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Conditional expression of human bone Gla protein in osteoblasts causes skeletal abnormality in mice

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ABSTRACT

Bone Gla protein (BGP), also known as osteocalcin, is one of the most abundant γ-carboxylated noncollagenous protein in bone matrix and plays important roles in mineralization and calcium ion homeostasis. BGP is synthesized specifically in osteoblasts; however, its precise function in bone metabolism has not been fully elucidated. To investigate the *in vivo* function of human BGP (hBGP), we generated CAG-GFP^{floxed}-hBGP transgenic mice carrying a transgene cassette composed of the promoter and a floxed GFP linked to hBGP cDNA. The mice were crossed with Coll-Cre mice, which express the Cre recombinase driven by the mouse collagen type 1a1 gene promoter, to obtain hBGP^{Coll} conditional transgenic mice that expressed human BGP in osteoblasts. The hBGP^{Coll} mice did not survive more than 2 days after birth. The analysis of the 18.5-day *post coitum* fetuses of the hBGP^{Coll} mice revealed that they displayed abnormal skeletal growth such as deformity of the rib and short femur and cranium lengths. Moreover, increased BGP levels were detected in the serum of the neonates. These findings indicate that hBGP expression in osteoblasts resulted in the abnormal skeletal growth in the mice. Our study provides a valuable model for understanding the fundamental role of BGP *in vivo*.

1. Introduction

Bone Gla protein (BGP), also known as osteocalcin, is a small (5.9 kDa) negatively charged (pI = 4.0) molecule that is one of the most abundant noncollagenous protein in bone [1,2]. The BGP protein is highly conserved among species and contains 3 γ-carboxyglutamic acid (gla) residues, which are the result of vitamin K-dependent posttranslational carboxylation [3]. Structural studies have indicated that the sequence region bearing the 3 gla residues forms an α-helix that binds calcium ions. This region is also important for the BGP to bind to hydroxyapatite [4,5]. It has been proposed that the calcium binding causes a conformational change that leads to increased affinity for hydroxyapatite [5].

BGP is synthesized specifically by osteoblast lineage cells and is established as a marker of differentiated mature osteoblasts. The level of BGP protein in bone is increased markedly during periods of intense skeletal growth [6]. Moreover, serum BGP concentrations fluctuate in diseases that are characterized by high bone turnover, such as osteoporosis [7].

Ducy et al. [8] generated null mice for the *BGP* genes. These mice were normal at birth, viable, and fertile but showed increased bone density and bone formation rate after 4.5 months without a comparable increase in osteoblast surface. Increased bone tissues had a beneficial effect on mechanical properties in the null mice; however, the null mice developed more severe osteopenia after ovariectomy. Thus, BGP is considered as an important protein in bone metabolism and development, although its precise role remains to be clarified.

Dacquin et al. [9] have reported the construction of a mouse transgenic line where the *Cre* gene is under the control of the 2.3-kb proximal fragment of the *Col1a1* promoter and expressed at high levels in osteoblasts throughout their differentiation. The transgenic mice were identified as Coll-Cre and expressed Cre at a level sufficient to induce recombination in osteoblasts [9].

To examine the role of human BGP (hBGP) during development, using Coll-Cre mice we generated conditional transgenic mice that express hBGP in osteoblasts. These mice displayed perinatal lethality and skeletal growth abnormalities. The serum level of BGP was

Abbreviations: BGP, bone Gla protein; hBGP, human bone Gla protein; Gla, γ-carboxyglutamic acid; Cre, Cre recombinase; GFP, green fluorescent protein.

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increased in the conditional transgenic mice compared with the wild-type mice. This mouse model shows the crucial role of hBGP in bone formation and is useful for understanding the *in vivo* function of hBGP.

2. Materials and methods

2.1. Construction of the transgene

A CAG-GFP^{floxed}-hBGP transgene plasmid was generated using a pCALwL vector that contained a composite construct consisting of a cytomegalovirus IE enhancer, a chicken β-actin promoter, 2 functional loxP sites, and a rabbit β-globin polyadenylation signal [10]. cDNAs for GFP fused to Histone H2B [11] and human BGP [12] were subcloned into sites between the 2 loxP sites and upstream of the polyadenylation signal, respectively.

2.2. Generation of transgenic mice

The plasmid CAG-GFP^{floxed}-hBGP was linearized with restriction enzyme digestion. Transgenic mice were produced by microinjecting the linearized plasmid into the pronuclei of the fertilized eggs from the C57BL/6 mice as described previously [13]. Transgenic mice were identified by PCR assays of genomic DNA extracted from the tail, and transgene expression was monitored by GFP fluorescence using a stereomicroscope. Primers derived from the 5′- and 3′-flanking sequences of GFP (5′-ACGTGCTGGTTGTTGTGCTGTCTCTCATCA-3′ and 5′-TGATTTGATCCCCGGGTACCGAGCGAC-3′) were used to amplify a 1.9-kb product. The ColI-Cre mice were kindly provided by Dr. G. Karsenty [9], and the ROSA26LacZ reporter mice were obtained from The Jackson Laboratory (Bar Harbor, ME). For the Cre transgenic mice, the primers derived from Cre (5′-CCTGGAAAATGCTTCTGTCCGTTTGCC-3′ and 5′-GAGTTGATAGCTGGCTGGTGGCAGATG-3′) were used to amplify a 653-bp product. For the ROSA26LacZ transgenic mice, the primers (5′-GCGAAGAGTTTGTCCTCAACC-3′, 5′-AAAGTCGCTCTGAGTTGTTAT-3′, and 5′-GGAGCGGGAGAAATGGATATG-3′) were used to amplify a 340-bp product for the transgene and a 650-bp product for the wild-type allele (The Jackson Laboratory). The CAG-GFP^{floxed}-hBGP transgenic male mice were mated with ColI-Cre transgenic female mice to obtain the conditional transgenic mice hBGP^{ColI}. The ColI-Cre/ROSA26LacZ reporter mice were generated by cross-breeding the ROSA26LacZ and ColI-Cre mice and used to track the activity of the *ColI* promoter throughout the ontogeny of the mouse. All the animal experiments were approved by the institutional animal care and use committee of the Saitama Medical University.

2.3. Fibroblasts preparation, adenoviral infection, and immunocytochemistry

Fibroblasts were prepared from the CAG-GFP^{floxed}-hBGP transgenic mice by culturing minced tail in Dulbecco's modified Eagle medium. A recombinant adenovirus expressing Cre recombinase (AxCANCre) under control of the CAG promoter [10] was amplified and titered in 293T cells, using standard methodologies [14]. The fibroblasts incubated on coverslips in serum-free medium were infected with the adenovirus at 10× multiplicity of infection to allow for efficient infection and *GFP* gene excision. After 48 h, the fibroblasts were fixed with 4% paraformaldehyde and used for immunocytochemical analysis with mouse anti-Cre (Nacalai Tesque, Kyoto, Japan) or rabbit anti-human BGP antibody. Then, the cells were visualized with secondary antibodies conjugated with Cy3 (The Jackson Laboratory).

2.4. Histological procedures

Assessment of β-galactosidase (β-gal) activity was performed according to the method of Hogan et al. [13] with a slight modification. The frozen sections (10 μm thick) of the femur and rib from the neonatal ColI-Cre/ROSA26LacZ mice (P0) were fixed with a 0.25% glutaraldehyde solution for 10 min. The sections were then washed 3 times and incubated with a staining solution [1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.1% phosphate buffer at pH 7.5, 5-mM potassium ferrocyanide, 5-mM potassium ferricyanide, 0.02% NP-40, 0.01% sodium deoxycholate, and 2-mM $MgCl_2$] for 4–5 h to visualize β-gal activity. Subsequently, the X-gal stained sections were fixed with 4% paraformaldehyde for 10 min, rinsed, and incubated for 24 h in a solution (1:10 naphthol AS-BI phosphate sodium salt, 0.025-M Tris–HCl at pH 8.5, 0.06% Fast Red TR salt) for alkaline phosphatase (ALP) staining [15]. *In situ* hybridization was performed using standard techniques [13]. Briefly, the frozen sections (10 μm thick) of the rib and femur from the hBGP^{ColI} neonates (P0) were hybridized with DIG-labeled anti-sense cRNA riboprobe for human BGP according to the manufacturer's instruction (Roche Molecular Biochemicals, Mannheim, Germany). Differential staining of the cartilage and bone of the 18.5-day *post coitum* (d.p.c.) mice by Alcian Blue and Alizarin Red was performed as described [13].

2.5. Assay of BGP levels in serum

Blood samples were collected from the hBGP^{ColI} neonates at P0 immediately after they were sacrificed. Because 30 μL of serum is required to quantify the intact-BGP level for an immunoradiometric assay (BML, Tokyo, Japan), the sera were pooled from the neonates according to genotype, either hBGP^{ColI} or control littermates (CAG-GFP^{floxed}-hBGP or ColI-Cre). Using the pooled sera, the measurements were repeated 3 and 5 times for the hBGP^{ColI} and control littermates, respectively. The results were shown as mean ± SD. The statistical analysis was performed using the Student *t* test. Significance was defined as $p < 0.05$.

3. Results

3.1. Generation of floxed GFP-BGP transgenic mice

To investigate the effects of the overexpression of BGP, we used a conditional transgenic mouse system to express the human BGP (hBGP) transgene. We first established floxed GFP-hBGP transgenic mouse lines bearing the CAG-GFP^{floxed}-hBGP transgene (Fig. 1A). The promoter used here has the potential for ubiquitous expression in various tissues; however, we expected that the mice bearing this construct would express GFP but not hBGP owing to the poly(A) signal sequence that is located immediately after the GFP sequence. In the presence of Cre recombinase, the GFP sequence would be deleted and hBGP would be expressed instead of GFP under the control of the promoter. The CAG-GFP^{floxed}-hBGP construct was introduced into pronuclear-stage eggs of the C57BL/6 mice by microinjection. We obtained CAG-GFP^{floxed}-hBGP transgenic mice. The fluorescent stereomicroscopic analysis of the tissues (Fig. 1B) from the transgenic mice confirmed that the GFP protein was expressed ubiquitously. Then, to assess whether the hBGP was expressed under the presence of Cre recombinase, tail fibroblasts from the transgenic mouse were infected with a Cre-expressing recombinant adenovirus (AxCANCre) and used for immunocytochemical detection of Cre and BGP. As shown in Fig. 1C, positive signals for hBGP protein were observed after the infection of AxCANCre, indicating that this conditional transgene expression system works well *in vitro*.

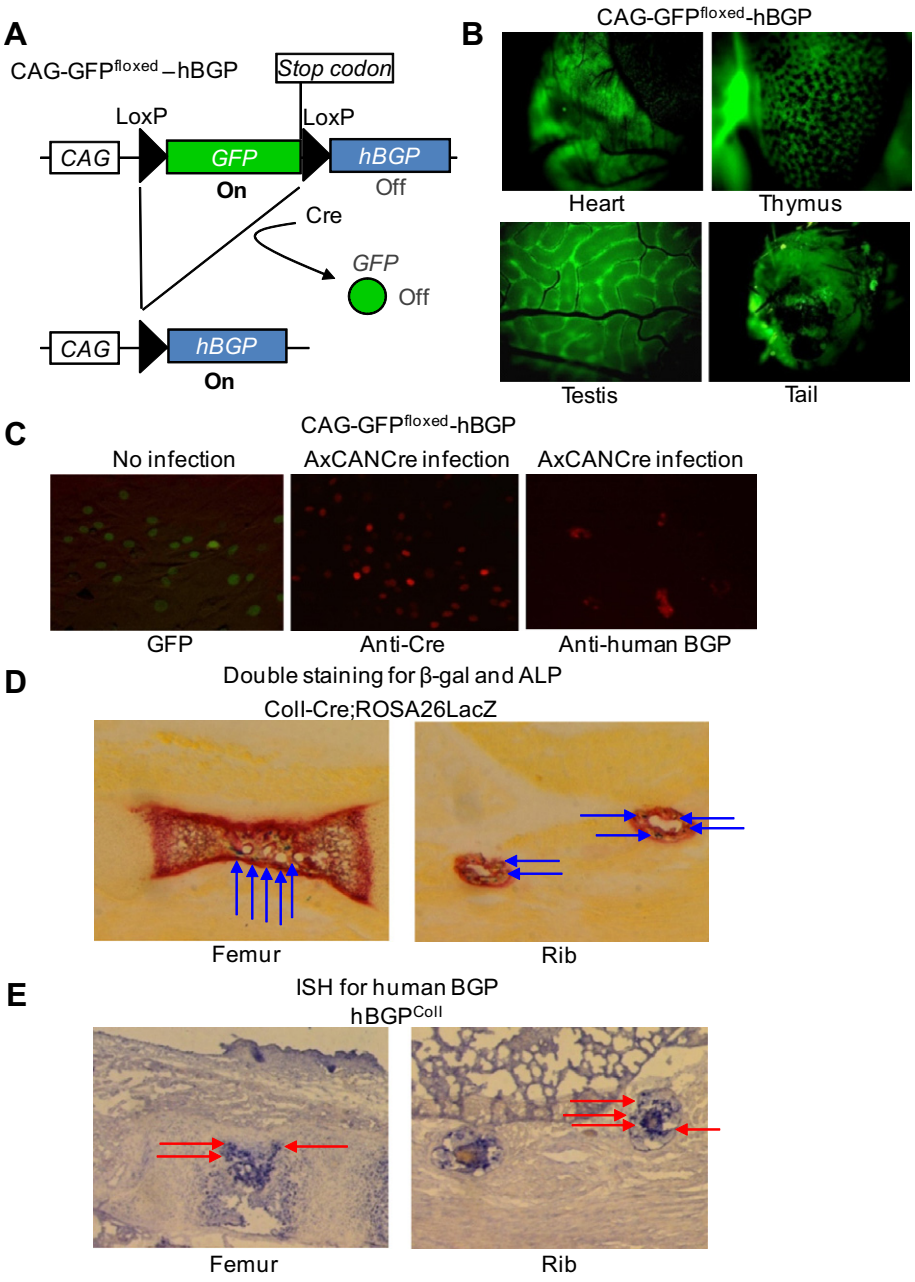

Fig. 1. Generation of conditional transgenic mice expressing BGP in osteoblasts. (A) Schematic representation of the CAG-GFP^{Floxed}-hBGP transgene. The construct expresses GFP but not human BGP (hBGP), as the loxP-flanked GFP gene has a stop codon at the end of the gene. In the presence of Cre recombinase, the GFP sequence is excised and hBGP is expressed instead of GFP under the control of the CAG promoter (Ref. [10]). (B) Stereomicroscopic image of the tissues from the CAG-GFP^{Floxed}-hBGP transgenic mouse. The GFP fluorescence (green) was visualized by a stereomicroscope. (C) Conditional hBGP expression in fibroblasts. The fibroblasts were prepared from the tails of the CAG-GFP^{Floxed}-hBGP transgenic mice and infected with a Cre-expressing recombinant adenovirus. The Cre and human BGP protein expressions were assessed by immunocytochemistry with anti-Cre and anti-human BGP antibodies, respectively, and visualized with secondary antibody conjugated with Cy3. The images were captured by fluorescence microscope (original magnification, 400×). (D) The Coll-Cre transgenic mouse was mated with the ROSA26LacZ tester mouse, and frozen sections of the femur and rib from the Coll-Cre + ROSA26LacZ + newborn mice (P0) were stained with X-gal followed by ALP staining. Double-positive signals (blue arrows) for X-gal (blue) and ALP (red) were detected in the osteoblasts. The images were collected using a microscope (original magnification, 200×). (E) *In situ* hybridization of the femur and rib from a hBGP^{Coll} neonate (P0). Human *BGP*-specific riboprobe was labeled with DIG and used for hybridization. Positive signals (red arrows) were detected in the osteoblasts. The images were collected using a microscope (original magnification, 200×).

3.2. Generation of conditional transgenic mice that express human BGP in osteoblasts

We used the Coll-Cre mouse [9] to achieve a conditional expression of hBGP in osteoblasts. To inspect the Cre expression in the osteoblasts, we generated mice heterozygous for Coll-Cre and ROSA26LacZ transgenes by breeding the homozygous ROSA26LacZ mice with the homozygous Coll-Cre mice. The femur and rib sections were prepared from the heterozygous neonates at P0 and examined by X-gal staining, followed by ALP staining as an osteoblast marker (Fig. 1D). As shown in the figure, LacZ-positive signals were detected in the ALP-positive osteoblasts in these tissues. Next, we generated conditional transgenic mice hBGP^{Coll} expressing hBGP protein in the osteoblasts. Using the femur and rib from

the hBGP^{ColI} neonates (P0), the expression of *hBGP* transgene was confirmed in the osteoblasts by *in situ* hybridization with a human *BGP*-specific riboprobe (Fig. 1E). These data indicate successful generation of conditional transgenic mice expressing human BGP in osteoblasts.

3.3. Skeletal abnormalities of mice expressing human BGP in osteoblasts

While we tried to expand this conditional transgenic mouse, we found that the hBGP^{ColI} genotype mouse did not survive to reproductive age. We crossed the homozygous CAG-GFP^{floxed}-hBGP male mice with the homozygous ColI-Cre female mice, and the survival of the offspring was monitored. As shown in Table 1a, the embryos were normally found *in utero* at 18.5 d.p.c.; however, a reduced number of living pups was observed at P0, and notably, all the pups were dead by P1. We confirmed that the stomachs of the living pups contained milk. On the other hand, when we intercrossed the heterozygous CAG-GFP^{floxed}-hBGP male mice and the homozygous ColI-Cre female mice, both genotypes of hBGP^{ColI} and ColI-Cre+; hBGP− embryos were detected equally at 18.5 d.p.c. (Table 1b). These data indicate that overexpression of hBGP in osteoblasts causes lethality in mice at around the time of birth.

Table 1a
The number of survival embryos or pups with hBGP^{ColI} genotype.

Day	Survival embryos or pups	Average	Number of breeding pairs
18.5 d.p.c.	24	4.8	5
P0	13	2.2	6
P1	0	0	6

Progenies were obtained from intercross between homozygous CAG-GFP^{floxed}-hBGP male and homozygous ColI-Cre female.

Table 1b
The number of 18.5 d.p.c. embryos obtained from 5 pairs of intercross between heterozygous CAG-GFP^{floxed}-hBGP male and homozygous ColI-Cre female.

Genotype	Number of embryos	Average
hBGP^{ColI}	15	3.0
ColI-Cre+; hBGP−	19	3.8

Subsequently, we examined the skeletal development of the hBGP^{ColI} mice using Alcian Blue and Alizarin Red stains. Fig. 2A shows that the hBGP^{ColI} embryos had severe skeletal deformities in their ribs and tails, although those of the other genotype littermates were normal. In addition, the femurs and craniums from the hBGP^{ColI} embryos were smaller than those of their wild-type littermates (Fig. 2B). These results indicate that conditional transgenic mice expressing human BGP in osteoblasts have impaired skeletal growth.

3.4. Higher BGP levels in serum in the hBGP^{ColI} neonates

Considering that the hBGP^{ColI} mice displayed skeletal malformation at 18.5 d.p.c., it was assumed that overexpression of hBGP in osteoblasts caused perturbations of bone formation during development. Therefore, we assessed serum BGP levels as a biochemical marker for bone metabolism. The serum BGP levels of the hBGP^{ColI} neonates were clearly higher than those of the other genotype littermates (Fig. 3).

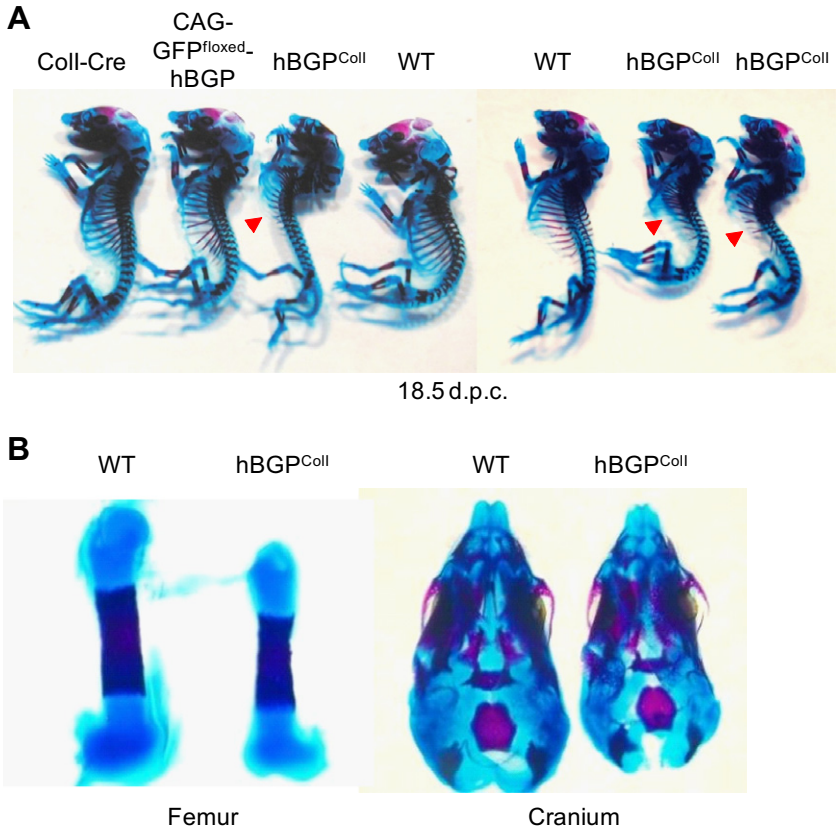

Fig. 2. Skeletal abnormality in the hBGP^{ColI} mice. Alcian Blue and Alizarin Red stainings were performed using 18.5 d.p.c. embryos of the hBGP^{ColI} and other genotypes. The hBGP^{ColI} mice showed apparent abnormalities of the rib (arrowheads) and tail (A), and smaller femur and cranium (B).

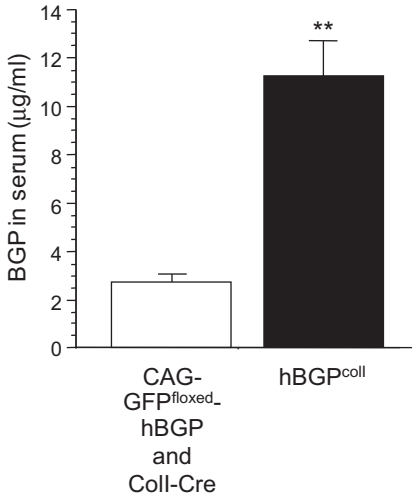

Fig. 3. Increased serum BGP levels in the hBGP^{Coll} mice. The intact-human-BGP level was determined by immunoradiometric assay using pooled serum collected from the hBGP^{Coll} and control littermates (CAG-GFP^{floxed}-hBGP or Coll-Cre+; hBGP−) at P0. Measurement was carried out 3 and 5 times for the hBGP^{Coll} and controls, respectively, and the results are presented as mean ± SD ($^{**}p < 0.01$).

4. Discussion

In the present study, we generated conditional transgenic mice using the Cre/loxP system to explore the function of human BGP in osteoblasts. The CAG-GFP^{Floxed}-hBGP transgenic mice, which expressed GFP but not hBGP, were generated and mated with the Coll-Cre mice to allow hBGP expression in osteoblasts [9]. The hBGP^{Coll} neonates had smaller and abnormal bone, and were dead by P1. Furthermore, serum intact-BGP level was significantly increased in the hBGP^{Coll} newborn mice compared with the control littermates. In humans, several studies have shown that increased levels of BGP in serum provide a sensitive and useful marker of skeletal growth [16] and bone metabolism in various metabolic bone diseases [17]. Therefore, these results suggest that the expression of hBGP in osteoblasts affects bone metabolism and causes skeletal growth abnormality in mice.

Ducy et al. [8] reported that *BGP*-deficient mice exhibited increased bone formation and bone strength, suggesting that BGP functions as a negative regulator of bone formation. In our conditional transgenic mouse model, the expression of human BGP in osteoblasts resulted in short stature and abnormal bone formation. This result would support the notion that BGP is a critical regulator for bone metabolism *in vivo*. In a study by Murshed et al. [18], a line of α1(I)Col-Osteocalcin transgenic mice was shown to exhibit no metabolic or histological abnormalities. We assume that the differences between their model and ours may result from the amounts and the species of exogenous BGP expressed in mice: Murshed et al. used a mouse BGP sequence, whereas we used a human BGP sequence.

In terms of species differences of BGP genes, there are three BGP-related genes in the mouse genome whereas only one BGP gene in the human genome. The mouse BGP cluster is composed of three genes; osteocalcin gene 1 (OG1), osteocalcin gene 2 (OG2), and osteocalcin-related gene (ORG) [19]. It has been shown that OG1 and OG2 are expressed only in the bone tissue, whereas ORG is predominantly transcribed in kidney. ORG mRNA has been predicted to encode nephrocalcin, but this molecule appears to be silent and unlikely to play a major role in mice [20]. Although mouse OG1 and OG2 encode identical amino acid sequences, the transcriptional regulation of these mouse genes may be distinct from that of human BGP gene, which may result in the species differences of BGP functions. In addition to the BGP genes, some of the BGP modulatory factors or target molecules may also be involved in the species differences of BGP functions. For instance, osteotesticular protein-tyrosine-phosphatase (OST-PTP), which influences metabolic bioactivity of BGP by regulating the level of γ-carboxylation [21], is expressed in mouse, but lost in human [22]. As another possibility, the biologically relevant receptor for BGP, GPRC6A, may be allosterically modulated by BGP in a species–specific manner [23]. Thus, it could be speculated that human and mouse BGP differentially influence the expression of BGP themselves, OSP-PTP or their receptor GPRC6A. Moreover, amino acid variations in human and mouse mature BGP sequences may also be important for the species differences. For example, cathepsin-sensitive proteolytic cleavage sites of human BGP, Gln39–Glu40 and Arg43–Arg44, are not conserved in mouse BGP [24]. Taken together, the transcription of BGP and its associated genes as well as the protein synthesis and turnover of BGP may be differentially regulated in mice and human.

In regard to the difference of transgene expression level, 2 of 3 lines of hBGP^{Coll} transgenic mice that we generated exhibited overexpression of BGP. The first line of our transgenic mice contained approximately 4-times higher concentration of BGP levels in serum compared with control mice (Fig. 3), assuming that the mice substantially produce human BGP protein also in osteoblasts. Notably, the neonates from this first line of hBGP^{Coll} mice did not survive more than 2 days after birth and the second line of transgenic mice also exhibited early postnatal lethality similar to the first line (data not shown). Interestingly, the third line with a lower level of human BGP mRNA expression compared to the first and second lines confirmed by qRT-PCR did not show any differences with WT mice. Based on our results, we consider that high expression of BGP in osteoblasts may cause the abnormal bone phenotypes in hBGP^{Coll} transgenic mice. In comparison with our study, the data of serum levels of BGP are not available in the study of α1(I)Col-Osteocalcin transgenic mice [18].

The hBGP^{Coll} mice showed perinatal lethality and did not survive for longer than 2 days after birth. Although the cause of this lethality remains unclear, their feeding behavior seemed to be intact as the living pups could drink milk. We speculate the following other reasons for perinatal death of the mice. First, these mice may exhibit respiratory insufficiency caused by the abnormal skeletal growth. The hBGP^{Coll} conditional transgenic mice showed a skeletal-patterning defect in their ribs, suggesting that they may have respiratory abnormality. In fact, pulmonary hypoplasia is sometimes found in patients with perinatal lethal osteogenesis imperfect (OI type II), or "brittle bone disease" [25]. A murine model for human OI died within 3–4 h after birth, and their phenotypes revealed respiratory distress and severe skeletal deformity [26]. Second, the mice are likely to have some abnormality in the central nervous system. Considering that the hBGP^{Coll} fetuses had substantially smaller craniums, these mice may have had impaired brain development. The third possibility may be derived from some metabolic defects of the mice. A previous report showed that BGP-lacking mice had higher blood glucose levels and lower serum insulin levels than wild-type mice, and showed decreases of insulin secretion, glucose tolerance, and energy expenditure [21]. The report also implied that BGP increases β-cell proliferation, stimulates insulin expression and secretion by pancreatic β cells, enhances energy expenditure, and increases the expression of adiponectin, an insulin-sensitizing hormone produced by adipocytes. Therefore, hBGP^{Coll} mice can be expected to have metabolic abnormalities. However, we could not observe significant differences in blood sugar between the hBGP^{Coll} conditional transgenic mice and the wild-type controls (data not shown).

In summary, we successfully developed a conditional transgenic murine model allowing human BGP expression in osteoblasts using

the Cre/loxP system. This model may be valuable for understanding the fundamental roles of human BGP. It has been well known that BGP expression is specifically localized to the cells of osteoblast lineage, including mature osteoblasts, osteocytes, and hypertrophic chondrocytes [27–30]. Furthermore, lower levels of BGP expression have been observed in megakaryocytes, platelets [31], and the brain [32]. The transgenic mouse model generated in the present study will help us to understand human BGP function in a variety of tissues.

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