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GPNMB Enhances Bone Regeneration by Promoting Angiogenesis and Osteogenesis: Potential Role for Tissue Engineering Bone

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ABSTRACT

Bone regeneration is a coordinated process involving the connection between blood vessels and bone cells. Glycoprotein non-metastatic melanoma protein B (GPNMB) is known to be vital in bone formation. However, the effect of GPNMB on bone regeneration and the underlying molecular mechanism are still undefined. Fibroblast growth factor receptor (FGFR)-mediating signaling is pivotal in bone formation and angiogenesis. Therefore, we assessed GPNMB function as a communicating molecule between osteoblasts and angiogenesis, and the possible correlation with FGFR-1 signaling. Recombinant GPNMB dose-dependently increased the differentiation of human bone marrow stromal cells (hBMSCs) into osteoblasts, as well as the mRNA levels of osteoblasts marker alkaline phosphatase (ALP) and osteocalcin (OCN). Furthermore, these increases depended on the activation of FGFR-1 signaling, as pretreatment with FGFR-1 siRNA or its inhibitor SU5402 dramatically dampened GPNMB-induced osteogenesis. Additionally, GPNMB triggered dose-dependently the proliferation and migration of human umbilical vein endothelial cells (hUVECs), FGFR-1 phosphorylation, as well as capillary tube and vessels formation in vitro and in vivo. Blocking FGFR-1 signaling dampened GPNMB-induced angiogenic activity. Following construction of a rodent cranial defect model, scaffolds delivering GPNMB resulted in an evident increase in blood vessels and new bone formation; however, combined delivery of GPNMB and SU5402 abated these increase in defect sites. Taken together, these results suggest that GPNMB stimulates bone regeneration by inducing osteogenesis and angiogenesis via regulating FGFR-1 signaling. Consequently, our findings will clarify a new explanation about how GPNMB induces bone repair, and provide a potential target for bone regeneration therapeutics and bone engineering. J. Cell. Biochem. 114: 2729–2737, 2013.

KEY WORDS: BONE GENERATION; GPNMB; FGFR-1; OSTEOGENESIS; ANGIOGENESIS

Approximately 5–10% of over 6 million annual fracture patients has been reported in the United States [Hunsaker et al., 2002]. The increasing aging population will augment the demand for the treatment of bone loss. It is generally believed that the repair of large bone defects ranks as a major clinical orthopedic and plastic surgery challenge. Although diverse therapies have been applied including autografts, allografts, and artificial materials, the side effects have shed light on the limitations of the current therapies. Recently, tissue engineering approaches reveal a promising method for bone regeneration based on the delivery of osteoinductive growth factors and angiogenic factors [Kanczler and Oreffo, 2008; Qu et al., 2011]. Accordingly, the delivery of promising agents is currently considered to be an effective approach for treating osseous defects and diseases.

Glycoprotein non-metastatic melanoma protein B (GPNMB), also known as osteoactivin, is a highly glycosylated type I transmembrane protein. Human GPNMB encompasses 560 amino acids, which are encoded by a gene localized at chromosome 7p15 [Kuan et al., 2006]. GPNMB is expressed in various tissues and possesses regulatory roles in numerous cellular functions, including cell proliferation, adhesion,

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and invasion [Abdelmagid et al., 2006; Metz et al., 2007]. GPNMB was initially discovered as a novel osteoblasts-specific protein, based on the notable up-regulation of GPNMB expression in osteoporotic bone [Safadi et al., 2001]. It has been reported that GPNMB is highly expressed during osteoblasts differentiation, its mutation significantly decrease osteoblasts differentiation and mineralization [Abdelmagid et al., 2008]. Additionally, a dramatical increase in GPNMB mRNA has been observed in rat fracture model, indicating that GPNMB may elicit a critical role in bone formation and serve as a positive regulator of fracture healing [Abdelmagid et al., 2010]. However, the effect of GPNMB-induced bone regeneration and the corresponding molecular mechanism remains undefined.

Skeletal development and fracture repair are considered to rely on the close spatial and temporal connection between blood vessels and bone cells [Kanczler and Oreffo, 2008]. Angiogenesis has a critical role in the regeneration of living bone. Once vascular supply decreases, the resulting limited nutrient availability will impairs bone healing [Cinotti et al., 2013]. Fibroblast growth factor receptors (FGFRs) are pivotal in angiogenesis through their interaction with fibroblast growth receptor (FGFs) [Murakami and Simons, 2008]. It has been demonstrated that FGFR-1 is expressed in endothelial cells, while blocking FGFR-1 expression results in a prominent impairment of blood vessel development and maintenance [Presta et al., 2005]. Similarly, the angiogenic activity of FGFR has been also confirmed in chick embryos and mice [Ardi et al., 2009; Lee et al., 2013]. In addition to its angiogenic effect, FGFR signaling also exerts a predominant function in chondrogenesis and skeletal development [Marie, 2012]. Genetic studies on the FGFR pathway suggest an essential mechanism involved in controlling bone formation. For example, FGFR mutation induces bone alterations during postnatal development [Jarzabek et al., 2012].

In this study, we aimed to assess the effect of GPNMB on bone regeneration, and the correlation between GPNMB and FGFR-1 pathway underlying this process. In this study, we will demonstrate a potential application of GPNMB in bone regeneration therapeutics.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

If not otherwise mentioned, all substances were purchased from Gibco (Grand Island, NY). SU5402 (FGFR-1 inhibitor) was bought from Calbiochem (San Diego, CA). Rabbit anti-extracellular signal-regulated kinase isoform 2 (ERK-2) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and mouse anti-FGFR-1 antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-GPNMB antibodies were purchased from Abcam (Cambridge, MA). Anti-phospho-ERK1/2 antibodies were bought from Sigma (St. Louis, MO). The monoclonal antibody against phospho-FGFR-1 was obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-human vWF polyclonal antibodies were from Dako Corporation (Carpenteria, CA). Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were from Dako Cytomation (Glostrup, Denmark).

CELL CULTURE AND TREATMENTS

All human materials were handled and obtained in a standardized fashion supervised by senior scientist in our laboratory. The study was conducted in compliance with the Helsinki Declaration; all the patients gave written informed consent. Human bone marrow stromal cells (hBMSCs) were isolated from the human crest as previously described [Huang et al., 2005]. Isolated cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and penicillin. Human umbilical vein endothelial cells (hUVECs) were isolated and pooled from umbilical cords. The obtained cells were grown in M199 supplemented with 10% FBS, 5 mM L-glutamine, endothelial cell growth supplement, and 0.25 μg/ml fungizone. Before incubation with various doses of human recombinant GPNMB, cells were pretreated with SU5402 (0.5 or 1 μM). All cells were maintained at 37°C with 5% CO_2. Cells of passages 6–10 were used for all experiments.

EXPRESSION OF GPNMB IN VITRO

Total RNA was isolated from human osteoblasts using the RNAiso plus kit (Roche Diagnostics, Mannheim, Germany). The obtained RNA (~4 μg) was reverse-transcribed to synthesize first strand cDNA with the Oligo (dT) 18 primer using the cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Then, 2 μl of cDNA was used as a template in the PCR reaction mixture with gene-specific primers (sense: 5′-GCGAATTCATGGAATGTCTCTACTATTTCCTGGGAT-3′, containing ATG translation start codon and *Eco*RI restriction enzyme site; anti-sense: 5′-CGCTCGAGTCATTAAGAAACTCCTTTAAATTCTTG-3′, containing *Xho*I restriction enzyme site) for GPNMB. Following digestion with *Eco*RI and *Xho*I restriction enzymes, the GPNMB cDNA was ligated into the *Eco*RI and *Xho*I cloning site of the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) to induce GPNMB expression. The recombinant GPNMB protein was purified using Ni-NTA magnetic beads according to the manufacturer's instructions (Qiagen, Santa Clarita, CA). The purified protein was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

SiRNA TRANSFECTION

To targeted silence FGFR-1, specific siRNA fragments of FGFR-1 were designed as previously described [Cheung et al., 2011]. The siRNA strands were synthesized (GeneChem, Shanghai, China). Human BMSCs and hUVECs were separately seeded in 24-well micro-plates to reach 40–50% confluence. Cells were then transfected with 2 μg/ml FGFR-1-siRNA or 1 ml Lipofectamine™ RNAi-MAX (Invitrogen) using the GeneSilencer® siRNA transfection reagent (GeneTherapy System, San Diego, CA). About 24 h later, cells were washed and then incubated with recombinant GPNMB; the transfection efficiency was analyzed by Western blotting.

OSTEOGENIC DIFFERENTIATION OF hBMSCs AND ALIZARIN RED S STAINING

Human BMSCs were plated at a density of 3×10^5 per well in 12-well plates. Three days later (50% confluence), the indicated doses of recombinant human GPNMB were introduced into the differentiation media DMEM containing 10% FBS, 100 μg/ml streptomycin and penicillin, and 0.3 mM inorganic (sodium) phosphate. Ten days later, osteogenic differentiation was detected by staining with Alizarin Red S.

RNA EXTRACTION AND REAL-TIME PCR

Total RNA was isolated from hBMSCs using the RNAiso plus kit (Roche Diagnostics), followed by the reverse-transcribed to

synthesize first strand cDNA using the cDNA Synthesis Kit (Fermentas). The obtained cDNA was subjected to real-time PCR with specific primers for alkaline phosphatase (ALP) (sense: 5′-gcaccatgatttcaccat-3′; anti-sense: 5′-ctgggccctcagaacaggac-3′) and osteocalcin (OCN) (sense: 5′-cgagacaccatgagagcc-3′; anti-sense: 5′-gagcgacaccctagaccg-3′). The reaction conditions consisted of 10 μmol/L specific primers and 2 μl of DNA made up to a final volume of 20 μl using SYBR Premix Ex Taq™ II Kit (Taka, Otsu, Japan). For normalization, β-actin mRNA was introduced. All of the samples were performed in triplicate.

WESTERN BLOTTING

After dissociating cells with RIPA lysis buffer (Beyotime, Nantong, China), the collected total protein concentrations were measured using the BCA assay (Pierce, Rockford, IL). About 100 μl of protein were electrophoresed by SDS–PAGE, and then transferred onto a polyvinylidene difluoride (PVDF) membrane in a semi-dry trans-blot apparatus. After blocking with 5% non-fat dry milk in PBS at 4°C overnight, the nitrocellulose membranes was respectively incubated with anti-GPNMB, anti-ERK-2, anti-phospho-ERK1/2, anti-FGFR-1, or anti-phospho-FGFR-1 antibodies for 1 h at room temperature. Following three washes with TBST buffer, HRP-conjugated secondary antibodies were introduced, and LumiGLo reagent (Pierce) was used to detect the bound antibodies.

MTT PROLIFERATION ASSAYS

After preconditioning with FGFR-1-siRNA or SU5402, hUVECs were plated into 96-well plates. Human BMSCs were plated at a density of 3×10^5 per well in 96-well plates. Cells were stimulated with various doses of GPNMB for 12 h. About 20 μl of MTT stock solution (5 mg/ml) was added into each well for 4 h, following the dissolving of the formazan production in 200 μl DMSO. Results were shown as the mean of optical density values at 490 nm.

CELL MIGRATION ASSAY

Migration of hUVECs was determined by a transwell system with 8.0 μm inserts coated with Matrigel (BD Bioscience, Bedford, MA). The M199 medium consisted of control or test compounds was placed in the bottom well of chamber. Cells were trypsinized and washed three times with M199 medium, and then 1×10^5 cells were added on Matrigel-coated PET membrane in the upper compartment at 37°C. Following incubation for 6 h, migrated cells were fixed and stained with methylene blue. Total numbers of migrated cells were quantified by counting nine high-powered fields in the center of each well using an inverted light microscope (Olympus, Long Island, NY).

TUBE FORMATION ASSAY

HUVEC cells (2×10^4 per well) were plated into 12-well plates coated with Matrigel Basement Membrane Matrix (BD Bioscience) and incubated for 15 h in M199 medium containing the indicated test compounds. Cells were fixed and stained with the Diff-Quik® staining set (DADE BEHRING, Inc., Newark, DE), according to the manufacturers′ instructions. After washing three times with distilled H_2O, four areas of each sample were photographed under an inverted light microscope, and the number of tube branches was counted in triplicates.

MATRIGEL PLUG ASSAY AND IMMUNOSTAINING

The Matrigel plug assay was adapted from the literature methods [Han et al., 2013]. Six C57BL/6 mice (8 weeks old) were subcutaneously injected in the abdomen with Matrigel plugs containing the test compounds. After implantation for 7 days, mice were anesthetized, and the Matrigel plug was harvested along with the overlying skin and peritoneal membrane. Following fixed in 4% paraformaldehyde and embedded in paraffin, sections were immunohistochemically stained with anti-CD31 antibody (1:1,000) to observe the vascular visualization. The positive microvessels were visualized under an FV1000 Olympus confocal microscope and counted in six random areas by three independent individuals. This protocol conformed to the guidelines of the Institutional Animal Care and Use Committee.

PREPARATION OF CO3Ap–COLLAGEN SPONGES

Carbonate-containing apatite (CO3Ap) was synthesized as previously described [Hamada et al., 2007]. After treatment with enzymes to minimize antigenicity, the Cellgen calfskin collagen solution was neutralized with 0.05 nM NaOH, and then mixed with CO3Ap. Following frozen at −80°C for 2 h and dried for 24 h, samples were subjected to ultraviolet radiation.

BONE REGENERATION IN A RAT CRANIAL DEFECT MODEL

Animal experiments were undertaken with the approval from the Institutional Animal Care and Use Committee. In this study, 22 male Lewis rats (12 weeks old) were used. After being anesthetized with pentobarbital, a 14 mm incision was created through the skin over the cranium and periosteum, and then a circular defect 8 mm in diameter was produced symmetrically on the bilateral sides of the midline in the rat cranium with a trephine bur.

A CO3Ap-collagen sponge containing GPNMB (5 μg) and/or SU5402 (1 μM) was implanted as a graft into the tissue defect created in the rat cranium. About 2–4 weeks later, three-dimensional microcomputed (μCT) was applied to analyze bone regeneration at the defective site. After fixed with formalin and embedded in paraffin, serial 5-mm-thick sections were obtained from each specimen. H&E staining was used to observe the histological changes. Bone ingrowth was analyzed by an iSolution DT analysis system (InTechnology, Daejeon, Republic of Korea), and bone volume was shown as a percentage of mineralized bone tissue relative to the total tissue.

ANALYSIS OF BLOOD VESSEL INGROWTH

To analyze the extent of blood vessel ingrowth, serial 5-mm-thick sections were incubated with anti-vWF primary antibodies (1:300), and then HRP-conjugated secondary antibodies were added. The blood vessels immunostained with vWF were determined manually at 100× magnification in the total implant area and normalized to the implant area. All images were visualized using a Nikon Eclipse E800 light microscope and a Spot RT digital camera (Sterling Heights, MI).

STATISTICAL ANALYSIS

All results are expressed as mean ± SEM. A typical image from at least three similar experiments was presented in this study. Statistical analysis was determined by an independent Student′s *t*-test. $P < 0.05$ was considered significant.

RESULTS

EXPRESSION OF GPNMB IN VITRO

Recombinant rGPNMB was expressed in vitro using the pcDNA3.1 (+) expression vector. Following electrophoresed with SDS–PAGE, Western blotting was used to identify the expression of purified rGPNMB. As shown in Figure 1, rabbit anti-human GPNMB serum exhibited strong reactivity with the recombinant GPNMB protein samples, but not with the control ones.

GPNMB STIMULATED THE DIFFERENTIATION OF HBMSCS INTO OSTEOBLASTS IN A DOSE-DEPENDENT MANNER

To analyze the effect of GPNMB on osteogenesis of human BMSCs, the purified GPNMB protein was applied. After exposure to various doses (0, 1, 10, 50, and 100 nM) of recombinant GPNMB, we firstly detected the roles of GPNMB on cell proliferation, and found that GPNMB treatment did not significantly affect cell proliferation (Fig. 2A). However, an obvious dose-dependent increase in the differentiation of hBMSCs on day 10, indicating that direct application of exogenous GPNMB dose-dependently triggered the differentiation and mineralization of osteoblasts, as demonstrated by Alizarin red staining (Fig. 2B). It is known that ALP and OCN are generally thought to be the markers of mature, matrix-producing osteoblasts [Cinotti et al., 2013]. Following stimulation with extracellular GPNMB, similar increases in the mRNA levels of ALP and OCN were further confirmed, suggesting that exogenous application of GPNMB was sufficient to induce osteogenic marker expression (Fig. 2C).

To further discuss the underlying mechanism of GPNMB-induced osteogenesis of hBMSCs, the FGFR-1 signaling pathway was assessed. Compared with the control group, GPNMB induced the activation of FGFR-1 signaling (Fig. 2D). This increased effect was ameliorated when precondition with the FGFR-1 inhibitor, SU5402. Moreover, treatment with FGFR-1 siRNA dramatically abrogated the expression of FGFR-1 (Fig. 2E). Silencing of FGFR-1 expression significantly decreased GPNMB-induced phosphorylation of FGFR-1 and the downstream effectors ERK1/2 (Fig. 2D). Additionally, the ability of GPNMB to induce the expression of the osteogenic markers ALP and OCN was strikingly attenuated by FGFR-1 siRNA or SU5402 pretreatment (Fig. 2F). Moreover, SU5402 treatment also resulted in an obvious dose-dependent decrease in GPNMB-induced mineralization, concomitant with a decrease in pretreatment with GPNMB siRNA groups (Fig. 2G). All of these results revealed that GPNMB extracellularly triggered the osteogenesis mainly through FGFR-1 signaling pathways.

Fig. 1. Western blotting analysis of purified rGPNMB protein. GPNMB cDNA was inserted into the pcDNA3.1 (+) expression vector to obtain the rGPNMB protein. Following electrophoresed with SDS–PAGE, rabbit anti-human GPNMB polyclonal antibodies were used as the primary antibody to identify rGPNMB expression by Western blotting.

GPNMB-INDUCED ANGIOGENESIS IN VITRO AND IN VIVO

It is well known that endothelial cell proliferation and migration is essential in angiogenesis. To investigate whether GPNMB could stimulate angiogenesis, hUVECs cells were used. As shown in Figure 3A, the proliferation rate of hUVECs gradually rose with the increased dose of exogenous GPNMB. Furthermore, recombinant GPNMB triggered cell migration in a dose-dependent manner, and was about 1.7-fold over control at 100 nM-treated groups (Fig. 3B). Tube formation is required for angiogenic potency. To address the roles of GPNMB in angiogenesis, a three-dimensional capillary tube formation assay was utilized. As shown in Figure 3C, GPNMB dose-dependently stimulated the well-organized, capillary-like networks compared with control groups. These results therefore indicated that GPNMB had angiogenic properties in vitro. Subsequently, we evaluated the angiogenic activity of GPNMB in vivo by a Matrigel plug assay. Abundant fluorescent signals labeled for CD31, an endothelial marker, were measured by immunostaining compared with control group, suggesting that GPNMB evidently stimulated blood vessels formation. Taken together, these data identified GPNMB as an angiogenic factor in vitro and in vivo.

GPNMB-INDUCED ANGIOGENESIS BY FGFR-1 SIGNALING

FGFR is expressed in endothelial cells, and believed to be pivotal in angiogenesis [Murakami and Simons, 2008]. To determine whether the pro-angiogenic activity of GPNMB is correlated with FGFR-1, we used GPNMB to activate the FGFR-1 pathway. Western blotting analysis confirmed that GPNMB treatment induced the phosphorylation of FGFR-1 and the corresponding downstream molecule ERK1/2 (Fig. 4A). To further assess the correlation between GPNMB and FGFR-1, we silenced FGFR-1 expression in hUVECs by specific siRNA or SU5402, which both almost abrogated the expression of FGFR-1 (Fig. 4B). Simultaneously, little phosphorylation signals of FGFR-1 pathway was demonstrated (Fig. 4A). Furthermore, preconditioning with FGFR-1 siRNA or SU5402 significantly inhibited the proliferation of hUVECs (Fig. 4C), as well as cell migration (Fig. 4D). As expected, disruption of FGFR-1 signaling with SU5402 blocked GPNMB-mediated organization of capillary networks (Fig. 4E). Furthermore, GPNMB was no longer capable to induce tube formation when pretreatment with FGFR-1 siRNA. Therefore, our results indicated that GPNMB-induced angiogenesis mainly through the activation of FGFR-1 pathway.

GPNMB PROMOTED BONE REGENERATION IN VIVO

Based on the above results, we then assessed the possible activity of GPNMB in bone healing in vivo. After constructing a rat cranial defect model, the CO3Ap-collagen sponge was used as scaffolds to deliver GPNMB and/or SU5402 into the defective sites. As shown in Figure 5A, about 60% of the areas displayed bone healing following treatment with GPNMB in the μCT assays, which was mitigated by SU5402 (Fig. 5A). These results identified GPNMB as a pro-bone regeneration molecule acting via FGFR-1 signaling. A similar bone repair effect of GPNMB in defective areas was also

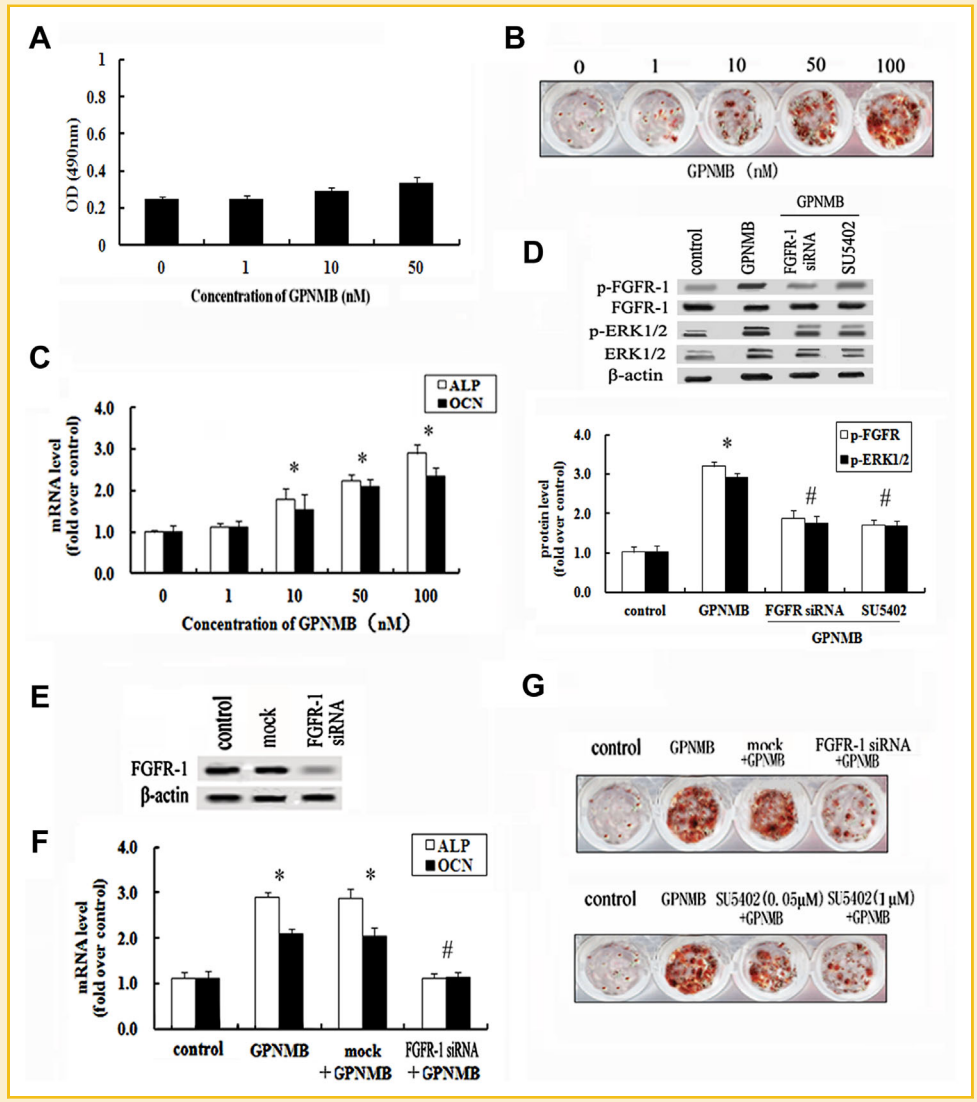

Fig. 2. GPNMB triggered the differentiation of hBMSCs into osteoblasts. HBMSCs were treated with various doses of recombinant GPNMB, and then cell proliferation was analyzed by MTT assay (A). Osteogenic differentiation and mineralization were detected 10 days later by alizarin red S staining (B). In addition, the mRNA levels of the osteogenic markers ALP and OCN were also analyzed by real-time PCR (C). The phosphorylation of FGFR-1 and its downstream effector ERK1/2 was assessed by Western blotting assay, and the corresponding quantified analysis was also performed (D). HBMSCs were transfected with FGFR-1 siRNA or pretreated with the FGFR-1 inhibitor SU5402, the corresponding silencing effect on FGFR-1 expression (E) and reduced phosphorylation of FGFR-1 signaling (D) were analyzed. Furthermore, precondition with FGFR-1 siRNA and SU5402 dampened the ALP and OCN mRNA levels (F), and decreased the differentiation of hBMSCs (G). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus GPNMB and mock-pretreated cells.

observed by H&E staining to observe the histological changes. Furthermore, vWF staining analysis indicated that GPNMB-induced abundant blood vessels formation compared with control. However, implantation of the scaffold containing SU5402 impeded GPNMB-induced angiogenesis (Fig. 5C). Thus, these data confirmed a pro-bone regeneration activity of GPNMB and the corresponding underlying mechanism in vivo.

DISCUSSION

Repair of bone defects is generally believed to be a major clinical orthopedic and plastic surgery challenge. It is known that bone is ranked as a highly vascularized tissue dependent on the cross-talk between blood vessels and bone cells. Numerous studies have focused on the combined application of pro-osteogenic and pro-angiogenic factors to promote bone formation, revealing a promising therapeutic roles for bone healing [Du et al., 2012; Franceschi, 2012; Cui et al., 2013]. Accordingly, to explore the molecules involved in angiogenesis and osteogenesis will have a potential clinical prospect in bone regeneration therapy.

Bone tissue development is a highly coordinated process, involving the interdependent processes of osteogenesis and angiogenesis [Götz et al., 2012; Portal-Núñez et al., 2012]. The multiple coordinated events between bone-forming osteoblasts and endothelial cells are vital in skeletal development and fracture repair. As an osteoblasts-specific protein, GPNMB has drawn the increasing interests in

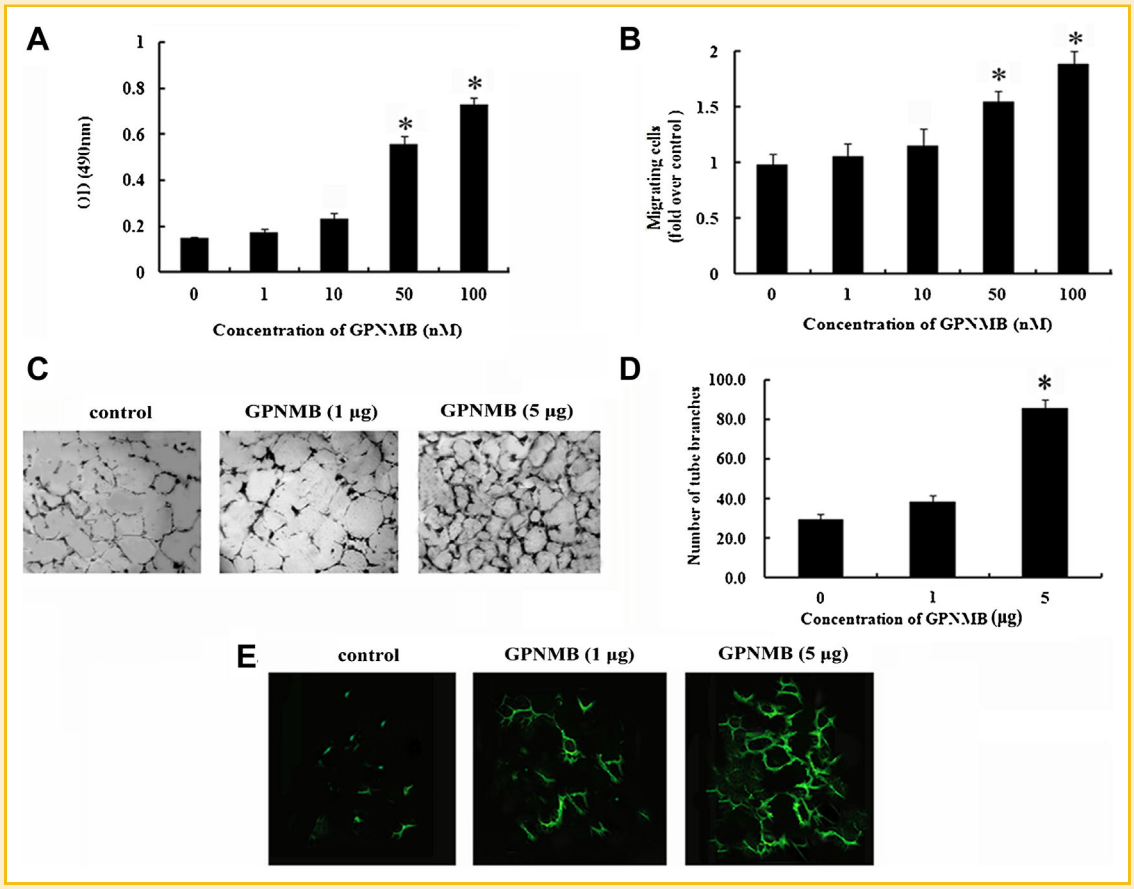

Fig. 3. GPNMB stimulated angiogenesis in vitro and in vivo. Following treatment with the indicated doses of GPNMB, cell proliferation (A) and migration (B) were analyzed by MTT assay and the transwell system. After incubation for 15 h in M199 medium containing the indicated test compounds, tube formation was monitored by Diff-Quik® staining (C, D). Matrigel plugs containing the test compounds were subcutaneously injected into the abdomen. Seven days later, the Matrigel plugs were harvested and vessels were labeled with an antibody against CD31 (E). $^{*}P < 0.05$ versus control group.

osteogenesis based on its high expression during osteoblasts differentiation [Abdelmagid et al., 2008]. In this study, exogenous recombinant GPNMB dose-dependently induced the differentiation and mineralization of hBMSCs into osteoblasts, as well as increasing expression of osteogenic markers ALP and OCN. These results indicate GPNMB function as an osteogenic factor to induce the osteoblasts differentiation of hBMSCs. Endothelial cells are considered to be a key regulator in blood vessels formation [Herbert and Stainier, 2011; Baumann, 2013]. It has been reported that GPNMB elicits a pivotal regulatory function in endothelial cell adhesion by binding to integrin [Shikano et al., 2001]. Additionally, in breast cancer, GPNMB exhibits an angiogenic activity [Rose et al., 2010]. Therefore, we assessed the possible function of GPNMB in angiogenesis during bone healing. After treatment with various doses of GPNMB, a notable increase in endothelial cell proliferation and migration was observed, ultimately leading to the formation of capillary tubes. CD31 is a known marker of blood vessels and its strong immunofluorescence signals were confirmed in vivo, suggesting a potential pro-angiogenic activity of GPNMB. To further confirm the function of GPNMB in bone healing, we constructed a rodent model of a cranial defect. As expected, scaffolds delivering GPNMB triggered obviously abundant new bone formation at the defect sites, concomitant with the generation of new vessels in vivo, indicating that GPNMB ameliorated the defective areas. Taken together, our results provide convincing evidence that application of exogenous GPNMB has a potential role in repairing bone defects through enhanced osteogenesis and angiogenesis.

Numerous studies have suggested that FGFR signaling is crucial for the regulation of skeletal repair and blood vessels generation [Du et al., 2012; Jarzabek et al., 2012]. New blood vessels formation modulates tissue healing by delivering oxygen, nutrients, soluble factors and numerous cell types to the impaired sites. FGFR-1 has been shown to function as a signaling receptor to mediate vasculogenesis [Kazenwadel et al., 2012; Wang and Miao, 2013]. Furthermore, FGFR-1 is a predominant regulator of prenatal and postnatal skeletal development [Karsenty and Wagner, 2002; Jarzabek et al., 2012]. Genomic studies also highlight the implication of molecular targets of FGF/FGFR signaling to mediate osteoblastogenesis [Du et al., 2012; Marie et al., 2012]. Strong phosphorylation signals of FGFR-1 and its downstream signaling molecule ERK1/2 were demonstrated in GPNMB-treated cells. Accordingly, we linked FGFR-1 signaling and GPNMB-induced

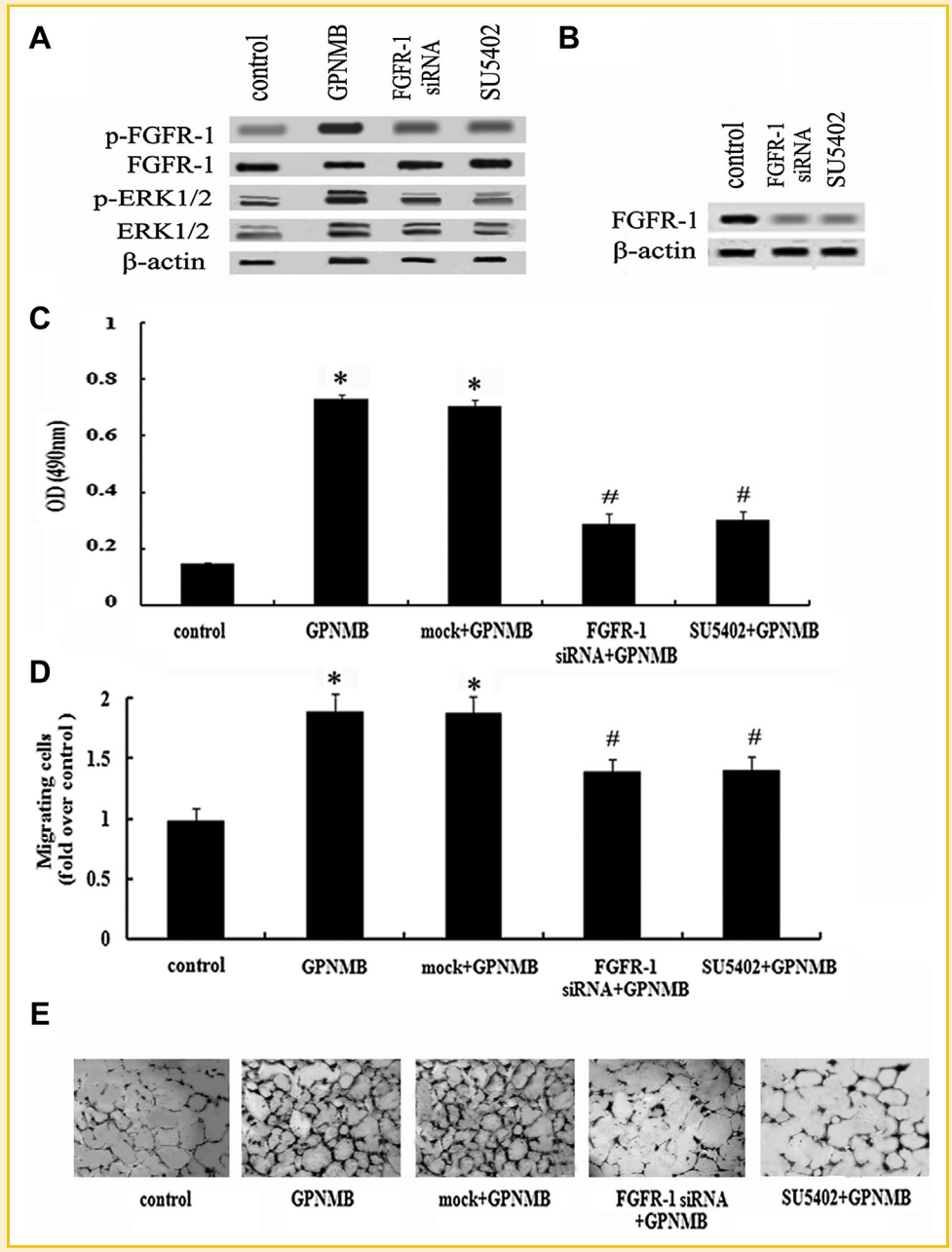

Fig. 4. GPNMB elicited its pro-angiogenic activity though FGFR-1 signaling. HUVECs were stimulated with various doses of GPNMB, and the phosphorylation of FGFR-1 and its downstream effector ERK1/2 was examined by Western blotting (A). Specific FGFR-1 siRNA was introduced to block FGFR-1 signaling (B). The corresponding activation of FGFR-1 pathway was confirmed by Western blotting (A). Following treatment with SU5402 for 1 h, or FGFR-1 siRNA, the increased cell proliferation (C) and migration (D) rates induced by GPNMB were blocked, as well as tube formation (E). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus GPNMB and mock-pretreated groups.

angiogenesis and osteogenesis together. To further investigate the correlation, we silenced the expression of FGFR-1 by FGFR-1 siRNA or SU5402. The activity of FGFR-1 signaling was significantly abrogated in GPNMB-administered hUVECs and hBMSCs. Moreover, blocking of the FGFR-1 pathway notably attenuated GPNMB-stimulated endothelial cell proliferation and migration, ultimately reducing tube formation. Consistently, FGFR-1 silencing also mitigated the GPNMB-mediated differentiation and mineralization of hBMSCs. More importantly, angiogenesis and bone regeneration failed to increase when a CO3Ap-collagen sponge containing the SU5402 inhibitor was implanted as a graft into the tissue defect created in rat cranial. Accordingly, we can conclude that FGFR-1 signaling is responsible for GPNMB-induced bone healing. However, how GPNMB interacts with FGFR? Does GPNMB activated FGFR-1 signaling directly or through other molecules? Whether GPNMB functions as a ligand for FGFR-1? All of these questions need us to further explore in the future.

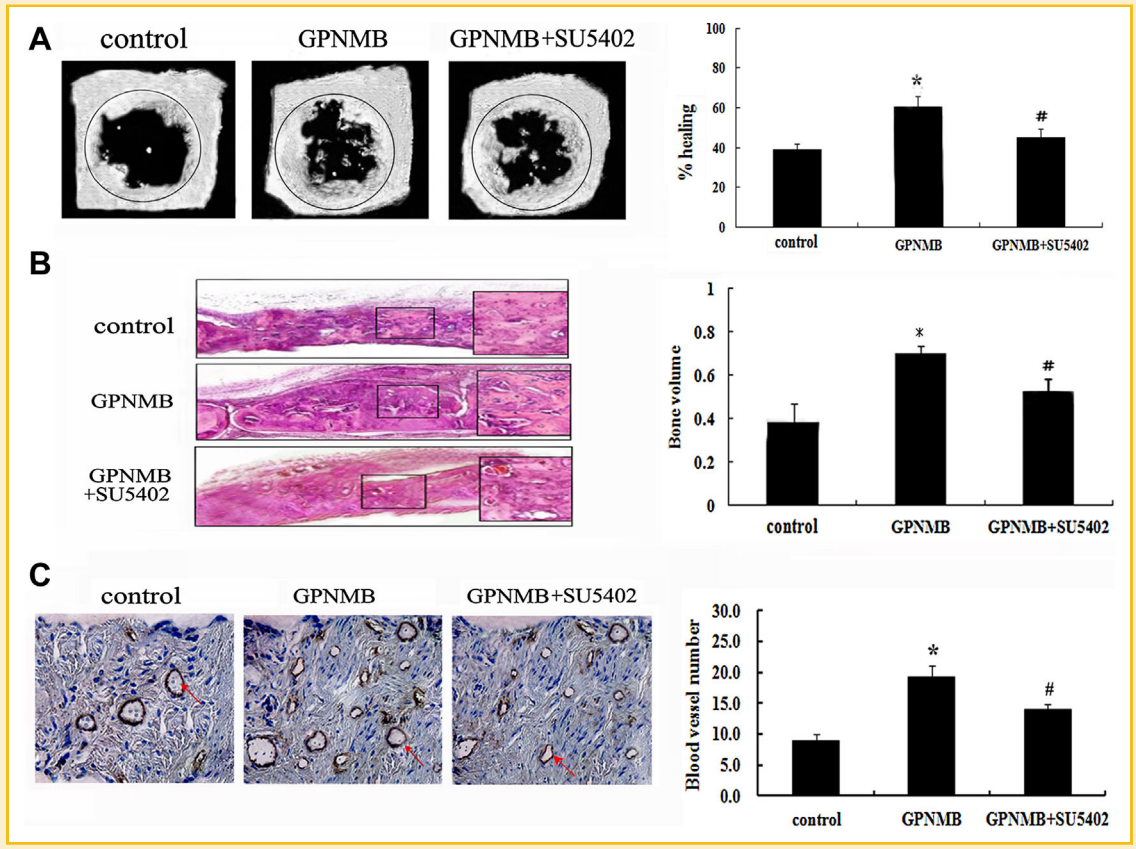

Fig. 5. GPNMB-induced bone regeneration in a rat cranial defect model. Following construction of a rat cranial defect model, the CO3Ap-collagen sponge scaffolds delivering GPNMB and/or SU5402 were implanted into the defective sites. Four weeks later, μCT was introduced to analyze bone healing (A). The sections of bone formation at defective sites were analyzed by H&E staining, and the increased bone formation was also quantified (B). To investigate the effect of GPNMB on blood vessels formation, vWF staining was used to label the new vessels before measuring the numbers of new formed vessels (C). $^{*}P < 0.05$, $^{\#}P < 0.05$.

In this study, GPNMB stimulated osteogenesis and angiogenesis via activation of the FGFR-1 pathway in vitro and in vivo, both of which are the key regulators for bone regeneration. These findings identify a new function of GPNMB as a pro-osteogenic and pro-angiogenic factor in bone healing. Thus, our study may provide a new therapeutic strategy for bone tissue regeneration engineering.

REFERENCES

Abdelmagid SM, Barbe MF, Arango-Hisijara I, Owen TA, Popoff SN, Safadi FF. 2006. Osteoactivin acts as downstream mediator of BMP-2 effects on osteoblast function. J Cell Physiol 210:26–37.

Abdelmagid SM, Barbe MF, Rico MC, Salihoglu S, Arango-Hisijara I, Selim AH, Anderson MG, Owen TA, Popoff SN, Safadi FF. 2008. Osteoactivin, an anabolic factor that regulates osteoblast differentiation and function. Exp Cell Res 314:2334–2351.

Abdelmagid SM, Barbe MF, Hadjiargyrou M, Owen TA, Razmpour R, Rehman S, Popoff SN, Safadi FF. 2010. Temporal and spatial expression of osteoactivin during fracture repair. J Cell Biochem 111:295–309.

Ardi VC, Van den Steen PE, Opdenakker G, Schweighofer B, Deryugina EI, Quigley JP. 2009. Neutrophil MMP-9 proenzyme, unencumbered by TIMP-1, undergoes efficient activation in vivo and catalytically induces angiogenesis via a basic fibroblast growth factor (FGF-2)/FGFR-2 pathway. J Cell Biochem 284:25854–25866.

Baumann K. 2013. Development: Growing a blood vessel network. Nat Rev Mol Cell Biol 14:127.

Cheung LW, Leung KW, Wong CK, Wong RN, Wong AS. 2011. Ginsenoside-Rg1 induces angiogenesis via non-genomic crosstalk of glucocorticoid receptor and fibroblast growth factor receptor-1. Cardiovasc Res 89:419–425.

Cinotti G, Corsi A, Sacchetti B, Riminucci M, Bianco P, Giannicola G. 2013. Bone ingrowth and vascular supply in experimental spinal fusion with platelet-rich plasma. Spine 38:385–391.

Cui Q, Dighe AS, Irvine J. 2013. Combined angiogenic and osteogenic factor delivery for bone regenerative engineering. Curr Pharm Des 19:3374–3383.

Du X, Xie Y, Xian CJ, Chen L. 2012. Role of FGFs/FGFRs in skeletal development and bone regeneration. J Cell Physiol 227:3731–3743.

Franceschi R. 2012. A novel approach to regeneration of bone: Using focused ultrasound for the spatiotemporal patterning of angiogenic and osteogenic factors. Ann Arbor: Michigan University.

Götz W, Reichert C, Canullo L, Jäger A, Heinemann F. 2012. Coupling of osteogenesis and angiogenesis in bone substitute healing—A brief overview. Ann Anat 194:171–173.

Hamada Y, Egusa H, Kaneda Y, Hirata I, Kawaguchi N, Hirao T, Matsumoto T, Yao M, Daito K, Suzuki M. 2007. Synthetic osteopontin-derived peptide SVVYGLR can induce neovascularization in artificial bone marrow scaffold biomaterials. Dent Mater J 26:487–492.

Han S-W, Jung Y-K, Lee E-J, Park H-R, Kim G-W, Jeong J-H, Han M-S, Choi J-Y. 2013. DICAM inhibits angiogenesis via suppression of AKT and p38 MAP kinase signaling. Cardiovasc Res 98:73–82.

Herbert SP, Stainier DY. 2011. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nat Rev Mol Cell Biol 12:551–564.

Huang YC, Kaigler D, Rice KG, Krebsbach PH, Mooney DJ. 2005. Combined Angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration. J Bone Miner Res 20:848–857.

Hunsaker FG, Cioffi DA, Amadio PC, Wright JG, Caughlin B. 2002. The American academy of orthopaedic surgeons outcomes instruments normative values from the general population. J Bone Joint Surg Am 84:208–215.

Jarzabek K, Wolczynski S, Lesniewicz R, Plessis G, Kottler M. 2012. Evidence that FGFR1 loss-of-function mutations may cause variable skeletal malformations in patients with Kallmann syndrome. Adv Med Sci 57:314–321.

Kanczler J, Oreffo R. 2008. Osteogenesis and angiogenesis: The potential for engineering bone. Eur Cell Mater 15:100–114.

Karsenty G, Wagner EF. 2002. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2:389–406.

Kazenwadel J, Secker GA, Betterman KL, Harvey NL. 2012. In vitro assays using primary embryonic mouse lymphatic endothelial cells uncover key roles for FGFR1 signalling in lymphangiogenesis. PLoS ONE 7:e40497.

Kuan C-T, Wakiya K, Dowell JM, Herndon JE, Reardon DA, Graner MW, Riggins GJ, Wikstrand CJ, Bigner DD. 2006. Glycoprotein nonmetastatic melanoma protein B, a potential molecular therapeutic target in patients with glioblastoma multiforme. Clin Cancer Res 12:1970–1982.

Lee SH, Lee J, Jung MH, Lee YM. 2013. Glyceollins, a novel class of soy phytoalexins, inhibit angiogenesis by blocking the VEGF and bFGF signaling pathways. Mol Nutr Food Res 57:225–234.

Marie PJ. 2012. Fibroblast growth factor signaling controlling bone formation. Gene 498:1–4.

Marie PJ, Miraoui H, Sévère N. 2012. FGF/FGFR signaling in bone formation: Progress and perspectives. Growth Factors 30:117–123.

Metz RL, Patel PS, Hameed M, Bryan M, Rameshwar P. 2007. Role of human HGFIN/nmb in breast cancer. Breast Cancer Res 9:R58.

Murakami M, Simons M. 2008. Fibroblast growth factor regulation of neovascularization. Curr Opin Hematol 15:215.

Portal-Núñez S, Lozano D, Esbrit P. 2012. Role of angiogenesis on bone formation. Histol Histopathol 27:559.

Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev 16:159–178.

Qu D, Li J, Li Y, Gao Y, Zuo Y, Hsu Y, Hu J. 2011. Angiogenesis and osteogenesis enhanced by bFGF ex vivo gene therapy for bone tissue engineering in reconstruction of calvarial defects. J Biomed Mater Res A 96:543–551.

Rose AA, Annis MG, Dong Z, Pepin F, Hallett M, Park M, Siegel PM. 2010. ADAM10 releases a soluble form of the GPNMB/Osteoactivin extracellular domain with angiogenic properties. PLoS ONE 5:e12093.

Safadi F, Xu J, Smock SL, Rico MC, Owen TA, Popoff SN. 2001. Cloning and characterization of osteoactivin, a novel cDNA expressed in osteoblasts. J Cell Biochem 84:12–26.

Shikano S, Bonkobara M, Zukas PK, Ariizumi K. 2001. Molecular cloning of a dendritic cell-associated transmembrane protein, DC-HIL, that promotes RGD-dependent adhesion of endothelial cells through recognition of heparan sulfate proteoglycans. J Cell Biochem 276:8125–8134.

Wang Y-Q, Miao Z-H. 2013. Marine-derived angiogenesis inhibitors for cancer therapy. Mar Drugs 11:903–933.