Involvement of Endogenous Bone Morphogenetic Protein (BMP) 2 and BMP6 in Bone Formation*

Fumitaka Kugimiya‡6, Hiroshi Kagawuchi5, Satoru Kamekura5, Hirotaka Chikuda5, Shinsuke Ohba5, Fumiko Yano5, Naoshi Ogata5, Takenobu Katagiri5, Yoshifumi Harada5, Yoshiaki Azuma5, Kozo Nakamura5, and Ung-il Chung††1

From the Divisions of ‡Tissue Engineering and §Sensory & Motor System Medicine, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo, 113-8655, the †Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical School, Yamane 1397-1, Hidaka, Saitama, 350-1241, and ††Teijin Co., Ltd., Asahigaoka 4-3-2, Hino, Tokyo 191-8512, Japan

Although accumulated evidence has shown the bone anabolic effects of bone morphogenetic proteins (BMPs) that were exogenously applied in vitro and in vivo, the roles of endogenous BMPs during bone formation remain to be clarified. This study initially investigated expression patterns of BMPs in the mouse long bone and found that BMP2 and BMP6 were the main subtypes expressed in hypertrrophic chondrocytes that induce endochondral bone formation. We then examined the involvement of the combination of BMP2 and BMP6 by generating the compound-deficient mice (Bmp2+/-;Bmp6-/-). Under physiological conditions, these mice exhibited moderate growth retardation compared with the wild-type (WT) littermates during the observation period up to 52 weeks of age. Both the fetal and adult compound-deficient mice showed a reduction in the trabecular bone volume with suppressed bone formation, but normal bone resorption, whereas the single deficient mice (Bmp2+/- or Bmp6-/-) did not. When a fracture was created at the femoral midshaft and the bone healing was analyzed, the endochondral bone formation, but not intramembranous bone formation, was impaired by the compound deficiency. In the cultures of bone marrow cells, however, there was no difference in osteogenic differentiation between WT and compound-deficient cells in the presence or absence of the exogenous BMP2. We thus concluded that endogenous BMP2 and BMP6 cooperatively play pivotal roles in bone formation under both physiological and pathological conditions.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily. BMPs were originally identified as molecules that induced ectopic bone formation when implanted into rodent muscles (1, 2). In accordance with such in vivo effects, BMPs have been shown to regulate osteogenic differentiation in vitro (3). However, naturally occurring or genetically engineered mice deficient in BMPs reported so far are either mesenchymal cell abnormalities, or die during early embryonic development, thus being non-informative as to the role of endogenous BMPs in bone formation (4, 5).

In mammals, there are two distinct modes of bone formation: intramembranous and endochondral (6). Most of the bones form via the latter process, which is characterized by the replacement of a cartilage mold by bone and bone marrow (7). During this process, cells in the mesenchymal condensations become chondrocytes, the primary cell type of cartilage; cells at the border of the condensations form a perichondrium. Chondrocytes have a characteristic shape, express a characteristic genetic program driven by SOX9 and other transcription factors, and secrete a matrix rich in type II collagen and proteoglycan. Cartilage enlarges through chondrocyte proliferation and matrix production. Chondrocytes in the center of the cartilage mold then stop proliferating, enlarge (hypertrophy), and change their genetic program to synthesize the type X collagen. Hypertrophic chondrocytes mineralize their surrounding matrix, attract blood vessels through the production of the vascular endothelial growth factor and other factors, and attract chondroclasts and osteoclasts. Moreover, these chondrocytes direct mesenchymal cells in the perichondrium and in the bone marrow to become osteoblasts, which form the bone collar and the primary spongiosa (8, 9). Thus, during the endochondral bone formation, hypertrophic chondrocytes link chondrogenesis to osteogenesis by inducing osteogenesis and angiogenesis (8, 9). Hypertrophic chondrocytes express a number of growth factors, cytokines, and matrix proteins. Among them, Indian hedgehog (Ihh) has been proven to be indispensable for the induction of osteogenesis by these chondrocytes (8, 10). Ihh alone, however, cannot induce bone formation (11), suggesting that other factors secreted from these chondrocytes may also be necessary for osteogenesis. Because some BMPs can induce ectopic bone formation when implanted into rodent muscles and promote osteogenic differentiation in vitro, they are strong candidates for the osteogenic factors secreted by these chondrocytes. Among them, BMP2 and BMP6 are known to be expressed by these chondrocytes (8). As is the case with the other BMPs, however, there is no direct evidence that the endogenous BMP2 and BMP6 are involved in bone formation, because homozygous Bmp2-deficient (Bmp2-/-) mice die during the early embryonic stage (12), and homozygous Bmp6-deficient (Bmp6-/-) mice show no skeletal abnormality except for a slight delay in the ossification of the sternum (13). We hypothesized that there might be a genetic redundancy between BMP2 and BMP6 in the regulation of bone formation. Hence, the present study generated compound knock-out mice lacking one allele of the Bmp2 gene and both alleles of the Bmp6 gene (Bmp2+/-; Bmp6-/-) and investigated the effect of their compound loss on bone metabolism under both physiological and pathological conditions.

**EXPERIMENTAL PROCEDURES**

**Animals**—Bmp2+/- mice were kindly provided by A. Bradley (Baylor College of Medicine, Houston, TX) (12); Bmp6-/- mice by E. Robertson (Harvard University, Cambridge, MA) (13). The mice were maintained in a C57BL/6 background. To generate Bmp2+/-;
Bmp6+/− mice, Bmp2+/+ mice were mated with the homozygous Bmp6+/− mice to obtain Bmp2+/+;Bmp6+/− mice. Bmp2+/−; Bmp6+/− mice were then mated with each other. Because Bmp2−/−; Bmp6−/− mice were embryonically lethal, two of 12 live mice were expected to be Bmp2+/−;Bmp6−/−. All experiments were performed on male mice in accordance with the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

Genotyping—Genomic DNA was isolated from the tail. 10 ng of genomic DNA was used for genotyping by PCR. The PCR primers were as follows: 5′-AGCATGACCCCTATGTTGGT-3′ (forward primer for Bmp2 wild-type (WT) and mutant alleles), 5′-GTGACATTAGGCTGTGAGTAA-3′ (reverse primer for Bmp2 WT allele), 5′-GAGACTAGTGACAGCTGACT-3′ (reverse primer for Bmp6 mutant allele), 5′-CCCCCATCATCAAGCAGA-3′ (forward primer for Bmp6 WT and mutant alleles), 5′-TCGCCCAACACAGCTCCTTG-3′ (reverse primer for Bmp6 WT allele) and 5′-CGGTGA-CAGGCGGAACAGCG-3′ (reverse primer Bmp6 mutant allele). PCR were performed at 94 °C for 1 min, at 58 °C for 1 min, and at 72 °C for 1 min for 35 cycles. The PCR product from the WT Bmp2 allele was 322 bp and that from the Bmp6 mutant allele, 367 bp. The PCR product from the WT Bmp6 allele was 112 bp and that from the Bmp6 mutant allele, 499 bp.

Skeletal Preparation—Embryos at E17.5 were eviscerated, fixed in 100% ethanol for 4 days, and then transferred to 100% acetone. After 3 days, they were rinsed with water and stained for 10 days in staining solution containing 1 volume of 0.1% Alizarin red S (Sigma), 95% ethanol; 1 volume of 0.3% Alcian blue 8GX (Sigma), 70% ethanol; 1 volume of 100% acetic acid, and 17 volumes of 100% ethanol. After rinsing with 96% ethanol, the specimens were kept in 20% glycerol, 1% KOH at 37 °C for 16 h and subsequently at room temperature until the skeletons became clearly visible. For storage, the specimens were transferred to 50, 80, and finally 100% glycerol (14).

Histological Analysis—For the histological analysis, embryonic limbs were fixed in 4% paraformaldehyde/phosphate-buffered saline for 1 h and embedded in paraffin for sectioning, according to the standard procedures. Sections (5 μm thick) were then stained with Hematoxylin and Eosin (H&E) for morphological study, with toluidine blue for detection of cartilage after surgery by asphyxiation with carbon dioxide, and their femurs were excised. The calcified area and the bone mineral content of the affected bone formation during fracture healing, the callus was divided into three equal portions, where bones mainly form through the intramembranous and endochondral bone formation. Tissues were fixed in 4% paraformaldehyde/phosphate-buffered saline overnight at 4 °C, processed, embedded in paraffin, and cut. The in situ hybridization was performed as previously described (15) using complementary 35S-labeled riboprobes for mouse BMP2, BMP4, BMP6, BMP7, GDF5 (kindly provided by E. Robertson, Harvard University) (13), and type I collagen (8).

Real-time RT-PCR—Total RNA was extracted using an ISOGEN Kit (Wako Pure Chemicals Industry, Ltd., Tokyo) and an RNeasy Mini Kit (Qiagen, Hilden, Germany), then treated with DNase I (Qiagen), according to the manufacturer’s instructions. One μg of RNA was reverse transcribed using a Takara RNA PCR Kit (AMV) version 2.1 (Takara Shuzo Co., Shiga, Japan) to generate the single-stranded cDNA. PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Each PCR consisted of 1 X QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.3 μM specific primers, and 500 ng of cDNA. The mRNA copy number of a specific gene in the total RNA was calculated with a standard curve generated using serially diluted plasmids containing PCR amplicon sequences and normalized to the human or rodent total RNA (Applied Biosystems) with mouse actin as the internal control. Standard plasmds were synthesized using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. All reactions were run in triplicate. The primer sequences are available upon request.

Radiological Analysis—Bone radiographs were taken with a soft x-ray instrument (CMB-2, SOFTEX, Kanagawa, Japan). A three-dimensional CT scan was taken with a composite x-ray analyzing system (NX-HCP, NS-ELEX Inc., Tokyo). The bone mineral density (BMD) was measured by single energy x-ray absorptiometry using a bone mineral analyzer (DCS-600R, Aloka Co., Tokyo).

Bone Histomorphometry—Eight 9-week-old mice were used in each group. For Villanueva-Goldner staining, tibias were fixed with ethanol, embedded in methyl methacrylate, and sectioned in 6-μm slices. For double labeling, the mice were subcutaneously injected with 8 mg/kg body weight of calcein at 3 and 10 days before sacrifice. Tartrate-resistant acid phosphatase-positive cells were stained at pH 5.0 in the presence of 1-(-)-tartaric acid with naphthol AS-MX phosphate (Sigma) in N,N-dimethyl formamide as the substrate. The specimens were subjected to histomorphometric analyses using an image analyzer (Histometry RT CAMERA, System Supply Co., Nagano, Japan). The parameters of the trabecular bone were measured in an area 1.2 mm in length from 0.5 mm below the growth plate at the proximal metaphysis of the tibias. The parameters of the cortical bone were measured at the midshaft of the tibias. The thickness of the growth plate was measured at the proximal tibias.

Fracture Model—Bone fractures were generated as previously described (16). Eight 9-week-old mice were used in each group. Briefly, under general anesthesia with xylazine (0.05 mg/10 g body weight) and ketamine (0.5 mg/10 g body weight, Sigma), a 15-mm incision was longitudinally made to expose the femur. A transverse osteotomy was performed with a bone saw (Volvere GX, NSK Nakanishi, Inc., Tochigi, Japan) at the middle of the femur. Fractured bones were repositioned, and then the full-length of the bone marrow cavity was internally stabilized with an intramedullary nail with the inner pin of a 23-gauge spinal needle. The animals were allowed activity, diet, and water ad libitum. For the histological analyses, the animals were killed at 5, 7, and 18 days after surgery by asphyxiation with carbon dioxide, and their femurs were excised. The calcified area and the bone mineral content of the entire femur were measured. The % gain of calcified area and the % gain of BMC were calculated, and the differences were compared between WT, Bmp2+/−, Bmp6−/−, and Bmp2+/−;Bmp6−/− mice. To distinguish between the intramembranous bone formation and endochondral bone formation during fracture healing, the callus was divided into three equal portions along the axis of the bone. The distal and proximal portions, where bones mainly form through the intramembranous...
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process (17), were designated as the peripheral part, and the middle portion, where bones mainly form through the endochondral process (17), was designated as the central part. In each part, we measured the ratio of the calcified area to the total area of the histological sections using NIH Image.

Serum and Urinary Biochemistry—Blood samples from 9-week-old WT and Bmp2+/-;Bmp6-/- mice (n = 6 for each group) were collected by heart puncture under anesthesia with nembutal (0.4 mg/10 g body weight) (Dainippon Pharmaceutical Co., Ltd., Tokyo). Urine samples were collected for 24 h before sacrifice in oil-sealed bottles in the metabolism cages (CL-0305, CLEA Japan, Inc., Tokyo). The levels of creatinine, calcium, and inorganic phosphorus in the serum were measured using a standard colorimetric technique, a Calcium HR Kit (Wako Pure Chemical Industries, Ltd.), and an Inorganic Phosphorus II Kit (Wako Pure Chemical Industries, Ltd.), respectively, by an autoanalyzer (Type 7170, Hitachi High-Technologies Co., Tokyo). Urinary deoxyypyridinoline was measured using a Pyriliks-D enzyme-linked immunosorbent assay kit (Mitra Biosystems, Inc., Mountain View, CA). The values were corrected for urinary creatinine measured by a standard colorimetric technique using the Type 7170 autoanalyzer.

In Vitro Bone Marrow Differentiation Assay—Bone marrow cells were isolated from WT and Bmp2+/-;Bmp6-/- mice at 3 weeks of age and inoculated at a density of 2 × 10^5 cells/well onto 24-well plates in α-minimal essential medium containing 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and ITS+1 liquid media supplement (Sigma) (osteogenic medium). For treatment with BMP, we added recombinant human (rh) BMP2 at 200 ng/ml. We changed the medium every 4 days with BMP2 being replenished each time. Two weeks after confluence, total RNA was extracted, and alkaline phosphatase (ALP), Alizarin red S, and von Kossa stainings were performed. For ALP staining, cells were fixed in 70% EtOH and stained for 10 min with a solution containing 0.01% naphthol AS-MX phosphate disodium salt (Sigma, 1% N,N-dimethyl formamide (Wako Pure Chemicals Industry, Ltd.), and 0.06% fast blue BB (Sigma). For the Alizarin red S staining, the cells were fixed in 10% formalin/phosphate-buffered saline and stained for 10 min with 2% Alizarin red S, pH 4.0, (Sigma) solution. For the von Kossa staining, the cells were fixed with 100% ethanol at room temperature for 15 min, stained with 5% silver nitrate solution (Wako Pure Chemicals Industry, Ltd.) under ultraviolet light for 10 min, and incubated for 5 min with 5% sodium thiosulfate solution (Wako Pure Chemicals Industry, Ltd.).

The numbers of total fibroblastic colonies (CFU-F), ALP positive colonies (CFU-ALP), and bone nodules were assessed as described (18–20). In brief, bone marrow cells isolated from WT and Bmp2+/-; Bmp6−/− mice at 3 weeks of age were disseminated into a six-well plate at a concentration of 5 × 10^5 cells/ml and cultured in osteogenic medium for 5, 10, and 15 days in the presence or absence of rhBMP2 at 200 ng/ml. Subsequently, cells were fixed with 10% neutral-buffered formalin and subjected to the ALP or von Kossa stainings as described above. Colonies consisting of more than 50 cells were defined as CFU-F, and ALP-positive CFU-F were defined as CFU-ALP. Bone nodules were counted on a grid under low power microscopy.

Statistical Analysis—The means of the groups were compared by analysis of variance, and the significance of differences was determined by post-hoc testing using Bonferroni method.

RESULTS

BMP Subtypes Expressed by Hypertrophic Chondrocytes—To determine the BMP subtypes expressed by hypertrophic chondrocytes, we performed in situ hybridization for BMPs known to be expressed in chondrocytes (5). Consistent with the previous report (8), the main BMP subtypes expressed by hypertrophic chondrocytes were BMP2 and BMP6 (Fig. 1A). As for the other BMPs examined, BMP4 was weakly expressed in the prehypertrophic chondrocytes, BMP7 in the proliferating chondrocytes, and GDF5 in the perichondral proliferating chondrocytes.

Analysis of Fetal Bmp2−/−;Bmp6−/− Mice—To investigate the roles of the endogenous BMP2 and BMP6 during bone formation, we generated their compound-deficient mice by appropriate mating. Because of the early embryonic lethality of Bmp2−/− mice, we studied the bone phenotypes of Bmp2−/−;Bmp6−/− mice. To macroscopically visualize bone and cartilage elements, whole embryos at E17.5 were double stained with Alizarin red and Alcian blue (Fig. 1B). The staining revealed a normal skeletal patterning in the single deficient (Bmp2+/− and Bmp6−/−) and the compound-deficient mice. However, the size of the compound-deficient mice was smaller than that of WT mice, whereas that of the single deficient mice was not. Temporal profiles of the body length and weight of the compound-deficient mice showed an ~5% decrease in axial growth and body weight throughout life (Fig. 1C).

Histological analysis of the growth plate of the proximal tibias from the E17.5 WT and Bmp2+/-;Bmp6−/− mice disclosed that the size of the compound-deficient growth plate was smaller than that of WT (643 ± 17 versus 723 ± 24 μm, p < 0.05), but the proportions of the distinct layers of the growth plate chondrocytes were not significantly different between the two groups (the layer of proliferating chondrocytes, 72.9 ± 2.3 versus 72.1 ± 2.6%; the layer of hypertrophic chondrocytes, 27.1 ± 0.8 versus 27.9 ± 0.9%) (Fig. 1D). Immunohistochemistry of the type II and X collagens showed no remarkable difference between the two groups (Fig. 1E).

To evaluate bone formation in the fetal growth plate, we performed immunohistochemistry and real-time RT-PCR detecting the type I collagen (Fig. 1F) and von Kossa staining (Fig. 1G). The type I collagen immunoreactivity and type I collagen mRNA expression in the tibia were similar between the compound-deficient and WT mice. The von Kossa staining revealed that the mineralized area in the primary spongiosa of the compound-deficient mice was reduced in comparison with that of WT, although the mineralized area in the hypertrophic layer was similar between the two groups. The number of osteoclasts of the compound-deficient mice was not significantly different from that of WT (data not shown). During endochondral bone formation, osteogenesis is influenced by chondrogenesis. To rule out the possibility that the reduced mineralization of the primary spongiosa of the compound-deficient mice was because of a cartilage defect, we investigated whether chondrocytes reached terminal differentiation by examining the onset of hypertrophic differentiation and cartilage mineralization. H&E staining, immunohistochemical analysis with anti-type X collagen and MMP-13 antibodies, and von Kossa staining of the metatarsal bone sections at E15.5 showed that neither hypertrophic differentiation nor cartilage mineralization occurred in WT or compound-deficient mice (Fig. 1H). At E16.5, chondrocytes in the center of the cartilage underwent hypertrophic differentiation, expressing type X collagen and mineralizing the surrounding matrix in both WT and Bmp2+/-;Bmp6−/− mice. MMP-13, a marker of terminally differentiated hypertrophic chondrocytes (21–23), just began to be expressed in both groups. At E17.5, MMP-13 expression was markedly up-regulated, the area of the mineralized cartilaginous matrix was increased, and the bone collar was formed in both groups. Thus, no abnormality of terminal differentiation of the chondrocytes was detected in the compound-deficient mice. Taken together, these data suggest that the differentiation/
function of osteoblasts is impaired by the compound deficiency of Bmp2 and Bmp6.

**Analysis of Adult Bmp2+/−:Bmp6−/− Mice**—We then investigated whether the compound deficiency of Bmp2 and Bmp6 had effects on the bone metabolism in adult mice. X-rays of the femur at 9 weeks of age showed that the lengths of the femur and the tibia were shorter and that the trabecular bone volume was reduced in Bmp2+/−:Bmp6+/− mice compared with that in WT (Fig. 2A). Quantitative analysis of the BMD disclosed that the femoral BMD of the compound-deficient mice was reduced compared with that of WT, whereas that of Bmp2+/−, Bmp6+/−, or Bmp2+/−:Bmp6+/− mice was not significantly different from that of WT (Fig. 2B). Three-dimensional CT analysis manifested a marked reduction in the trabecular bone volume of the compound-deficient mice (Fig. 2C). Next, to investigate the role of endogenous Bmp2 and Bmp6 in intramembranous bone formation, the calvarias were examined. X-ray of the calvarias at 9 weeks of age from WT, Bmp2+/−, Bmp6−/−, and Bmp2+/−:Bmp6−/− mice did not show any significant difference (Fig. 2D), which was confirmed by the quantitative analysis of the BMD (Fig. 2E).

Histological analysis with von Kossa staining of the proximal tibia revealed a trabecular bone loss in Bmp2+/−:Bmp6−/− mice compared with that in WT (Fig. 3A). Although there was no significant difference in the proportions of the growth plates or in the cartilage mineralization between the two groups, the mineralization in the primary spongiosa was notably reduced in the compound-deficient mice (Fig. 3B). To analyze the mechanism of the bone loss in detail, bone histomorphometric analysis was performed on the tibias (Fig. 3C). The bone volume and cortical thickness of the compound-deficient mice were found to be decreased compared with those of WT. Regarding the parameters of bone formation, the mineral apposition rate and bone
FIGURE 2. Radiological analysis of adult Bmp2+/−;Bmp6−/− mice. A, plain radiographs of the femurs (upper panels) and tibias (lower panels) from WT, Bmp2+/−; Bmp6−/−, and Bmp2+/−;Bmp6−/− mice at 9 weeks of age. B, BMD of the whole femurs from WT, Bmp2+/−; Bmp6−/−, Bmp6−/−, Bmp2+/−; Bmp6−/−, and Bmp2+/−;Bmp6−/− mice at 9 weeks of age. Data are expressed as mean ± S.E. for 12 bones/group. *, p < 0.01 versus the rest. C, three-dimensional CT analysis of the distal epiphysis of the femurs from WT (upper panels) and Bmp2+/−;Bmp6−/− mice (lower panels) at 9 weeks of age. D, plain radiographs of the calvarias from WT, Bmp2+/−; Bmp6−/−, Bmp6−/−, Bmp2+/−;Bmp6−/−, Bmp2+/−; Bmp6−/−, and Bmp2+/−;Bmp6−/− mice at 9 weeks of age. E, BMD of the calvarias from WT, Bmp2+/−; Bmp6−/−, Bmp6−/−, Bmp2+/−;Bmp6−/−, Bmp2+/−; Bmp6−/−, and Bmp2+/−;Bmp6−/− mice at 9 weeks of age.

FIGURE 3. Histological analysis of adult Bmp2+/−;Bmp6−/− mice. A, von Kossa staining of the tibia sections from WT and Bmp2+/−;Bmp6−/− mice at 9 weeks of age. Scale bar, 300 μm. B, toluidine blue staining (upper panels) and von Kossa staining (lower panels) of the tibia growth plate sections from WT and Bmp2+/−;Bmp6−/− mice at 9 weeks of age. Blue, green, and red bars indicate the layer of proliferating chondrocytes, the layer of hypertrophic chondrocytes, and the primary spongiosa, respectively. Scale bar, 20 μm. C, bone histomorphometric analysis of the tibias from WT and Bmp2+/−;Bmp6−/− mice at 9 weeks of age. BV/TV, bone volume per tissue volume; C.Th, cortical bone thickness; Ob.S/BS, osteoblast surface per bone surface; MAR, mineral apposition rate; BFR/BS, bone formation rate per bone surface; N.Oc/B.Pm, number of osteoclasts per 100 mm of bone perimeter; Oc.S/BS, osteoclast surface per bone surface; ES/BS, erosive surface per bone surface. Data are expressed as the mean ± S.E. of 8 mice per genotype. *, p < 0.05 versus WT.
formation rate per bone surface were markedly decreased with no remarkable difference in the osteoblast number expressed by Ob.S/BS. On the other hand, the parameters of bone resorption were normal. These data suggest that the bone loss in the compound-deficient mice is caused by the inhibition of the bone formation because of the impaired osteoblast function.

To rule out the possibility that the bone loss of these mice was caused by general conditions, such as renal failure or abnormal calcium homeostasis, the serum biochemical data were analyzed. There was no remarkable difference in the serum creatinine, calcium, or inorganic phosphorus between WT and compound-deficient mice (data not shown). Urinary deoxypyridinoline, a marker for bone resorption, showed no remarkable difference between the two groups, confirming the bone histomorphometric data (data not shown).

Bone Fracture Healing in Bmp2+/−;Bmp6−/− Mice—The data so far suggest that the combination of the endogenous BMP2 and BMP6 plays an important role in bone formation under physiological conditions. To further investigate the effects of the compound loss of Bmp2 and Bmp6 on bone formation under pathological conditions, we generated fractures at the midshaft of the femurs and compared the healing process among WT, Bmp2+/−, Bmp6−/−, and Bmp2+/−;Bmp6−/− mice. Radiological analysis at 18 days after the fracture showed substantial calcified callus formation in WT, Bmp2+/−, and Bmp6−/− mice (Fig. 4A). On the other hand, in the compound-deficient mice, fracture healing was delayed, and the size of the calcified callus was reduced. To quantify the extent of the callus formation, the % gain of the calcified area and the % gain of bone mineral content in the fractured and control femurs were measured using a bone densitometer (Fig. 4B). Although no significant difference was observed among WT, Bmp2+/−, and Bmp6−/− mice at 18 days after the fracture, both parameters were markedly reduced in the compound-deficient mice. When histological sections were stained with H&E and toluidine blue to distinguish between the bone and cartilage tissues, the total callus size was reduced, and a massive cartilaginous callus containing hypertrophic chondrocytes persisted in the compound-deficient mice. During fracture healing, new bone is known to be formed through two pathways: the endo-
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chondral bone formations in the center and the intramembranous bone formation in the periphery of the callus (17). To distinguish these two pathways, we divided the callus into three equal portions along the axis of the bone, and designated the distal and proximal 1/3 portions as the peripheral part, and the middle 1/3 portion as the central part. The measurement of the ratio of the calcified area to the total area (CA/TA) of the histological sections using NIH Image revealed that calcification in the central part, but not in the peripheral part, was significantly reduced in the compound-deficient mice compared with that in WT (Fig. 4D), indicating that the endochondral, not intramembranous, bone formation, was defective in the compound-deficient mice. To investigate whether the delayed fracture healing in Bmp2+/−;Bmp6−/− mice was caused by a delay in terminal differentiation of chondrocytes, we examined the earlier stages of fracture healing in WT and compound-deficient mice. H&E staining and immunohistochemical analysis with antitype X collagen and MMP-13 antibodies showed that no hypertrophic differentiation occurred in either group at 5 days after the fracture (Fig. 4E). At 7 days after the fracture, terminal hypertrophic differentiation of chondrocytes determined by expression of MMP-13 and intramembranous bone formation occurred in both groups.

In Vitro Bone Marrow Differentiation Assay—The current results support the view that the endogenous BMP2 and BMP6 play vital roles in bone formation under physiological and pathological conditions. In situ hybridization analysis indicated that, in comparison with the expression in hypertrophic chondrocytes, there was little, if any, expression of BMP2 and BMP6 in bone and bone marrow cells including osteoblasts that were marked by the strong expression of the type I collagen (Fig. 5A). However, we still could not rule out the possibility that a small amount of BMP2 and BMP6 secreted from bone and bone marrow cells might act in an autocrine or paracrine fashion to modulate the osteoblast function. To test this possibility, we isolated bone marrow cells including osteoblasts from 3-week-old mice, cultured them in serum-free osteogenic medium, and assessed their osteogenic ability. ALP, Alizarin red S, and von Kossa stainings revealed no difference in the basal osteogenic ability between WT and Bmp2+/−;Bmp6−/− cells (Fig. 5B). Upon treatment with rhBMP2 (200 ng/ml), both WT and the compound-deficient cells responded well to the same extent (Fig. 5B). The real-time RT-PCR analysis of osteopontin and osteocalcin, markers for osteoblasts, revealed no difference between the two genotypes (Fig. 5C). The quantitative analysis of the numbers of CFU-F, CFU-ALP, and bone nodules using WT and Bmp2+/−;Bmp6−/− bone marrow cells. Bone marrow cells were isolated from 3-week-old mice, then cultured in serum-free osteogenic medium in the presence or absence of exogenous rhBMP2 (200 ng/ml). Two weeks after confluence, ALP, Alizarin red S, and von Kossa stainings were performed. C, expression of osteopontin and osteocalcin mRNAs was determined by real-time RT-PCR on the above mentioned marrow cells. Data are expressed as the mean ± S.E. of 6 wells per group. *, p < 0.01, significant stimulation by rhBMP2. D, temporal profiles of the numbers of CFU-F, CFU-ALP, and bone nodules using WT and Bmp2+/−;Bmp6−/− bone marrow cells. Bone marrow cells were isolated from 3-week-old WT and Bmp2+/−;Bmp6−/− mice and cultured in serum-free osteogenic medium in the presence or absence of endogenous rhBMP2 (200 ng/ml). After 5, 10, and 15 days, ALP and von Kossa stainings were performed. Data are expressed as mean ± S.E. for 6 wells per group. *, p < 0.01, significant stimulation by rhBMP2.
focus on these chondrocytes. To obtain physiologically relevant data on the role of the endogenous BMPs during bone formation, we screened for BMPs expressed by these chondrocytes to find that BMP2 and BMP6 were the main subtypes and analyzed the effects of their loss on bone formation.

Fetal Bmp2+/−/Bmp6−/− mice exhibited a reduced bone formation in the primary spongosia, probably because of reduced bone formation and/or stimulation of bone resorption. We think the latter possibility rather unlikely, because the number of osteoclasts was not increased in the compound-deficient mice. The expression of the type I collagen, a marker for both early and late osteoclasts, showed no reduction, suggesting that the differentiation of osteoclasts from the precursor cells was not affected. The growth plates of these mice were smaller, but the proportions of distinct layers were maintained; in addition, the expressions of type II and X collagens were not changed, and hypertrophic chondrocytes mineralized the surrounding cartilaginous matrix to the same extent as WT. There was no difference in the onset of terminal differentiation. These data suggest that there is little, if any, abnormality in the chondrocyte differentiation. The data for adult mice concur with those of fetal mice; bone formation was reduced because of the impaired function of the osteoclasts with no change in their number, whereas the bone resorption markers were all normal. Taken together, these findings provide evidence that the combination of the endogenous BMP2 and BMP6 is vital for bone formation in both the fetal and adult life stages.

In line with our results are those of transgenic mice expressing a dominant-negative form of the BMP receptor 1B (BMPR-1B) under the control of the type I collagen promoter (24). They were smaller than WT and showed impairment of postnatal bone formation with the number of osteoblasts and the parameters of bone resorption unchanged, suggesting that the osteoblast function was impaired. In addition, transgenic mice lacking the BMP receptor 1A (BMPR-1A) specifically in osteoblasts using the Cre/loxP system under the control of the Og2 promoter also exhibited a low bone mass because of the impaired osteoblast function (25). The bone phenotypes of these genetically manipulated mice, of which the osteoblast could not transduce normal BMP signaling, are similar to those of Bmp2+/−/Bmp6−/− mice. These data suggest that osteoblasts require endogenous BMPs to provide their full function in vivo, but are not informative on the subtype and the origin of such BMPs. Our data suggest that BMP2 and BMP6 are two of them.

One question, however, still remained as to where BMP2 and BMP6 came from to act on the osteoblasts. There were three possibilities. First, they came from hypertrophic chondrocytes. Second, they came from other cell sources at a distance in an endocrine fashion. In situ hybridization analysis of the developing bone showed that BMP6 was expressed in hypertrophic chondrocytes and that BMP2 was strongly expressed in hypertrophic chondrocytes and marginally in the osteoblasts. Bone marrow cells obtained from Bmp2+/−; Bmp6−/− mice showed the same osteogenic ability as WT both at the basal status and in response to the exogenous BMP2. Furthermore, intramembranous bone formation in the calvaria was not affected in Bmp2+/−;Bmp6−/− mice. These data favor the first possibility. To strictly prove this, however, a further study using the tissue-specific ablation of the Bmp2 and Bmp6 genes in hypertrophic chondrocytes is needed. In addition, we should be careful in extrapolating the results of murine experiments to humans, because the growth plate, the source of hypertrophic chondrocytes, in humans disappears at puberty, whereas that of mice persists throughout life.

As for the small size of the growth plate, it may be related to the decreased axial growth of the mutant mice. It may be that the loss of BMP2 and BMP6 affects the size of the mesenchymal condensations and/or the proliferation rate of the chondrocytes. Alternatively, the osteoblast dysfunction caused by the compound loss of these BMPs may elicit a decrease in the axial bone growth, because there is a report that the ablation of the osteoblasts led to skeletal growth arrest (26). A further study is needed to clarify these issues.

In the fracture model, the cartilaginous callus was almost completely replaced by newly formed bone tissue in WT mice, whereas a massive cartilaginous callus persisted in Bmp2+/−/Bmp6−/− mice. When the callus was divided into the central and peripheral part, mineralization of the central part, where the endochondral bone formation prevailed, was reduced. These data suggest that the replacement of cartilaginous callus by bone is affected in the compound-deficient mice, which is in line with the data for physiological bone formation. It is noteworthy that the total callus size of the compound-deficient mice was smaller than that of WT. This may be because of the same cause of the smaller size of the growth plate.

A previous study of chimeric mice containing both WT and Ihh−/−; parathyroid hormone/parathyroid hormone-related peptide receptor−/− cells revealed that Ihh synthesized by the prehypertrophic and hypertrophic chondrocytes was locally required for the induction of bone formation in the adjacent perichondrium (8). In these chimeric mice, although both BMP2 and BMP6 were strongly expressed by the ectopic Ihh−/−;parathyroid hormone/parathyroid hormone-related peptide receptor−/− hypertrophic chondrocytes, the ectopic bone formation did not occur, suggesting that BMPs alone were not sufficient to induce physiological bone formation. In addition, blocking of Hh signaling inhibited the BMP2-induced osteogenic differentiation in mouse limb bud cell line MLB13MYC clone 17 (10), suggesting the presence of synergistic interactions between Ihh and BMPs. In the present study, the lack of one allele of the Bmp2 gene and both alleles of the Bmp6 gene caused a reduction in bone formation because of the osteoblast dysfunction. Taken together, we think it likely that BMP2 and BMP6 expressed by hypertrophic chondrocytes act in synergy with Ihh for physiological bone formation.

BMP2 and BMP6 have been reported to form a heterodimer, which was more potent for induction of osteogenic differentiation than the BMP2 homodimer or BMP6 homodimer (27). To assess the role of the Bmp2/Bmp6 heterodimer in vivo, we analyzed the bone formation of Bmp6−/− mice. The BMD of Bmp6−/− mice, which were thought to have no BMP2/BMP6 heterodimer, was similar to that of WT. In addition, the fracture model did not reveal any difference between WT and Bmp6−/− mice. These data suggest that, although the BMP2/BMP6 heterodimer is more potent than the BMP2 or BMP6 homodimers in in vitro or implant experiments, the BMP2/BMP6 heterodimer may not have a physiologically relevant role in the bone formation.

In conclusion, the combination of BMP2 and BMP6 play pivotal roles in bone formation under both physiological and pathological conditions. To the best of our knowledge, this is the first report to show that endogenous BMPs are important for in vivo bone formation.

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Involvement of Endogenous Bone Morphogenetic Protein (BMP) 2 and BMP6 in Bone Formation

Fumitaka Kugimiya, Hiroshi Kawaguchi, Satoru Kamekura, Hirotaka Chikuda, Shinsuke Ohba, Fumiko Yano, Naoshi Ogata, Takenobu Katagiri, Yoshifumi Harada, Yoshiaki Azuma, Kozo Nakamura and Ung-il Chung

J. Biol. Chem. 2005, 280:35704-35712.
doi: 10.1074/jbc.M505166200 originally published online August 18, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M505166200

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