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Original

Combined Administration of BMP-2 and HGF Facilitate Bone Regeneration through Angiogenic Mechanisms

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Abstract: Promotion of bone repair contributes to the shortening of the treatment period and improvement of the therapeutic effect in large bone defects. The purpose of the present study was to determine the optimal condition for the administration of growth factors to facilitate bone repair and explore the significance of angiogenesis in bone regeneration *in vivo*. Critical-size calvarial defects were created in the parietal bones of adult ddY mice, and the defects were treated with gelatin sponges impregnated with growth factors BMP-2, FGF-2, and HGF, alone or in combination. The bone regeneration at 1, 2, 3, and 4 weeks was evaluated using micro-computed tomography and histological observation. Critical-size calvarial defects were also created in Flk1-GFP mice to analyze angiogenesis in the early phase of bone repair. The proliferation of Flk1-GFP positive endothelial cells during bone repair was quantitatively evaluated using immunohistochemistry for Ki67. Treatment with a combination of BMP-2 and HGF (BMP-2+HGF) significantly induced more new bone formation within the bone defects compared with the other groups. Compared with BMP-2 alone, bone regeneration was rapidly increased by BMP-2+HGF from 3 weeks of the beginning of the treatment. The number of FLK1-GFP and Ki67 double positive cells in the defect areas of mice administered BMP-2+HGF at 1 week was larger than that in those administered BMP-2 alone. These results indicate that the combination of BMP-2 and HGF was markedly efficient in bone regeneration due to the promotion of angiogenesis in the early phase of bone repair.

Key words: Angiogenesis, BMP-2, Bone regeneration, Critical-size defect, HGF

Introduction

The repair of bone defects is a major clinical challenge for orthopedic and oral-maxillofacial surgeons. These days, the bone defects caused by injury, infection, tumor resection, and skeletal abnormalities are treated using autologous bone grafts, allografts, or bone prosthetic materials such as titanium, hydroxyapatites, synthetic meshes, or allogeneic demineralized bone. Autologous bone grafting is considered the gold standard because of its high safety due to the use of the patient's own tissues. Autologous grafts also show superior conduction ability and bone inductive potential for living bone cells. However, there are limitations to the quantity of bone that can be collected, and the risk of complications such as pain, deformity, infection, and postoperative absorption exists[1)]. Although quantity is not a limitation in allogeneic bone grafting, it is associated with infection, low quality of bone, and delayed healing[1)]. Bone prosthetic materials have low mechanical strength and lack bone inductive potential and symphysis[2)]. Therefore, new treatments for bone defects should be developed, which can be used as alternatives to autografting, allografting, and application of bone prosthetic materials.

Recently, one of the predominant goals in the treatment of bone defects has been to enhance bone regeneration using growth factors. Growth factors play an important role in cellular functions such as proliferation and differentiation under physical and pathological conditions, and various growth factors have been shown to promote the sequential bone development cascade[3,4)]. Among growth factors, BMP-2 is especially known to be a strong bone inducer[5-7)]. BMPs are a subfamily of the transforming growth factor (TGF)-β, and around 20 different proteins have been

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categorized as BMPs in humans. Among these, eight BMPs (BMP-1 to BMP-8a) have an osteochondral function[8] through phosphorylation of specific Smad proteins in mesenchymal stem cells[9,10]. Thus, BMPs has become an important object in biological and medical research fields associated with bone regeneration and development. Numerous studies demonstrated variable increases in bone regeneration after a single administration of BMP-2 in both large and small animal models with direct and indirect deliveries by viral or non-viral transfection[11], and the efficacy of BMP-2 on bone regeneration has been presented in clinical trials[12,13].

Additionally, to further potentiate the bone induction ability of BMP-2, a number of studies have investigated the use of other growth factors such as other BMPs, HGF, and FGFs, which have angiogenic properties, in combination with BMP-2, and demonstrated the synergistic effects either in vivo or in vitro[11,14-20]. Angiogenesis plays a vital role during bone repair/regeneration[21-23]. However, the efficacy of these growth factors on BMP-mediated osteo-/angiogenesis *in vivo* remains controversial, particularly in the field of cranial bone regeneration. Therefore, in this study, we aimed to determine the effective combination of growth factors to facilitate bone regeneration in vivo and clarified the significance of angiogenesis by using a mouse critical-size bone defect model.

Materials and Methods

Calvarial defects and surgical procedure

The mice (ddY, 8 weeks old, male) were obtained from Japan SLC (Shizuoka, Japan) and maintained in plastic cages in a room at an ambient temperature of 23 ± 1 °C with *ad libitum* access to water and standard laboratory pellet diet. Calvarial defects were created in the parietal bone of the mice using a high-speed dental drill (Portacare 21, Morita, Tokyo, Japan). The mice were anesthetized using 2.5 % isoflurane and 1L/min oxygen. After cleaning of the surgical site with povidone iodine solution, an incision was made just off the sagittal midline to expose the parietal bone. The periosteum on the parietal bone was carefully peeled off with fine forceps. Using a trephine bar (Sophia Tech, Inc. Tokyo, Japan) and saline irrigation, unilateral full-thickness calvarial defects were created in the non-suture-associated right and left parietal bone. Then, gelatin sponges (MedGel, Medgel Inc. Tokyo, Japan) with growth factors were implanted, and the skin and periosteum were restored and sewed. Regarding the size of the bone defects, since ≧ 2 mm was reportedly accepted to be the critical size for the mouse calvarial defect[24], additionally we reconfirmed that a defect 2.4 mm in size implanted with gelatin sponges and normal saline did not demonstrate significant radiographic or histological evidence of spontaneous healing after 12 weeks (data not shown). Thus, we determined that a diameter of 2.4 mm was the critical-size defect for this study. All experiments were conducted in accordance with the Protocols for the Humane Treatment of Animals of Iwate Medical University. The protocol of the animal experiment was approved by the Institutional Animal Care and Use Committee of Iwate Medical University, # 24-010, and the Institutional Recombinant DNA Experiments Safety Committee of Iwate Medical University, #308.

Preparation of gelatin sponges

MedGel PI5 or PI9 (MedGel, Medgel Inc. Tokyo, Japan) is a biodegradable gelatin hydrogel sheet for controlled release of growth factors and is degraded by hydrolysis in approximately 2–4 weeks in vivo[25-28]. Gelatin sponges were punched out as 2.4 mm diameter discs, and 5 µl of aqueous solution containing growth factors (BMP-2, 1 µg; FGF-2, 5 µg; HGF, 200 ng; and their combinations) was dropped onto the freeze-dried sponge, followed by incubation at 37℃ for 1 h before implantation. The resulting sponge was used as the gelatin incorporated with growth factors without washing. The gelatin sponge impregnated with growth factor-free normal saline was used as control.

Analysis of bone regeneration

Micro computed tomography (Micro CT) was performed at 0, 1, 2, 3, and 4 weeks after administration of growth factors using Laboratory MicroCT Scanner (GE Healthcare, London, Canada). Quantitative analysis of the bone regeneration was performed as previously described[29]. Briefly, the obtained digital images were imported into Adobe Photoshop CS2 (Adobe Systems Inc., CA). The region of interest typically encompassed 4×10^4 (200 × 200) pixels. The number of pixels of the areas with bone defects (black area) was determined using the magic wand tool (tolerance setting; 20, histogram pixel setting; cache level 1) by a single blinded investigator and confirmed by a second independent investigator. These data were then used to calculate the bone regeneration rate [(postoperative - preoperative)/ preoperative pixels × 100 %] in each injury site.

Histological observation

At 1, 2, 3, and 4 weeks, animals were sacrificed by CO_2 asphyxiation or cervical dislocation. The calvaria were harvested, formalin-fixed, decalcified in 8% EDTA, and paraffin-embedded or frozen section compound-embedded. Hematoxylin and eosin staining was performed on every 10th section throughout the sample to examine the histological features in a detailed manner.

Immunostaining

Immunostaining was performed as previously described[30,31]. Briefly, after adding blocking serum [5% normal house serum in phosphate buffer solution (PBS)], specimens were incubated with primary antibodies, Ki67 (1:100, Dako, Denmark) and ostepontin (1:100, R&D, MN), at 4°C overnight. After washing with PBS,

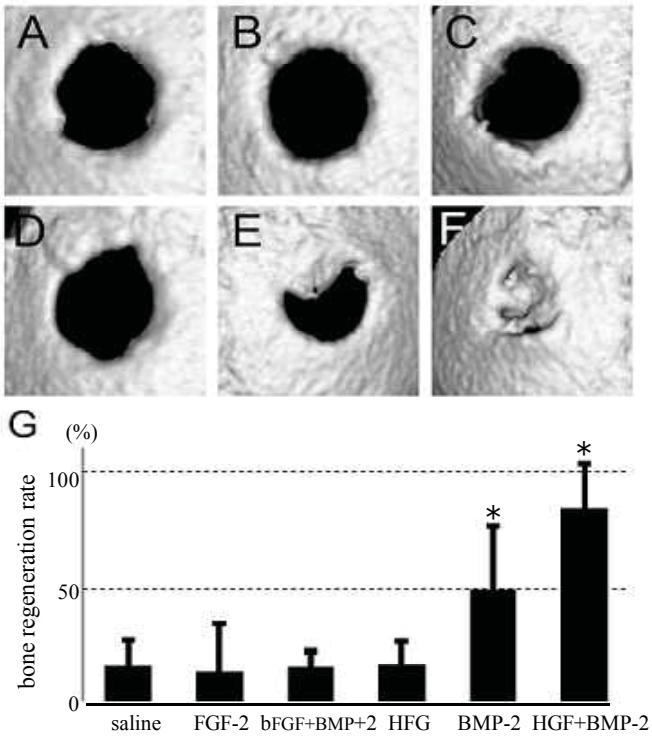

Figure 1. Calvarial healing by micro CT. Calvarial defects of 2.4 mm diameter were created in the parietal bone of ddY mice. After 4 weeks, quantitative micro CT analysis was performed. Treatment groups included normal saline (A), FGF-2; 5 μg (B), BMP-2; 1 μg + FGF-2; 5 μg (C), HGF 200 ng (D), BMP-2; 1 μg (E) and BMP-2; 1 μg + HGF; 200 ng (F). (G) Quantification of Micro CT. Bone regeneration ratio at 4 weeks was calculated from micro CT images. n = 3 per group. *P < 0.05.

specimens were incubated with a secondary antibody, Alexa Fluor 546 (1:500, Invitrogen, Japan) or MAX-PO (Rat) IgG conjugated Fab (Nichirei, Japan). Immunoreactivity was visualized with a DAB (3, 3'- diaminobenzidine) Substrate kit (Vector laboratories, Burlingame, CA, U.S.A) for 1–10 min and then counterstained lightly with hematoxylin. Specimens were observed using a BX51 microscope (Olympus, Tokyo, Japan) or confocal laser-scanning microscope (C1si, Nikon, Japan). Control specimens were incubated directly with secondary antibodies in the absence of primary antibodies and processed as above. No significant positive immunoreactivity was found in controls.

Flk1-GFP mice in vivo imaging

Flk1-GFP mice (8 weeks old, male) that were administered green fluorescent protein (GFP) combined with an Flk1 promoter[32-34] were used in the same osteogenesis experiment. On the third and seventh days, these mice were sacrificed. Their images were obtained using fluorescent stereo microscopy (MZ16FA, Leica, Germany) and confocal laser scanning microscopy (FV300, Olympus, Japan).

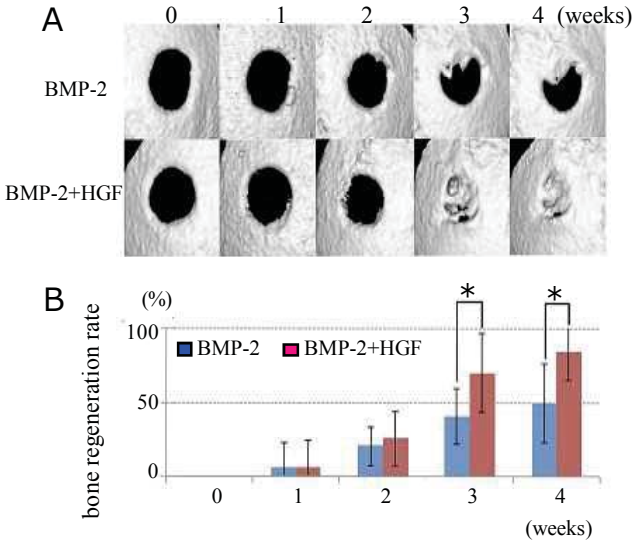

Figure 2. Comparison of the time course for calvarial healing with BMP-2+HGF and BMP-2 imaged by Micro CT. (A) Calvarial healing at 1-week intervals up to 4 weeks. (B) Quantification of healing of defects represented as an area of bone regeneration. n = 5 per group, *P < 0.05.

Statistical analysis

The mean ± standard deviation (SD) was calculated for all data. Statistical significance for the two groups was assessed using the Student's t-test. The probability level at which differences were considered significant was $P < 0.05$.

Results

Stimulatory effect of the combination of HGF and BMP-2 on bone repair

First, to find an effective combination of growth factors for bone regeneration, we implanted gelatin sponges with BMP-2, FGF-2, HGF, and their combinations in the bone defects. After 4 weeks, we assessed bone healing using micro CT. The defects treated with HGF alone (HGF), those treated with a combination of BMP-2 and FGF-2 (BMP-2+FGF-2), and those treated with FGF-2 alone (FGF-2) showed little to no healing, similar to those treated with normal saline (Fig. 1A, B, C and D). In comparison, the defects treated with BMP2 alone (BMP-2) showed some healing from the defect edges inward, but the majority of the defects showed no osseous healing (Fig. 1E). Finally, the defects treated with the combination of BMP-2 and HGF (BMP-2+HGF) showed robust bone regeneration and near complete healing as revealed by micro CT (Fig. 1F). Quantitative analysis demonstrated that the defects treated with normal saline, HGF alone, BMP-2+FGF-2, and FGF-2 alone healed by less than 20% after 4 weeks. The defects treated with BMP-2 alone showed a moderate increase in healing. Finally, the defects treated with BMP-2+HGF showed approximately 80% healing after 4 weeks of treatment (Fig. 1 G).

Next, we compared bone healing of the cranial defects treated with BMP-2 alone and those treated with BMP-2+HGF over time.

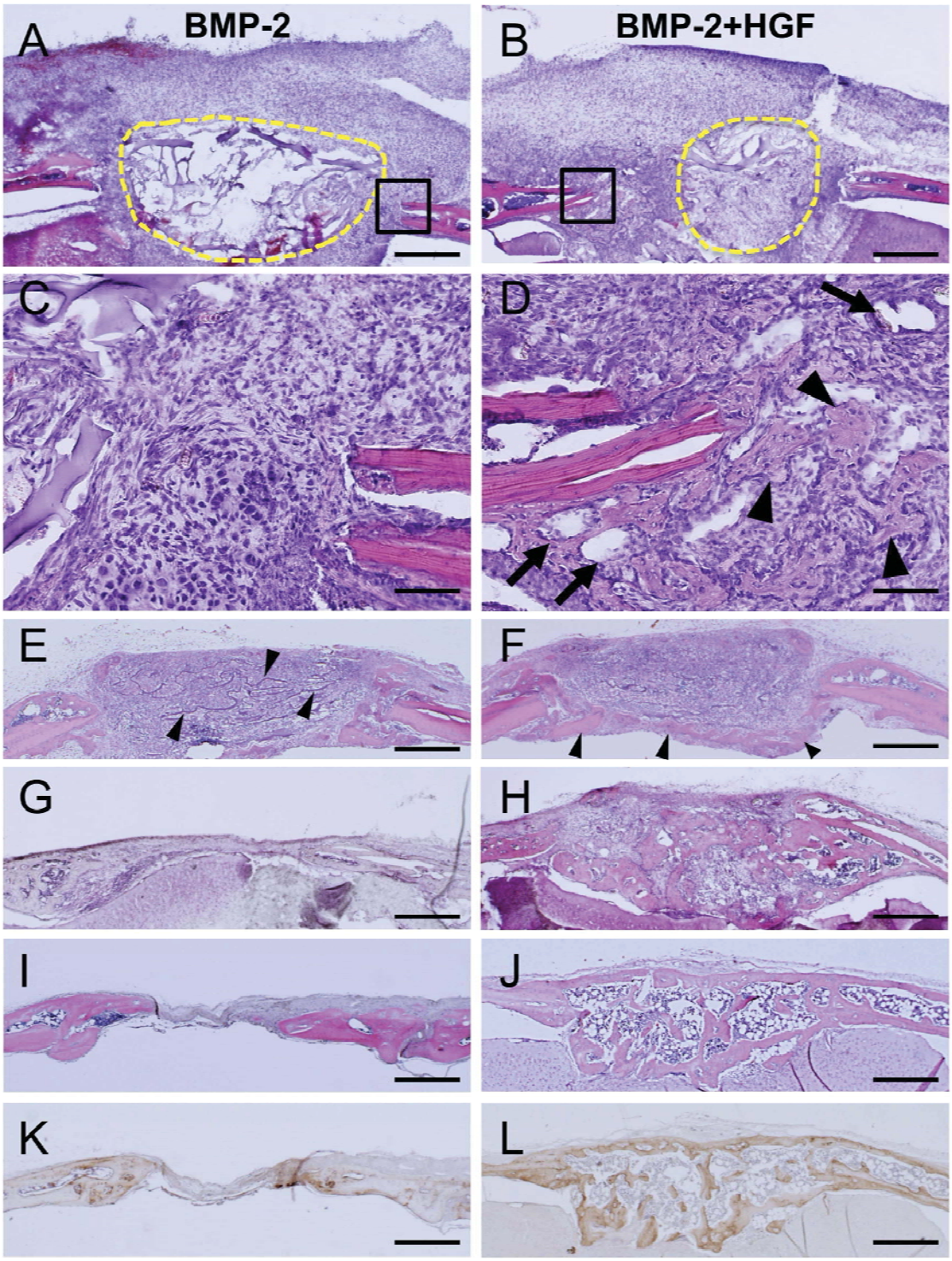

Figure 3. Calvarial healing by histology. Serial sections throughout the defect site were created at 1-week intervals up to 4 weeks postoperatively. Represented slides were stained with hematoxylin and eosin. Left side images are of the mice in the BMP-2 group, and right side images are of the mice in the BMP-2+HGF group at 1 week (A, B, C, D), 2 weeks (E and F), 3 weeks (G and H), and 4 weeks (I and J). (C) and (D) are highly magnified images of the black squares in A and B, respectively. (K) and (D) are the immunohistochemical images for osteopontin at 4 weeks. Bar = 500 μm (A, B, E, F, G, H, I, J, K and L), 100 μm (C and D)

The defects treated with both BMP-2 and BMP-2+HGF showed some increase in bone healing from defect edges inward until 2 weeks. However, while the defects treated with BMP-2 showed small peninsulas of bony regenerate and some healing of defect edges inward after 3 and 4 weeks, those treated with BMP-2+HGF showed rapid increase of bone healing inside the defect after 3 weeks, and the defect was almost filled with the reorganized mineralized tissues after 4 weeks (Fig. 2A). Quantitative analysis

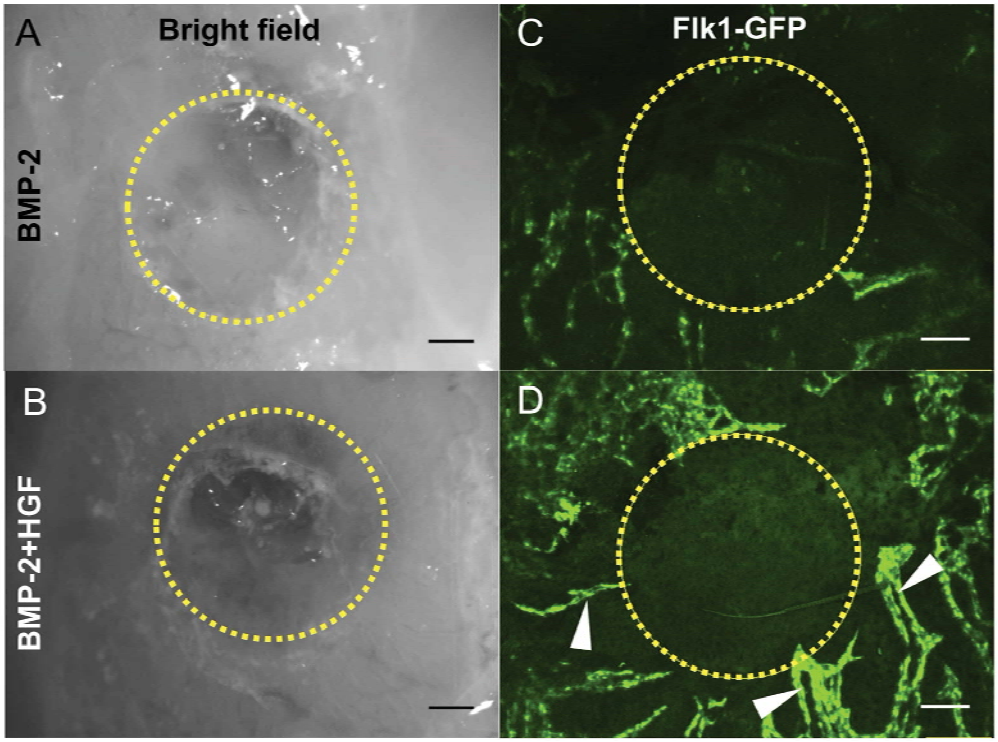

Figure 4. Flk1-GFP positive cells 3 days postoperatively. Bright field pictures were obtained by stereomicroscopy (A and B). GFP fluorescent images were obtained using confocal laser scanning microscopy in the same area (yellow dotted circles) (C and D). Arrowheads show Flk1-GFP positive cells. Bar = 500 μm

also demonstrated that BMP-2+HGF significantly increased the area of bone regeneration from 3 weeks compared with BMP-2 alone (Fig. 2B).

Histological observation of bone defects

To verify our micro CT findings and investigate the detailed effects of BMP-2+HGF and BMP-2 over time, we observed the histological changes. After 1 week, the area of the remaining gelatin sponge in the defects treated with BMP-2+HGF was smaller than those treated with BMP-2, and a massive cellular filtration into the gelatin sponge was observed in the defects treated with BMP-2+HGF (Fig. 3A and B region surrounded by a dotted yellow). Compared with those treated with BMP-2 (Fig. 3C), more formation of newly woven bone (Fig. 3D arrow heads) and blood vessels (Fig. 3D arrows) were also detected at the bony defect edge in the defects treated with BMP-2+HGF. After 2 weeks, whereas the gelatin sponge still remained in the defects treated with BMP-2 (Fig. 3E arrow heads), in those treated with BMP-2+HGF, the gelatin sponge had almost disappeared (Fig. 3F), and the formation of woven bone at the defect edge and the bottom of the regenerated tissue was much more apparent (Fig. 3F arrow heads). After 3 weeks, in the defects treated with BMP-2, whereas a little new bone formation was detected only at the defect edge, and the middle of the defect area was filled with fibrotic connective tissue (Fig. 3G), robust trabecular bone connected each side of the defect and was thickened vertically (Fig. 3H). In the defects treated with BMP-2+HGF, the bone defect area was completely filled with thick trabecular bone and bone marrow after 4 weeks (Fig. 3J). In contrast, in the defects treated with BMP-2, most of the defect area was filled with fibrous connective tissue (Fig. 3I). Furthermore, we confirmed that the regenerated bone tissue of the defects treated with BMP-2+HGF at 4 weeks was positive for osteopontin by immunostaining (Fig. 3L).

The effect of HGF on angiogenesis during bone regeneration in vivo

Next, we investigated how HGF promoted bone regeneration in vivo. Osteogenesis is closely related to angiogenesis, and HGF reportedly has strong angiogenic activity[35-37]. Besides, our data in Fig. 3 showed that BMP-2+HGF induced more blood vessels than BMP-2 alone in the early phase of bone healing (1 week). Hence we hypothesized that HGF potently facilitates bone healing by promoting angiogenesis during the early phase of bone regeneration in vivo. To verify this hypothesis, we performed the calvarial bone defect experiment using FLK1-GFP mice. On day 3, when we directly observed the defect sites, the emergence of a GFP positive area with a cord-like structure toward the bone defect edge was more prominent in the defects treated with BMP-2+HGF compared with those treated with BMP-2 (Fig. 4). On day 7, quantitative analysis of histological sections showed that the Flk1-

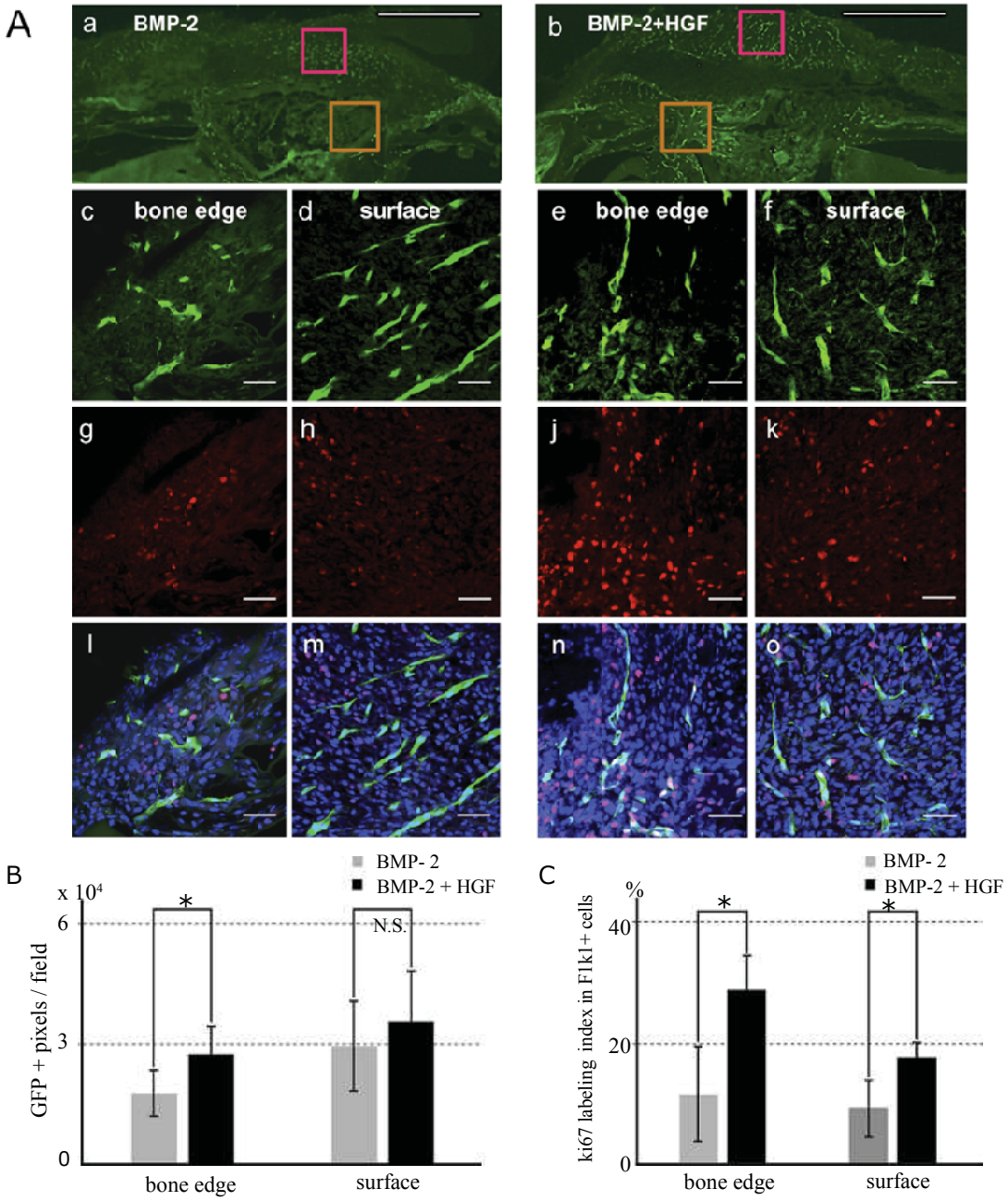

Figure 5. Location and proliferation of Flk1-GFP cells 1 week postoperatively
(A) Serial frozen sections throughout the defect site were created 1-week post operatively. Upper images (a and b) show representative low magnification images of Flk1-GFP fluorescence. Bar = 1 mm. Lower images show high magnification view of the boxed areas (orange, bone edge area; red, surface area) in the upper images. Flk1-GFP (c-f), Ki67 (g-k), and merged images of Flk1-GFP, Ki67 and DAPI (l-o). Bar = 50 μm. (B) Quantification of Flk1-GFP-positive pixel counts per field-of-view in bone edge area and surface area. (C) Quantification of the ratio of Ki67 positive cells in Flk1-GFP positive cells (Ki67 labeling index) per field-of-view in bone edge area and surface area. Data are expressed as the means ± standard deviation (n = 3 in each group). *P < 0.05.

GFP positive area was significantly wider in the defect edge area of the defects treated with BMP-2+HGF than in those treated with BMP-2 (Figure 5A and B). Moreover, the number of double positive cells for Flk-1 GFP and Ki67, which is a marker for proliferation, was higher both in the defect edge area and in the surface area of the defects treated with BMP-2+HGF than in those treated with BMP-2 (Fig. 5A and C).

Discussion

Long-term treatment of bone defects is a significant medical and social burden for patients. Therefore, one of the major goals for clinicians and scientists in the field of bone research today is

to identify new approaches to facilitate bone formation, evidenced by numerous completed and ongoing clinical trials as well as basic studies on growth factor administration for induction of osteogenesis. In our study, we sought to determine the optimal condition for the administration of single or combined growth factors in critical-size bone defects in mice, and demonstrated that the combination of BMP-2 and HGF was markedly efficient in bone regeneration due to the promotion of angiogenesis in the early phase of bone repair.

Our micro CT findings showed that the bone defects treated with BMP-2+HGF were filled with calcified tissue until 4 weeks after surgery, whereas the defects treated with other growth factors and their combinations were not. Further, HGF stimulated the proliferation of Flk1-GFP positive cells in the early phase of bone repair. The *Flk1 (VEGFR-2)* gene encodes a receptor for vascular endothelial growth factor A, C, D, and E[38] and is expressed abundantly in endothelial cells during the early stage of embryonic development[39]. In adults, *Flk1* expression is restricted to endothelial cells and transiently up-regulated during angiogenesis[40,41]. These results suggested that angiogenesis induced by HGF in the initial stage was a vital event to facilitate bone regeneration. HGF, originally identified as a growth-promoting factor for mature hepatocytes[42], is a multi-functional growth factor that simulates the proliferation and migration of endothelial cells and has angiogenic properties[43,44]. The HGF receptor c-Met is expressed in endothelial cells and is up-regulated after switching to angiogenic growth conditions[45]. Endothelial cells with increased c-Met expression were more responsive to HGF[45]. HGF implants in mouse subcutaneous tissue and rat corneas have resulted in an in-growth of new blood vessels[46]. It was demonstrated in a rabbit hind limb model of ischemia that HGF stimulated angiogenesis more efficiently than VEGF[47]. The growth and development of vasculature is one of the earliest events in organogenesis, and the factors and events that lead to the normal development of the embryonic vasculature are recapitulated during situations of neoangiogenesis in adults[48]. In an osteodistraction model, angiogenesis predominantly occurred before the onset of osteogenesis during bone lengthening[49]. Previous reports demonstrated that angiogenesis played a pivotal role in bone repair, that an effectual vascular supply was essential for bone regeneration to successfully occur[21-23,50], and that during the process, the early vascular response is highly sensitive to initial micromovement[51]. Conversely, inhibiting angiogenesis prevents bone fracture healing[52]. Together with these facts and our findings, it is suggested that in our experimental model, newly formed vasculature facilitated by HGF could first provide the necessary factors such as growth factors, hormones, chemokines, metabolites, and numerous cell types which contribute to osteogenesis, consequently promoting bone formation.

In addition to the angiogenic capacity, in the early phase of our bone defect model, absorption of the gelatin sponge was more accelerated in the defects treated with BMP-2+HGF compared with those treated with BMP-2. In the defects treated with BMP-2+HGF, most of the gelatin sponge was absorbed and replaced by newly formed bone with bone marrow four weeks after surgery. Since HGF is a potent antifibrotic agent[53] and facilitates the resorption of the biological scaffold[54] comprising extracellular matrix, HGF may help to promote the absorption of the gelatin sponge, which could improve the environment for bone regeneration in the defect area. However, the impact of HGF on the degradation of extracellular matrix is still under investigation.

Besides targeting endothelial cells, the influence of HGF on osteoblast differentiation is reported in several papers, although different mechanisms and even opposing effects have been described. HGF and its receptor, c-Met, are also expressed in both osteoblasts and osteoclasts[55,56]. HGF, in combination with vitamin D3, facilitates growth and differentiation of human mesenchymal stem cells into osteogenic cells[57]. At the fracture site, HGF induces the up-regulation of BMP receptors in mesenchymal cells during the early phase of fracture repair[17]. Furthermore, HGF was reported to stimulate osteoblast differentiation based on angiogenic properties in combination with hydroxyapatite[58,59]. Since endothelial cells can produce BMP-2[60] and osteoblasts secreted HGF[61] during bone repair, in our experimental model, endothelial cells activated by exogenous HGF may produce BMP-2 that can act on osteoblasts as an osteogenic signal, and in turn, the activated osteoblasts may secrete HGF to further promote angiogenesis. In contrast, inhibitory effects of HGF on osteoblast differentiation have also been proposed. HGF inhibited BMP-induced osteoblastogenesis from human mesenchymal stem cells in vitro[19]. HGF also displayed significantly reduced ectopic bone formation in muscle induced by BMP-2, while treatment with HGF prior to the administration of BMP-2 did not influence subsequent osteoblast differentiation induced by BMP-2[18]. These discrepancies may arise from the differences in experimental conditions such as cells and animals, the dose of growth factors, and variations in surgical procedures. Hence, the mechanism whereby HGF regulates osteoblast differentiation should be assessed further.

Our data indicate the potential application of the combination of HGF and BMP-2 for treating bone defects, and the importance of the induction of angiogenesis in the early phase of bone healing. Use of this method could shorten the duration of treatment for bone defect, which would achieve physical and mental well-being of patients and reduce their economic burden. However, before clinical application, challenges to be addressed still remain. Because the critical sequential timing and the optimal dose of the secreted factors such as HGF and BMP-2 tightly regulate the reparative/regenerative mechanism during bone repair, the appropriate timing and dosing should be elucidated, and a suitable

scaffold which releases the factors in a spatiotemporally regulated manner to promote osteogenesis should be developed. Safety concerns should also be evaluated, for example, whether or not to generate tumors and induce an intense inflammation response. To clarify the precise mechanism of bone regeneration, further studies are necessary to determine the molecular mechanism by which other factors regulate angiogenesis in the early phase of bone healing.

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References

1. Oreffo R and Triffitt J. Future potentials for using osteogenic stem cells and biomaterials in orthopedics. Bone 25: 5S-9S, 1999
2. Bauer TW and Muschler GF. Bone graft materials: an overview of the basic science. Clin Orthop Relat Res 371: 10-27, 2000
3. Teti A. Bone development: overview of bone cells and signaling. Curr Osteoporos Rep 9: 264-273, 2011
4. Long F. Building strong bones: molecular regulation of the osteoblast lineage. Nat Rev Mol Cell Biol 13: 27-38, 2012
5. Li X and Cao X. BMP signaling and skeletogenesis. Ann NY Acad Sci 1068: 26-40, 2006
6. Urist MR and Strates BS. Bone morphogenetic protein. J Dent Res 50: 1392-1406, 1971
7. Kim CS, Kim JI, Kim J, Choi SH, Chai JK, Kim CK and Cho KS. Ectopic bone formation associated with recombinant human bone morphogenetic proteins-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers. Biomaterials 26: 2501-2507, 2005
8. Even J, Eskander M and Kang J. Bone morphogenetic protein in spine surgery: current and future uses. J Am Acad Orthop Sur 20: 547-552, 2012
9. Ducy P and Karsenty G. The family of bone morphogenetic proteins. Kidney Int 57: 2207-2214, 2000
10. Chen G, Deng C and Li YP. TGF-β and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci 8: 272-288, 2012
11. Gothard D, Smith E, Kanczler J, Rashidi H, Qutachi O, Henstock J, Rotherham M, El Haj A, Shakesheff K and Oreffo R. Tissue engineered bone using select growth factors: A comprehensive review of animal studies and clinical translation studies in man. Eur Cells Mater 28: 166-208, 2014
12. Govender S, Csimma C, Genant HK, Valentin-Opran A, Amit Y, Arbel R, Aro H, Atar D, Bishay M and Börner MG. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg 84: 2123-2134, 2002
13. Katayama Y, Matsuyama Y, Yoshihara H, Sakai Y, Nakamura H, Imagama S, Ito Z, Wakao N, Kamiya M and Yukawa Y. Clinical and radiographic outcomes of posterolateral lumbar spine fusion in humans using recombinant human bone morphogenetic protein-2: an average five-year follow-up study. Int Orthop 33: 1061-1067, 2009
14. Maegawa N, Kawamura K, Hirose M, Yajima H, Takakura Y and Ohgushi H. 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, 2007
15. Nakamura Y, Tensho K, Nakaya H, Nawata M, Okabe T and Wakitani S. Low dose fibroblast growth factor-2 (FGF-2) enhances bone morphogenetic protein-2 (BMP-2)-induced ectopic bone formation in mice. Bone 36: 399-407, 2005
16. Singhatanadgit W, Salih V and Olsen I. Up-regulation of bone morphogenetic protein receptor IB by growth factors enhances BMP-2-induced human bone cell functions. J Cell Physiol 209: 912-922, 2006
17. Imai Y, Terai H, Nomura-Furuwatari C, Mizuno S, Matsumoto K, Nakamura T and Takaoka K. Hepatocyte growth factor contributes to fracture repair by upregulating the expression of BMP receptors. J Bone Miner Res 20: 1723-1730, 2005
18. Kawasaki T, Niki Y, Miyamoto T, Horiuchi K, Matsumoto M, Aizawa M and Toyama Y. The effect of timing in the administration of hepatocyte growth factor to modulate BMP-2-induced osteoblast differentiation. Biomaterials 31: 1191-1198, 2010
19. Standal T, Abildgaard N, Fagerli UM, Stordal B, Hjertner O, Borset M and Sundan A. HGF inhibits BMP-induced osteoblastogenesis: possible implications for the bone disease of multiple myeloma. Blood 109: 3024-3030, 2007
20. Samee M, Kasugai S, Kondo H, Ohya K, Shimokawa H and Kuroda S. Bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) transfection to human periosteal cells enhances osteoblast differentiation and bone formation. J Pharmacol Sci 108: 18-31, 2008
21. Cavadias A and Trueta J. An Experimental study of the vascular contribution to the callus of fracture. Surg Gynecol Obstet 120: 731-747, 1965
22. Carano RA and Filvaroff EH. Angiogenesis and bone repair. Drug Discov Today 8: 980-989, 2003
23. Glowacki J. Angiogenesis in fracture repair. Clin Orthop Relat Res 355: S82-S89, 1998
24. Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, Quarto N, Contag CH, Wu B and Longaker MT. Adipose-

derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol 22: 560-567, 2004

25. Tabata Y, Nagano A, Muniruzzaman M and Ikada Y. In vitro sorption and desorption of basic fibroblast growth factor from biodegradable hydrogels. Biomaterials 19: 1781-1789, 1998
26. Tabata Y, Nagano A and Ikada Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. Tissue Eng 5: 127-138, 1999
27. Isogai N, Morotomi T, Hayakawa S, Munakata H, Tabata Y, Ikada Y and Kamiishi H. Combined chondrocyte–copolymer implantation with slow release of basic fibroblast growth factor for tissue engineering an auricular cartilage construct. J Biomed Mater Res A 74: 408-418, 2005
28. Tatekawa Y, Kawazoe N, Chen G, Shirasaki Y, Komuro H and Kaneko M. Tracheal defect repair using a PLGA–collagen hybrid scaffold reinforced by a copolymer stent with bFGF-impregnated gelatin hydrogel. Pediatr Surg Int 26: 575-580, 2010
29. Xu Y, Hammerick KE, James AW, Carre AL, Leucht P, Giaccia AJ and Longaker MT. Inhibition of histone deacetylase activity in reduced oxygen environment enhances the osteogenesis of mouse adipose-derived stromal cells. Tissue Eng A 15: 3697-3707, 2009
30. Otsu K, Kishigami R, Oikawa-Sasaki A, Fukumoto S, Yamada A, Fujiwara N, Ishizeki K and Harada H. Differentiation of induced pluripotent stem cells into dental mesenchymal cells. Stem Cells Dev 21: 1156-1164, 2012
31. Otsu K, Kishigami R, Fujiwara N, Ishizeki K and Harada H. Functional role of rho-kinase in ameloblast differentiation. J Cell Physiol 226: 2527-2534, 2011
32. Ishitobi H, Matsumoto K, Azami T, Itoh F, Itoh S, Takahashi S and Ema M. Flk1-GFP BAC Tg mice: an animal model for the study of blood vessel development. Exp Anim 59: 615-622, 2010
33. Ema M, Takahashi S and Rossant J. Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. Blood 107: 111-117, 2006
34. Ema M, Faloon P, Zhang WJ, Hirashima M, Reid T, Stanford WL, Orkin S, Choi K and Rossant J. Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. Genes Dev 17: 380-393, 2003
35. Silvagno F, Follenzi A, Arese M, Prat M, Giraudo E, Gaudino G, Camussi G, Comoglio PM and Bussolino F. In vivo activation of met tyrosine kinase by heterodimeric hepatocyte growth factor molecule promotes angiogenesis. Arterioscler Thromb Vasc Biol 15: 1857-1865, 1995
36. Ishizawa K, Kubo H, Yamada M, Kobayashi S, Suzuki T, Mizuno S, Nakamura T and Sasaki H. Hepatocyte growth factor induces angiogenesis in injured lungs through mobilizing endothelial progenitor cells. Biochem Biophys Res Commun 324: 276-280, 2004
37. Siltanen A, Kitabayashi K, Lakkisto P, Makela J, Patila T, Ono M, Tikkanen I, Sawa Y, Kankuri E and Harjula A. hHGF overexpression in myoblast sheets enhances their angiogenic potential in rat chronic heart failure. PloS one 6: e19161, 2011
38. Ferrara N, Gerber HP and LeCouter J. The biology of VEGF and its receptors. Nat Med 9: 669-676, 2003
39. Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML and Rossant J. flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development 118: 489-498, 1993
40. Goede V, Schmidt T, Kimmina S, Kozian D and Augustin H. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 78: 1385-1394, 1998
41. Shweiki D, Itin A, Neufeld G, Gitay-Goren H and Keshet E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest 91: 2235–2243, 1993
42. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K and Shimizu S. Molecular cloning and expression of human hepatocyte growth factor. Nature 342: 440-443, 1989
43. Sengupta S, Gherardi E, Sellers LA, Wood JM, Sasisekharan R and Fan TP. Hepatocyte growth factor/scatter factor can induce angiogenesis independently of vascular endothelial growth factor. Arterioscler Thromb Vasc Biol 23: 69-75, 2003
44. Zhang YW, Su Y, Volpert OV and Woude GFV. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. Proc Natl Acad Sci USA 100: 12718-12723, 2003
45. Ding S, Merkulova-Rainon T, Han ZC and Tobelem G. HGF receptor up-regulation contributes to the angiogenic phenotype of human endothelial cells and promotes angiogenesis in vitro. Blood 101: 4816-4822, 2003
46. Bussolino F, Di Renzo M, Ziche M, Bocchietto E, Olivero factor which stimulates endothelial cell motility and growth. J Cell Biol 119: 629-641, 1992
47. Van Belle E, Witzenbichler B, Chen D, Silver M, Chang L, Schwall R and Isner J M. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. Circulation 97: 381-390, 1998
48. Carmeliet P. Angiogenesis in health and disease. Nat Med 9: 653-660, 2003
49. Sojo K, Sawaki Y, Hattori H, Mizutani H and Ueda M. Immunohistochemical study of vascular endothelial growth

factor (VEGF) and bone morphogenetic protein-2,-4 (BMP-2,-4) on lengthened rat femurs. J Craniomaxillofac Surg 33: 238-245, 2005

50. Rhinelander FW. The normal microcirculation of diaphyseal cortex and its response to fracture. J Bone Joint Surg 50: 784-800, 1968
51. Wallace A, Draper E, Strachan R, McCarthy I and Hughes S. The vascular response to fracture micromovement. Clin Orthop Relat Res 301: 281-290, 1994
52. Hausman M, Schaffler M and Majeska R. Prevention of fracture healing in rats by an inhibitor of angiogenesis. Bone 29: 560-564, 2001
53. Conway K, Price P, Harding KG and Jiang WG. The molecular and clinical impact of hepatocyte growth factor, its receptor, activators, and inhibitors in wound healing. Wound Repair Regen 14: 2-10, 2006
54. Goshima K, Nakase J, Xu Q, Matsumoto K and Tsuchiya H. Repair of segmental bone defects in rabbit tibia promoted by a complex of β-tricalcium phosphate and hepatocyte growth factor. J Orthop Sci 17: 639-648, 2012
55. Sato T, Hakeda Y, Yamaguchi Y, Mano H, Tezuka K I, Matsumoto K, Nakamura T, Mori Y, Yoshizawa K and Sumitani K. Hepatocyte growth factor is involved in formation of osteoclast− like cells mediated by clonal stromal cells (MC3T3− G2/PA6). J Cell Physiol 164: 197-204, 1995
56. Blanquaert F, Delany AM and Canalis E. Fibroblast Growth Factor-2 Induces Hepatocyte Growth Factor/Scatter Factor Expression in Osteoblasts. Endocrinology 140: 1069-1074, 1999
57. D'ippolito G, Schiller P, Perez-Stable C, Balkan W, Roos B and Howard G. Cooperative actions of hepatocyte growth factor and 1, 25-hydroxyvitamin D_3 in osteoblastic differentiation of human vertebral bone marrow stromal cells. Bone 31: 269-275, 2002
58. Zambonin G, Camerino C, Greco G, Patella V, Moretti B and Grano M. Hydroxyapatite coated with hepatocyte growth factor (HGF) stimulates human osteoblasts in vitro. J Bone Joint Surg 82: 457-460, 2000
59. Hossain M, Irwin R, Baumann M and McCabe L. Hepatocyte growth factor (HGF) adsorption kinetics and enhancement of osteoblast differentiation on hydroxyapatite surfaces. Biomaterials 26: 2595-2602, 2005
60. Bouletreau PJ, Warren SM, Spector JA, Peled ZM, Gerrets RP, Greenwald JA and Longaker MT. Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. Plast Reconstr Surg 109: 2384-2397, 2002
61. Taichman RS, Reilly MJ, Verma RS, Ehrenman K and Emerson SG. Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. Br J Haematol 112: 438-448, 2001