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Hypoxia Promotes Osteogenesis of Human Placental-Derived Mesenchymal Stem Cells

Qiaoli Gu,[1] Yanzheng Gu,[2] Qin Shi[1] and Huilin Yang[1]

[1]Department of Orthopaedic Surgery, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, People's Republic of China
[2]Jiangsu Institute of Clinical Immunology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, People's Republic of China

Placental-derived mesenchymal stem cells (pMSCs) are promising candidates for regenerative medicine because they possess high proliferative capacity and multi-differentiation potential. Human pMSCs are residing in an environment with low oxygen tension in the body. Heme oxygenase-1 (HO-1) is known to participate in the regulation of MSC differentiation. The present study aimed to investigate the impact of hypoxia on the osteogenic differentiation of human pMSCs, and to elucidate the role of HO-1 in the osteogenic differentiation of hypoxic pMSCs. Human pMSCs were cultured under normoxia (21% O_2) or hypoxia (5% O_2) for 3 days. We found that hypoxia maintained the morphology and immunophenotype of human pMSCs. The expression of stemness markers Oct4, Nanog, and Sox2 was increased under hypoxia. After a 5-day hypoxic culture, the proliferation ability of pMSCs was increased, which might be correlated with the increased expression of stem cell factor. During osteogenic induction, hypoxia increased the expression of osteogenic genes including osteopontin, osteocalcin, and alkaline phosphatase (ALP). Moreover, hypoxia increased the mineralization and ALP levels of human pMSCs as evidenced by Alizarin Red staining and ALP staining. Upregulation of HO-1 by cobalt-protoporphyrin treatment increased the osteogenic differentiation of pMSCs under hypoxia, while inhibition of HO-1 by Zn-protoporphyrin reduced the osteogenic differentiation of hypoxic pMSCs. Taken together, our data suggest that hypoxia can promote the osteogenic differentiation of human pMSCs. Upregulation of HO-1 can further increase the osteogenesis of human pMSCs under hypoxia. Our findings will highlight the therapeutic potential of MSCs in the tissue engineering of bones.

Keywords: heme oxygenase-1; hypoxia; osteogenic differentiation; placental-derived mesenchymal stem cells; proliferation
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Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into several cell types. For the reason, MSCs are considered an ideal source for tissue repair and regeneration (Doulatov and Daley 2013). Placental-derived mesenchymal stem cells (pMSCs) are an intermediate between embryonic and adult stem cells (Jung et al. 2013). Apart from the common characteristics of MSCs (self-renewal, proliferation, and differentiation), pMSCs have their distinctive features: ease to collect in massive numbers, rapid expansion potential, and strong immunosuppressive property (Li et al. 2007; Choi et al. 2016). Moreover, because placenta is often discarded as waste after birth, pMSCs can be easily obtained without ethical concerns. In recent years, human pMSCs are considered an excellent alternative to bone marrow (BM)-derived MSCs for regenerative medicine, especially in repair of bone fracture.

In most cases, MSCs are cultured under normoxic conditions (21% O_2) *in vitro*. However, MSCs often reside in niches of low oxygen *in vivo*: 1-7% in BM, 10-15% in adipose tissue, and 2.5-8.5% in placenta (Bizzarri et al. 2006; Schneider 2011; Boyette et al. 2014). Moreover, MSCs always participate in bone healing under low oxygen microenvironment *in vivo* (Dimitriou et al. 2005). It has been accepted that oxygen tension is a powerful signaling molecule that regulates MSC proliferation and differentiation. Increasing studies have been carried out to investigate the effects of hypoxia on MSC biology. However, the results remain conflicting. In addition, most studies have focused on BM or adipose tissue-derived MSCs. Few studies have focused on the hypoxic pMSCs until now. Therefore, the effect of hypoxia on pMSCs requires further investigation.

Heme oxygenase-1 (HO-1) is an anti-oxidant protein

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Correspondence: Huilin Yang, Department of Orthopaedic Surgery, The First Affiliated Hospital of Soochow University, 188 Shizi Road, Suzhou, Jiangsu 215006, People's Republic of China.
e-mail: suzhouspine@163.com

(Shibahara 2003). The inducible HO-1 is a rate-limiting enzyme that catalyzes the degradation of heme to carbon monoxide and biliverdin. It is well know that HO-1 can inhibit the cytotoxicity and apoptosis of MSC induced by low oxygen and oxidative stress (Hamedi-Asl et al. 2012), suggesting an important role of HO-1 under hypoxia. Recent studies have shown that HO-1 is important in regulating bone homeostasis. HO-1 can inhibit the maturation and mineralization of rat osteoblast (Lin et al. 2010). On the contrary, others reported that HO-1 can increase the osteogenic differentiation of human MSCs (Vanella et al. 2010). In addition, the activation of HO-1 regulates osteoclastogenesis and bone reabsorption during fracture repair (Komatsu and Hadjiargyrou 2004). However, the role of HO-1 in the osteogenesis of pMSCs under hypoxia has not been elucidated.

In the present study, we investigate the effect of 5% O_2 on the osteogenic differentiation of primary human pMSCs. Our data demonstrate that hypoxia can promote the osteogenic differentiation of human pMSCs. Upregulation of HO-1 can further increase the osteogenesis of human pMSCs under hypoxia.

Materials and Methods

Human pMSC culture and hypoxia treatment

The pMSCs were isolated from human term placenta and characterized as previously described (Park et al. 2011; Pilz et al. 2011). Small pieces of chorionic villi were dissected free of maternal decidua. Tissue samples were minced mechanically and enzymatically digested. Cells were passed through a nylon tissue mesh (45 μm) to obtain a single cell suspension. Next, cells were separated on a Percoll (Sigma-Aldrich, St. Louis, USA) discontinuous gradient for hematopoietic stem cell isolation and then seeded in tissue culture flask using Dulbecco's modified Eagle's medium (DMEM) (HyClone, South Logan, UT, USA) containing 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY). After 4 days, media were changed, and nonadherent cells were washed. MSCs were characterized for the absence of CD45, CD19 and CD34, and the presence of CD105 and CD90 (Dominici et al. 2006; Huang et al. 2010) (Fig. 1A). For hypoxia treatment, cells were cultured in a hypoxic chamber flushed with a mixture of 5% O_2, 5% CO_2, and 90% N_2 at 37°C.

Cell proliferation assay

Cell proliferation was detected using a BrdU flow kit (BD Biosciences, San Jose, CA) according to the manufacture's instruction. Briefly, cells were cultured in DMEM for 5 days (Fig. 1A). BrdU (10 μM) was added to cultures for 12 h, and then cells were processed for immunostaining.

Cell apoptosis assay

Cell apoptosis was examined using the Annexin V-FITC apoptosis Detection Kit I (BD Biosciences). Flow cytometric analysis of Annexin V-FITC and propidine iodide (PI) was performed according to the manufacture's instruction. Briefly, cells were cultured for 72 h (Fig. 1A), and then washed twice with cold PBS at a concentration of 1×10^6 cells/ml. The cells were added with 5 μl Annexin V-FITC and 5 μl PI, and incubated for 15 min at room temperature in the dark. All samples were analyzed by flow cytometry within 1 h.

Osteogenic differentiation of human pMSCs

For osteogenic differentiation, human pMSCs were cultured in DMEM culture medium supplemented with 10% fetal calf serum, 10 mM β-glycerol phosphate (Sigma-Aldrich), 0.1 mM L-ascorbic acid (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich), 2 mM glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). In some experiments, cobalt-protoporphyrin (CoPP) IX (2 μM) or Zn-protoporphyrin (ZnPP) IX (2 μM) (Frontier Scientific, Logan, UT) was added once every 3 days during osteogenic differentiation (Fig. 1C).

Alizarin red staining

Calcium deposits were detected by alizarin red S staining after 28 days of osteogenic induction (Fig. 1B, C). Cells were incubated with 2% alizarin red (pH 4.2) (Sigma-Aldrich) for 10 min and washed with distilled water. Cells were detected by phase-contrast microscopy at 28 days to verify the presence of mineralized nodules.

Alkaline phosphatase (ALP) staining

Human pMSCs were cultured in a 24 well plate, washed twice with PBS and fixed in 4% polyoxymethylene for 10 min. ALP staining was performed with a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Haimen City, China) according to the manufacturer's instructions (Fig. 1B, C).

Real-time quantitative PCR

Total RNA was extracted from each sample using TRIzol (Invitrogen) according to the manufacturer's instructions and reverse transcribed. Real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) and conducted with the Biorad CFX96 (Bio-Rad). Oligonucleotide primer sequences used in this study were listed in Table 1.

HO activity assay

HO activity was measured as previously described (Vanella et al. 2010) with minor modification (Fig. 1A, C). Cell homogenates were added to a reaction mixture containing 30 μM hemin (Sigma-Aldrich), 2 mg/ml rat liver cytosol, 1 mM $Mgcl_2$, 3 unit glucose-6-phosphatase dehydrogenase (Sigma-Aldrich), 1 mM glucose-6-phosphate (Roche, Basel, Switzerland), 2 mM NADPH (Beyotime), and 0.1 M potassium phosphate buffer (pH 7.4). The reaction was conducted at 37°C in the dark for 30 min and terminated by addition of 1 ml of chloroform. The extracted bilirubin was defined as the difference between 464 and 530 nm (ε = 40 $mM^{-1}\,cm^{-1}$). The results were expressed as nmol of bilirubin/5×10^6 cells/h.

Statistical analysis

The results were presented as means ± SEM. The significance between two groups was determined using Student's *t* test. One-way ANOVAs were used for multiple comparisons. *P* values less than 0.05 were considered as statistically significant.

Results

Hypoxia maintains the morphology and phenotype of human pMSCs

Human pMSCs displayed a fibroblast-like morphology under normoxic conditions (Fig. 2A). They highly

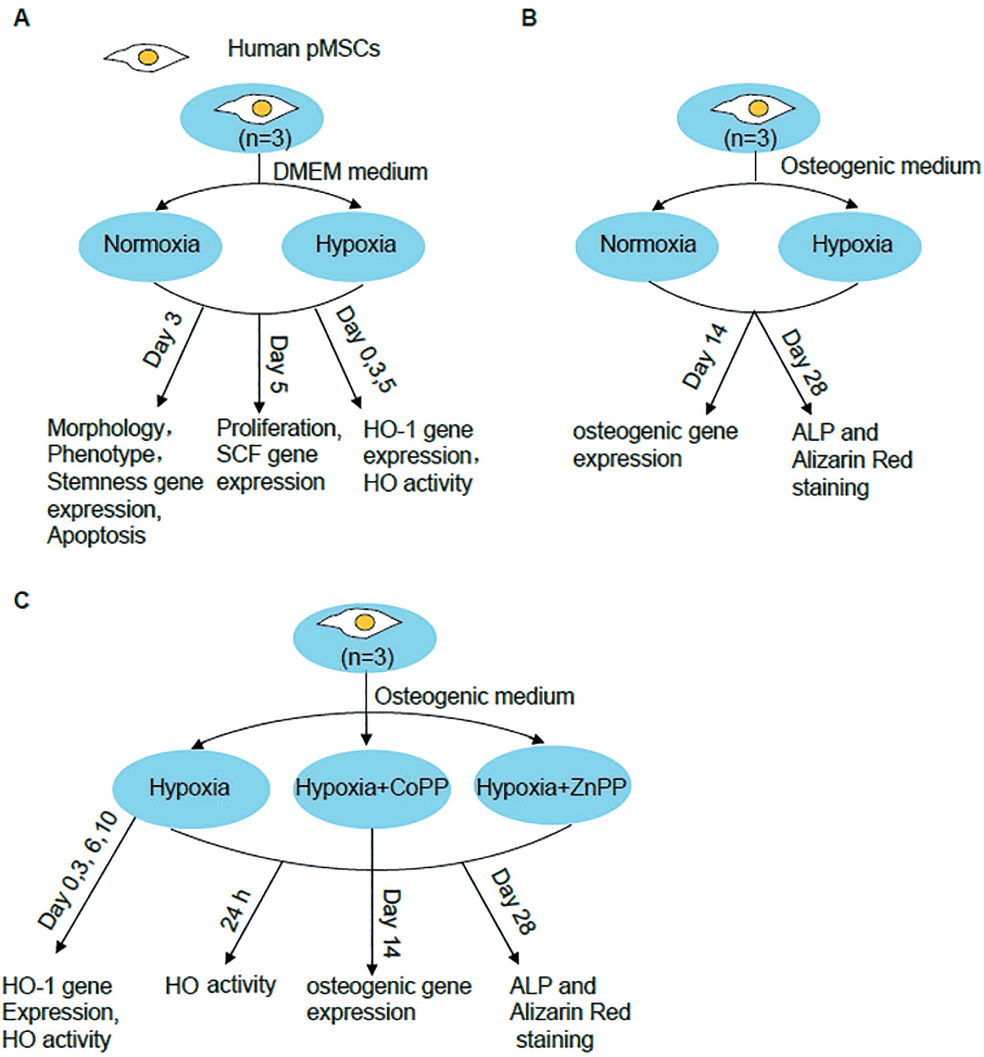

Fig. 1. Overall experimental design.
(A) Experimental setup to analyze the effects of hypoxia on the characteristics of human pMSCs. (B) Experimental setup to analyze the effects of hypoxia on the osteogenic differentiation of human pMSCs. (C) Experimental setup to analyze the effect of HO-1 on the osteogenic differentiation of pMSCs under hypoxia.

Table 1. Oligonucleotides used in quantitative real-time PCR.

Gene	Forward	Reverse
OCN	CACTCCTCGCCCTATTGGC	CCCTCCTGCTTGGACACAAAG
OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT
HO-1	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCG
SCF	CCAAAAGACTACATGATAACCCTCAA	CATCTCGCTTATCCAACAATGACT
Sox2	CCCAGCAGACTTCACATGT	CCTCCCATTTCCCTCGTTTT
Oct4	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT
Nanog	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
ALP	GGAACTCCTGACCCTTGACC	TCCTGTTCAGCTCGTACTGC
COL1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

expressed CD105 and CD90, and lack the expression of CD19, CD45, and CD34 (Fig. 2B). Cells cultured under hypoxia for 3 days showed no obvious changes in morphology and phenotype compared to normoxic cells (Fig. 2A, B).

Hypoxia increases stemness marker expression in human pMSCs

To investigate the effect of hypoxia on the self-renewing capacity of human pMSCs, we examined the expression of stemness markers by real-time PCR after 3 days of hypoxic culture. The expression levels of Oct4, Nanog, and Sox2 were significantly increased under hypoxia as compared to normoxia ($p < 0.05$; Fig. 3A). We next measured the apoptosis rates of human pMSCs by Annexin V/PI double staining after 3 days of hypoxic culture. There was no significant difference in the apoptosis rate (Annexin V^+PI^+) between hypoxia (2.67 ± 0.16%) and normoxia (2.81 ± 0.10%) ($p > 0.05$; Fig. 3B).

Hypoxia increases the proliferation and stem cell factor (SCF) expression in human pMSCs

After the initial 24 h attachment, human pMSCs were exposed to hypoxia for 5 days. The proliferation of pMSCs was examined by BrdU incorporation assay. The percentage of BrdU-positive pMSCs cultured under hypoxia was higher (22.17 ± 1.58%) than those of normoxia (15.7 ± 1.37%) ($p < 0.05$; Fig. 4A). SCF can regulate the proliferation of stem cells. We next analyzed the expression level of SCF in human pMSCs. There was a significant increase in the expression level of SCF under hypoxia as compared to normoxia ($p < 0.05$; Fig. 4B).

Hypoxia reduces HO-1 expression in human pMSCs

HO-1 expression can be induced by various environmental factors (Udono-Fujimori et al. 2004). We then examined the expression of HO-1 in human pMSCs after 0, 3, 5 days of hypoxic culture. The mRNA expression of HO-1 was significantly decreased under hypoxia as compared to normoxia ($p < 0.05$, $p < 0.01$; Fig. 5A). HO activity was also reduced after hypoxic culture ($p < 0.05$; Fig. 5B)

Hypoxia promotes the osteogenic differentiation of human pMSCs

After 14 days of osteogenic induction, we analyzed the mRNA expression of osteogenic genes in pMSCs. The results showed that the expressions of osteopontin (OPN), osteocalcin (OCN), and ALP were significantly increased under hypoxia as compared to normoxia ($p < 0.05$, $p < 0.01$; Fig. 6A). However, there was no significant difference in the expression of type I collagen (COL1A1) between

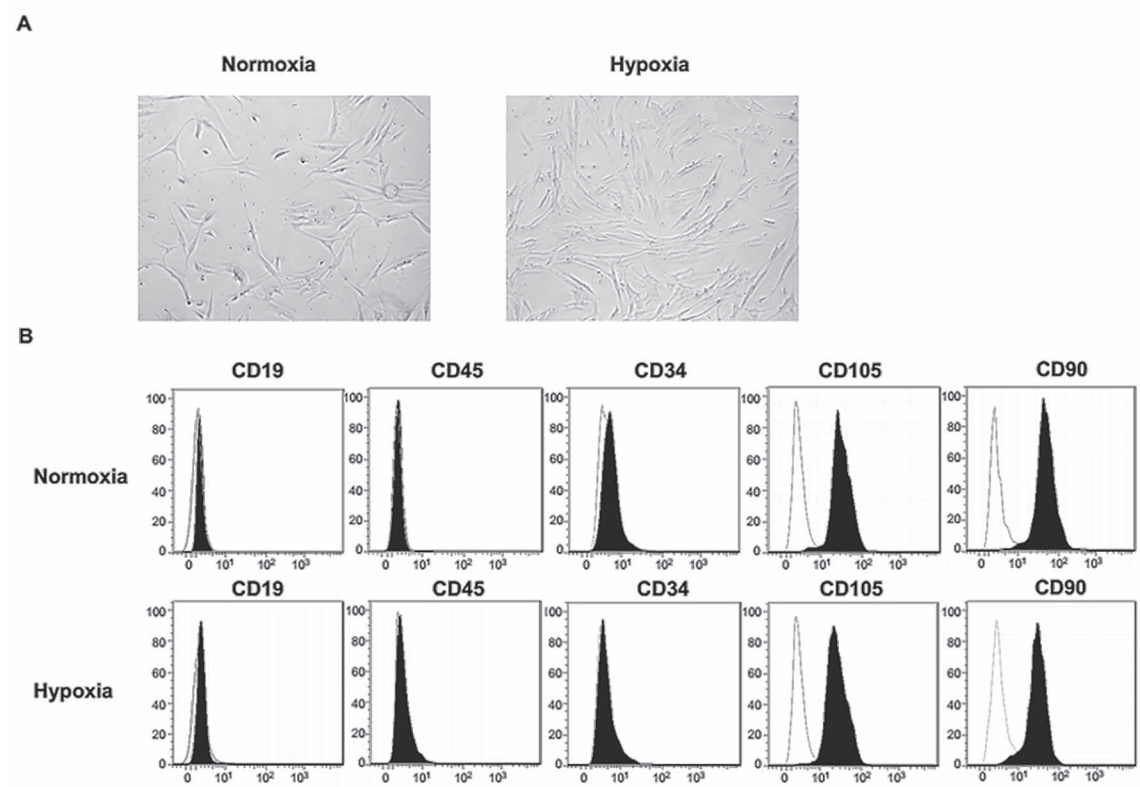

Fig. 2. The effect of hypoxia on the morphology and phenotype of human pMSCs.
Human pMSCs were cultured under normoxia or hypoxia for 3 days. (A) Human pMSCs displayed the fibroblast-like morphology under hypoxic and normoxic conditions (magnification 100×). (B) Flow cytometry analysis showed the expression of CD19, CD45, CD34, CD105, and CD90.

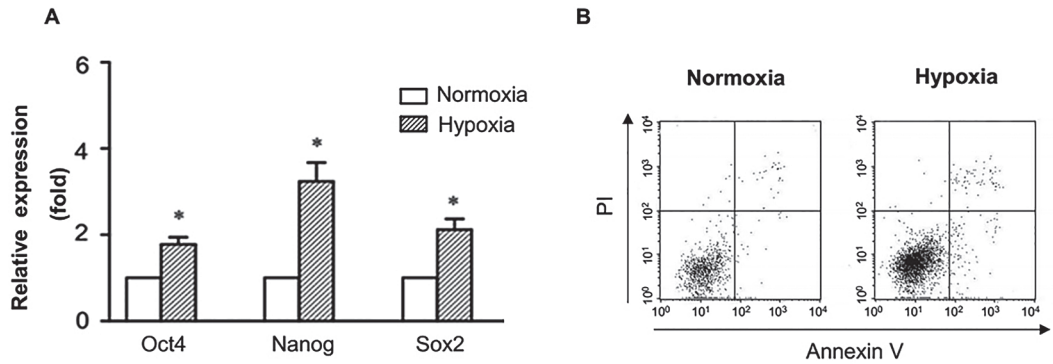

Fig. 3. The effect of hypoxia on the expression of stemness genes.
Human pMSCs were cultured under normoxia or hypoxia for 3 days. (A) The mRNA expression of stemness markers was analyzed using real-time PCR. (B) The apoptosis rate of human pMSCs was measured by Annexin V/PI staining. Data were shown as means ± SEM (n = 3), *$p < 0.05$.

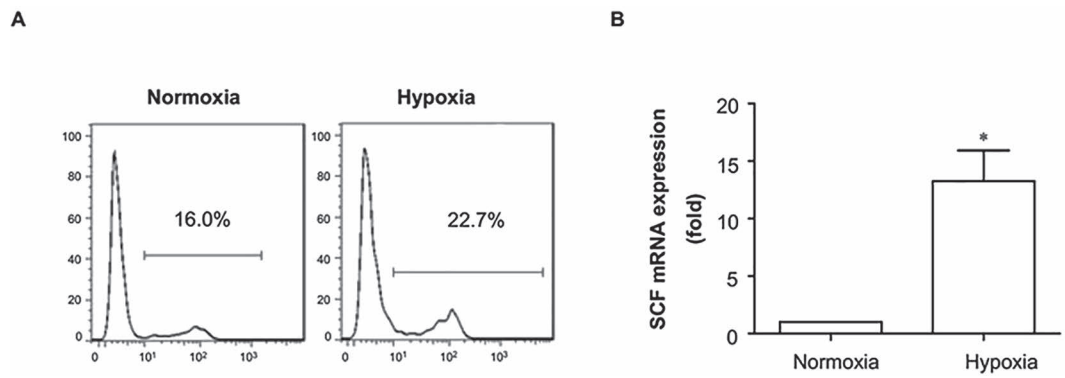

Fig. 4. The effect of hypoxia on the proliferation and SCF expression of human pMSCs.
Human pMSCs were cultured under normoxia or hypoxia for 5 days. (A) The proliferation rate of human pMSCs was analyzed by BrdU incorporation assay. (B) The mRNA expression of SCF was analyzed using real-time PCR. Data were shown as means ± SEM (n = 3), *$p < 0.05$.

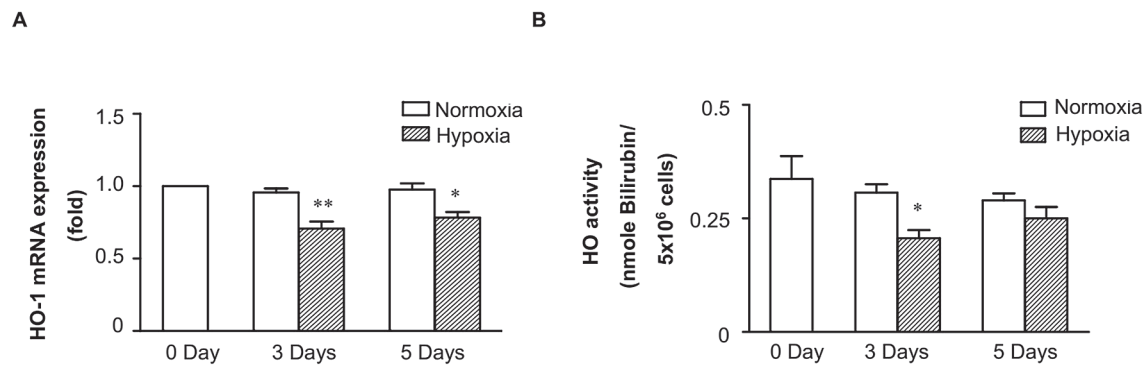

Fig. 5. The effect of hypoxia on HO-1 expression in human pMSCs.
(A) The expression of HO-1 was analyzed by real-time PCR at days 0, 3, 5 after hypoxic culture. (B) The activity of HO was detected at days 0, 3, 5 after hypoxic culture. Data were shown as means ± SEM (n = 3), *$p < 0.05$, **$p < 0.01$.

hypoxia and normoxia ($p > 0.05$; Fig. 6A). We next performed ALP and Alizarin Red staining after 28 days of osteogenic induction. The results showed that the mineralization and intracellular ALP levels in pMSCs were significantly enhanced under hypoxia as compared to normoxia (Fig. 6B).

HO-1 expression during osteogenic differentiation under hypoxia

HO-1 is important for the differentiation of MSCs. We first analyzed HO-1 expression in pMSCs after 0, 3, 6, 10 days of osteogenic induction under hypoxia. The results showed that HO-1 expression was downregulated at day 3,

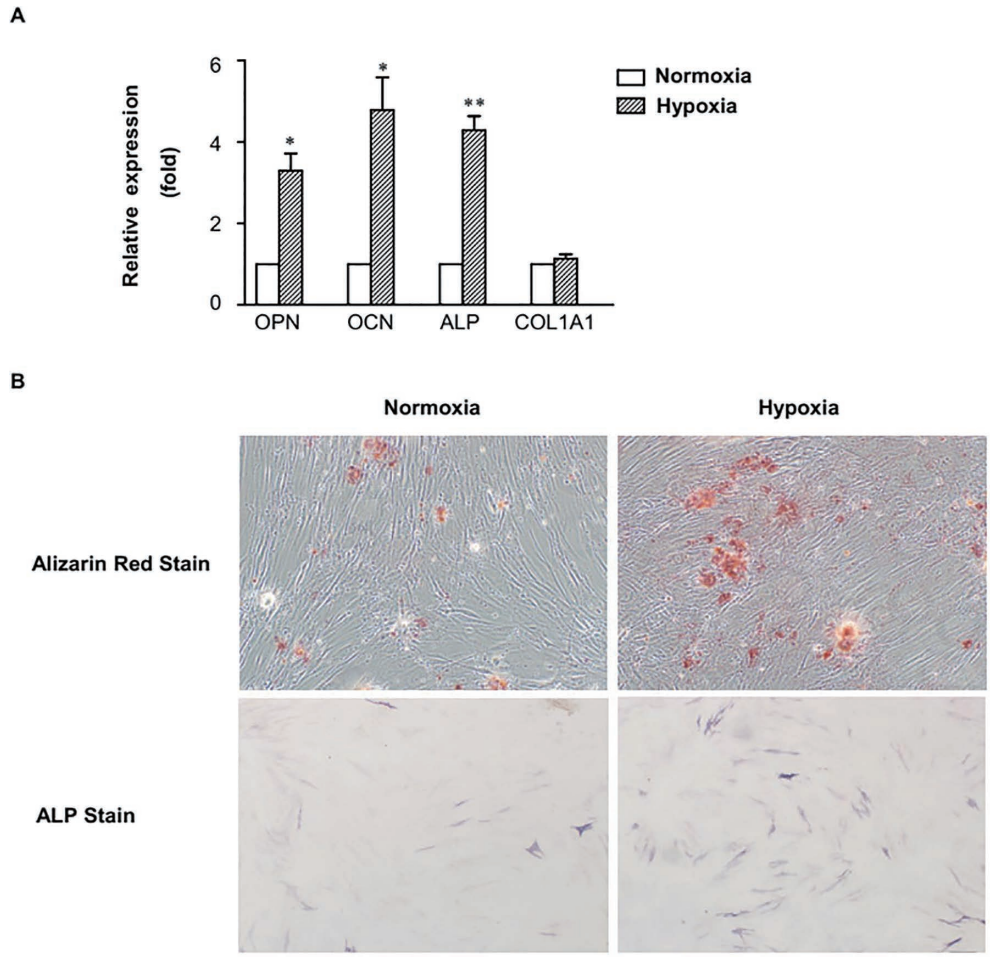

Fig. 6. The effect of hypoxia on the osteogenic differentiation of human pMSCs.
Human pMSCs were cultured in osteogenic medium under normoxia or hypoxia. (A) The expression of osteogenic differentiation gene was analyzed by real-time PCR at day 14. Data were shown as means ± SEM (n = 3), *$p < 0.05$, **$p < 0.01$. (B) Alizarin Red staining and ALP staining were performed at day 28.

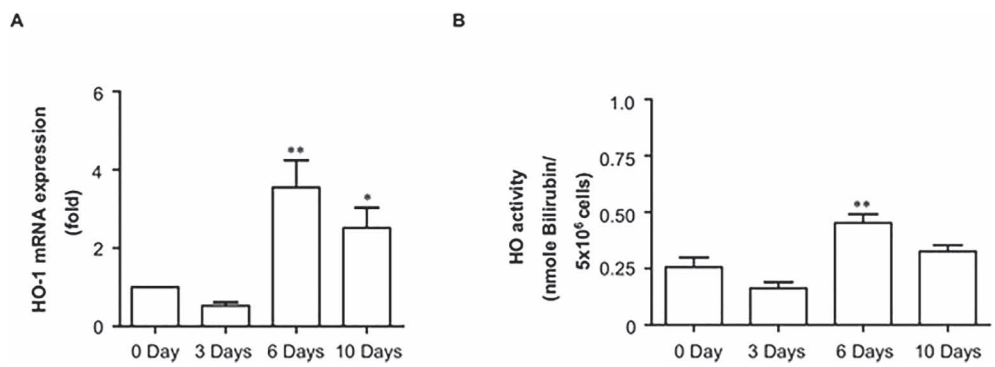

Fig. 7. HO-1 expression during the osteogenic differentiation under hypoxia.
Human pMSCs were cultured in osteogenic medium under hypoxia. (A) The expression of HO-1 was analyzed by real-time PCR at days 0, 3, 6, and 10 during osteogenic differentiation. (B) HO activity was detected at days 0, 3, 6, and 10 during osteogenic differentiation. Data were shown as means ± SEM (n = 5), *$p < 0.05$. **$p < 0.01$.

although not significantly. However, HO-1 expression was increased at day 6 and then slowly declined at day 10 (Fig. 7A). HO activity showed a similar pattern compared to the mRNA expression of HO-1 (Fig. 7B).

The effect of HO-1 on the osteogenic differentiation of human pMSCs after hypoxic culture

CoPP is an inducer of HO-1 activity. ZnPP is a specific inhibitor of HO-1 activity. To investigate the role of HO-1 in the osteogenesis of human pMSCs under hypoxia,

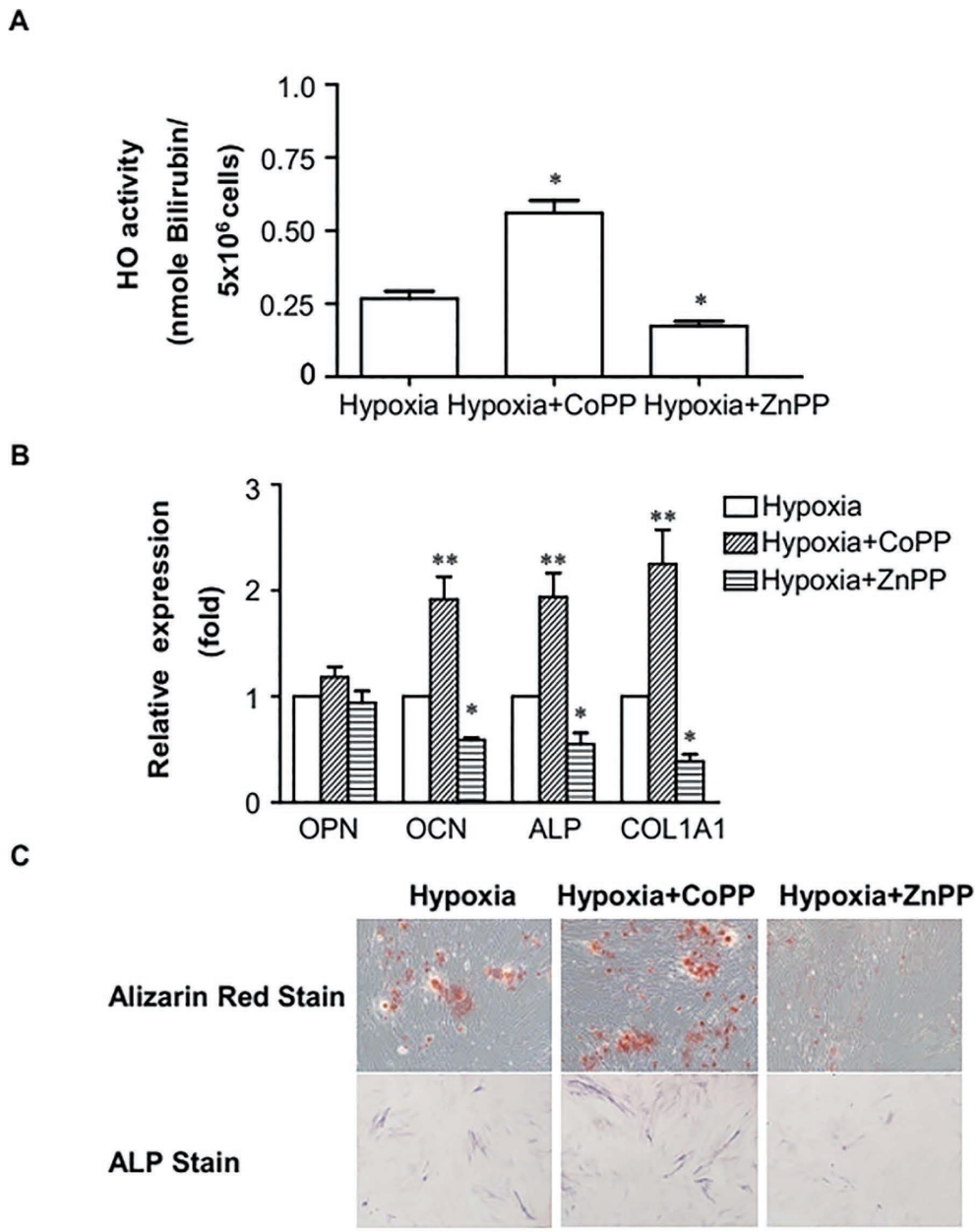

Fig. 8. The effect of HO-1 on the osteogenic differentiation of human pMSCs after hypoxic culture.
Human pMSCs were cultured in osteogenic medium under hypoxia. CoPP or ZnPP was added to the culture. (A) HO activity was analyzed 24 h after CoPP or ZnPP was added. (B) The osteogenic gene expression was analyzed by real-time PCR at day 14. (C) Alizarin Red staining and ALP staining were performed at day 28. Data were shown as means ± SEM (n = 5), *$p < 0.05$. **$p < 0.01$.

CoPP or ZnPP was added to the culture. The results showed that CoPP increased the HO-1 activity, while ZnPP reduced the HO-1 activity under hypoxia ($p < 0.05$; Fig. 8A). CoPP treatment increased the mRNA expression of OCN, ALP and COL1A1 in pMSCs under hypoxia ($p < 0.05$, $p < 0.01$; Fig. 8B). On the contrary, ZnPP treatment resulted in a decrease in the mRNA expression of OCN, ALP and COL1A1 ($p < 0.05$, $p < 0.01$; Fig. 8B). Both CoPP and ZnPP almost had no effect on the expression of OPN ($p > 0.05$; Fig. 8B). Moreover, CoPP treatment increased the mineralization and intracellular ALP levels of human pMSCs under hypoxia (Fig. 8C). On the contrary, ZnPP treatment inhibited the mineralization and intracellular ALP levels of human pMSCs under hypoxia (Fig. 8C). These data indicate that HO-1 may participate in the osteogenesis of pMSCs under hypoxia.

Discussion

MSCs are often found in a relatively low oxygen environment. Cultivation of MSCs under hypoxic conditions is an important prerequisite for investigating the biological properties of MSCs. Previous studies have demonstrated

conflicting results about the role of hypoxia on MSC differentiation. In the present study, we found that hypoxia (5% O_2) can promote the osteogenic differentiation of human pMSCs.

We first confirmed the immunophenotype of pMSCs used in our experiments. Hypoxia did not alter the morphology and immunophenotype of human pMSCs (Fig. 2), indicating that pMSCs can maintain the stem cell capabilities under hypoxia. Pluripotency marker genes are important for MSC self-renewal. We then analyzed the expression of stemness markers and found an increased expression of Oct4, Nanog, and Sox2 under hypoxia (Fig. 3A). Son et al. (2013) have reported that hypoxia can increase the apoptosis of MSCs. In contrast, the present study showed that hypoxia did not affect the apoptosis of pMSCs (Fig. 3B). Hypoxia-induced autophagy can prevent apoptosis (Zhang et al. 2011). It is possible that hypoxia can regulate the balance between apoptosis and autophagy in human pMSCs. Together, these results suggest that hypoxia is beneficial for the maintenance of pMSC properties.

We also found an increased BrdU incorporation in human pMSCs under hypoxia (Fig. 4A). This finding suggests that hypoxia can promote the growth of human pMSCs. Hung et al. (2012) have demonstrated that the increased proliferation of MSCs was due to proliferating cell nuclear antigen. However, we did not find the increased expression of proliferating cell nuclear antigen under hypoxia (data not shown). We observed a significant increase in the expression of SCF under hypoxia (Fig. 4B). SCF is a multifunctional factor. The membrane-bound form of SCF promotes the cell proliferation and is important in tumor growth and angiogenesis (Krasagakis et al. 2011). Hypoxia can increase the expression of SCF in breast cancer cells and astrocytes (Xu et al. 2007; Han et al. 2008). A recent study has shown that SCF can regulate pulmonary vascular cell proliferation under hypoxia (Young et al. 2016). These results suggest that SCF might participate in regulating the proliferation of human pMSCs under hypoxia.

Nakayama et al. (2000) reported that hypoxia inhibited HO-1 expression in human umbilical vein endothelial cells and astrocytes. In this study, we found that the expression of HO-1 was reduced by hypoxia in human pMSCs (Fig. 5A). In contrast, Some studies have reported that hypoxia can increase HO-1 expression in human retinal pigment epithelial cells, rat pulmonary arterial endothelial cells or hamster ovary cells (Murphy et al. 1991; Hartsfield et al. 1999; Udono-Fujimori et al. 2004) *in vitro*. These opposing results suggest that hypoxia may regulate HO-1 expression through different signaling mechanisms.

In the present study, we found that hypoxia promoted the osteogenic differentiation of human pMSCs. The expression of osteogenic genes including OPN, OCN, and ALP was increased significantly after hypoxic culture (Fig. 6A). Moreover, there was a significant increase in the amount of calcium deposition and ALP level under hypoxia (Fig. 6B), which further confirmed that hypoxia promoted the osteogenic differentiation of human pMSCs. Previous studies demonstrated that the expression of OCN and OPN was upregulated under 2% O_2 (Hung et al. 2012). Hypoxia can increase the ALP activity of BM-derived MSCs (Huang et al. 2011). These findings support the notion that hypoxia promotes the osteogenic differentiation of MSCs. However, contradictory findings were reported by others. Choi et al. (2014) showed that hypoxia attenuated osteogenic differentiation of human adipose-derived stem cells. Temporary hypoxia (48 h) inhibited osteogenic differentiation of human BM-derived MSCs (Potier et al. 2007). Moreover, hypoxia enhanced osteogenic differentiation of MSCs in the early stage, while inhibited osteogenesis in the latter stage (Ding et al. 2014). Taken together, the effects of hypoxia on MSCs are dependent on the cell source and species, the severity and duration of hypoxia, the isolation protocols and the method used for induction.

HO-1 plays an important role in bone metabolism. In our study, HO-1 expression was downregulated at day 3 after hypoxic culture, and then increased at days 6 and 10 (Fig. 7A). The results suggested that HO-1 might participate in the osteogenic differentiation of human pMSCs under hypoxia. When CoPP (an inducer of HO-1) was added to the culture under hypoxic conditions, the osteogenic differentiation was increased as evidenced by osteogenic gene expression, alizarin red staining and ALP staining (Fig. 8B, C). As expected, ZnPP (an inhibitor of HO-1) reduced the osteogenic differentiation (Fig. 8B, C). These results suggest that upregulation of HO-1 can increase the osteogenic differentiation of human pMSCs under hypoxia. NO can increase osteogenic differentiation of human periodontal ligament cells through the upregulation of HO-1 (Lee et al. 2009). However, Lin et al. (2010) reported that overexpression of HO-1 by Ad-HO-1 inhibited the maturation and mineralization of rat osteoblasts. This discrepancy may be attributed to different cell type and culture conditions. PI3K signaling is critical in the osteogenesis of MSCs (Baker et al. 2015). PI3K-deficient osteoblasts displayed reduced osteogenesis (Gamez et al. 2016). MAPK signaling also participates in osteogenic differentiation (Schindeler and Little 2006; Wu et al. 2015). Salicylideneamino-2-thiophenol can promote the osteogenic differentiation of MSCs through MAPK pathways (Kim et al. 2012). It has been reported that HO-1 can activate both MAPK and PI3k/Akt pathway (Alvarez-Maqueda et al. 2004; Rojo et al. 2006). It is possible that HO-1 can enhance the osteogenic differentiation of pMSCs under hypoxia through the activation of MAPK or PI3k/Akt pathway.

In the present study, using human placental-derived MSCs, we investigated the effects of hypoxia on the osteogenic differentiation of human pMSCs. Our results demonstrate that hypoxia can increase the osteogenic differentiation of human pMSCs. HO-1 may participate in the osteogenic differentiation of human pMSCs under hypoxia. We believe that our findings will highlight the therapeutic

potential of pMSCs in tissue engineering.

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Conflict of Interest

The authors declare no conflict of interest.

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