Cell Proliferation

Cell Prolif., 2015, **48**, 650–660 doi: 10.1111/cpr.12218

Effects of heterodimeric bone morphogenetic protein-2/7 on osteogenesis of human adipose-derived stem cells

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Received 22 April 2015; revision accepted 14 July 2015

Abstract

Objective: Roles of bone morphogenetic proteins (BMPs) on osteogenesis of human adipose-derived stem cells (hASCs) remain ambiguous. In this study, we evaluated *in vitro* and *in vivo* functional characteristics of BMPs of different dimerization types, with the aim of determining osteoinductive efficiency of heterodimeric BMP-2/7 on osteogenesis of hASCs.

Materials and methods: We explored osteoinductive effects of three different BMPs by using cell DNA assay, alkaline phosphatase (ALP) activity assay, alizarin red staining and mineralization assay, and real-time PCR, *in vitro*. Also, we examined ectopic bone formation in nude mice by using soft X-ray, histomorphometric and immunohistochemical analyses *in vivo*.

Results: In our dose–response study, we showed that BMPs with both dimerization types did not induce *in vitro* osteogenesis of hASCs without osteogenic medium (OM). In the presence of OM, BMPs significantly enhanced hASC osteogenesis in a dose-dependent manner. In *in vivo* experiments, our analyses indicated that BMPs promoted osteogenesis of hASCs without *in vitro* osteogenic induction. However, both *in vitro* and *in vivo*, there were no significant differences in hASC osteogenic induction between heterodimeric and homodimeric BMPs.

Conclusions: Heterodimeric BMP-2/7 significantly promoted osteogenesis of hASCs *in vitro* and *in vivo*. However, BMP-2/7 was not found to be a stronger inducer of osteogenesis compared to homodimeric either BMP-2 or BMP-7.

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Introduction

Compared to bone marrow-derived stem cells (BMSCs), human adipose-derived stem cells (hASCs) show promising potential as a cell source for bone tissue engineering, due to their easy accessibility, high yield efficiency and low donor-site morbidity (1,2).

Bone morphogenetic proteins (BMPs) have long been introduced into the field of bone tissue engineering. In particular, BMP-2 and BMP-7 have been approved for clinical use in the United States, Europe and Australia (3). It is, therefore, conceivable to be able to apply exogenous BMPs to promote osteogenesis of hASCs. However, direct effects of exogenous BMPs on hASC osteogenic differentiation are still controversial. Several isotypes of BMP have been demonstrated to play paramount roles on hASC osteogenesis (4–8), whereas other studies have shown that exogenous BMP-2 was unable to influence osteogenic fate of hASCs *in vitro* (9) or *in vivo* (10). It seems as if ability of exogenous BMPs to promote osteogenic differentiation of hASCs is highly dependent on numerous factors, such as BMP type, concentration, differentiation medium and administration time point (2). Consequently, tremendous amounts of investigation is still needed to modify application pattern of BMPs to facilitate their optimal promoting effects on hASC osteogenesis.

One such effort is to adopt different BMPs. Generally, most mature BMP molecules are composed of two monomers linked by a disulphide bridge. When they are derived

from the same BMP member, the BMP molecule is termed homodimeric BMP (11). Hitherto, almost all our knowledge in this aspect has mainly been based on homodimeric BMPs. In contrast, heterodimeric BMPs consist of two monomers that are derived from different BMP members (12). Application potential of heterodimeric BMPs is promising, as they exhibit higher efficiency at inducing osteogenesis of murine cells (13–21). Yet, up to now there have been no reports related to any biofunction of BMP-2/7 on human mesenchymal stem cells.

In the present study, we have aimed to unveil functions of heterodimeric BMP-2/7 on hASC osteogenic differentiation, by comparing inducing efficiency of heterodimeric BMP-2/7 with homodimeric BMP-2 and BMP-7 *in vitro and in vivo*.

Materials and methods

All materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and antibiotics were purchased from Gibco (Grand Island, NY, USA).

Ethics statement

hASCs from three different healthy human donors were purchased from ScienCell (7510; ScienCell, San Diego, CA, USA). The protocol used was approved by the Ethics Committee of the Peking University Health Science Center, Beijing, China (Permit Number: LA2014233). All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize animal suffering.

Cell culture

hASCs were cultured in proliferation medium (PM) containing fresh DMEM + 10% (v/v) FBS + antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37 °C, in an incubator, atmosphere of 95% air, 5% CO_2 and 100% relative humidity.

BMP concentration selection assay

To prepare stock BMP solutions, three different lyophilized BMPs were reconstituted at 10 μg/ml in sterile 4 mM hydrochloric acid (HCL) containing 0.1% bovine serum albumin (BSA) according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA). To assess hASC osteogenic differentiation in response to the variety of concentrations of different exogenous BMPs (5, 50, 100 and 200 ng/ml) and to identify optimal BMP concentration, dose–response assays of alkaline phosphatase (ALP) activities were performed as described in "ALP activity assay" and "Real-time quantitative PCR analysis" sections below. Administration of exogenous BMPs was in the presence of PM or osteogenic medium (OM) containing PM + 100 nM dexamethasone (DEX) + 0.2 mM ascorbic acid + 10 mM β-glycerophosphate (β-GP).

Proliferation and osteogenic differentiation of hASCs stimulated by BMPs in vitro

Experimental design. Fourth passage cells were used for the following experiments, and all experiments were repeated three times using hASCs from the three donors. hASCs were seeded into 12-well plates at a density of 5×10^4, and were seeded into six-well plates at a density of 10^5 for the studies below. Each group was performed in triplicate. Based on results of "BMP concentration selection assay", OM + 200 ng/ml BMPs were selected as experimental groups for *in vitro* experiments. hASCs were exposed to the following treatments: (i) negative control medium (PM); (ii) positive control medium (OM); (iii) OM with 200 ng/ml BMP-2; (iv) OM with 200 ng/ml BMP-7; (v) OM with 200 ng/ml BMP-2/7.

Cell proliferation assay. The hASCs were seeded in 12-well plates and divided into five groups as above. To investigate their proliferation in response to different BMPs, numbers of cells were calculated by content of cell DNA after stimulation for 1, 4 and 7 days according to the manufacturer's protocol (CyQUANT Cell Proliferation Assay Kit, Life Technologies, Carlsbad, CA, USA).

Cell differentiation assay. The hASCs were seeded in 12-well plates and divided into five groups as above. ALP staining was performed on 4th and 7th days of osteoinduction as described in detail previously (22). After 2, 4, 7 and 14 days induction, ALP activity was determined using an ALP kit according to the manufacturer's protocol, and normalized to total protein content, as described previously (22). To assess mineralization, alizarin red staining was performed on days 21 and 28 after stimulation. Cells were washed three times in phosphate-buffered saline (PBS), fixed in ethanol for 30 min, and then stained with alizarin red at room temperature. To quantitatively determine matrix calcification, alizarin red was destained using 10% cetylpyridinium chloride in 10 mM sodium phosphate for 30 min. Absorbance of released alizarin red was measured at 562 nm. Final mineralization levels in each group were normalized to total protein concentrations obtained from duplicate plates (22).

Table 1. Sequences of primers used for real-time PCR

Genes	Forward primer	Reverse primer
RUNX2	ACTACCAGCCACCGAGACCA	ACTGCTTGCAGCCTTAAATGACTCT
ALP	ATGGGATGGGTGTCTCCACA	CCACGAAGGGGAACTTGTC
COL-1A1	GTGCCAAGGGTCTGACTGGAA	ATCACACCAGCCTGACCACG
OPN	ACCCTGACCCATCTCAGAAGCA	CTTGGAAGGGTCTGTGGGGCTA
OC	AGCCACCGAGACACCATGAGA	GGCTGCACCTTTGCTGGACT
GAPDH	AAGGTCGGAGTCAACGGATTTG	TCCTGGAAGATGGTGATGGGAT

Real-time quantitative PCR analysis. hASCs were seeded in six-well plates and divided into five groups as above. After 2, 4, 7 and 14 days osteoinduction, total RNA was isolated according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and 2 μg RNA aliquots were reverse-transcribed according to the manufacturer's instructions (Roche, Basel, Switzerland). Real-time quantitative PCR assays were performed using Power SYBR Green PCR Master Mix and an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Primers for Runt-related transcription factor 2 (*RUNX2*), *ALP*, collagen 1A1 (*COL-1A1*), osteopontin (*OPN*) and osteocalcin (*OC*) were synthesized by Invitrogen

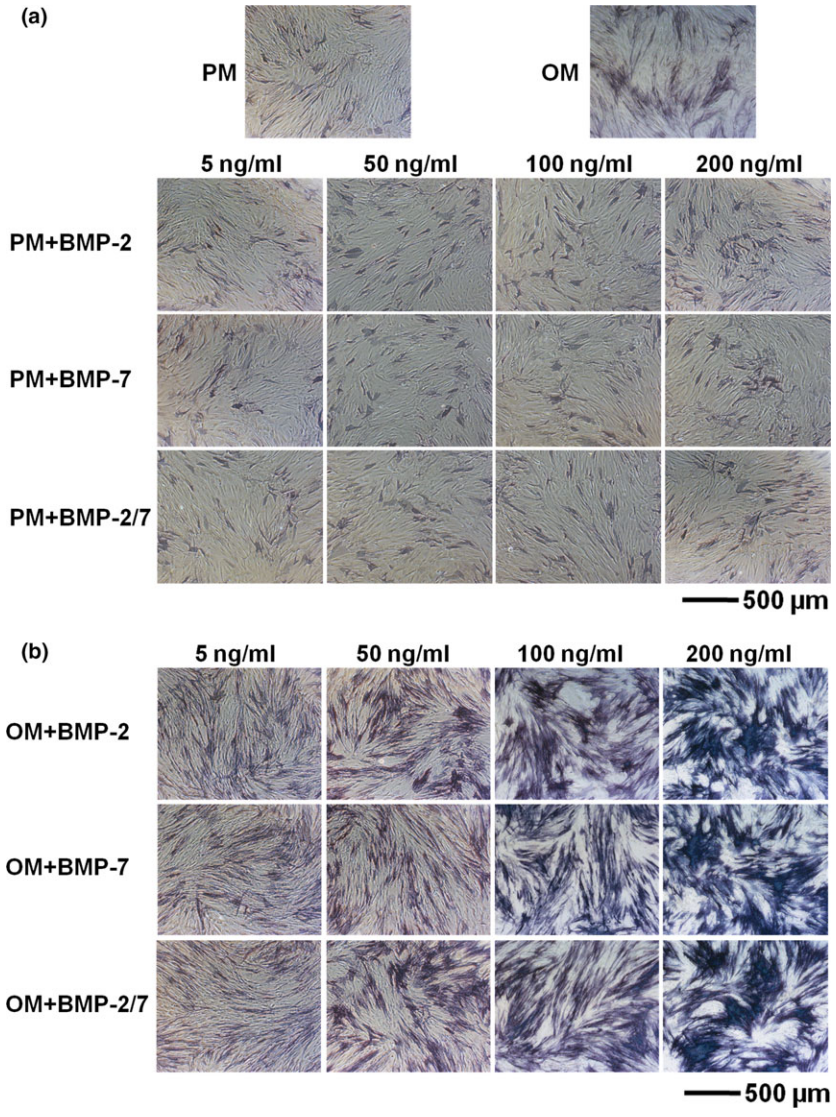


Fig. 1. ALP staining of hASCs induced by BMPs in presence of either PM or OM. (a) hASCs induced by BMPs in presence of PM did not express ALP. (b) hASCs induced by BMPs in the presence of OM stained ALP positively by 7 days of osteoinduction. However, at the same concentration level, there were no obvious differences between the three BMPs. hASCs, human adipose-derived stem cells; ALP, alkaline phosphatase; PM, proliferation medium; OM, osteogenic medium; BMP, bone morphogenetic protein.

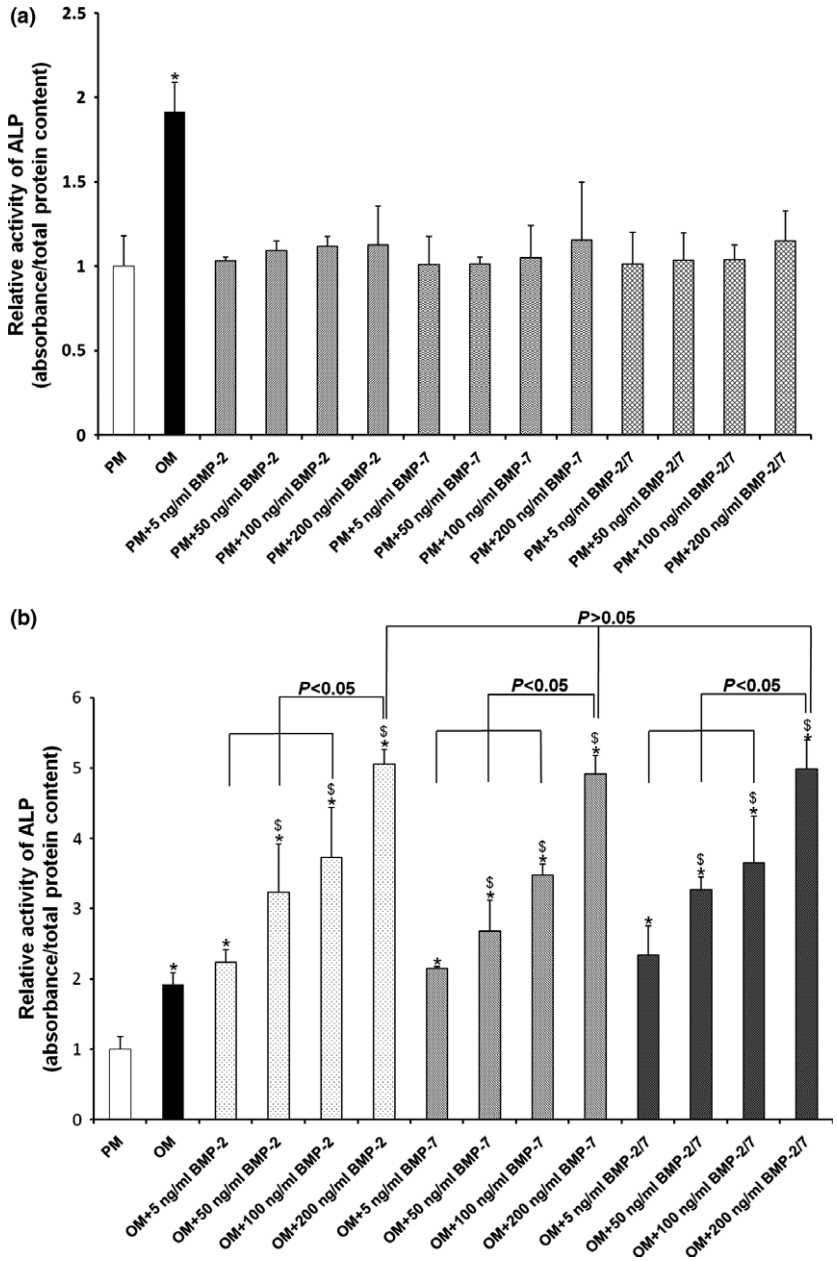


Fig. 2. ALP activities in hASCs induced by BMPs in presence of either PM or OM. (a) BMPs were unable to promote ALP activity in hASCs in the presence of PM. (b) BMPs significantly promoted hASC ALP activity in the presence of OM after 7 days osteoinduction. Moreover, the osteoinductive effect positively correlated to concentrations of BMPs. However, at the same concentration level, there were no significant differences between the three BMPs. $^{*}P < 0.05$ compared to PM, $^{\$}P < 0.05$ compared to OM. hASCs, human adipose-derived stem cells; ALP, alkaline phosphatase; PM, proliferation medium; OM, osteogenic medium; BMP, bone morphogenetic protein.

and are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as internal standard (23).

Subcutaneous transplantation in nude mice

Preparation of β-tricalcium phosphate (β-TCP) scaffolds containing BMPs. β-TCP (Bicon, Boston, MA, USA) was used for the scaffold. Based on the *in vitro* results, we demonstrated that BMP-2/7 exhibited osteoinductive effects similar to BMP-2, whereas inducing efficiency of BMP-7 was slightly weaker. Thus, the β-TCP + BMP-2 + hASCs complex was used as positive control group for *in vivo* experiments. Four groups were used to conduct the *in vivo* study: (I) β-TCP only (blank control); (II) β-TCP + hASCs; (III) β-TCP + BMP-2 + hASCs; (IV) β-TCP + BMP-2/7 + hASCs. For groups III and IV, 500 μl BMP-2-containing or BMP-2/7-containing suspension was absorbed on to β-TCP (40 mg) respectively. According to our previous experiments (24), final loading of BMPs was approximately 400 ng per β-TCP scaffold. The hASCs were cultured in PM for 1 week before the *in vivo* study, when they (4×10^5 cells) were resuspended directly into PM and then seeded on the β-TCP scaffold.

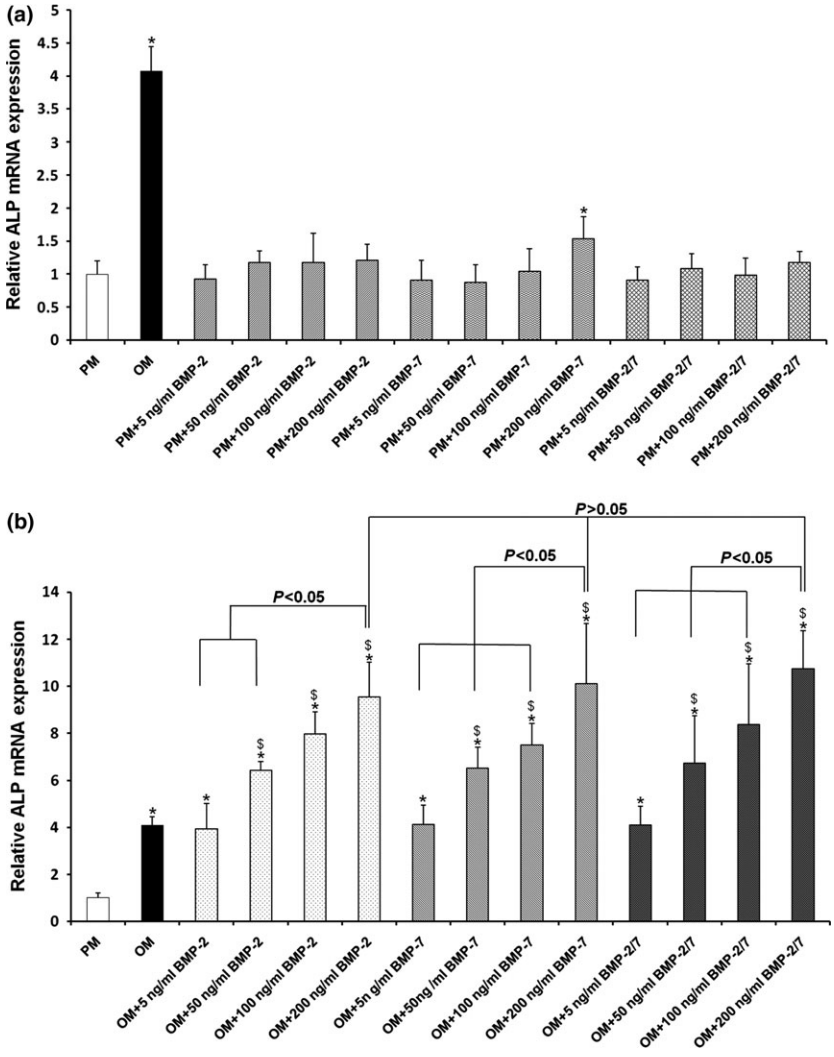


Fig. 3. *ALP* gene expression in hASCs induced by BMPs in presence of either PM or OM. (a) BMPs were unable to elevate *ALP* gene expression in hASCs in the presence of PM. (b) BMPs significantly elevated *ALP* gene expression in hASCs in the presence of OM after 7 days osteoinduction. Furthermore, inducing efficiency positively correlated with concentrations of BMPs. However, at the same concentration level, there were no significant differences between the three BMPs. $^{*}P < 0.05$ compared to PM, $^{\$}P < 0.05$ compared to OM. hASCs, human adipose-derived stem cells; ALP, alkaline phosphatase; PM, proliferation medium; OM, osteogenic medium; BMP, bone morphogenetic protein.

Animal experiments. 6-week-old male BALB/c homozygous nude (nu/nu) mice were purchased from Peking University Experimental Animal Center, and given 1 week to acclimatise to the housing facility. During housing, animals were monitored twice daily for health status during which no adverse events were observed. At the beginning of the *in vivo* experiment, the nude mice were anaesthetized with sodium pentobarbital. To avoid contamination from one group to another, four respective enclosed transplantation sites were prepared by means of haemostatic forceps in dorsal subcutaneous spaces. Subsequently, the four groups of β-TCP complexes were implanted aseptically into the four different sites ($n = 6$ per group).

Analyses of bone formation in vivo. As described in our previous study (25), specimens of each group were harvested 4 weeks after *in vivo* implantation, and animals in each group were sacrificed by CO_2 asphyxiation. Soft X-ray examinations were used to evaluate mineral density and relative grey scale was determined by Image J software (National Institutes of Health, Bethesda, MD, USA). Bone constructs were fixed in 4% paraformaldehyde and then decalcified for 10 days in 10% EDTA (pH 7.4). Following decalcification, specimens were dehydrated and subsequently embedded in paraffin wax. 5 μm tissue sections were stained with haematoxylin and eosin (HE) and Masson's trichrome. Osteogenesis was evaluated by immunohistochemical analysis for OC (primary antibody purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Statistical analysis

Data were analysed using SPSS software (Chicago, IL, USA) and statistical analysis of was performed by one-way analysis of variance (ANOVA). *Post hoc* testing for multiple comparisons was carried out using the Fisher LSD test. When variance was not homogeneous,

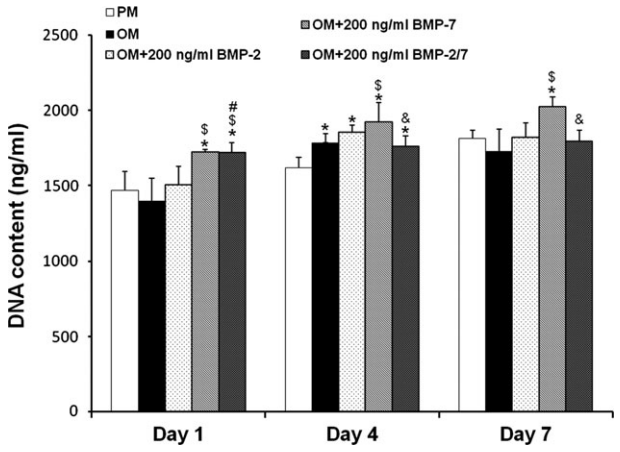


Fig. 4. DNA contents of hASCs induced by different BMPs at days 1, 4 and 7. BMP-2/7 and BMP-2 promoted cell proliferation at initial stages, and there were no significant differences compared to PM on day 7 ($P > 0.05$); whereas, BMP-7 promoted cell proliferation continuously ($P < 0.05$). $^{*}P < 0.05$ compared to PM, $^{\$}P < 0.05$ compared to OM, $^{\#}P < 0.05$ compared to OM + 200 ng/ml BMP-2, $^{\&}P < 0.05$ compared to OM + 200 ng/ml BMP-7. hASCs, human adipose-derived stem cells; PM, proliferation medium; OM, osteogenic medium; BMP, bone morphogenetic protein.

Kruskal–Wallis testing was used, followed by the Nemenyi test for multiple comparisons. For all tests, statistical significances were accepted for *P*-values lower than 0.05.

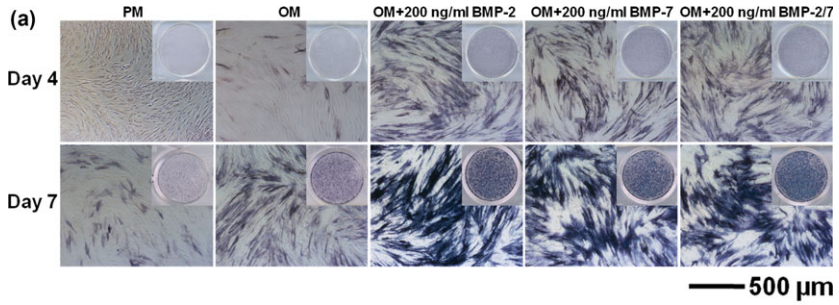
Results

BMP concentration selection assay

Our results showed that BMPs were unable to exert their osteoinductive effects in the presence of PM (Figs. 1–3), whereas they could induce osteogenic differentiation of hASCs in the presence of OM (Figs. 1–3). Moreover, osteoinductive effect of BMPs was positively correlated to concentrations. However, inducing effects of 50 ng/ml and 100 ng/ml BMPs were slightly higher than OM, whereas 200 ng/ml seemed to be an optimal concentration. Thus, we selected 200 ng/ml to perform *in vitro* assays. However, there were no significant differences in osteoinductive effects on hASCs between the three BMPs (Figs. 1–3), which needed to be verified further.

Comparison of proliferative capacity of hASCs stimulated by BMPs

BMP-2/7 and BMP-2 promoted cell proliferation in the initial stages, and there were no differences compared to PM on day 7 ($P > 0.05$); whereas, BMP-7 continuously promoted cell proliferation ($P < 0.05$) (Fig. 4).



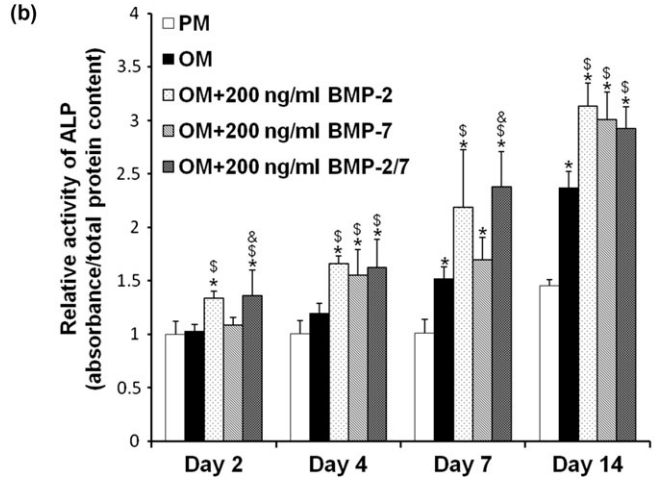


Fig. 5. ALP staining and activity of hASCs induced by different BMPs *in vitro*. (a) After 4 and 7 days induction, positive results for ALP staining confirmed the osteoinductive effect of BMPs. However, there were no notable differences between the three BMPs. (b) After 2, 4, 7 and 14 days induction, ALP activities showed that BMPs significantly enhanced ALP activities compared to PM and OM ($P < 0.05$). However, there were no significant differences between the three BMPs ($P > 0.05$). $^{*}P < 0.05$ compared to PM, $^{\$}P < 0.05$ compared to OM, $^{\&}P < 0.05$ compared to OM + 200 ng/ml BMP-7. hASCs, human adipose-derived stem cells; ALP, alkaline phosphatase; PM, proliferation medium; OM, osteogenic medium; BMP, bone morphogenetic protein.

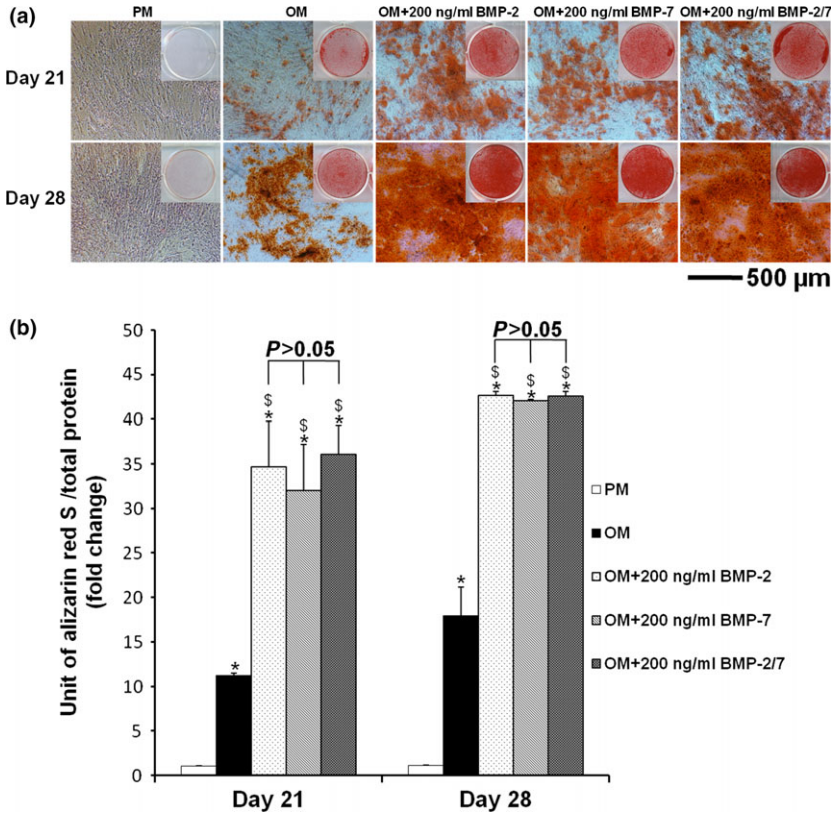


Fig. 6. Alizarin red staining and mineralization assay of hASCs induced by different BMPs *in vitro*. (a) After 21 and 28 days induction, positive results for alizarin red staining confirmed the osteoinductive effect of BMPs. However, there were no notable differences between the three BMPs. (b) After 21 and 28 days induction, mineralization assays demonstrated that hASC matrix calcifications were significantly enhanced by BMPs compared to PM and OM ($P < 0.05$). However, there were no significant differences between the three BMPs ($P > 0.05$). $^{*}P < 0.05$ compared to PM, $^{\$}P < 0.05$ compared to OM, $^{\&}P < 0.05$ compared to OM + 200 ng/ml BMP-7. hASCs, human adipose-derived stem cells; PM, proliferation medium; OM, osteogenic medium; BMP, bone morphogenetic protein.

Comparison of osteogenic potential of hASCs stimulated by BMPs in vitro

Positive results for ALP staining confirmed osteoinductive effects of the BMPs (Fig. 5a). However, there were no notable differences between the three BMPs by either days 4 or 7. ALP activity assay revealed that the BMPs significantly enhanced ALP activity compared to PM and OM ($P < 0.05$) (Fig. 5b). BMP-2/7 and BMP-2 seemed to exert a similar effect ($P > 0.05$), whereas inducing effect of BMP-7 was slightly weaker; however, the differences were not significant on days 4 and 14 ($P > 0.05$).

Alizarin red staining and mineralization assays demonstrated that hASC cell matrix calcifications were significantly elevated after 21 and 28 days induction by BMPs, compared to PM and OM ($P < 0.05$). However, there were no significant differences between the three BMPs ($P > 0.05$) (Fig. 6a,b).

Comparison of osteogenesis-associated gene expression in hASCs stimulated by BMPs

After 2 days induction with BMPs, expression of osteogenic genes (*RUNX2, ALP, COL-1A1*) was significantly up-regulated compared to PM and OM ($P < 0.05$). Moreover, the expression levels were continually elevated from day 2 to day 7 (Fig. 7). After 7 and 14 days induction, expression of *OPN* and *OC* genes was significantly up-regulated in BMP groups compared to PM and OM groups ($P < 0.05$) (Fig. 7). However, there were no significant differences ($P > 0.05$) in mRNA levels of these genes in hASCs induced by the three types of BMPs (Fig. 7).

Comparison of in vivo bone formation capability of hASCs induced by BMPs

Four weeks after *in vivo* transplantation, soft X-ray examination revealed that group III (β-TCP + BMP-2 + hASCs) and group IV (β-TCP + BMP-2/7 + hASCs) complexes had formed bone-like tissues with relatively higher density than control groups (Fig. 8a). However, there was no significant difference on the grey scale between these two groups ($P > 0.05$) (Fig. 8b).

HE staining showed that in groups III and IV, numbers of cells were significantly lower and eosinophilic bone-like tissues were found in the extracellular matrix (ECM) around the scaffold materials (Fig. 9a). However, there was no notable difference between the two groups. In control groups, cells were numerous and

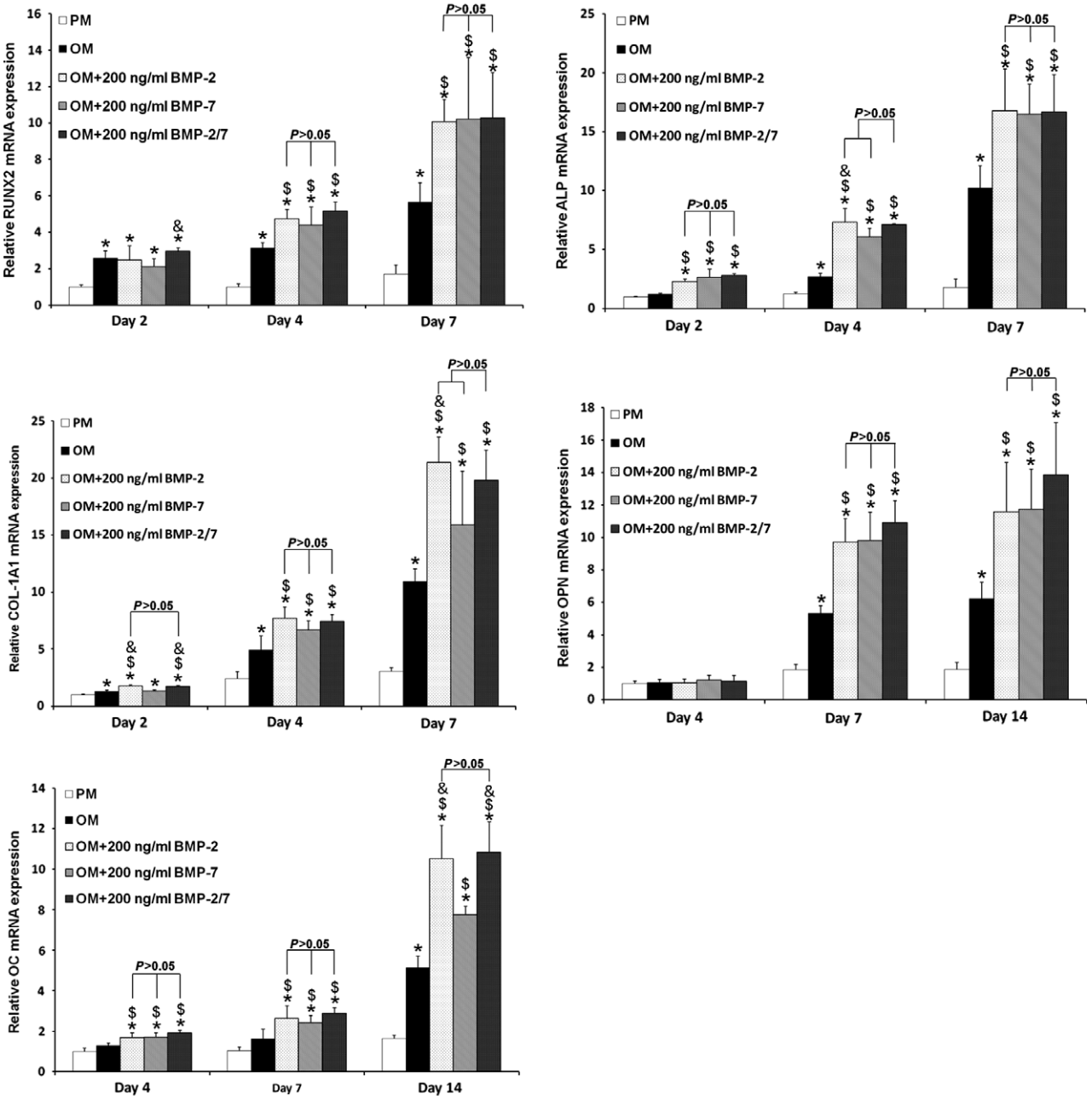


Fig. 7. Osteogenesis-associated gene expressions of hASCs induced by different BMPs. There were no significant differences in mRNA levels of *RUNX2*, *ALP*, *COL-1A1*, *OPN* or *OC* after osteoinduction by the three BMPs at 2, 4, 7 or 14 days ($P > 0.05$). $^{*}P < 0.05$ compared to PM, $^{\$}P < 0.05$ compared to OM, $^{\&}P < 0.05$ compared to OM + 200 ng/ml BMP-7. hASCs, human adipose-derived stem cells; PM, proliferation medium; OM, osteogenic medium; BMP, bone morphogenetic protein.

there were no typical bone-like structures in the ECM (Fig. 9a).

Masson's trichrome staining illustrated strong positive results in groups III and IV. More expression of collagen was observed in ECM compared to control groups (Fig. 9b). However, similar to HE staining, no notable difference were observed between the two groups. There was only a small amount of collagen expression in group II (β-TCP + hASCs) complex, and no obviously positive results were found in the blank control group (Fig. 9b).

Immunohistochemical staining showed that osteogenic marker OC was highly expressed in groups III and IV. Moreover, the two groups had much higher

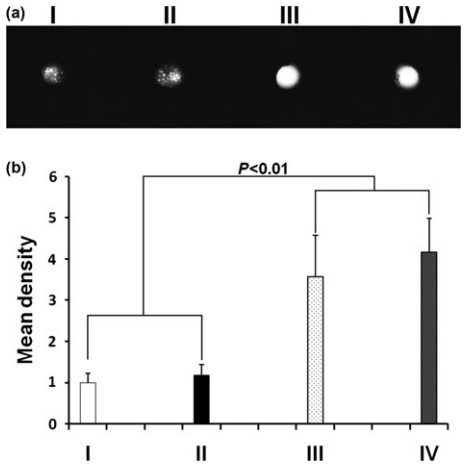


Fig. 8. Soft X-ray examination of β-TCP + BMPs + hASC complex implanted into nude mice for 4 weeks. (a) After 4-week *in vivo* transplantation, soft X-ray radiography showed that β-TCP + BMP-2 + hASC and β-TCP + BMP-2/7 + hASC complexes formed bone-like tissues with relatively higher density than control groups. (b) Relative grey scales were determined by Image J software. Mean density of β-TCP + BMPs + hASC complexes was significantly higher than control groups ($P < 0.01$). However, there was no significant difference between these two groups ($P > 0.05$). (I) β-TCP only (blank control); (II) β-TCP + hASCs; (III) β-TCP + BMP-2 + hASCs; (IV) β-TCP + BMP-2/7 + hASCs. hASCs, human adipose-derived stem cells; β-TCP, β-tricalcium phosphate; BMP, bone morphogenetic protein.

expression of OC than the control group. However, there was no significant difference in expression level between groups III and IV (Fig. 9c).

Discussion

Gradually, BMPs have been found to play more pleiotropic roles in promoting differentiation of pluripotent stem cells to different lineages, such as osteogenesis and adipogenesis (5,26,27). This phenomenon raises the question of whether BMPs can cause any certain differentiation direction in hASCs or not. In the absence of OM, irrespective of concentrations or dimerization type, BMPs did not significantly promote ALP activity *in vitro* (Figs. 1–3). In contrast, in the presence of OM, BMPs significantly enhanced hASC osteogenesis (Figs. 1–3). This result strongly suggests that BMPs did not commit hASCs to the osteogenic lineage, but enhanced osteogenic differentiation after committment. Even in the presence of OM, the effect of BMPs was controversial. Some previous studies have shown positive effects (5–8), whereas others were completely negative (9). One recent study indicated that even under the same culture conditions, exogenous BMP-2 promoted hASC osteogenesis from two donors, but inhibited it in hASCs from a further six donors, revealing donor variation (28). These results indicate that osteogenic efficacy of hASCs under certain osteogenic induction conditions varies largely between different donors.

In our concentration selection test, the promoting effect of BMPs on hASC osteogenic differentiation had increasing dose-dependent pattern. At 200 ng/ml, all three selected BMPs exhibited the highest effects for promoting ALP activity. On the basis of these results, we determined to use 200 ng/ml in the remaining tests. At the same concentration level, there were no significant differences in ALP activities after stimulation by BMPs. Consistently, the following ALP staining and quantitative assay, alizarin red staining and mineralization assay, and osteogenic gene expressions also showed no significant differences between these three BMPs. These results were not consistent with previous findings.

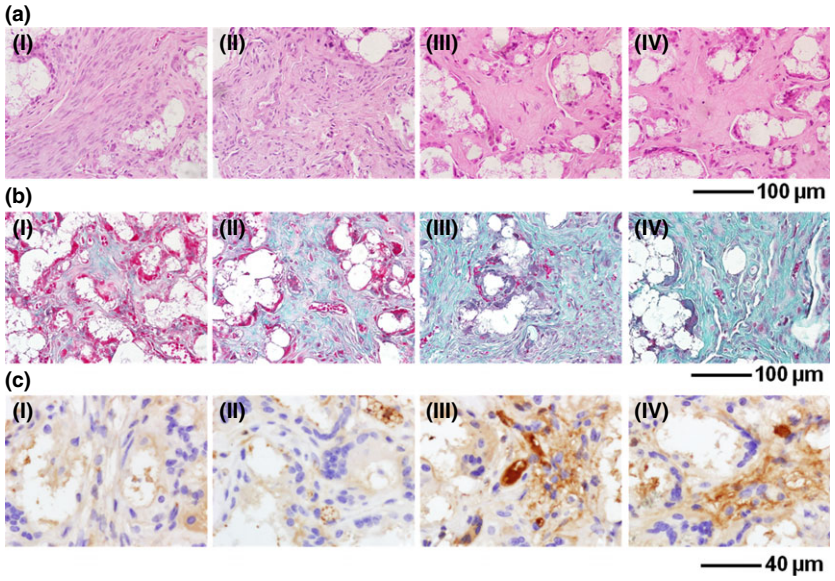


Fig. 9. Histological and immunohistochemical analyses of β-TCP + BMPs + hASC complexes implanted into nude mice for 4 weeks. (a) HE staining revealed eosinophilic bone-like tissues in group III and group IV animals after 4-weeks implantation, whereas there were no typical bone-like structures in the control groups. (b) Masson's trichrome staining showed strong positive results in groups III and IV. Higher expression of collagen could be observed compared to the control groups. (c) Immunohistochemical staining showed higher expression of OC in groups III and IV compared to control groups. (I) β-TCP only (blank control); (II) β-TCP + hASCs; (III) β-TCP + BMP-2 + hASCs; (IV) β-TCP + BMP-2/7 + hASCs. hASCs, human adipose-derived stem cells; β-TCP, β-tricalcium phosphate; BMP, bone morphogenetic protein.

Heterodimeric BMP has been frequently shown to be more potent than homodimeric BMPs in inducing osteogenesis of murine cells (13–18, 21). In previous studies, BMP-2/7 showed a different dose-dependent pattern from BMP-2 and BMP-7; BMP-2/7 started to take effect at 5 ng/ml and reached a plateau at 50 ng/ml; whereas, BMP-2 or BMP-7 started to take effect at 50 ng/ml and continued to increase osteogenic differentiation as the dose increased (19). However, in our study, induction of osteogenesis by BMP-2/7 at these low effective concentrations (5–50 ng/ml) disappeared. Thus, the mechanism for this phenomenon needs to be further investigated.

In the subsequent *in vivo* experiments, β-TCP + BMPs + hASCs complexes caused formation of ectopic bone structures, which suggests that *in vitro* pre-osteoinduction was not a requirement for *in vivo* bone formation. In contrast to their *in vitro* performance, BMPs induced hASC osteogenesis *in vivo* without OM; however, the mechanisms that account for their different performances remain to be unveiled.

Consistent with the *in vitro* findings, BMP-2/7 did not result in superior osteogenesis of hASCs compared to BMP-2 in the *in vivo* model. Our previous data have already corroborated that BMP-2/7 combined with pure scaffolds significantly accelerates and enhances regeneration of new bone compared to BMP-2 and BMP-7, in the mini pig (24). This finding suggests that BMP-2/7 was superior in inducing *in vivo* osteogenesis. However, caution needs to be taken when extrapolating the data to human cells. In the previous studies, BMP-2 was less efficient in inducing osteogenesis of human BMSCs compared to BMSCs from rats and mice (29). In addition, Carpenter *et al.* have indicated that combined AdBMP-2 and -7 gene transfer did not have any greater effect than using single AdBMP on osteogenic differentiation of human BMSCs (30). Species-specific differences may account for different sensitivities to BMP-2/7 between murine cells and hASCs. Furthermore, distinct BMP signalling patterns may be in underlying molecular mechanisms. Further investigation is needed in the future to provide a definitive answer to this question.

Selection of BMP concentration range in this study was mainly based on results of previous studies (19). According to earlier reports, osteoinductive efficiency of exogenous BMP-2/7 reaches its maximum at 200 ng/ml and significantly decreases by 250 ng/ml. Besides this, inducing effects of exogenous BMP-2 and BMP-7 also reached plateaux stages at 200 ng/ml. Although these results came from different cell lines, it has still provided a reference point for us. Furthermore, BMPs of higher concentration may not seem cost-effective in future clinical usage, which is very important for clinical translation. More importantly, we mainly wished to determine whether BMP-2/7 could exhibit superior osteoinductive effects compared to BMP-2 and BMP-7 over a relatively low concentration range. Consequently, we used maximum BMP concentration of 200 ng/ml. In addition, β-TCP was used as scaffold for *in vivo* transplantation due to its appropriate mechanical properties and good biological compatibility (22). Furthermore, to evaluate *in vivo* osteoinductive effects of BMPs more directly, numbers of hASCs loaded on to scaffolds was much lower than in our previous studies (22).

Limitations of this study are that a broader concentration range could be considered to completely exhibit characteristics of heterodimeric BMP-2/7 *in vitro*, and evaluation of bone formation *in vivo* could be performed at additional time points.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (2011/81170937 to Prof. Dr. Y. Zhou), the Program for New Century Excellent Talents in University from Ministry of Education (NCET-11-0026), and the Construction Program for National Key Clinical Specialty from National Health and Family Planning Commission of China (2011).

Conflicts of interest

None.

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