

Hypoxia suppresses osteogenesis of bone mesenchymal stem cells via the extracellular signal-regulated 1/2 and p38-mitogen activated protein kinase signaling pathways

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Received September 15, 2016; Accepted May 25, 2017

DOI: 10.3892/mmr.2017.7276

Abstract. There is a growing body of evidence indicating an association between osteoporosis and vascular diseases, which are associated with reduced blood supply. Decreased vascular flow results in a hypoxic gradient in the local microenvironment, affecting local bone remodeling. Bone mesenchymal stem cells (BMSCs) have been demonstrated to be the key to bone remodeling. To elucidate the molecular mechanisms involved in vascular supply and osteoporosis, the present study investigated the effect of hypoxia on BMSCs *in vitro* during osteogenesis. The BMSC osteogenesis process was evaluated by alkaline phosphatase (ALP) activity assay and the mRNA expression of the osteogenic markers runt-related transcription factor 2 (Runx2), ALP and osteocalcin. The function of extracellular signal-regulated kinase (ERK)1/2 and p38 kinase were studied under hypoxia using specific inhibitors. The results demonstrated that hypoxia reduces the osteogenic differentiation of BMSCs by inactivating Runx2, followed by decreased ALP activity and mRNA expression levels of ALP, collagen type I and osteocalcin. Furthermore, these data suggested that the ERK1/2 and p38-mitogen activated protein kinase signaling pathways might participate in hypoxia-induced

differentiation of BMSCs toward the osteogenic phenotype. Compared with ERK1/2, the p38-Runx2 signaling pathway might exert a relatively more prominent effect in the above process. These findings may help to elucidate the pathophysiology of osteoporosis caused by decreased vascular supply.

Introduction

Osteoporosis is a major health problem that affects millions of people around the world, including one in three postmenopausal women. Bone homeostasis requires a balance of activity between bone-resorbing osteoclasts and bone-forming osteoblasts. At menopause, a negative bone balance is established with an imbalance between excessive bone resorption and reduced bone formation, resulting in a loss of bone mass (1). It has been considered that estrogen deficiency is the pathogenic cause of postmenopausal osteoporosis, and treatment by estrogen replacement has positive effects. However, estrogen deficiency may not be the only pathogenic mechanism causing osteoporosis. Looker *et al* (2) demonstrated that bone loss begins immediately after attainment of peak bone mass, which is long before any decline in sex steroid production in women. Increasing evidence has therefore indicated that there are non-estrogen-associated pathogenic mechanisms for osteoporosis.

Recent findings have demonstrated that osteoporosis is associated with a reduced blood supply to the bone (3). It has been reported that the blood supply to the tibial metaphysis of rats is significantly reduced after ovariectomy, indicating that estrogen deficiency-induced osteoporosis is likely to be associated with a reduced blood supply to the bone (4). Decrease and interruption of the vascular flow results in a transient hypoxic gradient in the local microenvironment, and may initiate formation of a hyaline zone and subsequent bone resorption (5). Hypoxia is a prominent component of the microenvironment in ischemic diseases and tissue injuries (6). Alterations in O₂ tension may have important implications for bone remodeling.

Bone mesenchymal stem cells (BMSCs) are pluripotent stromal cells capable of detecting, transducing and responding to an extracellular stimulus (7). It is well known that the

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Key words: hypoxia, bone mesenchymal stem cell, osteogenesis, mitogen activated protein kinase, runt-related transcription factor 2

osteogenic ability of BMSCs is the key to bone remodeling. Not only are they the precursor cells of osteoblasts, but they are also involved in activation and differentiation of osteoclasts (8). Changes in osteogenic competence of BMSCs may contribute to the attenuated regeneration of bone, and osteoporosis is likely to be the consequence (9). However, the effects of hypoxia on osteogenic differentiation of BMSCs and the mechanisms involved are still not fully understood. Certain studies have suggested that under hypoxic conditions, BMSCs are recruited to participate in bone formation (10-11), indicating that hypoxia might be a positive stimulus in bone reconstruction. However, hypoxia may inhibit the growth and differentiation capacity of BMSCs (12).

Mitogen-activated protein kinases (MAPKs) are highly-conserved signaling kinases that respond to a wide range of stresses. They constitute a family of protein kinases with three isoforms: Extracellular-regulated kinase (ERK)1/2, p38 and c-Jun N-terminal kinase (JNK) (13). With regard to bone remodeling, MAPK signaling pathways also regulate bone mass as mediators of osteoblast differentiation. Mice expressing a dominant-negative mitogen activated protein kinase kinase (MEK1) mutant in osteoblasts display low bone mass of the clavicle and calvarium (14). The p38-MAPK pathway exerts its differentiation-promoting effects on osteoblasts at least in part through run-related transcription factor 2 (Runx2) phosphorylation (15). However, the involvement of the signaling MAPK pathway in the response of BMSCs to hypoxia remains to be fully elucidated.

Therefore, the present study aimed to investigate the impact of hypoxia on osteogenic differentiation of BMSCs, and the role of ERK1/2 and p38 MAPK pathways in the process. First, the effects of different periods of hypoxia on the osteogenic potential of BMSCs were investigated. Following this, the effects of hypoxia on ERK1/2 and p38 kinase activities were studied. Specific inhibitors were used to investigate the possible involvement of ERK1/2 and p38 kinase. The results of the present study may provide further experimental evidence to increase understanding of the mechanisms of bone remodeling under hypoxia.

Materials and methods

Harvest and culture of rat BMSCs. Rat BMSCs were isolated from the humeri and tibiae. A total of 12 female Sprague-Dawley rats (age, 6 weeks; weight, 200±10 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and placed in a temperature-(23±3°C) and humidity-(60±5%) controlled room with a 12-h light/dark cycle and with free access to food and water. The tibiae and femurs were dissected from the rats, and the bone marrow was flushed out using Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare, Chicago, IL, USA) supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. To discard the upper fat tissues, the washouts were collected and centrifuged at 500 x g for 10 min at room temperature. The precipitate was subsequently mixed with DMEM supplemented with 10% fetal bovine serum (HyClone; GE Healthcare) and antibiotics, and maintained at 37°C in a humidified incubator with 5% CO₂. Non-adherent cells were removed by changing the culture medium after

72 h. Thereafter, the medium was refreshed every 3 days. On reaching 70-80% confluence, the cells were serially passaged or plated for subsequent experiments. BMSCs at passages 3-5 were used in our experiments. The present study was approved by the ethics committee of Shanghai Ninth People's Hospital Affiliated to Shanghai Jiaotong University (Shanghai, China).

Hypoxia exposure. All hypoxia experiments were performed using a three-gas modular hypoxic incubator (IG750, Jouan, France). Cells were passaged and seeded into six-well plates at 2x10⁴/cm². The following day (designated as day 0) growth medium was replaced with osteogenic medium supplemented with 0.1 mM dexamethasone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 10 mM β-glycerophosphate (Sigma-Aldrich; Merck KGaA) and 50 mM ascorbic acid. After allowing the cells to settle overnight, BMSCs were then subjected to hypoxic conditions from day 1. According to the different O₂ concentrations, the cells were assigned to one of two groups: Normoxia (20% O₂) or the hypoxia (2% O₂). The medium was replaced every 3 days.

Quantification of alkaline phosphatase (ALP) activity. Total protein was extracted from BMSCs cultured under normoxic or hypoxic conditions on days 7 and 10. BMSCs were rinsed twice with pre-cooled phosphate-buffered saline (PBS), detached from the dishes and resuspended in ddH₂O, then lysed by three cycles of freezing and thawing. After lysis, the total protein content of these samples was determined by the Bicinchonic Acid method using a protein assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Finally, absorbance of the mixture at 405 nm was determined in a 96-well plate using a microplate reader. ALP activity was expressed as nmol p-nitrophenol produced/min/mg of total protein. Data are represented as fold changes over control group (day 0) at the respective time points.

ALP staining. After 14 days in culture, BMSCs were analyzed by ALP staining. Cells were rinsed with PBS three times and fixed with 4% paraformaldehyde at room temperature for 10 min. Color development was performed according to the manufacturer's protocol using a BCIP/NBT alkaline phosphatase color development kit (Beyotime Institute of Biotechnology, Jiangsu, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was reverse transcribed from mRNA using a RevertAid™ first strand cDNA synthesis kit (Fermentas, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR reactions were monitored using an ABI 7900HT system using SYBR® premix Ex Taq™ (Takara Bio, Inc., Otsu, Japan). The conditions were as follows: Initial denaturation at 95°C for 10 sec, followed 40 cycles of denaturation for 10 sec at 95°C, annealing for 30 sec at 60°C and a final dissociation stage at 95°C for 5 min. A dissociation curve was constructed to confirm that there was no non-specific amplification. The housekeeping gene β-actin was used as an endogenous control. Data were analyzed using the comparative Cq (2^{-ΔΔCq}) method (16) and expressed as a

fold change relative to the control. The primer sequences used in this study were as follows: β -actin forward, 5'-CACCCGCGAGTACAACCTTC-3' and reverse, 5'-CCCATACCCACCATCACACC-3'; Runx2 forward, 5'-ATCCAGCCACCTTCACTTACACC-3' and reverse, 5'-GGGACCATTGGGAAGTGA TAGG-3'; ALP forward, 5'-TATGTCTGGAACCGCACTGAAC-3' and reverse, 5'-CACTAGCAAGAAGAAGCCTTTGG-3'; collagen type I (COLI) forward, 5'-CAGGCTGGTGTGATGGGATT-3' and reverse, 5'-CCAAGGTCTCCAGGAACACC-3'; osteocalcin (OC) forward, 5'-GCCCTGACTGCA TTCTGCCTCT-3' and reverse, 5'-TCACCACCTTACTGC CCTCTG-3'.

Western blotting. Cells were lysed in ice-cold lysis buffer containing a protease inhibitor. Equal amounts of protein extract (20 μ g) for each sample were separated by 10% SDS-PAGE and electro-transferred onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk and sequentially probed with primary antibodies, followed by incubation with a horseradish peroxidase-linked IgG secondary antibody (1:1,000, Jackson Immuno Research Laboratories Inc., West Grove, PA, USA). The protein bands were visualized using an enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA). The primary antibodies used were as follows: Anti-Runx2 (mouse monoclonal, 1:500, Abcam, Cambridge, UK); anti-ERK1/2 (rabbit monoclonal anti-p44/42 MAPK, 1:500); anti-phosphorylated (p)-ERK1/2 (rabbit monoclonal anti-p-p44/42 MAPK [Thr 202/Tyr 204], 1:500); anti-p38 (rabbit monoclonal, 1:500); anti-p-p38 (rabbit monoclonal; Thr 180/Tyr 182; 1:500, all from Cell Signaling Technology, Boston, MA, USA). Runx2 levels were normalized using anti-GAPDH (1:500, Cell Signaling Technology), and p-ERK1/2 or p38 to the respective total protein levels.

Inhibition of MAPK signaling pathway. In order to assess the role of MAPK signaling pathways in the differentiation of BMSCs under hypoxia, the selective inhibitors U0126 and SB203580 (Cell Signaling Technology) were used. Preliminary experiments showed that the optimal concentrations of U0126 and SB203580 were 10 and 20 μ M, respectively. Cells were pre-incubated with inhibitors for 2 h prior to exposure to hypoxia, and they were present throughout the entire experiment.

Statistical analysis. Each experiment was performed a minimum of three times. All data are expressed as the mean \pm standard deviation. Significant differences were analyzed by two-way factorial analysis of variance followed by a Student-Newman-Keuls post hoc test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of hypoxia on osteogenic differentiation of BMSCs. To determine the role of hypoxia in the differentiation of BMSCs, the expression of the osteogenesis-associated markers ALP, COLI and OC under normoxic and hypoxic conditions were assessed. Compared with the normoxic

group, the expression of all three genes under hypoxic conditions was significantly lower at all time points tested (Fig. 1A). Runx2 mRNA expression levels in the hypoxic group remained at a sustained low level during differentiation (Fig. 1A). Furthermore, Runx2 protein expression levels were also downregulated in the hypoxic group, consistent with its mRNA level, with a significant decrease at day 7 (Fig. 1B). To confirm the early increase in osteogenesis, ALP levels were assessed by activity assay and staining. Consistent with the above results, BMSCs subjected to hypoxia demonstrated lower ALP activity (Fig. 1C), while the ALP staining of BMSCs under hypoxia was markedly decreased in comparison to those under normoxia (Fig. 1D). Taken together, these data suggested that hypoxia results in inhibition of the osteogenic differentiation of BMSCs.

Effects of hypoxia on induction of ERK1/2 and p38-MAPK signaling pathways. The MAPK signaling pathway during osteogenic differentiation of BMSCs was further studied under hypoxic conditions, in comparison to normoxia. As presented in Fig. 2A, following exposure to hypoxia, the phosphorylation level of ERK1/2 in BMSCs was enhanced relatively quickly, being rapidly upregulated to a maximum at day 1. Thereafter it dropped sharply by day 3, but still remained higher than in the normoxic group. By contrast, hypoxia led to a gradual activation of p38, with the phosphorylation level increasing gradually within 3 days of the start of hypoxia (Fig. 2B). These results indicated that both ERK1/2 and p38 were activated by hypoxia.

Effects of ERK1/2 inhibitor on osteogenic differentiation of BMSCs. To address whether the accumulation of ERK1/2 is involved in hypoxia-induced osteogenesis, BMSCs were treated with U0126, a selective inhibitor of ERK1/2 kinase (MEK1/2) under normoxia or hypoxia. Pre-treatment with the specific inhibitor U0126 significantly blocked the hypoxia-induced activation of ERK1/2 (Fig. 3A) but had no cytotoxic effect on the cells, as demonstrated by MTT viability assay (data not shown). Additionally, hypoxia-inhibited Runx2 protein expression levels were increased by U0126, but remained lower than in the normoxic group (Fig. 3B). Furthermore, U0126 moderately reversed the changes in expression of osteogenic-specific genes seen in hypoxic cells, especially at day 7 (Fig. 3C). Likewise, U0126 pretreatment enhanced hypoxia-restrained ALP activity at day 14 (Fig. 3D). These results suggested that the ERK1/2-Runx2 signaling pathway is involved in the transduction of external hypoxic signals.

Effects of p38 inhibitor on osteogenic differentiation of BMSCs. In addition to ERK1/2, the present study observed an induction of p38 during osteogenic differentiation of BMSCs under hypoxia. Thus, whether p38 has any functional effect in this process was investigated by using the specific p38 inhibitor SB203580 to block activation of p38 in BMSCs. Pre-treatment with SB203580 was able to significantly inhibit the hypoxia-induced activation of p38 (Fig. 4A). However, its effect on the expression levels of osteogenesis-associated genes was more obvious (Fig. 4B and C). Changes in Runx2 mRNA and protein expression levels were both significantly

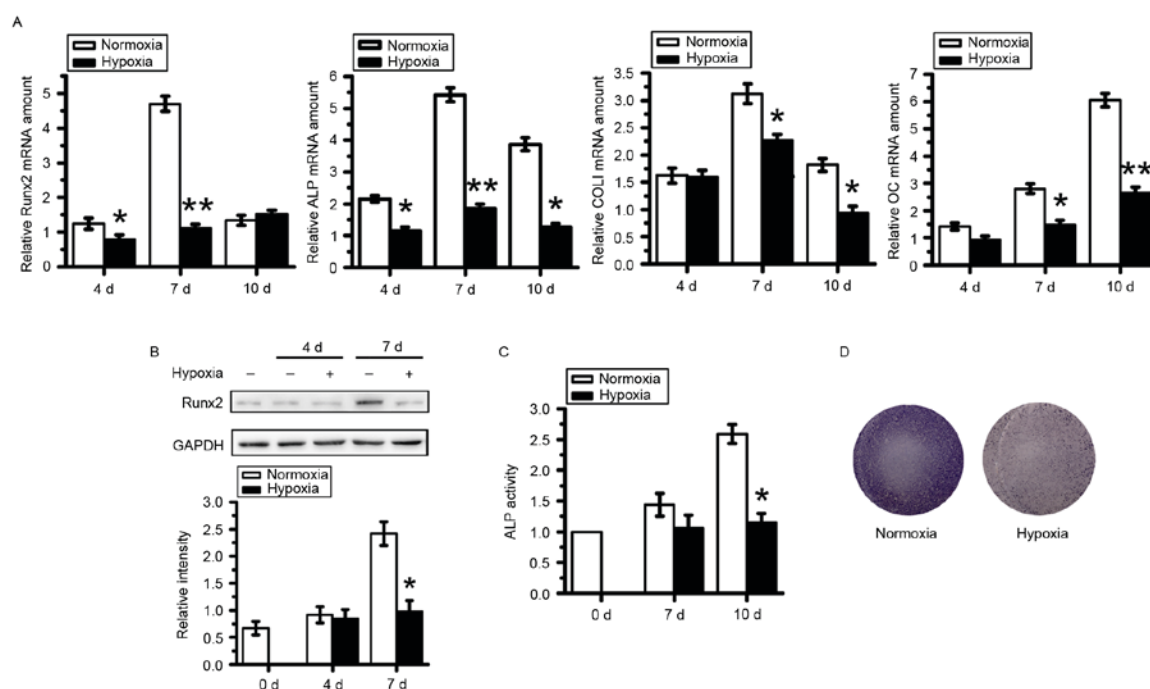


Figure 1. Effects of hypoxia on the osteoblastic differentiation of BMSCs. (A) mRNA expression levels of specific osteoblastic genes at different time points. (B) Protein expression levels of Runx2 under hypoxia. GAPDH served as an internal control. (C) ALP activity of BMSCs at days 4 and 7. (D) ALP staining at day 14. Data are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. normoxia group at the same time point. BMSCs, bone mesenchymal stem cells; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; COL1, collagen type I; OC, osteocalcin.

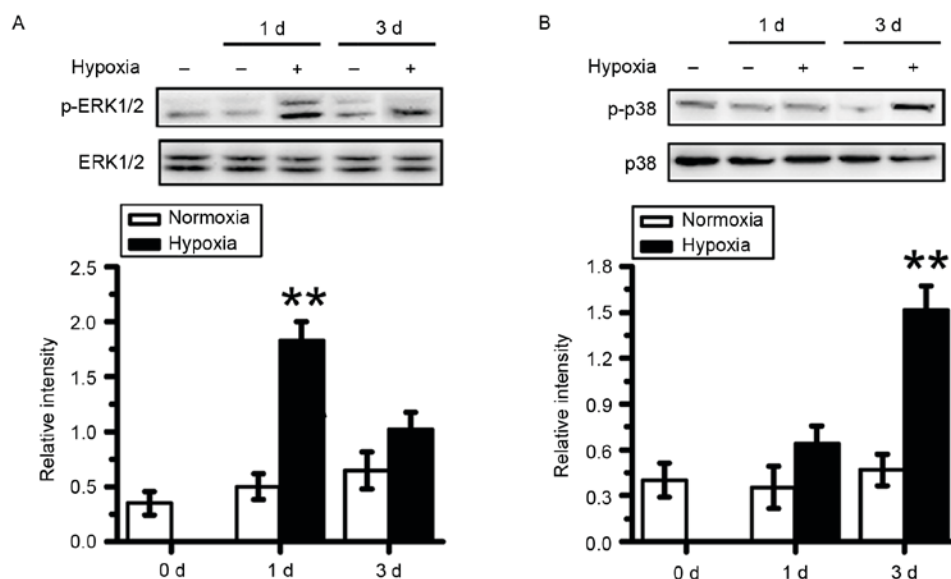


Figure 2. Effects of hypoxia on induction of the mitogen activated protein kinase signaling pathway. Representative western blot images and quantification of phosphorylation of (A) ERK1/2 and (B) p38. Data are expressed as the mean \pm standard deviation of three independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. normoxia group at the same time point. ERK, extracellular signal-regulated kinase; p, phosphorylated.

reversed in the presence of inhibitor, compared with the hypoxic group. ALP staining was also performed, and the results demonstrated that SB203580 significantly augmented the hypoxia-inhibited ALP activation (Fig. 4D). Taken together, these data demonstrated that, similar to ERK1/2, the reduction in osteogenic ability of BMSCs caused by hypoxia can be increased by treatment with a p38 inhibitor. These findings suggested that the p38-Runx2 signaling pathway also participates in the hypoxic regulation of osteogenesis;

however, compared with ERK1/2, it might exert a relatively more prominent effect.

Discussion

In recent years, it has become increasingly clear that O_2 is not only an obviously important substrate, but it is also a regulatory signal that controls expression of a specific genetic program. A change of O_2 tension, or hypoxia, might activate

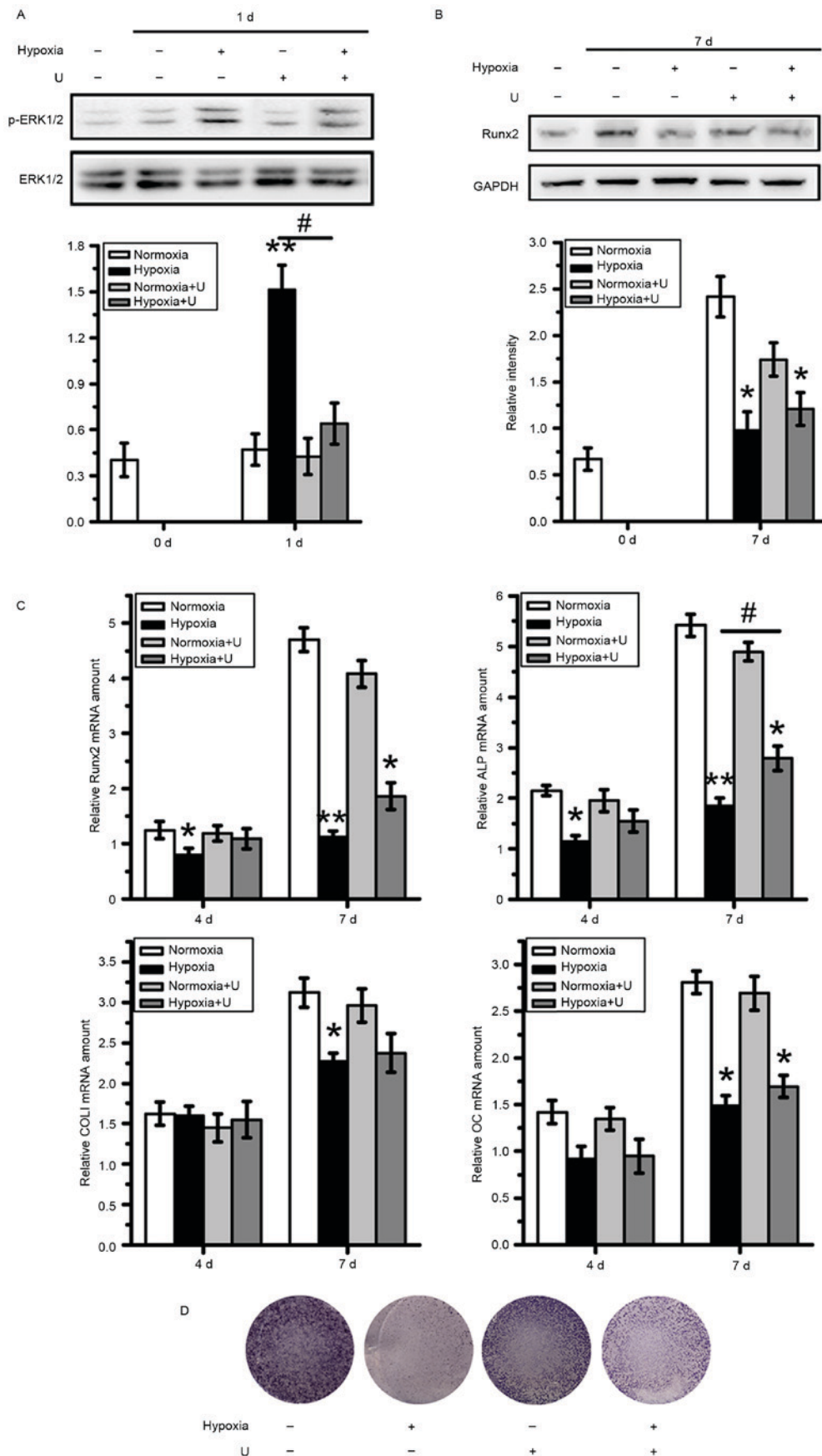


Figure 3. Involvement of the ERK-mitogen activated protein kinase signaling pathway in hypoxia-induced osteoblastic differentiation of BMSCs. Representative western blot images and quantification of protein expression levels of (A) ERK1/2 and (B) Runx2 following treatment with U0126. (C) mRNA expression levels of osteogenesis-associated genes in BMSCs at days 4 and 7 following U0126 treatment. (D) Alkaline phosphatase staining at day 14. Data are expressed as the mean \pm standard deviation of three independent experiments (n=3). *P<0.05, **P<0.01 vs. normoxia group at the same time point; #P<0.05 vs. hypoxia group at the same time point. ERK, extracellular signal-regulated kinase; p, phosphorylated; BMSCs, bone mesenchymal stem cells; Runx2, runt-related transcription factor 2; COL1, collagen type I; OC, osteocalcin; U, U0126.

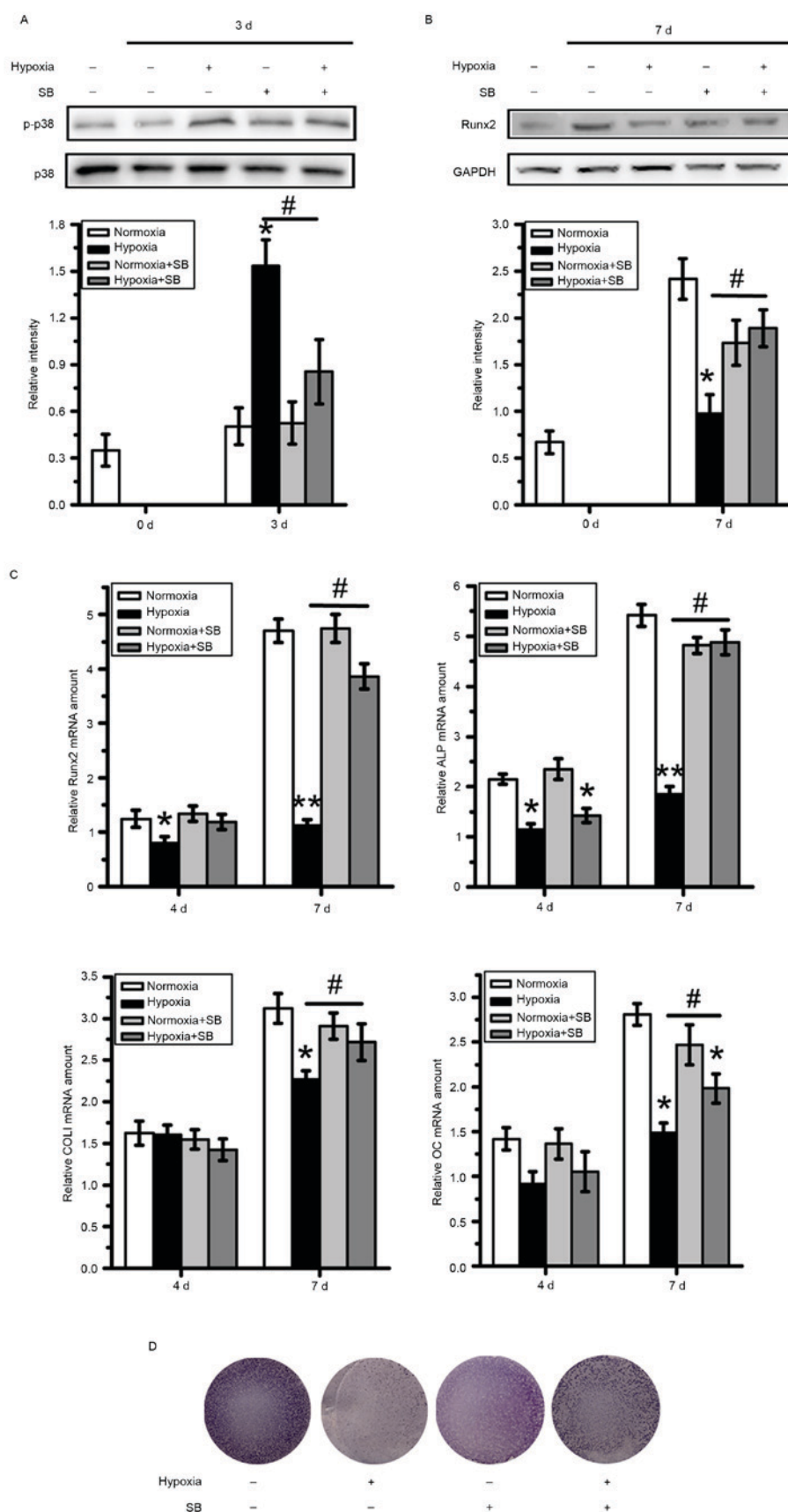


Figure 4. Effects of p38-mitogen activated protein kinase signaling pathway on osteogenic differentiation of BMSCs following application of hypoxia. Representative western blot images and quantification of protein expression levels of (A) p-p38 and (B) Runx2 following treatment with SB203580. (C) mRNA expression levels of osteoblastic genes in BMSCs at days 4 and 7 following SB203580 treatment. (D) ALP staining at day 14. Data are expressed as the mean \pm standard deviation of three independent experiments. * P <0.05, ** P <0.01 vs. normoxia group at the same time point; # P <0.05 vs. hypoxia group at the same time point. p, phosphorylated; BMSCs, bone mesenchymal stem cells; Runx2, runt-related transcription factor 2; COL1, collagen type I; OC, osteocalcin; SB, SB203580; ALP, alkaline phosphatase.

a transcriptional response that has an important role both in normal development and in pathological conditions, such as ischemia and osteoporosis (17). It has been documented that hypoxia may be a mechanism underlying not only osteoporosis due to decreased vascular supply but also senile osteoporosis, as blood flow in long bones and bone marrow declines with age (18). Vasodilator therapy using nitroglycerin has been demonstrated to be as effective as standard estrogen replacement therapy in prevention of oophorectomy-induced bone loss (19).

Although previous studies have demonstrated that hypoxia can be a potential or powerful trigger of bone remodeling, it is difficult to clarify the role of hypoxia on osteogenic differentiation of BMSCs. Previous studies have reported that hypoxia exhibits no or even negative effects on the osteogenic differentiation of BMSCs (20-21). In contrast, other findings indicated that hypoxia was beneficial in stimulating the osteogenic response of BMSCs (22). The differences in reported effects of hypoxia on the cellular behaviors of BMSCs may be due to discrepancies in O_2 concentration and exposure time between studies. Hung *et al* (23) identified lower cell numbers when human MSCs were exposed to 1% O_2 for short periods, while Grayson *et al* (24) reported that human MSCs also exhibit increased proliferation under hypoxia (2% O_2). Long-term or chronic exposure of MSCs to hypoxia was reported to enhance proliferation and inhibit osteogenic differentiation (25). It is notable that the concentrations of O_2 in these reports vary but are all quite low (<2% O_2). In contrast, Lennon *et al* (22) demonstrated that 5-20% O_2 could promote the osteogenic responses of BMSCs. These data may infer that a critical concentration (2%) of O_2 may exist. Consequently, in the present study, a 2% O_2 tension was adopted for the hypoxia group to investigate its influences on the osteogenic potency of BMSCs *in vitro*.

Osteogenic differentiation is controlled by a hierarchy of sequentially-expressed transcription factors, and Runx2 has been demonstrated to be the principal osteogenic transcription factor in regulating osteogenic differentiation (26-27). Heterozygous mutations in the Runx2 gene are responsible for the inherited human disease cleidocranial dysplasia (28). Furthermore, expression of Runx2 can induce the synthesis of ALP, COLI (early markers of osteoblast differentiation) and OC (late marker of osteoblast differentiation) (29), and is controlled by various extracellular signaling pathways, including MAPKs (30). To investigate whether hypoxia could induce BMSCs to adopt an osteogenic phenotype, the present study assessed the expression levels of Runx2, ALP, COLI and OC under hypoxic conditions. The present study provided evidence that, compared with the normoxic group, the mRNA and protein expression levels of Runx2 in the hypoxic group remained at a sustained low level throughout the process. Furthermore, the application of hypoxia significantly decreased ALP, COLI and OC expression levels, in line with the change in Runx2 expression. The inhibition of osteogenic differentiation was further confirmed by ALP staining. All these data demonstrated that osteoblastic differentiation of BMSCs was delayed under hypoxia.

As a family of serine and threonine kinases that serve important roles in signal transduction in response to external changes (31), the MAPKs have been extensively demonstrated

to be involved in the regulation of cell proliferation, growth and differentiation (32). ERK activation induces the osteogenic differentiation of MSCs via Runx2 phosphorylation and p38 MAPK, and is responsible for responses to environmental stimuli such as oxidative stresses (33). In the present study, both ERK1/2 and p38 were revealed to be phosphorylated upon stimulation by hypoxia. The phosphorylation level of p38 was enhanced gradually up to day 3 under hypoxic conditions. Similar results were observed for the protein of p-ERK1/2, which sharply increased to reach its maximum level at day 1. After confirming the activation of ERK1/2 and p38, specific inhibitors of these two key pathways were introduced. The results demonstrated that pre-treatment with U0126 or SB203580 could significantly reverse the hypoxia-inhibited gene expression of osteogenic markers. Furthermore, the most notable result of this study was the finding that inhibition of the p38 signaling pathway increased the osteogenic potential of BMSCs suppressed by hypoxia to a greater extent than ERK1/2 inhibition. Therefore, compared with ERK1/2, p38-Runx2 may be the dominant signaling pathway in the hypoxia-induced inhibition of cellular osteogenic responses.

In conclusion, the present study demonstrated that exposure of BMSCs to hypoxia significantly reduces the osteogenic responses of BMSCs. Furthermore, activation of the ERK1/2 and p38-MAPK signaling pathways was involved in hypoxia-mediated osteogenesis, and this regulation may be mediated by the activation and expression of the transcriptional factor Runx2. Upregulation of ERK1/2 and p38-MAPK signaling cascades, which transfer MAPK into the nucleus, could inhibit the activation of Runx2 and subsequently the osteogenic regulatory genes of ALP, COLI and OC. These results suggested that, compared with ERK1/2, the p38-Runx2 signaling pathway may exert a relatively more prominent effect in the above process. These findings may offer a theoretical basis to further understand the mechanisms of hypoxia-induced osteogenesis in bone remodeling.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81371121, 11342005, 30901698, 10972142 and 81570950), the Collaborative Foundation of Medical and Engineering Science of Shanghai Jiaotong University (grant no. YG2016QN16), Shanghai Sailing Program (grant no. 16YF1406800) and the 'Chen Xing' project from Shanghai Jiaotong University and Shanghai Summit & Plateau Disciplines.

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