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ORIGINAL RESEARCH PAPER

BMP-2, VEGF and bFGF synergistically promote the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells

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Abstract Mesenchymal stem cells (MSCs) were treated with bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) dose-dependently and time-dependently. Together they caused a strong synergistic effect on the osteogenic differentiation of MSCs, with lower concentrations of each factor being enough to show the synergistic promotion (50 ng BMP-2/ml, 1 ng VEGF/ml and 10 ng bFGF/ml). When both VEGF and bFGF were added in the early proliferating stage (the first 7 days) and BMP-2 was added in the late differentiation stage (the last 7 days), osteogenic differentiation of MSCs could be enhanced more effectively.

Keywords BMP-2 · bFGF · MSC · Osteogenic differentiation · VEGF

Introduction

Mesenchymal stem cells (MSCs) differentiate into a variety of cell types, such as osteoblasts, chondrocytes and adipocytes (Pittenger et al. 1999). Because of their good osteogenic differentiation capability and easy isolation, one of the best candidates for bone tissue engineering (Petite et al. 2000).

Differentiation of MSCs depends on culture conditions including supplementation of agents, hormones and growth factors (Bianco and Robey 2001). Bone morphogenetic proteins (BMPs) are the most potent inducers of osteoblastic differentiation (Chen et al. 2004). Although there are a large number of distinct forms of BMPs, BMP-2 is the most widely studied. BMP-2 induces osteogenic differentiation of multipotential mesenchymal cells and osteoblasts. (Kim et al. 2012; Song et al. 2011). VEGF, an angiogenic factor, is also involved in osteogenesis and bone repair by stimulating survival, recruitment and migration of major bone forming cells (Orlandini et al. 2006; Kempen et al. 2009). bFGF, a mitogen for various cells, regulates proliferation, differentiation and mineralization of bone cells (Huang et al. 2010; Fei et al. 2011).

Although many studies have used BMP-2, VEGF and bFGF to enhance the osteogenic differentiation of MSCs, the dose-dependent and time-dependent effects of these factors on osteogenic differentiation of MSCs have been little investigated. The differentiation of MSCs requires the synergistic action of several growth factors at distinct stages. Therefore, temporal relationships of growth factors supplied are very important, which could potentially hasten bone formation. In this study, we investigated the optimal dose and timing

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for ternary combination of BMP-2, VEGF and bFGF for the osteogenic differentiation of rat MSCs.

Materials and methods

Reagents

BMP-2, VEGF and bFGF were purchased from Pepro-Tech Inc. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium containing low glucose (DMEM-LG) were purchased from Gibco BRL. Fisher 344 rats were purchased from Experimental Animal Center of Sichuan University. All other chemicals and reagents were supplied by Sigma-Aldrich.

MSCs isolation and culture

MSCs were obtained from the bone marrow of femurs and tibias of 6 week-old rats. Briefly, adherent soft tissues and epiphyses were removed. The marrow were harvested by inserting a syringe needle into one end of the bone and flushing with complete medium (DMEM-LG supplemented with 1 % w/v antibiotics and 10 % v/v FBS) into a 60 mm culture dish. The cells were then centrifuged, counted, seeded at 5×10^7 per 100 mm culture dish and cultured at 37 °C in 95 % humidified air and 5 % CO_2. The third passage cells were used in all the experiments.

Induction of osteogenesis

For osteogenic induction, cells were plated and cultured in the osteogenic media (OM: DMEM media containing 1 % antibiotics, 10 % FBS, 0.28 mM ascorbic acid 2-phosphate, 100 nM dexamethasone and 10 mM β-glycerophosphate). To examine the dose-dependent effects of combined treatments, MSCs were treated with three conditions: (1) a fixed dose of VEGF/bFGF (10/10 ng/ml) and three doses of BMP-2 (10, 50 and 100 ng/ml); (2) a fixed dose of BMP-2/bFGF (50/10 ng/ml) and three doses of VEGF (1, 10 and 25 ng/ml); (3) a fixed dose of BMP-2/VEGF (50/25 ng/ml) and three doses of bFGF (1, 10 and 20 ng/ml). In addition, to assess the time-dependent effects of combined treatments, MSCs were treated continuously with any two factors of BMP-2, VEGF and bFGF (50 ng BMP-2/ml, 10 ng VEGF/ml and 10 ng bFGF/ml) for 14 days and the former factor was added under three conditions: (1) treated from days 1 to 7; (2) treated from days 8 to 14; and (3) treated from days 1 to 14.

ALP activity and Alizarin red S staining

To quantify alkaline phosphatase (ALP) enzymatic activity, MSCs were cultured for 7 days, washed twice with PBS and lysed with a lysis buffer. Their enzymatic activities were evaluated at 520 nm using ALP activity kits. For ALP activity staining, MSCs layers were washed twice and fixed with 4 % paraformaldehyde, then rinsed and stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. For Alizarin red S staining, MSCs were washed twice with PBS and fixed with 4 % paraformaldehyde, then rinsed and stained with Alizarin red S for 10 min. They were extracted using 10 % (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for quantification. The concentration was evaluated at 450 nm. All values are expressed as the-fold changes over the control.

RNA extraction and real-time PCR

Type-1 collagen and osteocalcin gene expression were examined by real-time quantitative PCR. Total RNA was extracted from MSCs with Trizol. Then, total RNA was reverse transcribed into cDNA and used for the PCR template. The special primer sequences and product sizes were listed in Supplementary Table 1. To account for differences in the efficiency of reverse transcription between samples, GAPDH was used as an internal control.

Statistical analysis

All results were expressed as mean values ± standard deviation. Statistical differences were analyzed by two-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

Results

Dose-dependent effects of BMP-2, VEGF and bFGF combination on the osteogenic differentiation of MSCs

When different doses of BMP-2 (10, 50 and 100 ng/ml) were used along with a fixed dose of VEGF/bFGF

(10/10 ng/ml), 100 ng BMP-2/ml significantly enhanced ALP activity compared with the control (treatment with VEGF/bFGF only, Fig. 1a, c). BMP-2/ml at 50 ng significantly enhanced mineralization (Fig. 1a, d) and expression of type-1 collagen and osteocalcin (Fig. 1e, f). Although BMP-2 had no significant effect on MSCs proliferation (Supplementary Fig. 1a), synergistic effects of BMP-2 and VEGF/bFGF on the osteogenic differentiation of MSCs were clearly demonstrated.

When different doses of VEGF (1, 10 and 25 ng/ml) were treated along with a fixed dose of BMP-2/bFGF (50/10 ng/ml), 25 ng VEGF/ml significantly increased ALP activity compared with the control (treatment with BMP-2/bFGF only, Fig. 2a, c). VEGF at 1 ng/ml significantly improved mineralization, type-1 collagen and osteocalcin expression (Fig. 2a–f). All three doses of VEGF significantly enhanced MSCs proliferation (Supplementary Fig. 1b).

When different doses of bFGF (1, 10 and 20 ng/ml) were treated along with a fixed dose of BMP-2/VEGF (50/10 ng/ml), 10 ng bFGF/ml significantly increased ALP activity and osteocalcin expression compared with the control (treatment with BMP-2/VEGF only, Fig. 3a, c, f). bFGF significantly enhanced mineralization (Fig. 3a, d) and proliferation (Supplementary Fig. 2c) of MSCs.

Time-dependent effects of BMP-2, VEGF and bFGF combination on the osteogenic differentiation of MSCs

When VEGF and bFGF were constantly added, BMP-2 added for the last 7 days was more effective than added for the first 7 days and whole 14 days, showing a strong synergistic effect (Fig. 1b, g–j). Conversely, when BMP-2 and bFGF were constantly added, application of VEGF in the first 7 days had a significant effect on MSCs osteogenic differentiation (Fig. 2b, g–j). When BMP-2 and VEGF were constantly treated, bFGF supplied in the first 7 days significantly enhanced the osteogenic differentiation of MSCs (Fig. 3b, g–j).

Discussion

The results of single application of BMP-2, VEGF or bFGF suggested that the osteogenic differentiation of MSCs could be promoted by BMP-2, VEGF or bFGF, respectively (Supplementary Fig. 2). The ternary combination of BMP-2, VEGF and bFGF exhibited a strong synergistic effect on the osteogenic differentiation of MSCs, with the low concentration of each factor (50 ng BMP-2/ml, 1 ng VEGF/ml and 10 ng bFGF/ml) being enough to show the synergistic promotion. These results indicated that the osteogenic differentiation of MSCs may be stimulated by the concerted actions of these growth factors and the positive interactions between these factors. Biological effects of VEGF, bFGF and BMP-2 were modulated by each other and the complex inter-relationships among these growth factors undoubtedly altered cell gene expression and cell function.

As shown in Fig. 4, the VEGF–VEGFRs interaction activated the MAPK/ERK pathway, which ultimately affected cells proliferation and gene expression of bFGF and BMP-2 in nucleus (Ball et al. 2007; Peng et al. 2005). bFGF–FGFRs interaction also activated the MAPK/ERK pathway and finally promoted cells proliferation and gene expression of VEGF, BMP/R and the bone-related transcription factor Runx2 (Deschaseaux et al. 2009; Dudley et al. 1999). BMP–BMPRs interaction activated and phosphorylated Smad 1, 5 and 8. The activated R-Smads could be assembled into a heteromeric complex with Smad4 in the cytoplasm. The heteromeric complex was translocated into nucleus and participated in the modulation of target gene expression, including bFGF, VEGF, Runx2/Cbfa 1 (Miyazono et al. 2005; Ryoo et al. 2006). Therefore, the coordination of BMP, VEGF and FGF might be accomplished by the regulation of each other's signaling pathways. All these complex interactions of different factors ultimately resulted in the synergistic promotion of MSCs differentiation.

When both VEGF and bFGF were added in the early proliferating stage and BMP-2 was added in the late differentiation stage, osteogenic differentiation of MSCs could be enhanced effectively. Interestingly, continuous supplementation of BMP-2, which is known as the most potent osteoblastic inducer during the entire culture period, did not show significant stimulating effects on the osteogenic differentiation of the MSCs. These results demonstrated the importance of VEGF and bFGF addition in the proliferating phase of MSCs culture, which might result from the positive effects of VEGF and bFGF on MSCs proliferation and some osteogenic genes expression. On one hand,

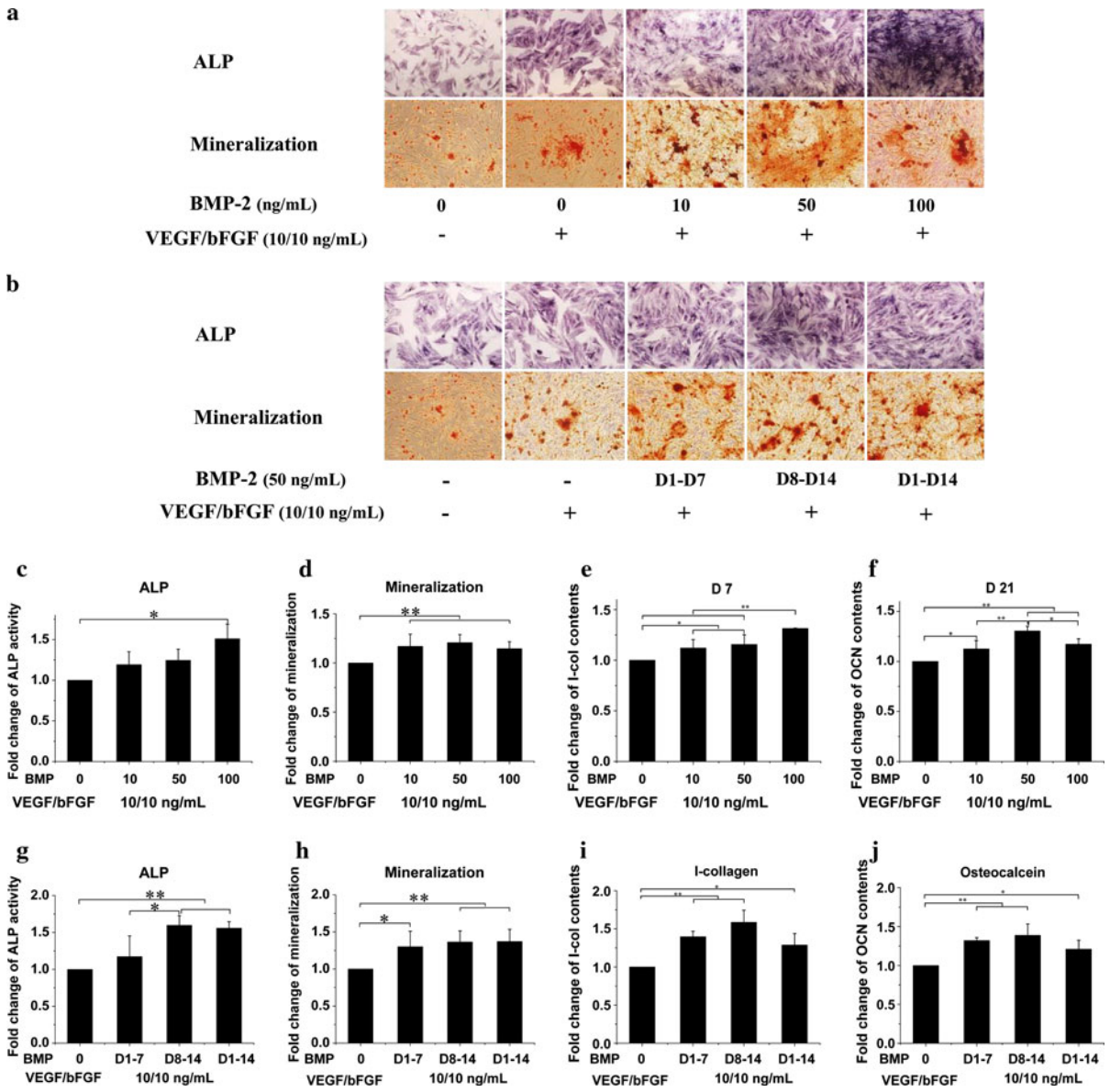

Fig. 1 Dose-dependent and time-dependent effects of BMP-2 combination on VEGF/bFGF. **a** Dose-dependent effects of BMP-2 on ALP staining on the 7th day and Alizarin red S staining on the 21st day. **b** Time-dependent effects of BMP-2 on ALP staining and Alizarin red S staining on the 14th day. **c–f** Dose-dependent effects of BMP-2 on ALP activity, mineralization, gene expression of type-1 collagen and osteocalcin. **g–j** Time-dependent effects of BMP-2 on ALP activity, mineralization, gene expression of type-1 collagen and osteocalcin ($N = 6$; $^{**}P < 0.01$; $^{*}P < 0.05$)

effects of VEGF and bFGF on proliferation in the early stage increased the total number of cells, which were competent for osteogenic differentiation and maturation. On the other hand, VEGF and bFGF may also increase the expression of BMP-2 receptors on cell surfaces, which have higher binding capacity to subsequent addition of BMP-2 (Maegawa et al. 2007; Fakhry et al. 2005). Therefore, VEGF and bFGF had dual functions in early osteogenic differentiation of MSCs and they were augmented by BMP-2 in the late phase of MSCs culture.

Based on these results, the possible process of MSCs mineralization could be divided into four stages (seen in Fig. 5): (A) proliferation stage: VEGF and bFGF enhanced proliferation, recruitment and migration of MSCs. (B) Early differentiation stage: the stage

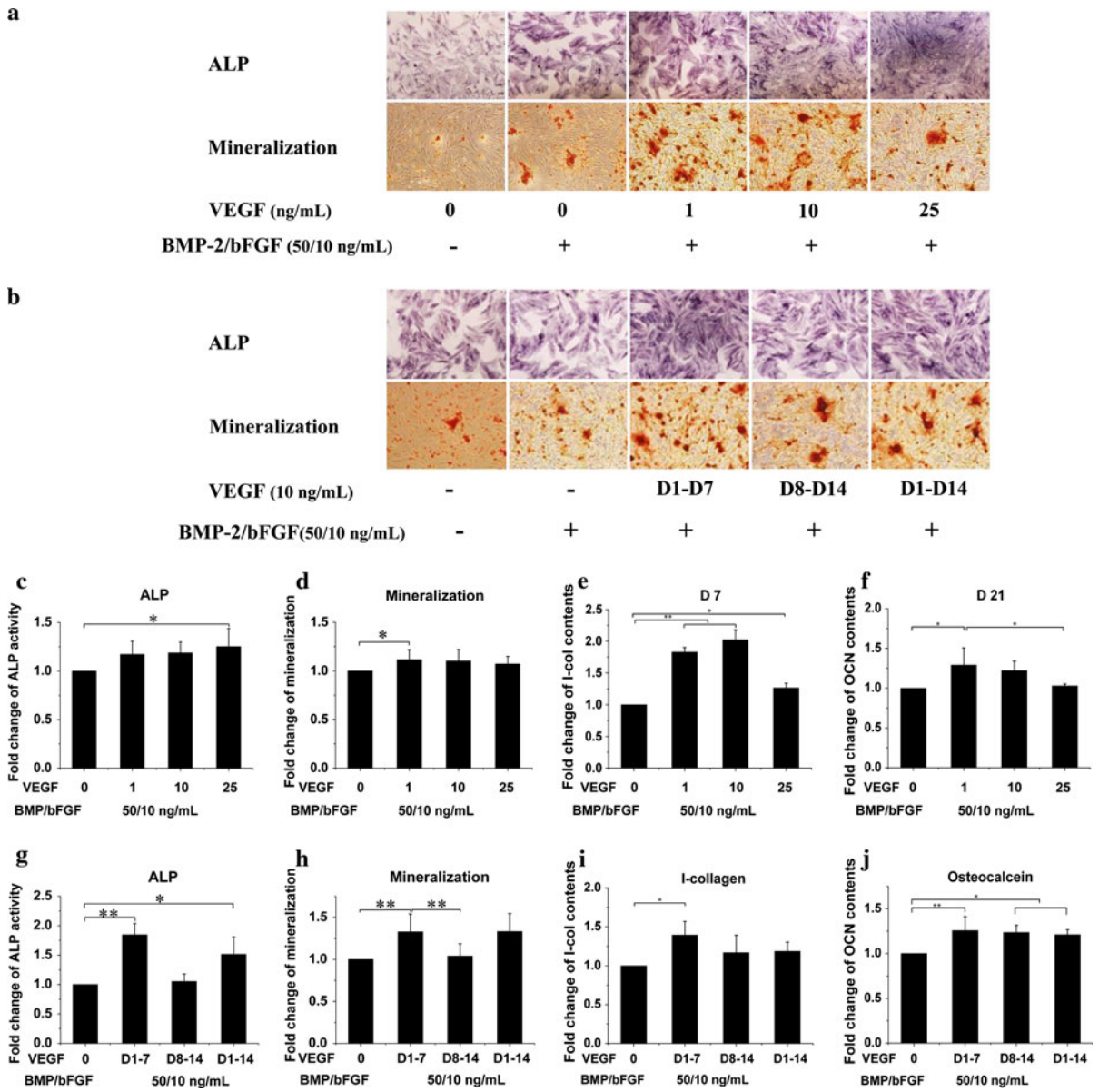

Fig. 2 Dose-dependent and time-dependent effects of VEGF combination on BMP-2/bFGF. **a** Dose-dependent effects of VEGF on ALP staining on the 7th day and Alizarin red S staining on the 21st day. **b** Time-dependent effects of VEGF on ALP staining and Alizarin red S staining on the 14th day. **c–f** Dose-dependent effects of VEGF on ALP activity, mineralization, gene expression of type-1 collagen and osteocalcin. **g–j** Time-dependent effects of VEGF on ALP activity, mineralization, gene expression of type-1 collagen and osteocalcin ($N = 6$; $^{**}P < 0.01$; $^{*}P < 0.05$)

was regulated by VEGF, bFGF and BMP-2. VEGF and bFGF consistently promoted MSCs proliferation and recruitment. BMP-2 induced Runx2 expression, which was the switch of MSCs differentiation. In this stage, ALP was the first expression gene and followed by collagen expression (Hulliger 2003). (C) Late differentiation stage: BMP-2 played the most important role in the stage. It not only promoted osteocalcin, osteopontin and collagen expression, but also stimulated growth factors synthesis, including IGF and VEGF. Calcium was delivered from the mitochondria into the extracellular matrix (ECM) to prepare the matrix for calcification and ECM became mature in the stage. (D) Calcium nodules formation:

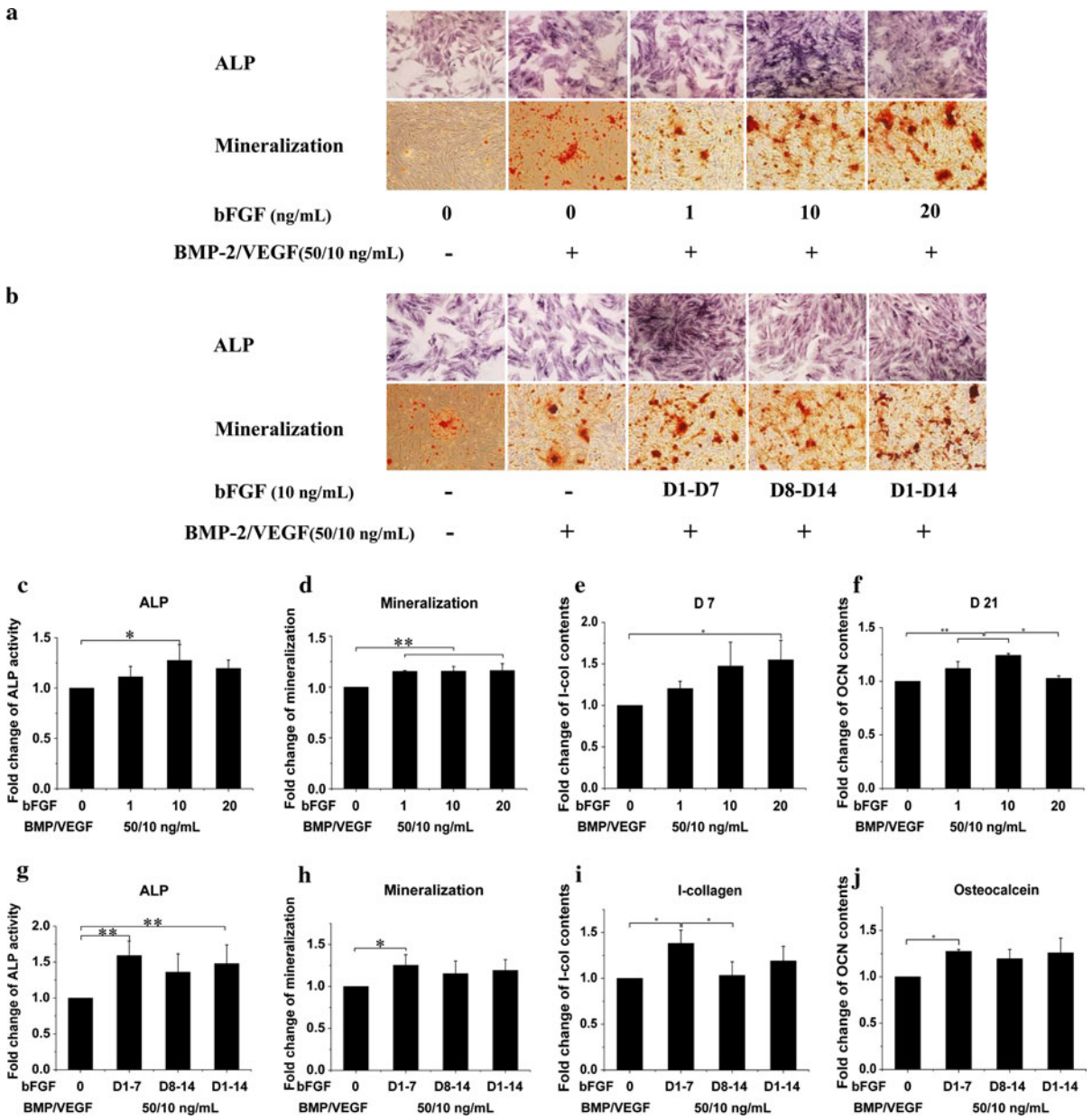

Fig. 3 Dose-dependent and time-dependent effects of bFGF combination on BMP-2/VEGF. **a** Dose-dependent effects of bFGF on ALP staining on the 7th day and Alizarin red S staining on the 21st day. **b** Time-dependent effects of bFGF on ALP staining and Alizarin red S staining on the 14th day. **c–f** Dose-dependent effects of bFGF on ALP activity, mineralization, gene expression of type-1 collagen and osteocalcin. **g–j** Time-dependent effects of bFGF on ALP activity, mineralization, gene expression of type-1 collagen and osteocalcin ($N = 6$; $^{**}P < 0.01$; $^{*}P < 0.05$)

the process included deposition of collagen and mineralization of ECM. The phosphatases provided phosphate ions to precipitate with the calcium delivered from the mitochondria and finally formed calcium nodules (Einhorn 2005). The calcium nodules consisted of low crystallized carbonate apatite, which actually existed in natural bone matrix. Thus, the mineralization process was not only a simple calcification process but also an in vitro bone formation process. Based on these results, the culture of rat MSCs with the three growth factors in a dose and time restricted manner could increase the areas or volume

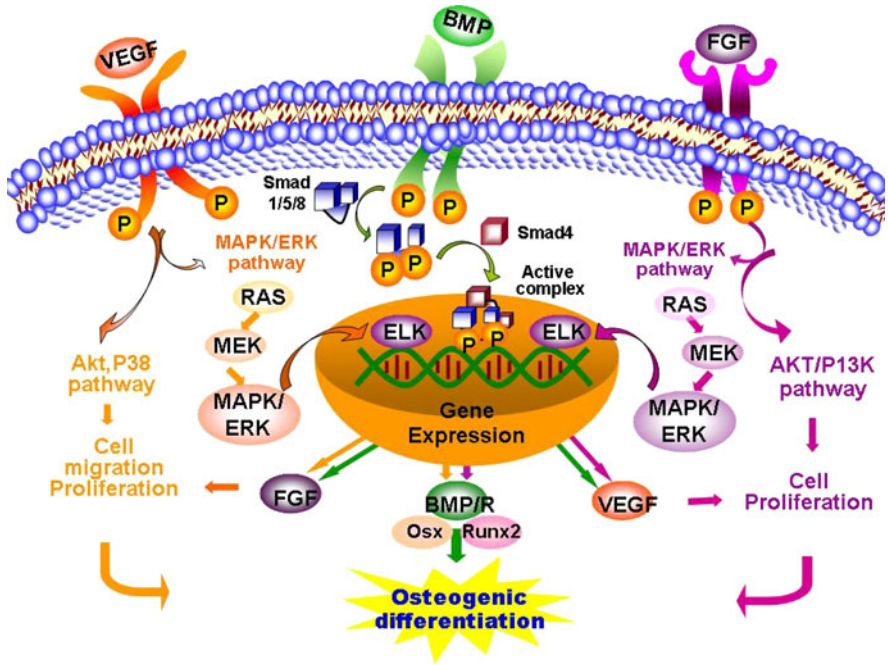

Fig. 4 Signaling pathways of VEGF, bFGF, BMP-2 and interactions between all three factors on osteogenic differentiation. VEGF, bFGF and BMP-2 regulate transcription of target gene via their respective signaling pathways and the complex interactions between these factors

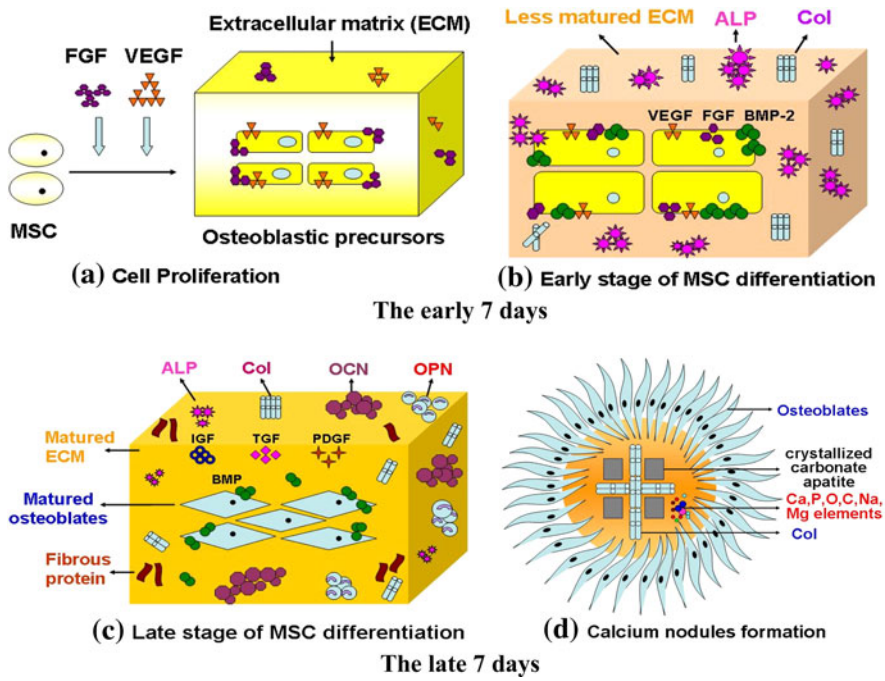

Fig. 5 Process of MSCs mineralization includes four stages. **a** Proliferation stage of MSCs. **b** Early stage of MSCs differentiation. **c** Late stage of MSCs differentiation. **d** Calcium nodules formation

of the cultured bone on the biomaterials and have great therapeutic effects in bone tissue engineering. Besides, our results had an important significance for the design of sequential delivery devices which release the best combinations of multiple growth factors at the right dose and kinetics.

In conclusion, the ternary combination of BMP-2, VEGF and bFGF could synergistically promote the osteogenic differentiation of MSCs, with low concentrations (50 ng BMP-2/ml, 1 ng VEGF/ml and 10 ng bFGF/ml) being enough to show the synergistic promotion. When both VEGF and bFGF were added

in the early proliferating stage (the first 7 days) and BMP-2 was added in the late differentiation stage (the last 7 days), the osteoblastic differentiation of MSCs could be enhanced significantly. These results might provide a basis to the development of sequential delivery systems of multiple growth factors in bone tissue engineering.

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References

Ball SG, Shuttleworth CA, Kielty CM (2007) Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. J Cell Biol 177:489–500

Bianco P, Robey PG (2001) Stem cells in tissue engineering. Nature 414:118–121

Chen D, Zhao M, Mundy GR (2004) Bone morphogenetic proteins. Growth Factors 22:233–241

Deschaseaux F, Sensébé L, Heymann D (2009) Mechanisms of bone repair and regeneration. Trends Mol Med 15:417–429

Dudley AT, Godin RE, Robertson EJ (1999) Interaction between FGF and BMP signalling pathways regulates development of metanephric mesenchyme. Genes Dev 13:1601–1613

Einhorn TA (2005) The science of fracture healing. J Orthop Trauma 19:S4–S6

Fakhry A, Ratisoontorn C, Vedhachalam C, Salhab I, Koyama E, Leboy P, Pacifici M, Kirschner RE (2005) Effects of FGF-2/-9 in calvarial bone cell cultures: differentiation stage-dependent mitogenic effect, inverse regulation of BMP-2 and noggin, and enhancement of osteogenic potential. Bone 36:254–266

Fei Y, Xiao L, Doetschman T, Coffin DJ, Hurley MM (2011) Fibroblast growth factor 2 stimulation of osteoblast differentiation and bone formation is mediated by modulation of the Wnt signaling pathway. J Biol Chem 286:40575–40583

Huang ZN, Ren PG, Ma T, Smith RL, Goodman SB (2010) Modulating osteogenesis of mesenchymal stem cells by modifying growth factor availability. Biomaterials 51: 305–310

Hulliger J (2003) Connective tissue polarity unraveled by a markov-chain mechanism of collagen fibril segment self-assembly. Biophys J 84:3501–3507

Kempen DHR, Lu L, Heijink A, Hefferan TE, Creemers LB, Maran A, Yaszemski MJ, Dhert WJA (2009) Effect of local sequential VEGF and BMP-2 delivery on ectopic and orthotopic bone regeneration. Biomaterials 30:2816–2825

Kim S, Kang YQ, Krueger CA, Sen M, Holcomb JB, Chen D, Wenke JC, Yang YZ (2012) Sequential delivery of BMP-2 and IGF-1 using a chitosan gel with gelatin microspheres enhance early osteoblastic differentiation. Acta Biomater 5:1768–1777

Maegawa N, Kawamura K, Hirose M, Yajima H, Takakura Y, Ohgushi H (2007) Enhancement of osteoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2). J Tissue Eng Regen Med 1:306–313

Miyazono K, Maeda S, Imamura T (2005) BMP receptor signaling: transcriptional targets regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16: 251–263

Orlandini M, Spreafico A, Bardelli M, Rocchigiani M, Salameh A, Nucciotti S, Capperucci C, Frediani B, Oliviero S (2006) Vascular endothelial growth factor-D activates VEGFR-3 expressed in osteoblasts inducing their differentiation. J Biol Chem 281:17961–17967

Peng H, Usas A, Olshanski A, Ho AM, Gearhart B, Cooper GM, Huard J (2005) VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone healing through modulation of angiogenesis. J Bone Miner Res 20: 2017–2027

Petite H, Viateau V, Bensaid W, Meunier A, Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G (2000) Tissue-engineered bone regeneration. Nat Biotechnol 18: 959–963

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Ryoo HM, Lee MH, Kim YJ (2006) Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. Gene 366:51–57

Song I, Kim BS, Kim CS, Ima GI (2011) Effects of BMP-2 and vitamin D3 on the osteogenic differentiation of adipose stem cells. Biochem Biophys Res Commun 408:126–131