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Skeletal analysis of the long bone abnormality (lbab/lbab) mouse, a novel chondrodysplastic C-type natriuretic peptide (CNP) mutant

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Abstract

Long bone abnormality (lbab/lbab) is a strain of dwarf mice. Recent studies revealed that the phenotype is caused by a spontaneous mutation in the Nppc gene, which encodes mouse C-type natriuretic peptide (CNP). In this study, we analyzed the chondrodysplastic skeletal phenotype of lbab/lbab mice. At birth, lbab/lbab mice are only slightly shorter than their wild-type littermates. Nevertheless, lbab/lbab mice do not undergo a growth spurt, and their final body and bone lengths are only ~60% of those of wild-type mice. Histological analysis revealed that the thickness of the growth plate in lbab/lbab mice, especially that of the hypertrophic chondrocyte layer, was significantly thinner than in wild-type mice. Overexpression of CNP in the cartilage of lbab/lbab mice restored their thinned growth plate, followed by the complete rescue of their impaired endochondral bone growth. Furthermore, the bone volume in lbab/lbab mouse was severely decreased, and was recovered by CNP overexpression. On the other hand, the thickness of the growth plate of lbab/+ mice was not different from that of wild-type mice; accordingly, impaired endochondral bone growth was not observed in lbab/+ mice. In organ culture experiments, tibial explants from fetal lbab/lbab mice were significantly shorter than those from lbab/+ mice, and were elongated by addition of $10^{-7}$ M CNP to the same extent as lbab/+ tibiae treated with the same dose of CNP. These results demonstrate that lbab/lbab is a novel mouse model of chondrodysplasia caused by insufficient CNP action on endochondral ossification.
Key words

C-type natriuretic peptide (CNP), long bone abnormality (*lbab*), chondrodysplasia, endochondral bone growth, organ culture
Introduction

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family, and exerts its biological actions through the accumulation of intracellular cyclic GMP via a subtype of membranous guanylyl cyclase receptor, guanylyl cyclase-B (GC-B) [1, 2]. We had previously demonstrated that the CNP/GC-B system is a potent stimulator of endochondral bone growth: transgenic mice with targeted overexpression of CNP in cartilage under the control of type II collagen promoter [3], or those with elevated plasma CNP concentrations under the control of human serum amyloid P component promoter [4], exhibit a prominent skeletal overgrowth phenotype. On the other hand, the physiological importance of the CNP/GC-B system on endochondral bone growth has been revealed by the phenotypes of hypomorphs. We generated complete CNP or GC-B null mice, and demonstrated that they exhibit an impaired bone growth phenotype [5, 6]. We have also reported that two lines of spontaneous mutant mice with disproportionate dwarfism, cn/cn and slw/slw, are caused by loss-of-function mutations in the murine GC-B gene [7, 8]. The skeletal phenotypes of these mutant mice resemble those of GC-B knockout mice. Furthermore, recent studies have elucidated that loss-of-function mutations in the human GC-B gene are the causes of acromesomelic dysplasia type Maroteaux (AMDM), one form of skeletal dysplasias with disproportionate short stature phenotype [9]. The impaired skeletal growth phenotype observed in patients suffering from AMDM is similar to the skeletal phenotype of cn/cn, slw/slw, or GC-B knockout mice.

The long bone abnormality (lbab/lbab) mouse was first identified in The Jackson Laboratory as a spontaneous autosomal recessive mutant mouse characterized by impaired growth of long bones [10]. Recent studies have elucidated that the impaired growth of lbab/lbab mouse is caused by a hypomorphic mutation in the mouse CNP gene; Jiao et al. found that its impaired growth phenotype is associated with a single point mutation in the mouse CNP gene [11], and we showed that this phenotype is completely recovered by CNP overexpression [12]. Yoder et al. characterized the mutant CNP in lbab/lbab mouse, and exhibited that it is less biologically active than authentic CNP; in whole cell cGMP elevation and membrane guanylyl cyclase assays, 30-fold to greater than 100-fold more mutant CNP is required to activate GC-B as compared to authentic CNP [13]. We also confirmed that
the mutant CNP in lbab/lbab mouse retains only about 10% activity to induce cyclic GMP production through GC-B compared to authentic CNP in in vitro transfection assay using COS-7 cells [12]. Collectively, lbab/lbab mouse is a novel chondrodysplastic mouse model with insufficient CNP action on endochondral bone growth. Nevertheless, the skeletal phenotypes of lbab/lbab mouse have only been partially described in short reports including our own brief communication [11-13], and have not yet been fully studied. In this study, we performed further analyses of the skeletal phenotypes of lbab/lbab mouse.
Materials and Methods

Mice
Heterozygous (Lbab+/+) mice (C57BL/6J background) were obtained from The Jackson Laboratory, and the strain was maintained by sib mating of heterozygotes. Transgenic mice with targeted overexpression of CNP in the growth plate chondrocytes under the control of the mouse pro-\( \alpha_1(II) \) (Col2a1) promoter (CNP-Tg) were created as reported previously [3]. To perform genetic rescue of \( lbab/lbab \) mice, CNP-Tg mice were mated with \( lbab/+ \) mice, and F1 offspring heterozygous for the transgene and for the \( Lbab \) allele were mated with that only for the \( Lbab \) allele to generate \( lbab/lbab \) mice with the transgene expression (\( lbab/lbab\cdot\text{CNP-Tg/+} \) mice) [12]. Genotypes for the CNP transgene and the \( Lbab \) allele were determined by PCR analysis using mouse genomic DNAs extracted from tails. Because there was no tendency of gender differences in the growth of each genotype (data not shown), we used only female mice in our experiments. Animal care and all experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Skeletal analysis
For 10 weeks after birth, body lengths of female mice were measured weekly. Body length was measured as the length from the nose to the anus (naso–anal length) or that from the nose to the tip of the tail (naso–tail length). Body weights were also measured weekly. Skeletal analysis was performed as previously described [14]. Briefly, mice were subjected to soft X-ray analysis (30 kVp, 5 mA for 1 min, Softron type SRO-M5, Softron, Tokyo, Japan), and lengths of the bones were measured on the X-ray films. CT scanning of the humerus was performed using a ScanXmate-L090 Scanner (Comscantecno Co., Ltd., Yokohama, Japan). Three-dimensional microstructural image data were reconstructed and structural indices were calculated using TRI/3D-BON software (RATOC System Engineering Co., Ltd., Tokyo, Japan).
**Histological examination**

Tibiae were fixed in 10% formalin neutral buffer, decalcified in 10% EDTA, and embedded in paraffin. Five-micrometer-thick sections were sliced and stained with Alcian blue (pH 2.5) and hematoxylin–eosin. For immunohistochemistry, sections were incubated with rabbit anti-type X collagen antibody (LSL, Tokyo, Japan), goat anti-Indian hedgehog (Ihh) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-matrix metalloproteinase 13 (MMP-13) antibody (Thermo Fisher Scientific Inc., MA), and mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Copenhagen, Denmark). Immunostaining was performed using the Histofine MOUSESTAIN KIT (Nichirei Biosciences Inc., Tokyo, Japan) according to the manufacturer’s instruction. Peroxidase activity was visualized using diaminobenzidine. The sections were counterstained with hematoxylin, dehydrated, and then mounted with Malinol (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). To confirm antibody specificity, normal rabbit serum (Sigma–Aldrich Co., St. Louis, MO), normal goat IgG (Santa Cruz Biotechnology Inc.), and mouse IgG (Dako) were used as first antibodies for negative controls, respectively.

**Organ culture**

Organ culture of fetal mouse tibiae or third metatarsi was performed as described previously [15]. Tibial or metatarsal explants from *Ibab/+* mice and their *Ibab/Ibab* littermates at 16.5 day post coitus were cultured for 4 days with vehicle or $10^{-7}$ M CNP (Peptide Institute, Inc., Minoh, Japan). Medium was changed every day. Before and after the culture, the maximal longitudinal lengths of tibiae were measured as the total tibial length, the sum lengths of proximal and distal cartilaginous primordia (CP), and the length of osteogenic center (OC), by using a linear ocular scale mounted on an inverted microscope. For histological analysis, explants were fixed in 10% formalin neutral buffer and embedded in paraffin. Five-micrometer-thick sections were sliced and stained with Alcian blue (pH 2.5) and hematoxylin–eosin. Immunohistochemical staining of incorporated bromodeoxyuridine (BrdU) was performed using 5-Bromo-2’-deoxy-uridine Labeling and Detection Kit II (Roche Applied
Science) according to the manufacturer’s protocol.

**Statistical analysis**

Data were expressed as the mean ± SEM. The statistical significance of differences between mean values was assessed using Student’s *t* test.
**Results**

**Analyses of skeletal growth of *lbab/lbab* and *lbab/+* mice**

As previously reported, *lbab/lbab* mice developed severe dwarfism characterized by short tails and extremities [11, 12]. At birth, *lbab/lbab* pups were slightly shorter than their wild-type littermates: the naso-anal and the naso-tail lengths of *lbab/lbab* mice were 88% and 83% of those of their wild-type littermates, respectively (Fig. 1a and Supplemental Fig. 1). The ratios of naso–anal and naso–tail lengths of *lbab/lbab* mice to those of wild-type mice sharply decreased to 65% and 55%, respectively, by the age of 3 weeks. After 5-week-old, these ratios stabilized at to 66–72% and 57–62%, respectively (Fig. 1a and Supplemental Fig. 1). The body weight of *lbab/lbab* mice was 68% of that of their wild-type littermates at birth, and was decreased to 46% by the age of 3 weeks. The ratio did not increase until 5 weeks of age, but was then increased and became ~60 % after 7-week-old (Fig. 1b).

On the other hand, the *lbab/+* mice were indistinguishable from their wild-type littermates at birth, and grew almost similarly as their wild-type littermates (Fig. 1a, b, and Supplemental Fig. 1). Soft X-ray analysis revealed that longitudinal growth of vertebrae, tail, and extremities was affected in *lbab/lbab* mouse at the age of 2 weeks, but not affected in *lbab/+* mice (Fig. 1c). Histological analysis revealed that at the age of 3 days, the thickness of the tibial growth plate, especially that of the hypertrophic chondrocyte layer, of *lbab/lbab* mice was apparently thinner than that of wild-type mice (Fig. 1d). On the other hand, the thickness of tibial growth plate of *lbab/+* mice was not different from that of wild-type mice (Fig. 1d).

**The Effect of CNP overexpression on impaired endochondral bone growth of *lbab/lbab* mice**

In order to further characterize the impaired skeletal growth of *lbab/lbab* mice, we analyzed how their impaired endochondral bone growth recovered in response to targeted overexpression of CNP in the cartilage *in vivo* [12]. We crossed *lbab/lbab* mice with cartilage-specific CNP transgenic mice under the control of type II collagen promoter (CNP-Tg mice), and obtained *lbab/lbab* mice with transgenic expression of CNP in cartilage (*lbab/lbab*•CNP-Tg mice) [12]. At the first week after birth, the
naso–anal length of lbab/lbab•CNP-Tg mice was almost the same as that of lbab/lbab mice, and considerably smaller than that of wild-type mice: naso–anal lengths of wild-type, lbab/lbab, and lbab/lbab•CNP-Tg mice were 4.38 ± 0.06, 3.87 ± 0.37, and 4.00 ± 0.12 cm, respectively. Subsequently, lbab/lbab•CNP-Tg mice began to grow larger than lbab/lbab mice, and promptly caught up with wild-type mice; although the naso–anal length of lbab/lbab•CNP-Tg mice was still considerably smaller than that of wild-type mice until 3 weeks of age (5.70 ± 0.57 and 6.71 ± 0.10 cm, respectively, at the age of 3 weeks), it became almost comparable to that of wild-type mice after 4-week-old (7.38 ± 0.48 and 7.61 ± 0.10 cm, respectively, at the age of 4 weeks). Further, the body weight of lbab/lbab•CNP-Tg mice was almost the same as that of lbab/lbab mice and was smaller than that of wild-type mice until the age of 3 weeks, but was then promptly increased to a level comparable to that of wild-type mice (Supplemental Fig. 2).

Soft X-ray analyses revealed that at the age of 2 weeks, the impaired growth of bones formed through endochondral ossification in lbab/lbab mice was partially recovered by targeted overexpression of CNP in cartilage in lbab/lbab•CNP-Tg mice (Fig. 2a): the recoveries in the longitudinal length of cranium and the lengths of humerus, radius, ulna, femur, tibia, and vertebra, were 35, 73, 68, 37, 51, 63, and 27%, respectively (Fig. 2b). Furthermore, at the age of 10 weeks, the impaired endochondral bone growth in lbab/lbab mice was almost completely recovered by targeted overexpression of CNP in cartilage, as observed in lbab/lbab•CNP-Tg mice (Fig. 2c, d). On the other hand, there were no significant differences in the width of cranium, which is formed via intramembranous ossification, among the three genotypes at either 2 or 10 weeks (Fig. 2b, d).

Histological analysis showed that both the thickness of proliferative chondrocyte layer and that of hypertrophic chondrocyte layer, positive for immunohistochemical staining for type X collagen, were significantly decreased in lbab/lbab mice compared to those in wild-type mice at the age of 2 weeks, as previously reported [12] (Fig. 3a, b). The thinner proliferative chondrocyte layer in lbab/lbab growth plate was completely recovered by targeted overexpression of CNP as observed in lbab/lbab•CNP-Tg growth plate (Fig. 3c). The thinner hypertrophic chondrocyte layer in lbab/lbab growth plate was also considerably recovered in lbab/lbab•CNP-Tg growth plate, although the extent
of the recovery was weaker than in the case of the proliferative chondrocyte layer (Fig. 3d). Immunohistochemical staining for PCNA revealed that the number of PCNA-positive cells is severely decreased in the proliferative chondrocyte layer of lbab/lbab growth plate (Fig. 3e). The number of PCNA-positive cells did not recover in the proliferative chondrocyte layer of lbab/lbab•CNP-Tg growth plate, whereas the thinner proliferative chondrocyte layer in lbab/lbab growth plate was almost completely recovered in lbab/lbab•CNP-Tg growth plate (Fig. 3c). The area positive for immunostaining of Ihh, one of markers of hypertrophic differentiation, was decreased in lbab/lbab growth plate compared to that in wild-type growth plate (Fig. 3f). The smaller size of the area positive for Ihh in lbab/lbab growth plate was almost completely recovered in lbab/lbab•CNP-Tg growth plate (Fig. 3f). Immunohistochemical staining of MMP-13, a useful marker for the terminal hypertrophic chondrocytes, was not changed between three genotypes, indicating that the progression through the hypertrophy program was not accelerated in lbab/lbab growth plate (Fig. 3g).

At the age of 10 weeks, the tibial growth plate of lbab/lbab mice continued to be thinner than that of wild-type mice, and was also completely recovered by overexpression of CNP in cartilage (Fig. 4).

**Recovery of decreased bone volume in lbab/lbab mouse by CNP overexpression**

Three-dimensional CT analysis manifested a marked reduction in bone volume of the humerus in lbab/lbab mouse, and considerable recovery in lbab/lbab•CNP-Tg mouse (Fig. 5). At the age of 10 weeks, the quantified bone volume (BV/TV) and trabecular thickness (Tb. Th) of humerus in lbab/lbab mouse were 2.4% and 34.5 μm, respectively, whereas those in wild-type mouse were 4.1% and 40.3 μm, respectively. The decreased BV/TV and Tb. Th in lbab/lbab mouse were increased to 5.4% and 37.0 μm, respectively, in lbab/lbab•CNP-Tg mouse.

**Organ culture experiments of tibiae from lbab/lbab mice**

In order to further analyze the impaired endochondral ossification of lbab/lbab mice, we preformed organ culture experiments using tibial explants from fetal mice (Fig. 6a) [15]. Because skeletal
phenotypes of mice heterozygous for the lbab allele were not different from those of wild-type mice, we compared the growth of tibial explants from lbab/lbab mice with that from lbab/+ mice. At the beginning of culture, both the total length and the sum length of cartilaginous primordia (CP) of lbab/lbab tibiae are significantly smaller than those of lbab/+ tibiae (3.80 ± 0.04 vs. 4.25 ± 0.03 mm and 2.19 ± 0.02 vs. 2.43 ± 0.01 mm, respectively, n = 8–12, each) (Fig. 6b, c). Tibial explants from lbab/lbab mice grew to the same extent as those from lbab/+ mice during a 4-day culture period; the difference in the total length or in the length of CP between lbab/lbab and lbab/+ explants at the end of culture was comparable to that at the beginning of culture (Fig. 6b, c). There was no significant difference in the length of the osteogenic center (OC) between two genotypes before and after the culture period (data not shown).

The treatment of CNP at the dose of 10⁻⁷ M stimulated the growth of both lbab/lbab and lbab/+ tibiae (Fig. 6b, c). CNP stimulated the growth of lbab/lbab tibiae more potently than that of lbab/+ tibiae; in the presence of 10⁻⁷ M CNP, the difference between the total length of lbab/+ tibiae and that of lbab/lbab tibiae was decreased (Fig. 6b), and furthermore, the CP length of lbab/lbab tibiae became almost the same as that of lbab/+ tibiae (Fig. 6c). The growth of OC was not stimulated by CNP in either lbab/lbab or lbab/+ explants (data not shown).

Histological examination at the end of culture period revealed that the length of the primordial growth plate (Fig. 7a), especially that of the hypertrophic chondrocyte layer positive for type X collagen immunostaining (Fig. 7b, c), was smaller in lbab/lbab explants than in lbab/+ explants. The area positive for immunostaining for Indian hedgehog (Ihh), one of markers for chondrogenic differentiation [16], tended to be a little decreased in lbab/lbab explants compared to that in lbab/+ explants, although the intensities of the immunostaining were not changed between two genotypes (Supplemental Fig. 3). Immunohistochemical detection of BrdU-incorporated chondrocytes revealed that BrdU-positive chondrocytes tended to be decreased in lbab/lbab explants compared to those in lbab/+ explants (Fig. 7d). Addition of CNP prominently increased the lengths of primordial growth plates (Fig. 7a) and their hypertrophic chondrocyte layers (Fig. 7b, c) of both lbab/+ and lbab/lbab explants. The lengths of primordial growth plate and its hypertrophic chondrocyte layer of lbab/lbab
explants treated with $10^{-7}$ M CNP became comparable to those of $lbab/+ \text{ explants treated with the same dose of } CNP (\text{Fig. 7a–c}). CNP increased the areas positive for Ihh immunostaining both in $lbab/+ \text{ and } lbab/lbab explants. By addition of CNP, the sizes of the areas positive for, and the intensities of, Ihh immunostaining became not different between in $lbab/+ \text{ and } lbab/lbab explants (\text{Supplemental Fig. 3}). CNP did not increased BrdU-positive chondrocytes in $lbab/lbab explants (\text{Fig. 7d}).

Further we explored whether CNP controls the progression of growth plate chondrocytes through the different stages of maturation or not. Because the process of endochondral ossification occurs delayed in metatarsus compared to that in tibia in an individual, we performed organ culture of metatarsi as well as tibiae from fetal mice at 16.5 day post coitus, and examined the expression of type X collagen and Ihh. In case of $lbab/+ \text{ organ culture, the area positive for immunostaining of type X collagen was reduced and that of Ihh was localized near the ossification center in metatarsal explants compared with those in tibial explants, indicating that metatarsal growth plate represents earlier stage of endochondral ossification than tibial growth plate (Fig. 8). The area positive for immunostaining of type X collagen was greatly reduced in $lbab/lbab metatarsal explants compared with that in $lbab/+ metatarsal explants, and recovered by addition of $10^{-7}$ M CNP to the same extent to that in $lbab/+ metatarsal explants treated with vehicle. The area positive for immunostaining of Ihh became closer to ossification center in $lbab/lbab metatarsal explants than in $lbab/+ metatarsal explants, and was returned to the same position as $lbab/+ metatarsal explants by addition of CNP (Fig. 8).
Discussion

Previously we and other groups had reported in brief communications that the short stature phenotype of \textit{lbab/lbab} mouse is caused by a mutation in the mouse CNP gene [11-13]. Here we further analyzed the skeletal phenotypes of \textit{lbab/lbab} mouse, and reported the results in this full-length manuscript.

Analysis of the growth curves of naso–anal and naso–tail lengths revealed that the shortness of \textit{lbab/lbab} mice is mild at birth, but rapidly progresses by the age of 3 weeks, and then after 4-week-old, the ratio of the length of \textit{lbab/lbab} mice compared to that of wild-type mice becomes almost constant. This suggests that CNP is especially crucial for the skeletal growth spurt that occurs in early life. Since CNP is expressed in the growth plate cartilage and works as an autocrine/paracrine regulator [5], CNP might affect the endochondral bone growth potently when the volume of growth plate cartilage is relatively abundant.

We confirmed the thinness of the growth plate of \textit{lbab/lbab} mice, especially in its hypertrophic chondrocyte layer, followed by the impaired growth of long bones. The thinness of the growth plate of \textit{lbab/lbab} mice was almost completely recovered by targeted overexpression of CNP in the growth plate by the age of 2 weeks. On the other hand, the recovery of the shortness of the total length of \textit{lbab/lbab} bones by CNP was only partial at 2-week-old, becoming complete at the age of 10 weeks. This finding suggests that the recovery is evident earlier in the thickness of the growth plate than in the total bone length. In addition, immunohistochemistry for PCNA revealed that at the age of 2 weeks, the proliferation of growth plate chondrocyte is decreased in \textit{lbab/lbab} mice, and the decreased proliferation is not rescued by CNP overexpression, even though the thickness of the growth plate does fully recover. The reason why the decreased proliferation of chondrocytes in \textit{lbab/lbab} growth plate was not rescued by CNP overexpression in chondrocytes is not clear, but it may because of the weak and slow expression of the CNP-transgene owing to the weak power of the promoter region. On the other hand, CNP could not increase the proliferation of growth plate chondrocytes in \textit{lbab/lbab} explants in organ culture experiment in this study. The effect of CNP on chondrocyte proliferation might be so mild that other effects of CNP on growth plate chondrocytes, e.g., the stimulatory effect on matrix synthesis as we had previously reported [3, 4], for example, might
proceed ahead to recover the thinned growth plate of \textit{lbab/lbab} mouse. The discrepancy between the effects on proliferation and matrix synthesis may explain in part the delayed recovery of bone length relative to growth plate thickness. On the other hand, immunohistochemical staining of type X collagen and Ihh in explanted growth plates at two different stages of endochondral ossification suggested that the progression of proliferative chondrocytes to hypertrophic chondrocytes was delayed in the \textit{lbab/lbab} growth plate and recovered by addition of CNP. In addition to the result that the expressions of MMP-13 were not different between in the terminal hypertrophic chondrocytes of wild-type, \textit{lbab/lbab}, and rescued growth plates, CNP might promote the hypertrophic differentiation of proliferative chondrocytes, but not accelerate the terminal differentiation of hypertrophic chondrocytes.

In this study, we investigated the character of calcified bones of \textit{lbab/lbab} mouse using three-dimensional CT analysis: the bone volume of \textit{lbab/lbab} mouse was substantially decreased compared to that of wild-type mouse, and was recovered by cartilage-specific CNP overexpression. The mechanism of decrease in bone volume of \textit{lbab/lbab} mouse is still unknown, but it would be postulated that CNP may be expressed in and affects on cells other than chondrocytes, i.e., osteoblasts or osteoclasts, in bone. Although overexpression of CNP was targeted to chondrocytes in our rescue experiments, early onset of the CNP-transgene expression from cartilaginous primordium might have been able to affect bone metabolism at the earlier stage of skeletogenesis [17], and may have continued to affect osteoblasts or osteoclasts near the growth plate cartilage in the later stage of skeletogenesis. Whereas several \textit{in vitro} effects of CNP on osteoblastic cell lineages or osteoclasts are reported [18-28], \textit{in vivo} effects of CNP on bone metabolism still remain elusive, and further experiments are now ongoing in our laboratory.

We previously discovered that two strains of dwarf mice, \textit{cn/cn} and \textit{slw/slw}, are caused by spontaneous mutations in the GC-B gene [7, 8]. In humans, AMDM has been identified to be caused by spontaneous loss-of-function mutations in the GC-B gene [9, 29]. The \textit{lbab/lbab} mouse, of which skeletal phenotype we have closely analyzed in the present paper, has a spontaneous loss-of-function mutation in the mouse CNP gene; by analogy to the GC-B gene, some forms of human skeletal dysplasias might be identified to be caused by mutations in the CNP gene in future. Thus far, no such
conditions have been discovered [30]. In the event such a discovery is made, the \( lbab/lbab \) mouse would then be a novel mouse model of a form of human skeletal dysplasias caused by a mutation in the CNP gene.

In contrast to mice homozygous for the \( lbab \) allele, the growth and skeletal phenotype of mice heterozygous for the \( lbab \) allele were not different from those of wild-type mice, as is the case with heterozygous CNP knockout mice. This confirms that haploinsufficiency for the CNP gene does not exist in mice. Likewise heterozygotes for the GC-B knockout, the \( cn \) allele, or the \( slw \) allele exhibit no skeletal abnormalities [6-8]; thus, haploinsufficiency of the GC-B gene also does not exist in mice. Nevertheless, haploinsufficiency of the GC-B gene does exist in humans: heterozygous carriers of AMDM are reported to be shorter than expected for their population of origin [31]. The reason for the discrepancy is not clear at present, but may be due to differences between species or some other unknown mechanisms. We will have to perform further investigations on the skeletal phenotypes of the aforementioned lines of GC-B mutant mice; such experiments are now ongoing in our laboratory.

In summary, in this study we more closely investigated the skeletal phenotypes of a novel CNP mutant mouse, \( lbab/lbab \). The results of this study will be useful not only for further elucidation of the physiological role of CNP on endochondral bone growth, but also for the prediction of pathophysiology of a hypothetical chondrodysplasia caused by a mutation in the human CNP gene, which has not yet been discovered.
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Disclosure of financial conflicts of interest

The disclosure of a manuscript by authors E.K., A.Y., T.T., T.F., M.M., N.K., N.T., H.A., T.K., and K.N. may appear as follows:

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peptide-induced differentiation of osteoblasts is switched from type B- to type C-natriuretic peptide receptor with aging. J Cell Biochem 103:753-764


Figure Legends

**Fig. 1**
Growth and skeletal phenotype of *lbab/+* and *lbab/lbab* mice.
(a), (b) Naso–anal lengths (a) and body weights (b) of female wild-type (Wt, ◊), *lbab/+* (□), and *lbab/lbab* (△) mice (n = 2–8). (c) Whole skeletons of wild-type, *lbab/+*, and *lbab/lbab* mice at 2 weeks of age. Wt, wild-type. Scale bar, 1 cm. (d) Histological analysis of the tibial growth plates of 3-day-old mice. Arrows indicate hypertrophic chondrocyte layers. Alcian blue and hematoxylin–eosin staining. Scale bar, 100 µm

**Fig. 2**
The Effect of CNP overexpression on impaired endochondral bone growth of *lbab/lbab* mice.
Whole skeletons (a, c) and bone lengths measured on soft X-ray films (b, d) of female wild-type (Wt), *lbab/lbab*, and *lbab/lbab•CNP-Tg* mice at the age of 2 weeks (a, b) and 10 weeks (c, d). (a), (c) Scale bar, 1 cm, each. (b), (d) White bars, wild-type mice; black bars, *lbab/lbab* mice; gray bars, *lbab/lbab•CNP-Tg* mice. CW, width of cranium; CL, longitudinal length of cranium; HL, humeral length; RL, radial length; UL, ulnar length; FL, femoral length; TL, tibial length; VL, vertebral length. n = 2–7 (b) and 3–5 (d)

**Fig. 3**
Histological analysis of tibial growth plates from 2-week-old wild-type (Wt), *lbab/lbab*, and *lbab/lbab•CNP-Tg* mice.
(a) Alcian blue and hematoxylin–eosin staining. Yellow bars (depicted as “P”) indicate proliferative chondrocyte layers, and red bars (depicted as “H”) indicate hypertrophic chondrocyte layers. (b) Immunohistochemical staining for type X collagen. Scale bar in (a), (b), 100 µm, each. (c), (d) Heights of the proliferative (c) and hypertrophic (d) chondrocyte layers. n = 3, each. *, P < 0.05; **, P < 0.01. (e) The proportion of PCNA-positive chondrocytes in proliferative chondrocyte layers. n =
3−4. **, $P < 0.01$. (f), (g) Immunohistochemical staining of Ihh (f) and MMP-13 (g). Scale bar in (f), (g), 50 μm, each.

**Fig. 4**

Histological analysis of tibial growth plate from female 10-week-old wild-type (Wt), lbab/lbab, and lbab/lbab•CNP-Tg mice.

(a) Alcian blue and hematoxylin−eosin staining. Arrows indicate the width of growth plates. Scale bar, 50 µm. (b) Total heights of the growth plates. n = 2−5, each

**Fig. 5**

Micro CT analysis of humeri from wild-type (Wt), lbab/lbab, and lbab/lbab•CNP-Tg mice at the age of 10 weeks. Scale bar, 1 mm

**Fig. 6**

The effect of CNP on cultured tibiae from fetal lbab/+ or lbab/lbab mice.

(a) A representative picture of a tibial explant from a fetal mouse. Total longitudinal length (Total) and the sum lengths of cartilaginous primordia (CP) are indicated. (b), (c) Graphs of Total (b) and CP (c) lengths of cultured tibiae from lbab/+ and lbab/lbab mice treated with vehicle (veh.) or $10^{-7}$ M CNP (CNP) for 4 days. Circles indicate lbab/+ tibiae, and squares indicate lbab/lbab tibiae. At the end of culture, closed symbols indicate tibiae treated with vehicle, and open symbols indicate those treated with CNP. n = 8−12, each

**Fig. 7**

Histological analyses of the growth plates of tibial explants from fetal lbab/+ or lbab/lbab mice treated with vehicle (veh.) or $10^{-7}$ M CNP (CNP) for 4 days.

(a), (b) Alcian blue and hematoxylin−eosin staining (a) and immunohistochemical staining for type X collagen (b). Yellow bars in (a) indicate lengths of cartilaginous primordia and red bars in (b) indicate
heights of hypertrophic chondrocyte layers. Scale bar, 200 µm in each panel. (c), (d) Height of hypertrophic chondrocyte layer (c) and proportion of BrdU-positive cells (d) of the growth plate of tibial explant from fetal lbab/+ or lbab/lbab mice treated with $10^{-7}$ M CNP or vehicle at the end of the 4-day culture period. n = 3, each. **, P < 0.01 in (c), and n = 2–3 each in (d).

**Fig. 8**

Immunohistochemical staining of type X collagen (upper panels) and Ihh (lower panels) of the growth plates of metatarsal explants from fetal lbab/+ or lbab/lbab mice treated with vehicle (veh.) or $10^{-7}$ M CNP for 4 days. Scale bar, 50 µm.
Fig. 2
Fig. 4

(a) Images showing growth plates of different genotypes: Wt, lbab/lbab, and lbab/lbab·CNP-Tg.

(b) Bar graph comparing growth plate thickness across genotypes:
- Wt: 150 ± 10 μm
- lbab/lbab: 100 ± 10 μm
- lbab/lbab·CNP-Tg: 200 ± 10 μm
Fig. 5
Fig. 6
Fig. 7
$lbab^{+/+} + \text{veh.} \quad lbab^{+/}lbab + \text{veh.} \quad lbab^{+/}lbab + \text{CNP}$

**Fig. 8**

*col X*

$Ihh$
Supplemental Fig. 1

The graph shows the naso-tail length (cm) over age (weeks) for different genotypes: Wt, lbab/+, and lbab/lbab. The y-axis represents the naso-tail length in centimeters, and the x-axis represents age in weeks. The data points are connected by lines to illustrate the growth pattern over time.
Supplemental Fig. 2

The figure shows the body weight (g) of different genotypes over time (weeks). The genotypes include Wt, lbab/lbab, and lbab/CNP-Tg, with the body weight plotted against age (weeks). The graph indicates a trend of increasing body weight with age for all genotypes, with Wt showing a slightly higher weight compared to the other genotypes.
Supplemental Fig. 3

Vehicle (veh.) vs. CNP

**Ibab/+**

**Ibab/Ibab**