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RESEARCH PAPER

Heterodimeric BMP-2/7 exhibits different osteoinductive effects in human and murine cells

Xiao Zhang[a,b,c,d*], Si Chen[a,b,c,d*], Yunsong Liu[a,b,c,d], Ping Zhang[a,b,c,d], Yanjun Ge[a,b,c,d], Jing Guo[e], Gang Wu[f] and Yongsheng Zhou[a,b,c,d]

[a]Department of Prosthodontics, Peking University School and Hospital of Stomatology, Beijing, China; [b]National Engineering Lab for Digital and Material Technology of Stomatology, Beijing, China; [c]National Clinical Research Center for Oral Diseases, Beijing, China; [d]Beijing Key Laboratory for Digital Stomatology, Beijing, China; [e]Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), Research Institute MOVE, VU University and University of Amsterdam, LA Amsterdam, The Netherland; [f]Department of Oral Implantology and Prosthetic Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), Research Institute MOVE, VU University and University of Amsterdam, LA Amsterdam, The Netherland

ABSTRACT

As robust osteoinductive cytokines, bone morphogenetic proteins (BMPs) play a significant role in bone tissue engineering. Constituted of two different polypeptides, heterodimeric BMPs are more effective than the homodimers in bone formation. While most studies focused on the murine cell lines, such as murine preosteoblasts MC3T3-E1, the role of heterodimeric BMPs in the osteogenic differentiation of human cells remains uncertain, which hinders their application to practical treatment. In this study, we compared the osteoinductive effects of BMP-2/7 heterodimer in human adipose-derived stem cells (hASCs) with their homodimers BMP-2 and BMP-7, in which MC3T3-E1 cells were utilized as a positive control. The results indicated that BMP-2/7 was not a stronger inducer during the osteogenic differentiation of hASCs as that for MC3T3-E1, and extracellular-signal-regulated kinase signaling played a role in the different effects of BMP-2/7 between hASCs and MC3T3-E1. Our study demonstrates the osteoinductive effects of heterodimeric BMP-2/7 present in a cell-specific pattern and cautions should be taken when applying heterodimeric BMP-2/7 to clinical practice.

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Bone morphogenetic proteins; heterodimer; human adipose-derived stem cells; MC3T3-E1; osteogenic differentiation

Introduction

Large-size bone defect, caused by trauma, infection, or oncological resection, is a prevailing challenge in reconstructive surgery. Tissue engineering technology has become one of the most prospective strategies for bone regeneration in clinical practice (Guan et al., 2012; Petite et al., 2000). Compared with human bone marrow-derived stem cells (hBMSCs), human adipose-derived stem cells (hASCs) show more potential as another cell source, owing to their easy accessibility, high-yield efficiency, and low donor-site morbidity (Zuk et al., 2002). hASC-based bone tissue engineering appears to be quite prospective for osseous restoration of large-size bone defects (Zhou et al., 2010).

Bone morphogenetic proteins (BMPs) belong to the superfamily of transforming growth factor-beta (TGF-β; Bragdon et al., 2011). The discovery of BMPs in the pioneering work by Urist was a landmark in the development of bone tissue engineering (Urist, 1965). Owing to the continuous efforts over the last 50 years, several isoforms of BMPs have been demonstrated to play paramount roles in the osteogenic differentiation of mesenchymal stem cells in various models such as human, mouse, rat, and rabbit (Zhang et al., 2014). In particular, BMP-2, 4, 6, and 7 have been studied for their potential clinical application to spinal fusions, long-bone defects, non-union bone fractures, and periodontal bone loss (Boyce et al., 2009; Lin et al., 2009; Valera et al., 2010).

Generally, most BMP molecules consist of two monomers derived from the same BMP member, termed as 'homodimeric BMPs' (Miyazono et al., 2010). In contrast, heterodimeric BMPs are composed of two monomers from different BMP members (Guo & Wu, 2012). As robust osteoinductive cytokines, BMP heterodimers might be more potent than their respective

CONTACT Gang Wu g.wu@acta.nl Department of Oral Implantology and Prosthetic Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), Gustav mahlerlaan 3004, 1081 LA Amsterdam, the Netherland; Yongsheng Zhou kqzhouysh@hsc.pku.edu.cn Peking University School and Hospital of Stomatology, 22 Zhongguancun Avenue South, Haidian District, Beijing 100081, China
*These authors contributed equally to this work.

homodimers in promoting bone formation. Israel et al. first discovered that co-expression of BMP-2 and -7 yielded a 20-fold higher alkaline phosphatase (ALP) activity than BMP homodimers in Chinese hamster ovary cells (Israel et al., 1996). Subsequently, researchers observed a similar phenomenon with other rodent cell lines, including C3H10T1/2 (murine pluripotent mesenchymal cell line), MC3T3-E1 (murine preosteoblast cell line), and rat BMSCs (Wang et al., 2010; Zhao et al., 2005; Zheng et al., 2010). However, our previous research reported no significant differences in hASCs osteogenesis between heterodimeric BMP-2/7 and the homodimeric BMPs (Zhang et al., 2015), hindering the application of BMP-2/7 into clinical practice. To minimize the differences caused by cell culture conditions, and so on, we introduced MC3T3-E1 as a positive control to further explore the osteogenic ability of BMP-2/7 in human cells.

Materials and methods

Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Minimum essential medium (α-MEM), fetal bovine serum (FBS), and antibiotics were purchased from Gibco (Grand Island, NY). Recombinant BMPs were purchased from R&D systems (R&D systems, Inc., Minneapolis, MN).

Cell culture

Primary hASCs from three different healthy human donors were purchased from Sciencell (San Diego, CA). MC3T3-E1 cell line was purchased from Cell Resource Center, Peking Union Medical College (Beijing, China). Cells were cultured at 37 °C in an incubator with an atmosphere of 95% air and 5% CO_2, with 100% relative humidity. For osteogenic differentiation, hASCs and MC3T3-E1 cells were cultured in osteogenic medium (OM) containing fresh α-MEM with 10% (v/v) FBS, 1% antibiotics (100 U/mL penicillin G and 100 µg/mL streptomycin), 100 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate.

BMPs administration

hASCs and MC3T3-E1 cells were seeded in six-well plate at a density of 10^5 for the following experiments. Cells between three and five passages were used for the experiments, and all the experiments were replicated in triplicate. We adopted this experimental set-up based on the conclusion from our previous study. In that study, we have already shown that in proliferative medium (un-induced controls), BMPs did not significantly promote ALP activity in hASCs irrespective of BMP concentration or dimerization type (Zhang et al., 2015). The aim of this study was to investigate the different osteoinductive abilities of BMP-2/7 between murine MC3T3-E1 cells and human ASCs. Therefore, we excluded the un-induced controls that had no effects on osteogenesis in our study. Three different lyophilized BMPs (BMP-2, BMP-7, BMP-2/7) were reconstituted at a stock concentration of 10 µg/mL in sterile 4 mM hydrochloric acid (HCL) containing 0.1% bovine serum albumin (BSA). Exogenous BMPs were administered in the presence of OM at a concentration of 50 ng/mL, which was validated to be effective for hASCs osteogenesis in our previous dose–response study (Zhang et al., 2015). hASCs and MC3T3-E1 cells were exposed to the following treatments: (i) control medium (OM); (ii) OM with 50 ng/mL BMP-2; (iii) OM with 50 ng/mL BMP-7; and (iv) OM with 50 ng/mL BMP-2/7. The media were replaced every 3 d.

Chemical administration

In order to assess the role of extracellular-signal-regulated kinase (ERK) signaling pathway in the different effects of BMP-2/7 in hASCs and MC3T3-E1, the selective ERK1/2 inhibitor U0126 was obtained from Selleck (S1102, Houston, TX). U0126 was dissolved in dimethylsulfoxide (DMSO), and then administrated at a concentration of 10 µM according to our previous research, and cells treated with the same concentration of DMSO were used as a control group (Zhang et al., 2017).

ALP staining assay

ALP staining and activity were performed on the 4th and 7th day of osteoinduction, as described previously (Lv et al., 2015). Briefly, cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, following which ALP staining was performed according to the protocol of the NBT/BCIP staining kit (CoWin Biotech, Beijing, China). Macroscopic images were obtained with a scanner (HP Scanjet G4050, Palo Alto, CA, USA), and microscopic images were captured with a microscope (TE2000-U, Nikon, Tokyo, Japan).

ALP quantification assay

After osteogenic induction for 4 and 7 d, cells were washed three times with cold PBS and lysed with 1%

TritonX-100 for 10 min on ice. The cell lysate was collected and then centrifuged at 12,000 rpm for 30 min at 4 °C. Total protein content was determined in the same sample by the bicinchoninic acid (BCA) method by using a Pierce Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). ALP activity was measured using an ALP quantification kit (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. ALP activity relative to the control treatment was calculated after normalization to the total protein content.

Alizarin red staining and mineralization assay

To assess extracellular mineralization, alizarin red staining was performed on the 14th and 21st day of stimulation. Cells were washed three times with PBS, fixed with 4% paraformaldehyde for 30 min, and stained with 1% alizarin red (pH 4.2) at room temperature. Macroscopic and microscopic images were obtained with a scanner and a microscopy, respectively. To quantitatively determine the degree of matrix calcification, alizarin red was destained with 100 mM cetylpyridinium chloride for 30 min and quantified by spectrophotometric absorbance at 562 nm. The final mineralization level in each group was normalized to the total protein concentration obtained from duplicate plates.

Real-time polymerase chain reaction (PCR) analysis

Total cellular RNAs were isolated on the 4th and 7th day of stimulation with TRIzol reagent (Invitrogen, Carlsbad, CA) and 2 μg aliquots of RNA were reverse-transcribed according to the manufacturer's instruction (Roche, Basel, Switzerland). Real-time quantitative PCR assay was performed using a Power SYBR Green PCR Master Mix and an ABI PRISM 7500 sequence-detection system (Applied Biosystems, Foster City, CA). The following thermal settings were used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The following primers were used for human and mouse, and *GAPDH* or *Gapdh* was used for normalization. *RUNX2* (human), forward primer: 5′-GCACAAACATGGCCAGATTCA-3′, reverse primer: 5′-AAGCCATGGTGCCCGTTAG-3′; *ALP* (human), forward primer: 5′-GAGATGGTATGGGCGTCTC-3′, reverse primer: 5′-GTTGGTGTTGTACGTCTTGGA-3′; *GAPDH* (human), forward primer: 5′-CTGGCACCACA-CCTTCTACA-3′, reverse primer: 5′-GGTACGACCAGA-GGCATACA-3′; *Runx2* (mouse), forward primer: 5′-ACTACCAGCCACCGAGACCA-3′, reverse primer: 5′-ACTGCTTGCAGCCTTAAATGACTCT-3′; *Alp* (mouse), forward primer: 5′-ATGGGATGGGTGTCTCCACA-3′, reverse primer: 5′-CCACGAAGGGGAACTTGTC-3′; *Gapdh* (mouse), forward primer: 5′-CATGTACGTT-GCTATCCAGGC-3′, reverse primer: 5′-CTCCTTAATG-TCACGCACGAT-3′. Data were analyzed using the $2^{-\triangle\triangle Ct}$ relative expression method.

Enzyme-linked immunosorbent (ELISA) assay

The cell supernatants were collected on the 14th day of stimulation. For quantitative determination of osteocalcin (OCN) secretion at protein level, a human OCN ELISA kit (eBioscience, Inc., San Diego, CA) and a mouse OCN ELISA kit (Biomedical Technologies, Stoughton, MA) were utilized. OCN secretion of each group was determined according to the manufacturer's protocol.

Western blot assay

After 7 d of osteoinduction, cells were washed with cold PBS, and lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail (Roche, Basel, Switzerland). After centrifugation of the cell lysate at 12,000 rpm at 4 °C for 30 min, the protein concentrations were determined using the BCA protein assay. Total protein (25 μg) of each sample was subjected to 10% SDS-PAGE, and proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA) after electrophoresis. Primary antibodies against p-Smad1/5/8, Smad1, GAPDH (Abcam, Cambridge, UK), p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, and p38 (Cell Signaling Technology, Beverly, MA) were diluted 1:1000 and incubated with the membranes at 4 °C overnight. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were diluted 1:10,000 and incubated with the membranes at room temperature for 1 h. The membranes were then visualized using an ECL kit (CWBIO), and GAPDH was used as the internal control. Band intensities were quantified using the ImageJ software (https://imagej.nih.gov/ij/).

Statistical analysis

Data were analyzed using SPSS 20.0 software (Chicago, IL), and expressed as mean ± SD of three independent experiments. Differences between two groups were analyzed with one-way analysis of variance (ANOVA). For multiple comparisons, Bonferroni post-hoc test was then implemented. The level for statistical significance was set at a p-value of $<.05$.

Results

BMP-2/7 does not promote early osteogenesis compared to homodimers in hASCs

After 4 and 7 d of stimulation, ALP staining and ALP quantification revealed that BMP-2/7 and the homodimers BMP-2 and BMP-7 significantly promoted the osteogenic differentiation of both hASCs and MC3T3-E1 compared with the control group (Figure 1(A–C)). However, no notable difference was observed in the effects of the three BMPs on either day 4 or 7 in hASCs ($p > .05$) (Figure 1(A,C)), while BMP-2/7 exhibited a higher osteoinductive efficiency than BMP-2 and BMP-7 in MC3T3-E1 on both day 4 and 7 ($p < .05$) (Figure 1(B,C)).

BMP-2/7 does not promote hASCs late mineralization compared with homodimers

Alizarin red staining and mineralization assay demonstrated that BMP-2/7 and the homodimers BMP-2 and BMP-7 significantly enhanced the cell matrix mineralization of both hASCs and MC3T3-E1 after 14 and 21 d of induction compared with the control group (Figure 2(A–C)). However, there was no significant difference in effects of the three BMPs in hASCs on either day 14 or 21 ($p > .05$) (Figure 2(A,C)). While, BMP-2/7 induced deposition of calcium more effectively than BMP-2 or BMP-7 for the MC3T3-E1 cells on both day 14 and 21 ($p < .05$) (Figure 2(B,C)).

BMP-2/7 does not enhance the expression of osteogenesis-associated genes in hASCs compared with homodimers

After stimulation by BMPs for 4 and 7 d, qRT-PCR showed that the expression levels of *RUNX2* (or *Runx2*) and *ALP* (or *Alp*) were significantly upregulated in both hASCs and MC3T3-E1 compared with the control group ($p < .05$) (Figure 3(A,B)). Moreover, the expression levels of osteogenesis-associated genes were continually elevated from day 4 to day 7. However, there was no significant difference in the expression levels of *RUNX2* and *ALP* in hASCs induced by the three types of BMPs ($p > .05$) (Figure 3(A,B)), while upregulation of osteogenesis-associated gene expression was more remarkable in BMP-2/7 group than in BMP-2 or BMP-7 group for MC3T3-E1 cells ($p < .05$) (Figure 3(A,B)).

BMP-2/7 does not induce more OCN secretion of hASCs compared with homodimers

After 14 d of induction with BMPs, the secretion of OCN was upregulated significantly in both hASCs and MC3T3-E1 compared with the control group ($p < .05$) (Figure 4(A,B)). The OCN secretion of hASCs in BMP-2/7 group showed an insignificant increase compared with BMP-2 ($p > .05$), but exhibited a minor increase compared with BMP-7 ($p < .05$) (Figure 4(A)). Meanwhile, for MC3T3-E1 cells, the OCN secretion was highest in BMP-2/7 group compared with the other two BMPs ($p < .05$) (Figure 4(B)).

ERK signaling is involved with the different effects of BMP-2/7 in hASCs and MC3T3-E1

We further determined the Smad-dependent signaling pathways and Smad-independent signaling pathways which modulated BMP activity. In MC3T3-E1, BMP-2/7 induced significantly higher levels of phosphorylated ERK1/2 than BMP-2 or BMP-7. Three BMPs induced a similar level of phosphorylated JNK. The phosphorylated Smad1/5/8 and p38 induced by BMP-2/7 were only significantly higher than BMP-2 but not than BMP-7 (Figure 5(A,C–F)). In contrast, in hASCs, BMP-2/7 did not induce significantly upregulation of phosphorylated ERK1/2, p38, or JNK in comparison with the control group. BMP-2/7 only induced an elevated phosphorylated Smad1/5/8, which was, however, significantly lower than BMP-2 or BMP-7 (Figure 5(B,C–F)). To further testify the different involvement patterns of ERK signaling in hASCs and MC3T3-E1 cells, we performed ALP activity and quantification using a well-established ERK inhibitor — U0126. As the results showed, U0126 could decrease the ALP activity triggered not only by OM but also by all the three BMPs in hASCs (Figure 6(A,C)). In contrast, U0126 could only significantly compromise the ALP activity induced by BMP-2/7 but not by BMP-2 or BMP-7 or OM in MC3T3-E1 cells (Figure 6(B,D)).

Discussion

BMPs have been introduced into the field of bone tissue engineering for several decades. BMP-2 and 7 have been approved for clinical practice in the United States, Europe, and Australia (Makino et al., 2005). However, the clinically effective doses of homodimeric BMPs to promote bone formation are extremely high (in milligrams), which leads to a heavy economic burden for patients and causes a series of potential side effects such as overstimulation of osteoclastic differentiation and topical bone formation at unintended sites (Kanoko et al., 2000; Zara et al., 2011). Heterodimeric BMP-2/7 is a potential substitute for homodimeric

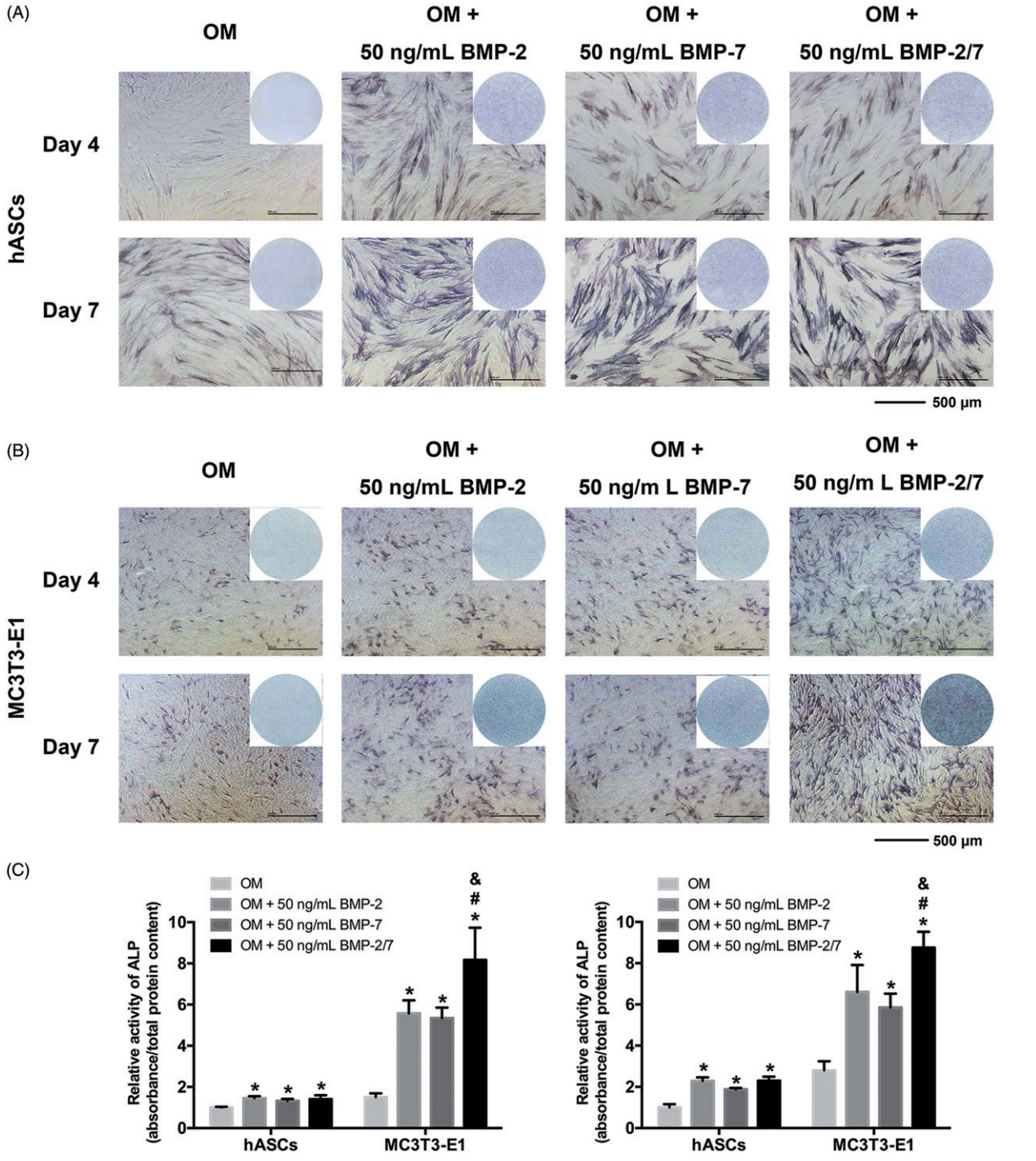

Figure 1. ALP staining and activity of hASCs and MC3T3-E1 induced by different BMPs. (A) ALP staining during the osteogenic differentiation of hASCs on day 4 and 7 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. Bars = 500 μm. (B) ALP staining during the osteogenic differentiation of MC3T3-E1 on day 4 and 7 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. Bars = 500 μm. (C) ALP quantification during the osteogenic differentiation of hASCs and MC3T3-E1 on day 4 and 7 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. $^{*}p < .05$ compared to OM, $\#p < .05$ compared to OM +50 ng/mL BMP-2, $\&p < .05$ compared to OM +50 ng/mL BMP-7.

BMPs, as it showed much higher osteoinductive potency in several murine cell types (Israel et al., 1996; Zhao et al., 2005; Zheng et al., 2010; Zhu et al., 2004). However, our previous research demonstrated that BMP-2/7 was not a stronger inducer of osteogenesis than homodimeric BMPs when applied to human cells.

To observe the osteoinductive effects of BMP-2/7 on MC3T3-E1 and hASCs simultaneously, we set up

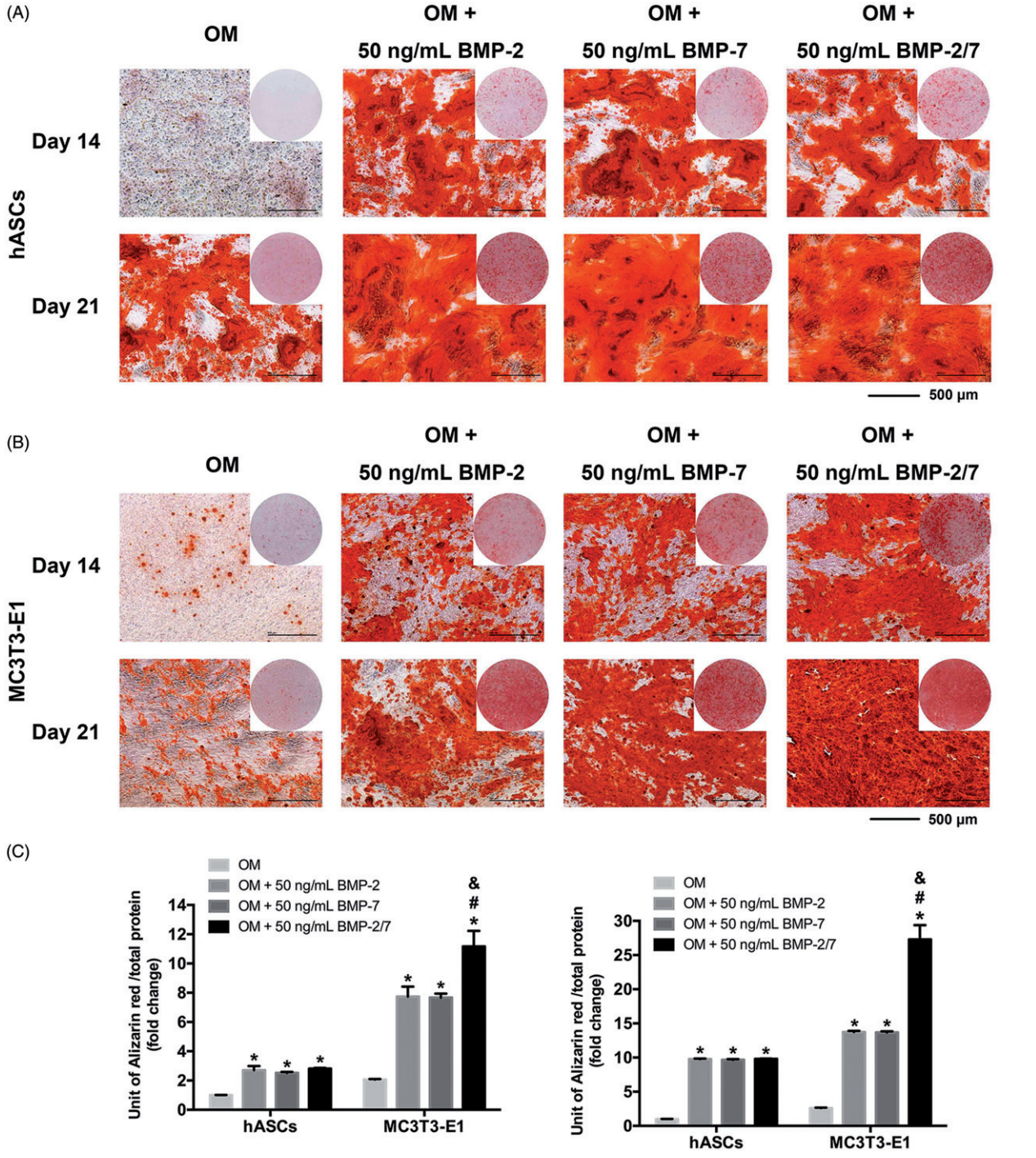

Figure 2. Alizarin red staining and mineralization assay of hASCs and MC3T3-E1 induced by different BMPs. (A) Alizarin red staining during the osteogenic differentiation of hASCs on day 14 and 21 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. Bars = 500 μm. (B) Alizarin red staining during the osteogenic differentiation of MC3T3-E1 on day 14 and 21 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. Bars = 500 μm. (C) Alizarin red mineralization assay during the osteogenic differentiation of hASCs and MC3T3-E1 on day 14 and 21 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. $^{*}p < .05$ compared to OM, #$p < .05$ compared to OM +50 ng/mL BMP-2, &$p < .05$ compared to OM + 50 ng/mL BMP-7.

50 ng/mL as the optimal concentration. According to a previous study, exogenous BMP-2/7 started to show an effect at 5 ng/mL and reached a plateau at 50 ng/mL during the osteogenic differentiation of MC3T3-E1 (Zheng et al., 2010). However, our recent data showed that BMP-2/7 started to show an effect at 50 ng/mL during the osteogenic differentiation of hASCs (Zhang et al., 2015). With the BMP dosage of 50 ng/mL, MC3T3-E1 cells are being stimulated with dose which is maximally osteogenic, while hASCs are

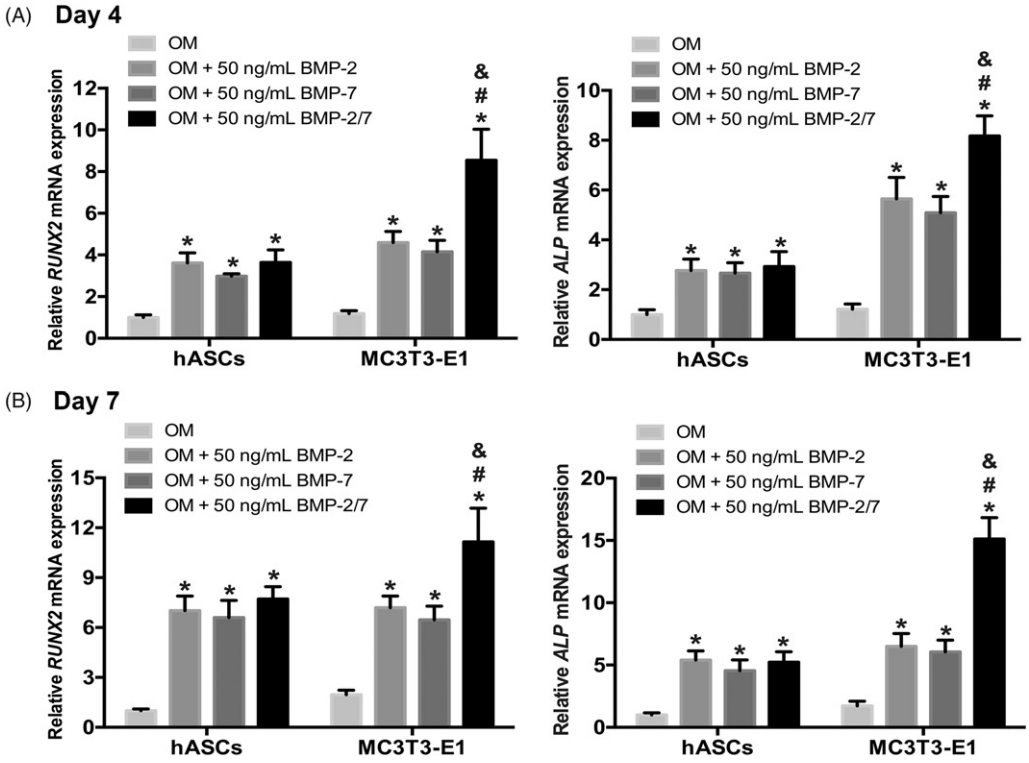

Figure 3. Osteogenesis-associated gene expressions of hASCs and MC3T3-E1 induced by different BMPs. (A) Relative mRNA levels of RUNX2 (Runx2) and ALP (Alp) measured by real-time PCR on day 4 during the osteogenic induction of hASCs and MC3T3-E1 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. GAPDH (Gapdh) was used for normalization. (B) Relative mRNA levels of RUNX2 (Runx2) and ALP (Alp) measured by real-time PCR on day 7 during the osteogenic induction of hASCs and MC3T3-E1 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. GAPDH (Gapdh) was used for normalization. $^{*}p < .05$ compared to OM, $\#p < .05$ compared to OM +50 ng/mL BMP-2, $\&p < .05$ compared to OM + 50 ng/mL BMP-7.

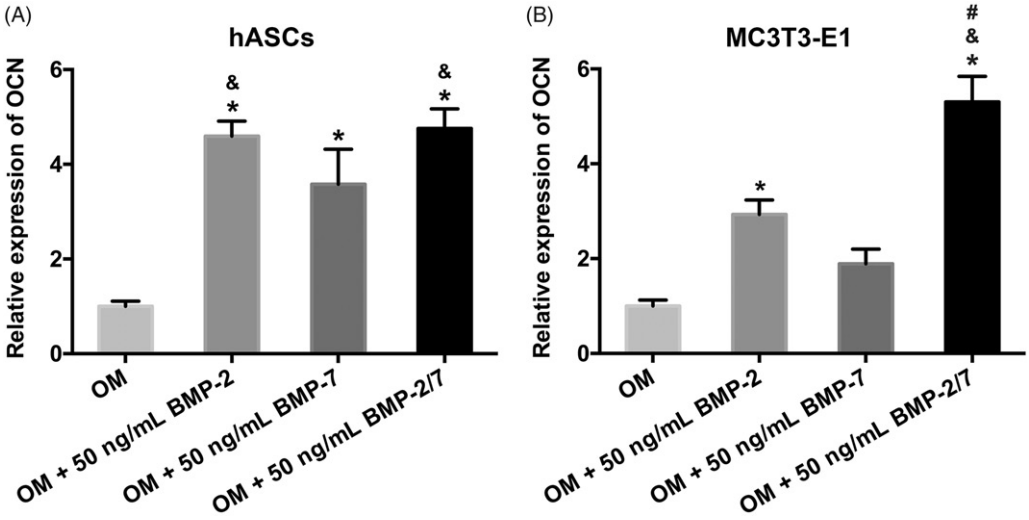

Figure 4. OCN secretion of hASCs and MC3T3-E1 cultured with different BMPs on day 14. (A) ELISA assay of OCN secretion on day 14 during the osteogenic differentiation of hASCs in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. (B) ELISA assay of OCN secretion on day 14 during the osteogenic differentiation of MC3T3-E1 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group. $^{*}p < .05$ compared to OM, $\#p < .05$ compared to OM +50 ng/mL BMP-2, $\&p < .05$ compared to OM +50 ng/mL BMP-7.

getting a dose that is suboptimal. For example, in Figures 1 and 2 induction of ALP and alizarin red staining, respectively, is extremely low in hASCs compared to MC3T3 cells. Our study mainly focused on the differential effects of BMP-2/7 and their homodimers in the osteogenesis of hASCs compared with

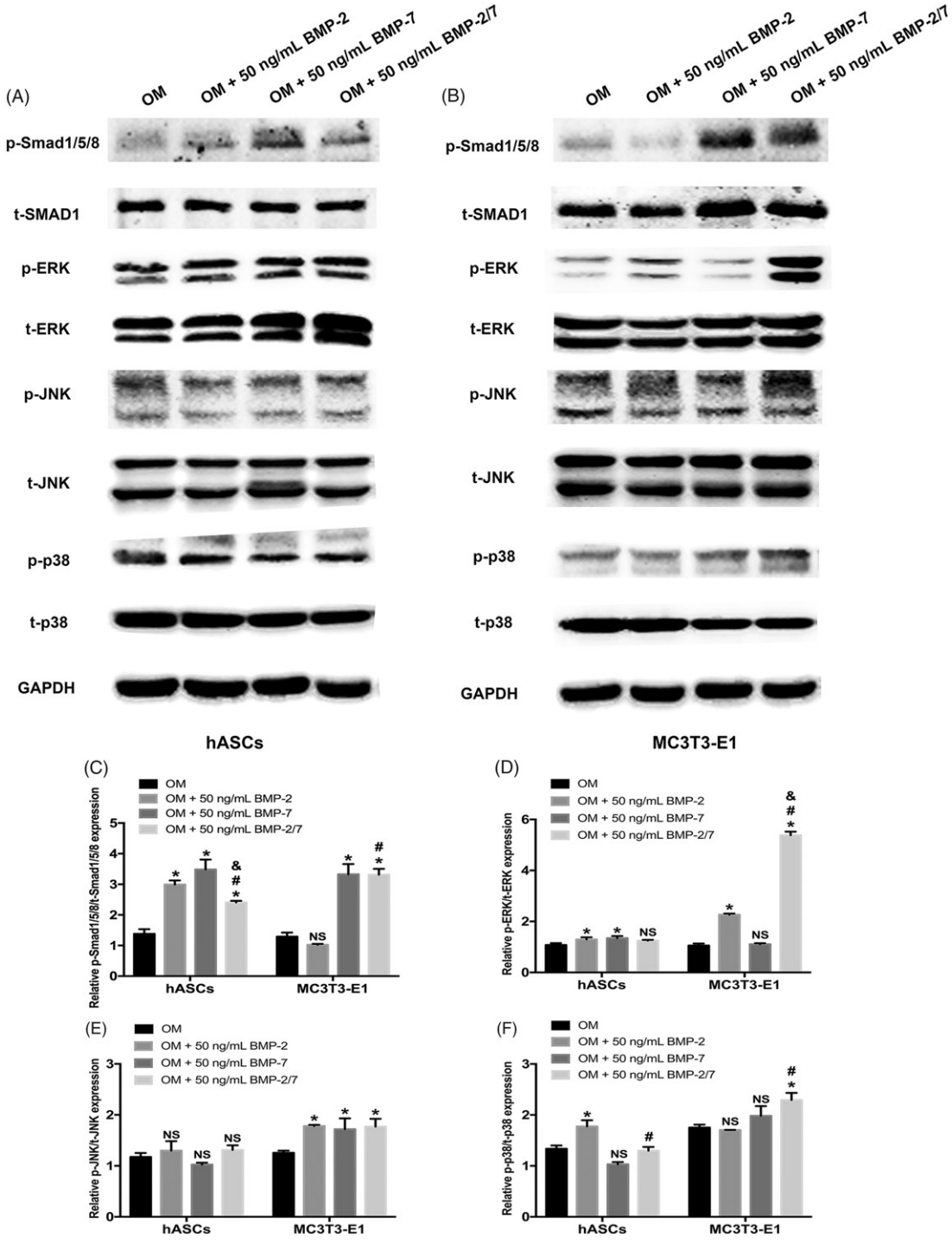

Figure 5. Smad-dependent and Smad-independent signaling of hASCs and MC3T3-E1 cultured with different BMPs on day 14. (A) Western blot of phosphorylated-Smad1/5/8, total-Smad1/5/8, phosphorylated-ERK, total-ERK, phosphorylated-JNK, total-JNK, phosphorylated-p38, and total-p38 on day 14 during the osteogenic differentiation of hASCs in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group, GAPDH was utilized as the internal control. (B) Western blot of phosphorylated-Smad1/5/8, total-Smad1/5/8, phosphorylated-ERK, total-ERK, phosphorylated-JNK, total-JNK, phosphorylated-p38, and total-p38 on day 14 during the osteogenic differentiation of MC3T3-E1 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group, GAPDH was utilized as the internal control. (C–F) Quantification of band intensities in phosphorylated-Smad1/5/8, total-Smad1/5/8, phosphorylated-ERK, total-ERK, phosphorylated-JNK, total-JNK, phosphorylated-p38, and total-p38 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) group, GAPDH was utilized as the internal control. NS: no significant difference, $^{*}p < .05$ compared to OM, $\#p < .05$ compared to OM +50 ng/mL BMP-2, $\&p < .05$ compared to OM +50 ng/mL BMP-7.

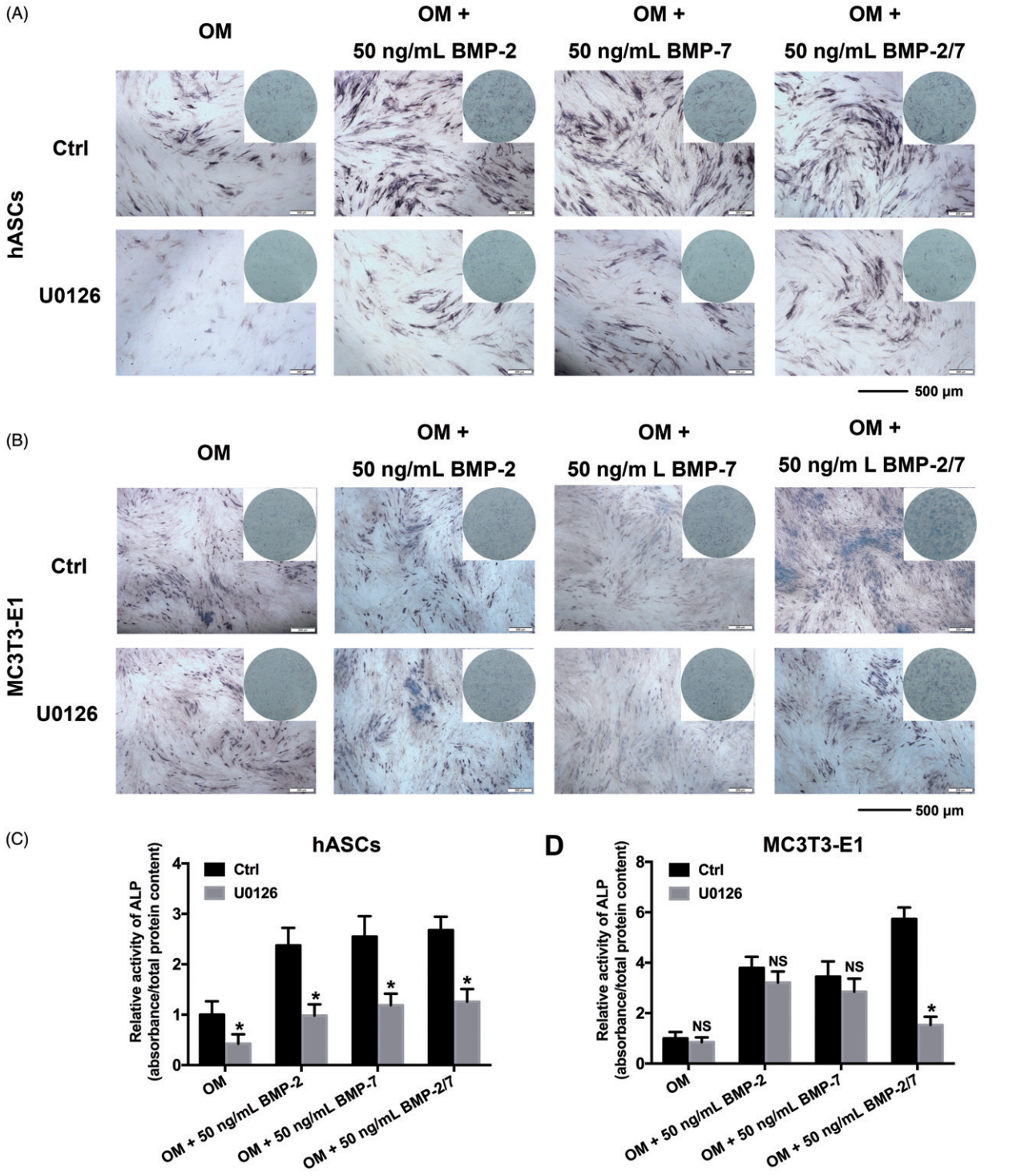

Figure 6. ERK signaling is involved with the different effects of BMP-2/7 in hASCs and MC3T3-E1. U0126 was delivered to each group at 10 μM. (A) ALP staining during the osteogenic differentiation of hASCs on day 7 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) groups with or without U0126. Bars = 500 μm. (B) ALP staining during the osteogenic differentiation of MC3T3-E1 on day 7 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) groups with or without U0126. Bars = 500 μm. (C) ALP quantification during the osteogenic differentiation of hASCs on day 7 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) groups with or without U0126. (D) ALP quantification during the osteogenic differentiation of MC3T3-E1 on day 7 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) groups with or without U0126. NS: no significant difference, $^{*}p < .05$ indicated the significance between the treatment with and without U0126 in the OM, OM + BMP-2 (50 ng/mL), OM + BMP-7 (50 ng/mL), and OM + BMP-2/7 (50 ng/mL) groups.

MC3T3-E1. Therefore, exogenous BMPs were administrated with OM at a concentration of 50 ng/mL in this study, and the results indicated that though BMP-2/7 promoted the osteogenic differentiation of MC3T3 compared with BMP-2, and BMP-7, similar enhancement was not observed when it comes to hASCs.

The primary outcomes of the ALP staining, ALP activity assay, alizarin red staining, and mineralization assay indicated that BMP-2/7 could significantly enhance osteogenesis in MC3T3-E1 and exhibit higher potency than the homodimers, which are consistent with the findings of a previous study (Zheng et al., 2010). Although BMP-2/7 could promote osteogenesis of hASCs, there was no significant difference among the effects of the three BMPs. The real-time PCR results demonstrated that BMP-2/7 upregulated the expression of osteogenesis-associated genes more effectively than BMP-2 or BMP-7 in MC3T3-E1, but this effect was not observed in hASCs; the secretion of OCN showed a similar tendency. These results strongly indicated that for hASCs, heterodimeric BMP-2/7 might not act as a more potent osteoinductive factor than BMP-2 or 7 as it does for MC3T3-E1. Our findings are also consistent with previous reports that gene transfer which combined BMP-2 and 7 did not have a greater effect on the osteogenic differentiation of human BMSCs than use of a single BMP (Carpenter et al., 2010).

The cellular effects of BMPs are mediated by their downstream signaling pathways that are initiated by binding of BMP to transmembrane serine/threonine kinase receptors. Two types of BMP receptors exist: type I and type II (Zhang et al., 2014). Receptors of both types are indispensable to form a functional complex to initiate downstream signaling events. BMPs can trigger two main downstream signaling pathways through binding to different receptor complexes: Smad-dependent and Smad-independent signaling pathways. In Smad-dependent signaling pathway, BMP receptors phosphorylate Smad1/5/8, which subsequently assembles into a complex with Smad4 and translocated to the nucleus, regulating the transcription of target genes, such as *RUNX2* and *Osterix*. In addition, Smad-independent pathways, including ERK, c-Jun N-terminal kinase (JNK), and p38 are also activated (Derynck & Zhang, 2003). Hitherto, it remains unclarified how heterodimeric BMP induces significantly higher osteoblastogenesis of MC3T3-E1 pre-osteoblasts. A hypothesis – ligand/receptor binding affinity – has been put forward to explain the phenomenon. Different homodimeric BMP ligands exhibit different affinities to both types of receptors. For example, homodimeric BMP-2 bears high affinities to type I receptors, whereas low affinities to type II receptors (Kirsch et al., 2000; Sebald et al., 2004). In contrast, homodimeric BMP-6 and BMP-7 have high affinities to type II receptors, whereas medium or low affinity to type I receptors (Allendorph et al., 2007; Isaacs et al., 2010). It has already been shown that heterodimeric BMP-2/6 simultaneously bears high affinity to both type I and type II receptors (Isaacs et al., 2010). Consequently, it has been speculated that the simultaneous possession of high affinity to both types of receptors could confer more rapid and stable formation of receptor complexes.

In this study, BMP-2/7 induced a higher level of phosphorylated Smad1/5/8 and p38 than BMP-2, but not than BMP-7 in MC3T3-E1. Three BMPs induced a similar level of phosphorylated JNK, which was significantly higher than the control (no BMP). Furthermore, BMP-2/7 induced significantly higher levels of phosphorylated ERK1/2 than BMP-2 or BMP-7 (Figure 5). In contrast, in hASCs, the phosphorylated Smad1/5/8 induced by BMP-2/7 was lower than BMP-2 or BMP-7. No significantly elevated levels of phosphorylated ERK1/2 or p38 or JNK were detected in hASCs under the stimulation of BMP-2/7. Two potential mechanisms might account for these phenomena: (1) the BMP receptor components on hASCs might be different from those on MC3T3-E1 cells; (2) the phosphorylation duration of the main signaling pathway proteins might be affected by some intracellular molecules in hASCs. Further studies are highly needed to clarify the potential molecular mechanisms.

In fact, ERK signaling pathway was shown to be highly important in the process of osteogenic differentiation. For example, when BMSCs were exposed to continuous mechanical strain (CMS), the phosphorylated level of ERK1/2 increased significantly accompanied with enhanced osteogenic differentiation, and inhibition of ERK signaling with U0126 blocked the osteogenic events (Zhang et al., 2012). Moreover, priming with tumor necrosis factor alpha (TNF-α) can promote the osteogenesis of hASCs, and stimulated ERK and p38 signaling pathways, and inhibition of ERK signaling reduced the endogenous BMP-2 levels and osteogenic differentiation (Lu et al., 2013). In line with these findings, our research demonstrated U0126 inhibited the osteogenic effects of hASCs triggered by BMP-2/7 and its homodimers. However, ERK signaling seemed to be a signaling that was generally important for the osteogenic differentiation of hASCs but not specifically for the effects of BMPs irrespective of their dimerization types. In contrast, U0126 failed to

compromise the osteogenic differentiation of MC3T3-E1 cells induced by OM or BMP-2 or BMP-7 although BMP-2/7 could significantly enhance the phosphorylated level of ERK1/2 in this type of cells. This finding suggested that ERK signaling might not be important for the general or BMP-homodimer-induced osteogenic differentiation of MC3T3-E1 cells as that in hASCs. On the other hand, U0126 could significantly compromise the enhancing effect of BMP-2/7 on the osteogenic differentiation of MC3T3-E1 cells, which indicated that ERK signaling was specifically of paramount importance for the osteogenic effect of BMP-2/7. This finding was consistent with the results that phosphorylated ERK1/2 was much more dramatically enhanced by BMP-2/7 than by BMP-2 or BMP-7 in MC3T3-E1, while BMP-2/7 was not advantageous in inducing phosphorylated ERK1/2 over BMP-2 or BMP-7 in hASCs. The different signaling pattern may account for the different effects of BMP-2/7 in inducing the osteogenic differentiation of MC3T3-E1 and hASCs.

Conclusion

In conclusion, our study indicated that heterodimeric BMP-2/7 was not a stronger osteogenic inducer when compared with BMP-2 or BMP-7 in hASCs as that for MC3T3-E1, and ERK signaling pathway might play a role in the different effects of BMP-2/7 between hASCs and MC3T3-E1. Our results demonstrated that when using heterodimeric BMP-2/7 as an osteoinductive agent, it is important to take the cell type into consideration before drawing a conclusion.

Disclosure statement

No potential conflict of interest was reported by the authors.

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