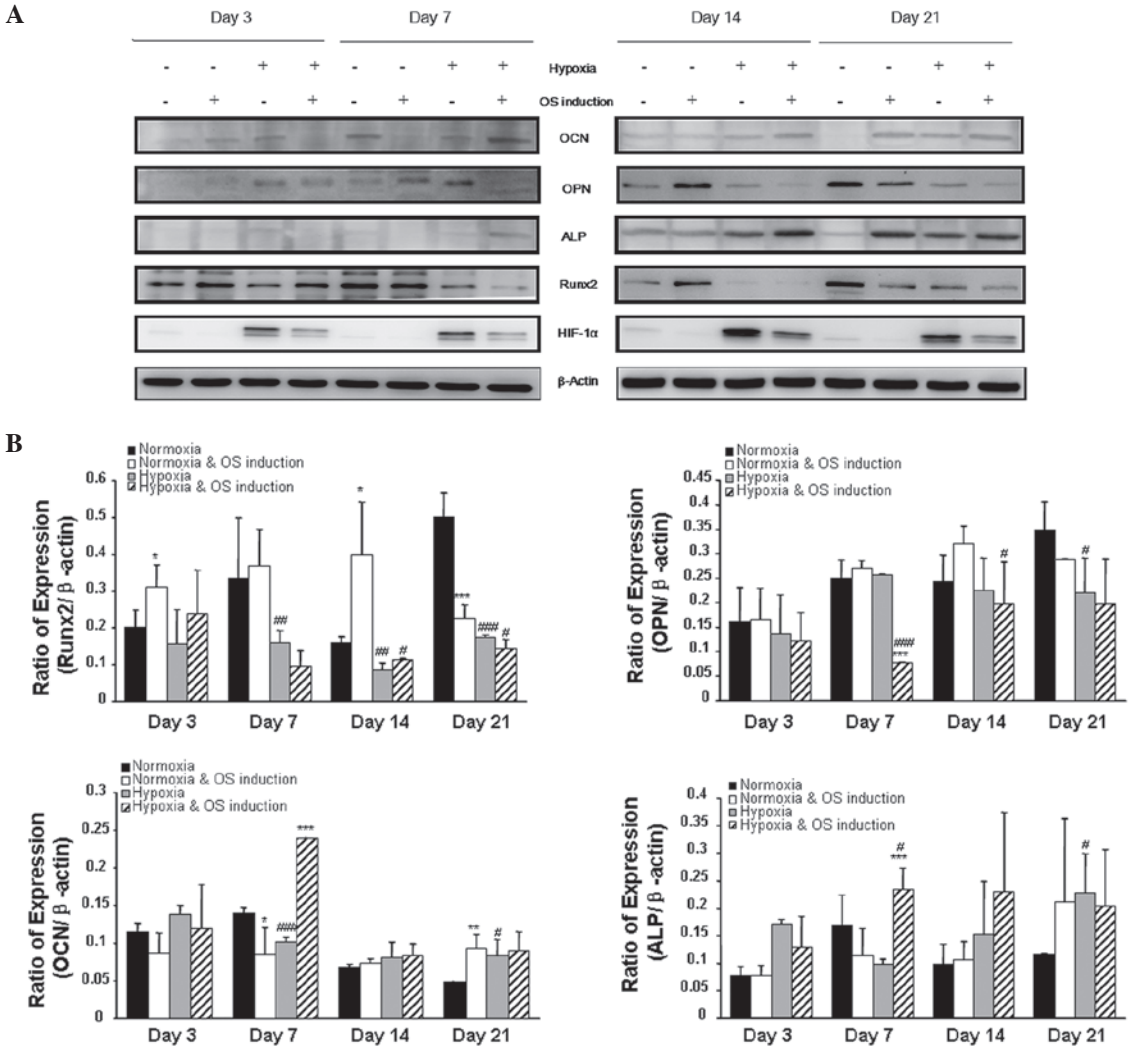


Figure 4. Western blot analysis of osteogenesis-related biomarkers. (A) The pattern of expression of osteogenic biomarkers at the protein level was shown on days 3, 7, 14 and 21, with β-actin expression set as the control. (B) The protein bands were quantified using Quantity One image analysis software. The expression of Runx2 was downregulated on days 7, 14 and 21 when cells were cultured under hypoxia. The expression of OPN was similar on day 3 in all groups, and the level of OPN was comparable when cells were exposed to OS induction under normoxia or hypoxia. The levels of OCN were similar on days 3 and 14 in all groups of cells; however, levels were higher in hypoxia-treated rat mesenchymal stromal cells. In terms of ALP expression, the level of ALP was higher on day 21 when the cells were treated by hypoxia. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 versus the non-OS induction group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 versus the corresponding normoxia group. OS, osteogenic; Runx2, runt-related transcription factor 2; OPN, osteopontin; OCN, osteocalcin; ALP, alkaline phosphatase.

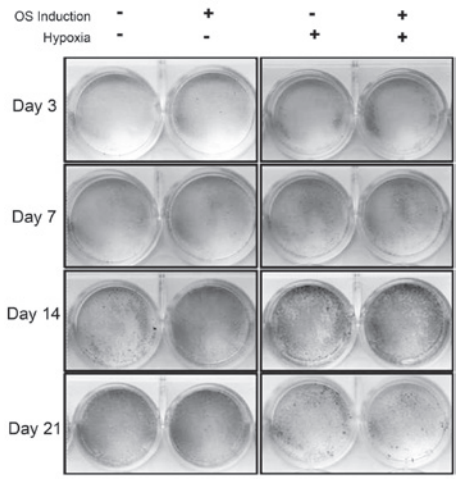


Figure 5. Alizarin Red staining. The results of the Alizarin Red staining indicated that the osteogenic potential was enhanced in hypoxia-treated rat mesenchymal stromal cells on days 3, 7 and 14. However, osteogenesis was impaired by continuous hypoxia up to day 21. OS, osteogenic.

genesis was comparable between the cells on the third day of culture in hypoxic conditions and those on the seventh day of culture in normoxic conditions, and similarly between the cells on the seventh and 14th days of hypoxia and normoxia treatment, respectively, and those on the 14th and 21st days of hypoxia and normoxia treatment, respectively (Fig. 5). It was therefore speculated that the osteogenic differentiation of rMSCs was accelerated following culture in 1% oxygen. However, when the cells were maintained in hypoxic culture until day 21, osteogenesis was impaired. It may therefore be concluded that hypoxia can regulate the osteogenic capacity of rMSCs in a time-dependent manner.

## Discussion

MSCs are widely used as precursor cells for regenerative medicine and *in vitro* tissue engineering. Preliminary promising results have been obtained from the application of cytotherapy with MSCs in the treatment of osteonecrosis,

cartilage defects and myocardial infarction (21-23). Due to the hypoxic environment *in vivo* (primary normoxia), it is speculated that cells under normoxia *in vitro* may undergo considerable cellular stress upon transplantation into *in vivo* conditions (24). Therefore, the study of the characteristics of the survival, differentiation and apoptosis of MSCs under hypoxia is highly significant.

It has previously been indicated that oxygen concentration plays a key role in the determination of cell fate and the maintenance of 'stemness' in adipose- and bone marrow-derived MSCs (25). However, whether hypoxia plays a positive or negative role in the proliferation and differentiation of MSCs remains controversial. A previous study by Grayson *et al* (18) found that hMSCs maintained under 2% $O_2$ for seven passages *in vitro* could result in a 30-fold higher level of hMSC expansion without the loss of multi-lineage differentiation capacity (18). Furthermore, it has been demonstrated that hypoxia can promote chondrogenesis of MSCs (26) through the transcriptional activity of SRY (sex determining region Y)-box 9 and HIF-1α (27). In the context of tissue engineering, when MSCs were seeded onto fibrin glue and cultured in a 3% $O_2$ atmosphere, Oct-4 was shown to be stably expressed and an increase in directed differentiation was observed (28). Conversely, a negative effect of hypoxia on MSCs has also been noted (9). Physiological oxygen tension (1% oxygen) during the *in vitro* culture of hMSCs slowed down cell cycle progression and differentiation, which led to the accumulation of MSCs in $G_1$ phase (16). Similarly, the effect of hypoxia on other precursors, including embryonic, neural and induced pluripotent stem cells, is also the subject of great controversy (29-31). It is currently believed that the effect of hypoxia on MSCs is dependent on the severity and duration of hypoxia, the cell origin and the method used for induction.

The effect of hypoxia on the differentiation of rMSCs was evaluated in the present study by consecutive assessment of the osteogenic biomarkers Runx2, OPN, OCN and ALP. The results shown in Figs. 3 and 5 consistently demonstrate that hypoxia regulates the osteogenesis of MSCs in a time-dependent manner. ALP was analyzed as a biomarker of mature osteogenesis, and was observed to peak in expression level faster when cells were cultured under hypoxia. However, the data from mRNA expression analysis differed from those from the analysis of individual protein expression, which indicated that the post-translational modification of osteogenic biomarkers was a critical step in the differentiation of MSCs.

Alizarin Red staining indicated that 1% oxygen could accelerate the osteogenic progress of rMSCs. However, culture in continuous hypoxia resulted in reduced osteogenesis. The results suggested that osteogenic induction had a protective function for hypoxia-treated rMSCs, indicating that cells could be protected from the effects of physiological hypoxia when transplanted into an osteogenic environment *in vivo*. The effect of hypoxia on the osteogenic differentiation of rMSCs may be in part dependent on the duration of hypoxia. This study, to the best of our knowledge, is the first to report of a time-dependent effect of hypoxia on MSCs, although this effect has been preliminarily described in previous studies (9,16-19,32).

The underlying molecular mechanisms of the hypoxia-regulated differentiation of multipotent cells has attracted the focus of a large number of studies, which have revealed that HIF-1α plays an important role in the progress of differentiation. HIFs regulate a number of stem cell effectors, including Notch, Wnt and octamer-binding protein 4, that are involved in the control of stem cell proliferation, differentiation and pluripotency (6,7,33). It has been shown that hypoxia-induced HIF-1α expression exerted a protective effect on rMSCs, which may have contributed to the accumulation of cells under hypoxia. Although previous findings indicated that HIF had a limited role in enhancing MSC self-renewal and growth factor secretion under hypoxia, the function of HIF proved to be pivotal; HIF promoted self-renewal through enhancing the preservation of early colony-forming progenitor cells and maintaining the undifferentiated phenotype of MSCs (34).

At present, investigations in the field of stem cell research are focusing on identifying therapeutic targets, developing therapeutic tests, exploring cell differentiation and the underlying physiological mechanisms, determining the optimal culture conditions for pluripotent stem cells and ensuring efficacy and safety (35). The aim of the present study was to identify the optimal prerequisite for the improved application of MSCs in *in vitro* engineering and cytotherapy. Since hypoxia is key factor in the pathogenesis of osteonecrosis, it is of great significance to study the effect of hypoxia on the biological behavior of MSCs. As a result of these findings and those from previous studies, traditional tissue engineering under normoxia may undergo numerous changes in the near future.

The limitations of the present study should also be noted. Only a selection of representative osteogenic biomarkers was investigated, and their intrinsic associations are not completely understood. Furthermore, the transcriptional characteristics of these biomarkers were not investigated; this is an aspect to be investigated in future studies. The effects of hypoxia on the behavior of MSCs, including the effects on migration, intercellular communication and aging, also need to be taken into account (36,37).

In conclusion, 1% oxygen was able to regulate the osteogenic differentiation of rMSCs. Under the given conditions, the osteogenesis of rMSCs was accelerated in the early period; however, long-term hypoxia resulted in poor osteogenesis. The effect of hypoxia on the osteogenic differentiation of rMSCs is time-dependent. Therefore, when MSCs are used for tissue engineering and cytotherapy, the duration of hypoxia pretreatment should be controlled accurately to achieve improved efficiency and the expected outcome.

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