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Hypoxia Inhibits the Spontaneous Calcification of Bone Marrow-Derived Mesenchymal Stem Cells

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are the popular seed cells for regenerative medicine, and there has been a rapid increase in the number of BM-MSC-based clinical trials. However, the safety of these cells should also be closely studied. In this study, spontaneous calcification of BM-MSCs from rats was evaluated in normoxia (20% O_2) without osteogenic medium after continuous culture for 21 days; obvious mineralized nodules were observed, which were positive for Alizarin Red, collagen-I (Col-I), osteocalcin (OC) and alkaline phosphatase (ALP), and mainly consisted of C, O and Ca elements. Interestingly, hypoxia (2% O_2) significantly inhibited this spontaneous calcification. In addition, the ALP and calcium content of rBM-MSCs were sharply reduced. Based on RT-PCR results, the expression of osteogenic genes (Cbfa1/Runx2, Col-I, ALP, and OC) was reduced compared to that in normoxia. These results demonstrate a natural and unique characterization of rat BM-MSCs in normoxia after continuous culture and highlight the inhibiting effects of hypoxia. Finally, this study contributes to the information regarding the application of BM-MSCs in the regeneration of various tissues. J. Cell. Biochem. 113: 1407–1415, 2012.

KEY WORDS: BONE MARROW; MESENCHYMAL STEM CELLS; SPONTANEOUS CALCIFICATION; NORMOXIA; HYPOXIA

Research on mesenchymal stem cells (MSCs) has provided new and exciting opportunities for regenerative medicine. MSCs were initially isolated from bone marrow [Pittenger et al., 1999]. Although subsequent studies have identified their presence in other tissues [Zvaifler et al., 2000; Zuk et al., 2002; Shih et al., 2005; Parolini et al., 2008], bone marrow-derived MSCs (BM-MSCs) are still the popular candidate stem cells for regenerative medicine. Furthermore, there has been a rapid increase in BM-MSC-based animal experiments and clinical trials with encouraging results and few deleterious effects [Toma et al., 2002; Dezawa et al., 2008; Nöth et al., 2008; Uccelli et al., 2008; Husein and Chris, 2010].

However, largely because of the natural characteristics and possible transformation of BM-MSCs, their use could be associated with two major risks: tumorigenesis [Zhu et al., 2006; Hall et al., 2007; Karnoub et al., 2007] and spontaneous calcification or ossification [Yoon et al., 2004; Breitbach et al., 2007]. The former issue remains controversial and is poorly understood, but spontaneous calcification has been clearly demonstrated in vivo. First, in a rat model, Yoon and colleagues showed that direct transplantation of unselected bone marrow cells into acutely infarcted myocardium induced significant intramyocardial calcification and demonstrated that the calcified area was surrounded by exogenous bone marrow cells [Yoon et al., 2004]. Subsequently, this research was further developed by Breitbach and colleagues who found that 51.2% of their animal population suffered severe intramyocardial calcification, which originated from BM-MSCs

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[Breitbach et al., 2007]. In addition, after ectopic transplantation, most BM-MSC pellets mineralized and underwent endochondral ossification with matrix calcification when induced by chondrogenic medium for 3–7 weeks in vitro [Choi et al., 2010]. Furthermore, compared to tendon stem cells, BM-MSCs formed bone rather than tendon-like tissue [Bi et al., 2007]. These phenomena may be due to a set of osteogenesis-related genes that are abundantly expressed in BM-MSCs [Panepucci et al., 2004]. Taken together, these data demonstrate that BM-MSCs retain a tendency for spontaneous calcification in vivo; this calcification may benefit bone regeneration but could led to adverse events in other tissues, such as cartilage, tendons, the heart and blood vessels [Price and Birdwell, 1978; Rostand et al., 1988; Yang et al., 1996; Giachelli, 2005]. Nevertheless, to date, no study has been designed to determine the inducers of spontaneous calcification or to investigate approaches that inhibit this process in vitro.

In this study, a continuous culture without osteogenic medium was used to build a spontaneous calcification model for BM-MSCs, and we hypothesized that hypoxia would be effective at suppressing this calcification because of the following reasons: (1) the average physiological oxygen tension is approximately 4–7%, and the BM-MSC niche is a hypoxic (2–8%) environment [Packer and Fuehr, 1977; Kofoed et al., 1985]; (2) BM-MSCs proliferated faster, exhibited greater colony-forming ability and maintained their stemness better in hypoxia (<3% O_2) compared to normoxia (20% O_2) [Grayson et al., 2007; Volkmer et al., 2009; Dos Santos et al., 2010]; and (3) exposure of BM-MSCs to hypoxia resulted in a negative impact on their osteogenic differentiation when cultured in traditional osteogenic medium [D'Ippolito et al., 2006; Potier et al., 2007]. The goal of our study was to define the spontaneous calcification of rat BM-MSCs in normoxia and to evaluate the effects of hypoxia on this particular characteristic in vitro.

MATERIALS AND METHODS

CELL ISOLATION AND CULTIVATION

Ten Sprague–Dawley rats were obtained from the West China Medical Center Laboratorial Animal Center of Sichuan University and used in this study. All experimental procedures were approved by Laboratory Animal Management Committee of Sichuan Province (Permit No.: SYXK-2008-119). Bone marrow-derived MSCs were isolated as our previous description [Zhou et al., 2010]. Briefly, bone marrow cells were obtained from the femurs and tibias by flushing with 5 ml complete medium (DMEM-HG with 10% FBS and 1% penicillin/streptomycin). The released cells were collected in a 25-cm^2 culture flask, and incubated at 37°C with 5% CO_2. After reaching 80–90% confluence, the cells were collected using 0.25% Trypsin/EDTA treatment at a dilution of 1:3. The surface antigens and differentiation potential of the cells were well characterized to ensure that they were BM-MSCs. Cells from the 4th passage were used in the subsequent assays.

IN VITRO MODEL OF SPONTANEOUS CALCIFICATION

Spontaneous calcification of rBM-MSCs was established with a continuous culture method in normoxia in the complete medium (DMEM-HG with 10% FBS and 1% penicillin/streptomycin) without traditional osteogenic medium. BM-MSCs were plated in 6-well plates at an initial cell density of 10,000 cells/cm^2. They were then incubated with complete medium at 37°C with 5% CO_2/95% air; the complete medium was changed every 3 days. Spontaneous calcification was assessed on days 3, 7, 14, and 21 using relevant experiments.

EXPERIMENTAL HYPOXIC CONDITIONS

After incubation in complete medium and normoxia for 24 h, the cells were cultured in hypoxia and processed in parallel to cells that were cultured in normoxia for comparison at the following time points: 3, 7, 14, and 21 days. The hypoxia system was generated in hypoxia incubator chambers (Thermo Fisher Scientific, USA) with humidified gas mixtures of 2% O_2, 5% CO_2, and 93% N_2.

OBSERVATION OF CALCIFICATION AND ALIZARIN RED STAINING

The calcification of rBM-MSCs cultured in normoxia and in hypoxia without osteogenic medium was observed under a light microscope on days 3, 7, 14, and 21. Then, the cells were fixed with 75% alcohol at room temperature (RT) and incubated with an Alizarin Red solution for 30 min at 37°C. The mineralized nodules were photographed.

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Slides containing rBM-MSCs from the two groups at the various time points were fixed with cold acetone for 30 min at RT and incubated with blocking solution for 30 min at 37°C. Subsequently, incubation with the primary antibodies, anti-Col-I and anti-OC (1:100), was performed overnight at 4°C. The next day, the cells were rinsed with PBS and incubated at 37°C for 40 min in the dark with FITC or TRIC conjugated secondary antibodies (1:50). In addition, the nuclei were stained with 5 μg/ml DAPI. Microscopic analysis was performed using laser confocal microscopy.

ALP STAINING AND ASSAY

A Gomori modified calcium-cobalt method was used to stain for alkaline phosphatase (ALP) expression in BM-MSCs that were cultured in normoxia and hypoxia for 3, 7, 14, and 21 days. Cells that were positive for ALP were stained brown or tan. BM-MSCs cultures from the two groups ($n = 6$ from each group) and at the various time points were lysed with three freeze-thaw cycles in distilled H_2O. Then, they were stored at −80°C until the assays could be performed on all of the samples. ALP activity was assessed using p-nitrophenyl phosphate as a substrate according to the manufacturer's instructions (Millipore, USA) and was read on a plate reader (Patents, USA) at 405 nm. ALP activity was normalized to the total protein content which was quantified with a BCA Protein Assay Kit (Keygen, China).

SCANNING ELECTRON MICROSCOPY (SEM) AND ENERGY DISPERSIVE X-RAY (EDX) SPECTROMETRY ANALYSIS

Fixed slides from the two groups at the various time points were dehydrated in a graded ethanol series; they were then air-dried, gold sputtered and viewed with a SEM (FEI INSPECT F, USA) operating at 20 kV. The elemental composition of the calcification areas on the slides was analyzed with EDX (Oxford INCA PentaFET X3, UK) running INCA-point & ID software.

CALCIUM CONTENT QUANTIFICATION

After fixation in 75% ethanol for 20 min and washing three times in distilled H_2O, the representative slides of the two groups (n = 6 from each group) were stored dry at 4°C. When all samples (n = 48) were collected, the calcium in each sample was extracted by shaking overnight (rpm = 50) with 1 ml of 0.6 N HCl. Afterward, the supernatants were used for calcium detection using an OCPC (o-cresolphthalein complexone) method according to the manufacturer's instructions (Roche, USA).

RNA ISOLATION AND RT-PCR

Total mRNA from the rBM-MSCs cultured in normoxia and hypoxia (n = 6 from each group) in the absence of osteogenic medium on days 3, 7, 14, and 21 was extracted by adding 1 ml TRIzol reagent (TAKARA, Japan). Then, 1 μg of RNA was used for cDNA synthesis with oligo dT and random 6-mer primers in a 20-μl reaction volume according to the manufacturer's protocols. Subsequently, the SYBR® Premix Ex Taq™ || kit (TAKARA, Japan) was then used with the cDNA samples in a iCycler iQ™ Multicolor real-time PCR Detection System (Bio-Rad). Amplification was conducted in a 25-μl final volume consisting of 2 μl of cDNA, 12.5 μl of SYBR® Premix Ex Taq™, 8.5 μl of sterile water, 1 μl of forward primer, and 1 μl of reverse primer as in our previous description [Zhou et al., 2010]. PCR conditions included initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 30 s. Finally, Optical System Software, version 3.1, was used to analyze the fluorescence data to obtain cycle threshold values, and all mRNA expression data were normalized to endogenous GAPDH mRNA. The relative expression (Cbfa1/Runx2, Col-I, ALP, and OC) was then calculated using the $2^{-\Delta\Delta CT}$ method. Thus, all data were expressed as the fold-change from the hypoxia group, which equaled 1.

STATISTICAL ANALYSIS

All values were presented as mean ± SD from three independent experiments. The Student's t-test was used to compare numeric data between the normoxia and hypoxia rBM-MSCs cultures at the various time points. One-way ANOVA was used to compare numeric data among the four time points, and the difference between groups

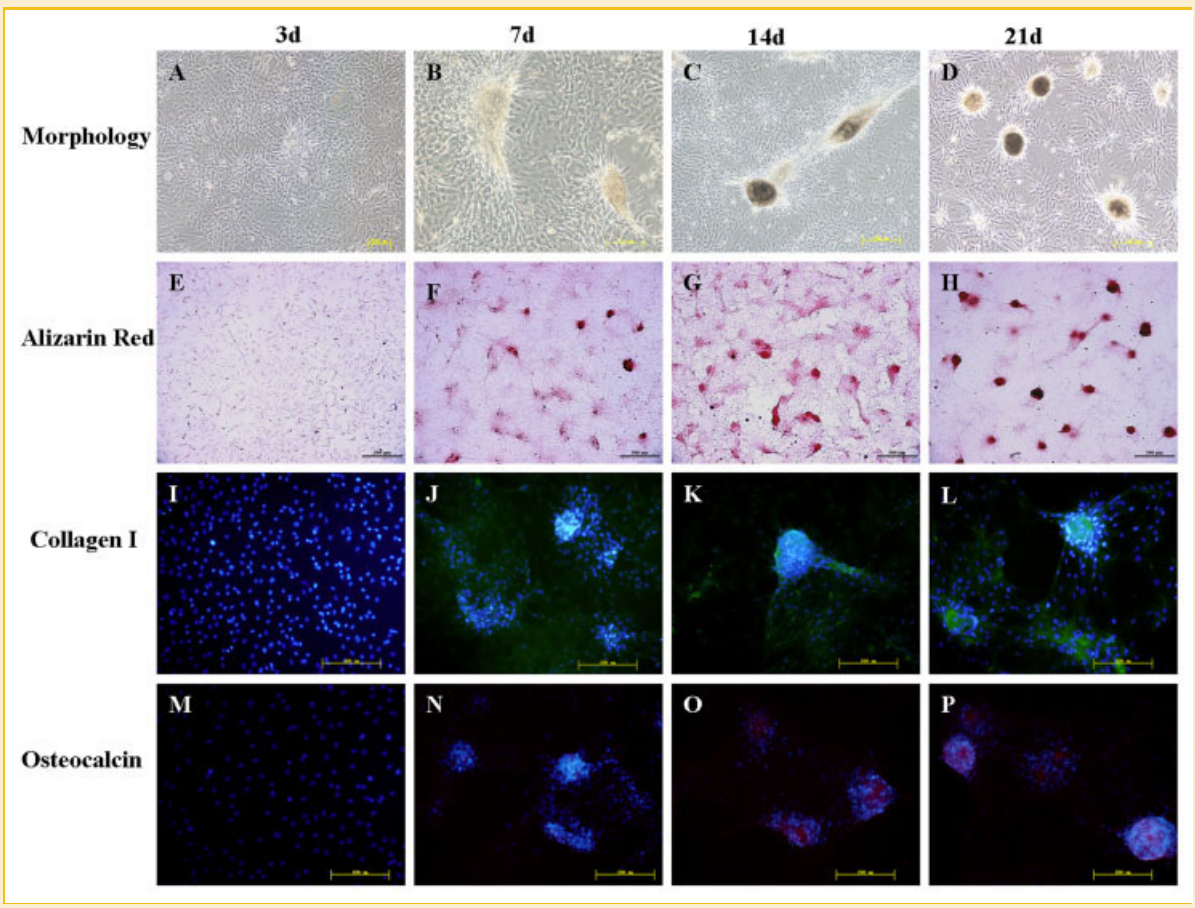

Fig. 1. Spontaneous calcification of rBM-MSCs in normoxia. A–D: Phase contrast images of rBM-MSCs on days 3, 7, 14, and 21. Scale bar = 200 μm. E–H: Alizarin Red staining was positive in the mineralized nodules of rBM-MSCs after a 7-day culture. Scale bar = 500 μm. I–L: Fluorescence micrographs showed immunostaining of Collagen I, which was labeled with FITC (green), and the nuclei were stained with DAPI (blue). Scale bar = 200 μm. M–P: Fluorescence micrographs showed immunostaining against osteocalcin, which was labeled with TRIC (red), and the nuclei were stained with DAPI (blue). Scale bar = 200 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

was determined with additional analysis with the LSD-Test. Statistical significance was accepted when $P < 0.05$.

RESULTS

rBM-MSCs CHARACTERIZATION

During the first 2 days, the cells grew slowly, but only displayed a fibroblast-like morphology and developed into visible symmetric colonies after 5 days of culture. Flow cytometry analysis revealed that these cells were uniformly positive for CD29, CD44H, CD54, CD73, and CD90, but negative for the hematopoietic surface markers CD31, CD34, and CD45. Furthermore, at the end of osteogenic and adipogenic induction, the cells were positive for Alizarin Red and Oil Red O, respectively [Zhou et al., 2010].

SPONTANEOUS CALCIFICATION OF rBM-MSCs

rBM-MSCs showed spindles with slim, fibroblast-like bodies that proliferated well in complete medium over 3 days and were negative for Alizarin Red, Col-I, and OC as shown in Figure 1A, E, I, and M. However, the cells aggregated and were weakly positive for Alizarin Red and Col-I after 7 days of culture (Fig. 1B, F, J, and N). After a 14-day continuous culture, obvious cellular aggregation and typical mineralization were observed; the cells were brightly positive for Alizarin Red, Col-I, and OC (Fig. 1C, G, K, and O). Furthermore, more mineralized nodules and significant aggregation were illustrated in Figure 1D, and rBM-MSCs displayed significant Alizarin Red, Col-I, and OC positivity after 21 days of continuous exposure to normoxia (Fig. 1H, L, and P). Thus, these data confirm that spontaneous calcification occurs when rBM-MSCs are cultured for long periods in normoxia.

HYPOXIA INHIBITS THE SPONTANEOUS CALCIFICATION OF rBM-MSCs

The rBM-MSCs did not exhibit spontaneous calcification in hypoxia at all time points, as observed through the expression of Alizarin Red, Col-I, and OC (Fig. 2). As illustrated, rBM-MSCs seldom aggregated or formed typical mineralized nodules, and they still maintained a fibroblast-like morphology (Fig. 2A–D). Furthermore, they were negative for Alizarin Red, Col-I, and OC at all time points based on histological analysis (Fig. 2E–P). These data clearly indicate that hypoxia negatively affects the spontaneous calcification of rBM-MSCs in this experimental design.

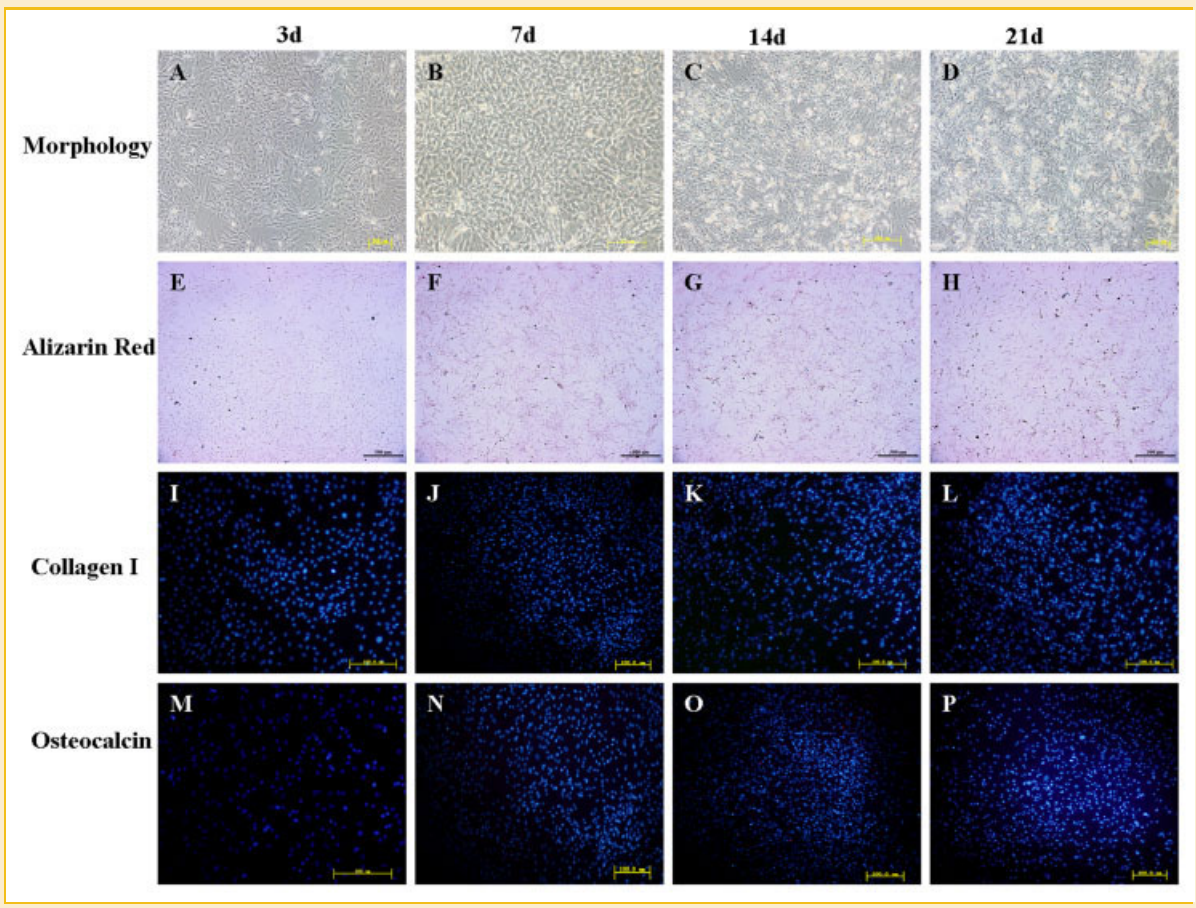

Fig. 2. Hypoxia inhibits the spontaneous calcification of rBM-MSCs. A–D: Phase contrast images of rBM-MSCs cultured in hypoxia on days 3, 7, 14, and 21. Scale bar = 200 μm. E–H: Alizarin Red staining was negative on rBM-MSCs at all time points. Scale bar = 500 μm. I–L: Fluorescence micrographs showed immunostaining for Col-I, rBM-MSCs at all time points; all were negative for Col-I. Scale bar = 200 μm. M–P: Fluorescence micrographs showed immunostaining for OC in rBM-MSCs at all time points; all showed negative for OC. Scale bar = 200 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

HYPOXIA REDUCES ALP EXPRESSION IN rBM-MSCs

In addition, qualitative and quantitative assays for ALP expression in rBM-MSCs in hypoxia and normoxia were performed. By day 3, rBM-MSCs in normoxia stained positive for ALP as evidenced by small brown or tan areas clearly seen in the cytoplasm of the cells. Similarly, this positive staining was also observed on day 7. From day 14, the positive area was clearly observed encompassing the mineralized nodules (Fig. 3A). However, almost no staining occurred in the hypoxic groups; slight ALP positivity was observed on days 3 and 7. After day 14, the rBM-MSCs in the hypoxia group were negative for ALP (Fig. 3A). As shown in Figure 3B, compared to air, low oxygen dramatically inhibited the total protein content at most of the time points (days 7, 14, and 21; $P < 0.01$). This may reflect the difference in cell number. Normalized to the total protein, ALP activity of rBM-MSCs grown in air was significantly higher than that of cells cultured in hypoxia at all of the evaluated time points (3 days: 1.8-fold; 7 days: 9.0-fold; 14 days: 1.6-fold; 21 days: 1.9-fold). Furthermore, the ALP activity was highest on day 7 of normoxia ($P < 0.01$). Therefore, hypoxia inhibits ALP expression in rBM-MSCs for 21 days.

HYPOXIA DECREASES THE CALCIUM CONTENT OF rBM-MSCs

Consistent with our previous description, the SEM results presented in Figure 4A showed that after continuous culture for 7, 14, and 21 days, rBM-MSCs spontaneously calcified in normoxia, whereas hypoxia substantially inhibited the appearance of spontaneous calcification. And the mineralized nodules of rBM-MSCs cultured in normoxia for 21 days could be macroscopically observed, while no nodule formation was found in the hypoxic group (Fig. 4B). As shown in Figure 4B, a significant amount of aggregation and mineralized nodules could be observed when rBM-MSCs were cultured in normoxia for 21 days, and SEM also revealed that the typical nodules, which were raised from the culture surface, were between 80 and 100 μm in diameter (Fig. 4A). EDX analysis showed strong peaks of Ca, C, and O in mineralized nodules (Fig. 4C–E), indicating that the spontaneous calcification of rBM-MSCs occurred after continuous culture. In addition, a significant decrease in the calcium content of rBM-MSCs was observed when the cells were stimulated with hypoxia for 14 or 21 days ($P < 0.01$). The Ca content of the cells cultured in normoxia for 14 and 21 days were 24.04 ± 2.64 and 24.08 ± 1.50 μg, respectively, whereas the Ca content of the cells cultured in hypoxic conditions for 14 and 21 days were 19.12 ± 0.94 and 21.56 ± 1.11 μg, respectively (Fig. 4F). These results determine that the mineralized nodules mainly consisted of Ca, and hypoxia suppressed the total calcium content thereby preventing the spontaneous calcification of rBM-MSCs after continuous culture.

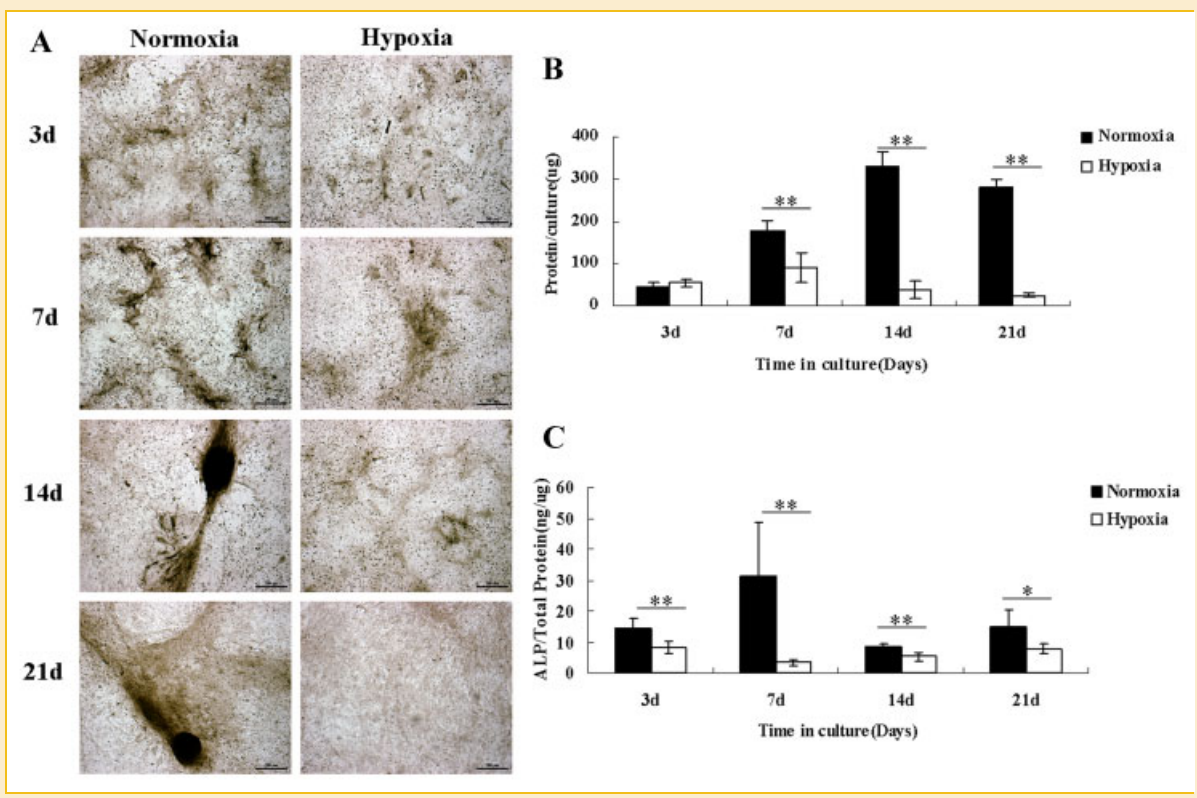

Fig. 3. Hypoxia reduces alkaline phosphatase (ALP) expression. A: ALP expressed by rBM-MSCs in hypoxia and in normoxia over 21 days was assessed. Scale bar = 200 μm. B: Total protein content of rBM-MSCs on days 3, 7, 14, and 21 in hypoxia and normoxia ($^{**}P < 0.01$, rBM-MSCs in normoxia vs. hypoxia). C: ALP expression was increased in rBM-MSCs (normalized to total protein content) when they were cultured in normoxia compared to hypoxia ($^{**}P < 0.01$, rBM-MSCs in normoxia vs. hypoxia; $^{*}P < 0.05$, rBM-MSCs in normoxia vs. hypoxia). All values are presented as mean ± SD. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

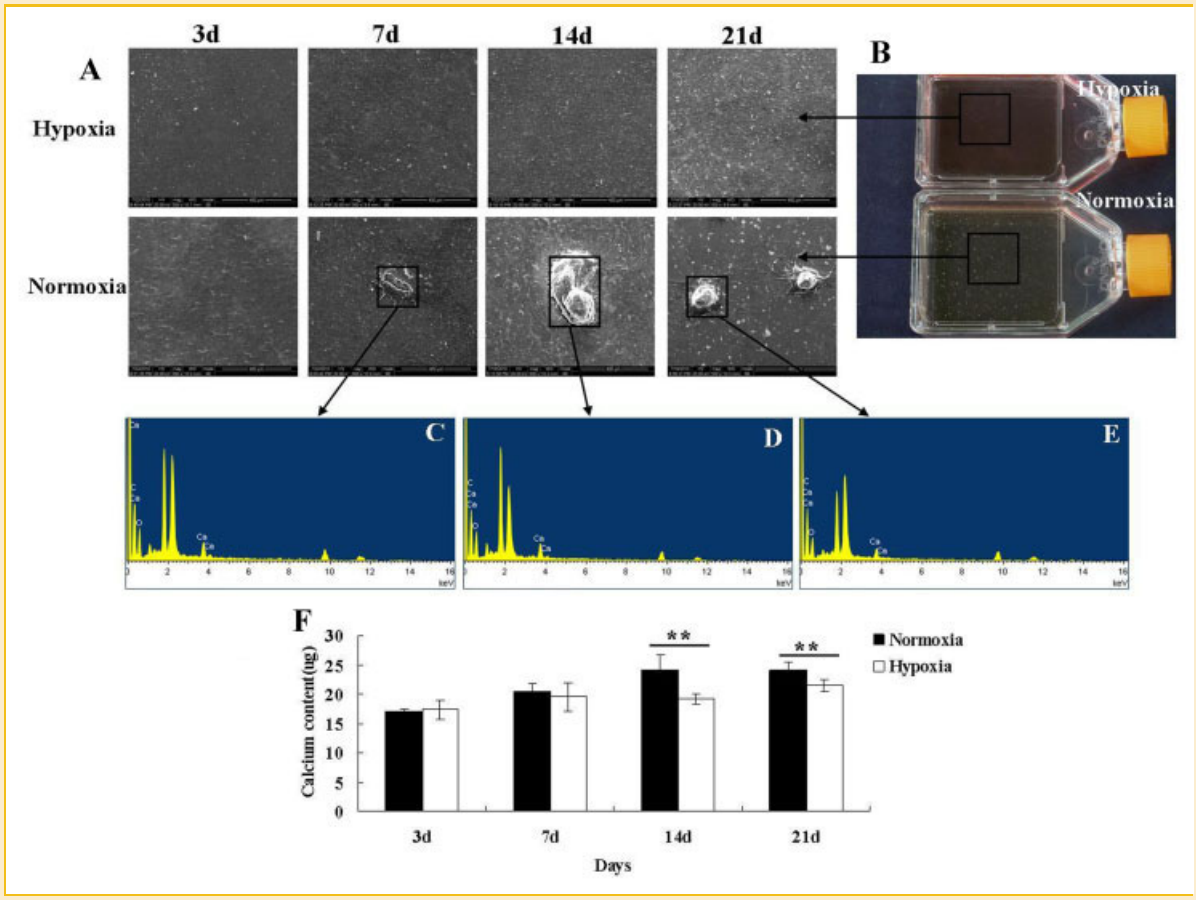

Fig. 4. Characterization of mineralization and calcium deposition analysis. A: SEM images of rBM-MSCs in normoxia and hypoxia demonstrated that no mineralized nodule was found in the hypoxia-cultured cells on days 3, 7, 14, and 21. Scale bar = 400 μm. B: Significant aggregation and mineralized nodules were observed when rBM-MSCs were cultured in normoxia for 21 days, while no mineralized nodule was found in the hypoxic group. C–E: EDX analysis of the elemental components revealed that the mineralized nodules formed on days 7, 14, and 21 and consisted of C, O, and Ca. F: Analysis of the calcium deposition showed higher calcium deposition in normoxia-cultured cells compared to hypoxia-cultured cells from days 14 to 21 ($^{**}P < 0.01$, rBM-MSCs in normoxia vs. hypoxia). All values are presented as mean ± SD. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

GENES EXPRESSION OF rBM-MSCs IN HYPOXIA VERSUS NORMOXIA

Finally, we extracted the RNA after 3, 7, 14, and 21 days and performed RT-PCR for Cbfa1/Runx2, Col-I, ALP, and OC. A decrease in Cbfa1, Col-I, ALP, and OC expression was observed when the rBM-MSCs were cultured in hypoxia for 3 days (Fig. 5A; $P < 0.05$). Sequentially, inhibition of Col-I expression (an 8.41 ± 3.16-fold change) was detected as early as 7 days after treatment ($P < 0.01$), but hypoxia did not reduce the expression of Cbfa1, ALP, or OC ($P > 0.05$; Fig. 5B). On day 14, the related gene expression levels of rBM-MSCs in normoxia were all greater than those in hypoxia, and the differences were statistically significant ($P < 0.01$; Fig. 5C). Specifically, Cbfa1 had a 7.70 ± 2.74-fold enhancement, Col-I had a 22.45 ± 2.92-fold enhancement, ALP had a 12.65 ± 0.78-fold enhancement and OC had a 6.39 ± 1.88-fold enhancement. On the last test day, similar gene expression results were obtained for both culture conditions. Indeed, higher values of rBM-MSC gene expression in normoxic conditions ($P < 0.01$) compared to hypoxic conditions (Cbfa1: 6.67-fold; Col-I: 5.82-fold; ALP: 21.49-fold; and OC: 5.21-fold change) were observed (Fig. 5D). To summarize, these data showed that hypoxia dramatically inhibits osteogenesis-related genes expression to inhibit the spontaneous calcification of rBM-MSCs.

DISCUSSION

Lack of any definitive marker to identify this heterogeneous population of stem cells has hindered the progress of BM-MSC research. To address this problem, the International Society for Cellular Therapy has proposed a set of criteria for defining human BM-MSCs [Dominici et al., 2006], and these minimum standards have been widely accepted. In the current study, after a 14-day continuous culture without traditional osteogenic stimulation, rat BM-MSCs spontaneously calcified, and all of the mineralized nodules stained positive for Alizarin Red, Col-I, OC, and ALP as confirmed with immunohistochemistry. The cells also expressed osteogenic related genes (Cbfa1, Col-I, ALP, and OC) as confirmed with RT-PCR, and calcified nodules consisted mainly of C, O, and Ca

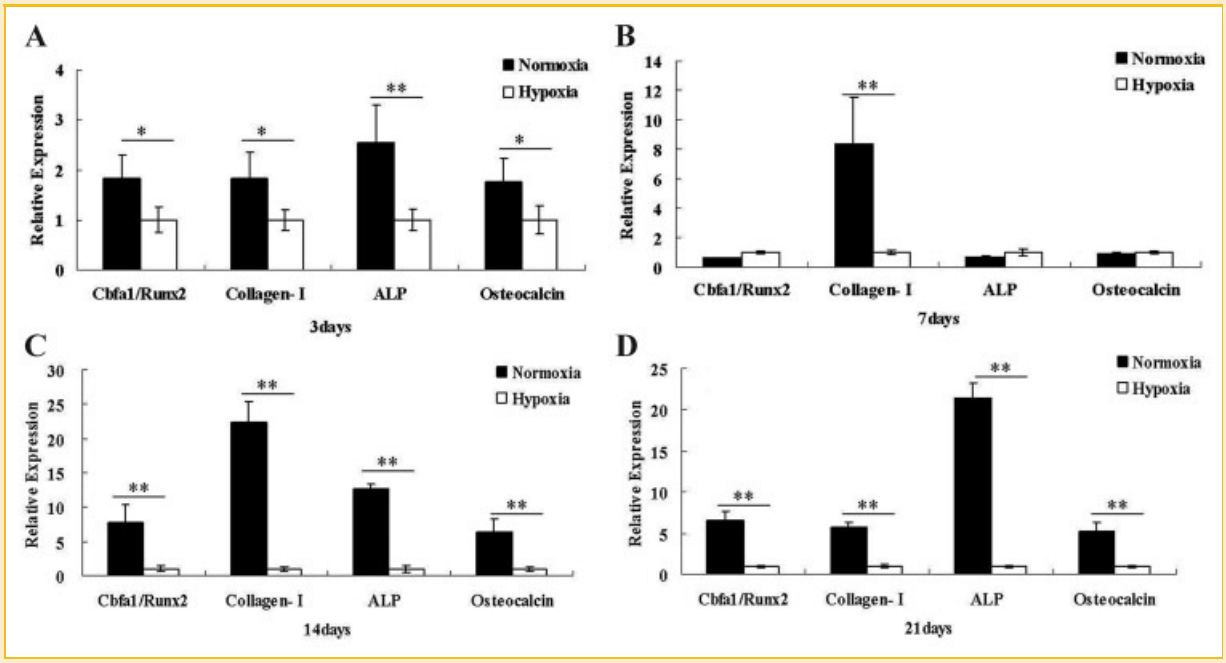

Fig. 5. Osteogenic genes expression of rBM-MSCs in normoxic and hypoxic conditions. Quantitative analysis of Cbfa1, Col-I, ALP, and OC expression in rBM-MSCs cultured in normoxia versus hypoxia was conducted with RT-PCT on days 3 (A), 7 (B), 14 (C), and 21 (D). All values are presented as mean ± SD ($^{**}P < 0.01$, rBM-MSCs in normoxia vs. hypoxia; $^{*}P < 0.05$, rBM-MSCs in normoxia vs. hypoxia).

as confirmed with EDX. This is, to our knowledge, the first report that shows this specific and natural characterization of rBM-MSCs in vitro. Consistent with the minimal criteria, this spontaneous calcification may become a new feature for the identification of BM-MSCs in vitro, but further investigation is required to detect the spontaneous calcification in BM-MSCs derived from humans and various animal species. In effect, it has been reported that rodent BM-MSCs are different from human BM-MSCs in the synthesis of extracellular matrix and differentiation potential [Phinney and Prockop, 2007; Prockop, 2009]. Hence, for clinical relevance, it is extremely important to further identify whether human BM-MSCs reveal spontaneous calcification in this culture system. In addition, a comprehensive comparison of MSCs derived from other tissues should also be conducted [De Bari et al., 2001; Zuk et al., 2002; Shih et al., 2005; Parolini et al., 2008; Huang et al., 2009].

This study showed that hypoxia significantly inhibited spontaneous calcification, and all of these qualitative findings were consistent with previous reports that hypoxia suppressed the osteogenic differentiation of BM-MSCs [D'Ippolito et al., 2006; Potier et al., 2007]. Additionally, hypoxia also decreased the ALP content per total protein unit at all time points and suppressed the total calcium quantity on days 14 and 21, further demonstrating that low pO_2 is an effective negative regulator of rBM-MSC calcification. A significant down-regulation of Cbfa1/Runx2 gene expression occurred after rBM-MSC exposure to hypoxia for 3, 14, and 21 days. Cbfa1/Runx2 plays an essential role in dominating the osteogenic differentiation of BM-MSCs and its inhibition is associated with a decrease in the rate of ossification [Toshihisa, 2003]. In this study, the inhibition of gene expression of Cbfa1/Runx2 was recorded in the hypoxic BM-MSCs which may be the inducer for the inhibiting of spontaneous calcification. Similar long-lasting inhibition of Col-I, which is the main component of bone matrix, plays a central role in BM-MSCs mineralization [Young, 2003]. The inhibition of gene expression persisted for 21 days after the end of the hypoxia. ALP expression levels were strongly reduced by hypoxia on days 3, 14, and 21, basically consistent with the results of the ALP content measurements. In addition, the inhibition of OC, a late osteogenic differentiation marker of BM-MSCs, also confirmed that the inhibition of spontaneous calcification can be attributed to hypoxia. On day 7, the inhibition of Cbfa1/Runx2, ALP, and OC expression was not detected; this may due to the balance between rBM-MSC proliferation and differentiation in normoxia; this finding requires further investigation. All of the above data provide evidence that hypoxia is an effective method of inhibiting spontaneous calcification in vitro.

The possible mechanism is that BM-MSCs maintained better stemness in hypoxia. For example, Tsai et al. examined hypoxia and the senescence and stem cell properties of human MSCs. They demonstrated that MSCs maintained better stemness in hypoxia through the down-regulation of E2A-p21 by HIF-1α-TWIST and these expanded BM-MSCs showed normal telomerase activity, karyotyping and intact genetic integrity, and did not form a tumor [Tsai et al., 2011]. Similar conclusion has been documented by other groups [Grayson et al., 2006, 2007; Volkmer et al., 2009]. However, the detailed mechanism of inhibiting effect of hypoxia on this spontaneous calcification required further investigation, which is the mainly limitation we should acknowledge. The HIF-1α pathway centrally regulates of the adaptive response of cells to hypoxia and involves in the skeletal development [Semenza, 2007]. In mice model, it has been reported that HIF-1α activation in osteoblasts had

significantly increased angiogenesis and bone formation [Wan et al., 2008]. This improvement was also found in the HIF-1α transduced rat BM-MSCs with higher level of angiogenic and osteogenic gene expression [Zou et al., 2011]. Similar result was documented in rabbit BM-MSCs [Huang et al., 2011]. However, the opposite result was observed in human BM-MSCs which showed that HIF-1α was implicated in the down-regulation of osteogenesis by Betacellulin [Genetos et al., 2010]. This finding was consistent with the previous description [Potier et al., 2007]. Hence, both the positive and negative roles of HIF-1α in the osteogenesis of BM-MSCs have been documented; this difference may be due to the species-specific regulation of HIF-1α. Therefore, as a central regulator of cells to low oxygen, the role of HIF-1α pathway is in the priority order for mechanism study of this inhibition; nevertheless, the species-specific difference should also be seriously considered.

Spontaneous calcification of rBM-MSCs shared some features with traditional osteogenic differentiation. First, rBM-MSCs aggregated and developed into dense mineralized nodules. All nodules stained positive for Alizarin Red, Col-I, OC, and ALP; this process is similar to the formation of traditional mineralized nodules in BM-MSCs induced by osteogenic medium [Gentleman et al., 2009]. Despite these consistencies, using EDX spectroscopy, we identified C, O, and Ca peaks in the spectra of the mineralized nodules from the continuous rBM-MSCs cultures. However, a P peak, which is a key element of bone, was not observed on the surface of the nodules. In bone formation, the calcium and phosphonium are the major components of hydroxyapatite [LeGeros, 2008]. On the basis of the EDX data, we suggest that the existence of calcium on the surface of the mineralized nodules after continuous culture without traditional osteogenic medium should be inorganic substance, which may be the major difference with the mineralized nodules formed by BM-MSCs induced by osteogenic medium [Gentleman et al., 2009]. Standard biological techniques, such as morphological evaluation and immunohistochemistry, provided limited information to distinguish between the mineralized nodules formed by spontaneous calcification and those formed by traditional osteogenic differentiation; therefore, a more systematic and materials-based analytical approach (such as micro-Raman spectroscopy) is urgently required in further studies.

In summary, spontaneous calcification of rat BM-MSCs was observed, and their biological performance was similar to that of traditional osteogenic differentiation. However, this spontaneous calcification was significantly inhibited by hypoxia. Taken together, this study illustrates the dramatic influence of hypoxia and supports its feasibility for use as a negative regulator of spontaneous calcification in BM-MSCs. Understanding the natural and unique characterization of BM-MSCs and their inhibition by hypoxia provides useful evidence for their safe and effective application.

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