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Hypomorphic mutation in mouse *Nppc* gene causes retarded bone growth due to impaired endochondral ossification

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ABSTRACT

Long bone abnormality (*lbab*/*lbab*) is a spontaneous mutant mouse characterized by dwarfism with shorter long bones. A missense mutation was reported in the *Nppc* gene, which encodes C-type natriuretic peptide (CNP), but it has not been confirmed whether this mutation is responsible for the dwarf phenotype. To verify that the mutation causes the dwarfism of *lbab*/*lbab* mice, we first investigated the effect of CNP in *lbab*/*lbab* mice. By transgenic rescue with chondrocyte-specific expression of CNP, the dwarf phenotype in *lbab*/*lbab* mice was completely compensated. Next, we revealed that CNP derived from the *lbab* allele retained only slight activity to induce cGMP production through its receptor. Histological analysis showed that both proliferative and hypertrophic zones of chondrocytes in the growth plate of *lbab*/*lbab* mice were markedly reduced. Our results demonstrate that *lbab*/*lbab* mice have a hypomorphic mutation in the *Nppc* gene that is responsible for dwarfism caused by impaired endochondral ossification.

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family, which includes two other members in mammals: atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) [1]. *Nppc* gene encodes the 126 amino acids of pre-pro CNP, which is cleaved to yield the biologically active form [1]. CNP exerts its effect by elevating the intracellular level of cGMP produced by a receptor for CNP, natriuretic peptide receptor B (NPRB) [1]. The most obvious physiological role of CNP is to regulate bone growth [2]. CNP has been shown to have the ability to regulate the proliferation, differentiation and matrix synthesis of chondrocytes [3,4]. Furthermore, CNP-deficient mice develop dwarfism due to impaired endochondral ossification [5]. Targeted expression of CNP in chondrocytes rescues the dwarfism of CNP-deficient mice, indicating that CNP acts as a local regulator of longitudinal bone growth [5].

Long bone abnormality (*lbab*/*lbab*) is an autosomal recessive mutation of mice that is characterized by dwarfism with shorter long bones [6]. Recently, a missense mutation in the *Nppc* gene has been found in *lbab*/*lbab* mice, suggesting that the mutation is associated with dwarfism in *lbab*/*lbab* mice [7]; however, the mutation screening was carried out in only the coding regions of genes within the *lbab* locus. Thus, it is possible that other mutations may exist in unknown functional regions near the *Nppc* gene. Furthermore, although *lbab*/*lbab* mice is expected to be a useful model for human hereditary skeletal disorders involving CNP signals, the abnormalities of *lbab*/*lbab* mice have not been fully analyzed. In the present study, we first investigated whether the mutation in the *Nppc* gene was actually responsible for skeletal abnormalities of *lbab*/*lbab* mice by rescue experiments and by in vitro measurement of CNP activity. Then, we assessed the tibial growth plates of *lbab*/*lbab* mice by histological analysis.

Materials and methods

Mice. Heterozygous (*lbab*/+) mutant mice were obtained from the Jackson Laboratory, and the strain was maintained by sib mating of heterozygotes. Transgenic mice overexpressing CNP were described previously [5].

Rescue experiment with transgenic mice. Heterozygous (*lbab*/+) mice were crossed with transgenic mice to obtain F_1 mice that carried the *lbab* allele and the transgene. Homozygous (*lbab*/*lbab*) mice with the transgene were generated by mating the F_1 mice and heterozygous (*lbab*/+) mice. For 10 weeks after birth, body length was measured weekly as the length from the nose to the anus of female mice. All data are expressed as means ± SD, and the statistical significance of differences was determined by using Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

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Transient expression of the Nppc gene and cGMP measurement in COS-7 cells. COS-7 cells were obtained from the RIKEN cell bank (Tsukuba, Japan). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine medium (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO_2 in air. The cells were plated at a density of 5×10^3 cells/well in 96-well plates and used for transfection after incubation for 24 h.

Nppc cDNA was amplified by RT-PCR with tibial RNA extracted from *lbab*/*lbab* or +/+ mice using primers 5′-CACCATGCACCTCTCCC AGC-3′ and 5′-GCTGCACTAACATCCCAGACCG-3′. PCR was performed at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 20 s and 72 °C for 30 s. Amplified fragments were ligated into the pGEM-Teasy vector (Promega, Madison, USA) and then religated into the EcoRI site of pcDNA3.1 expression vector (Invitrogen, Carlsbad, USA). An expression vector that containing *Npr2* cDNA used in this report was described previously [8]. COS-7 cells were transfected with the DNA constructs by using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) and then incubated at 37 °C for 48 h. The cells were washed twice with phosphate-buffered saline (PBS) and incubated in DMEM containing 0.1% FBS and 0.5 mM 3-isobutyl-1-methyl-xanthine at 37 °C for 30 min. Thereafter, the amount of cGMP was measured using a cGMP enzyme immunoassay Biotrak System (GE Healthcare, Amersham, UK).

Western blotting. Cell extracts were prepared by boiling the cells in sample buffer (100 mM Tris–HCl, 4% SDS, 20% glycerol and 12% mercaptoethanol) for 5 min, and protein samples were separated by SDS–PAGE. Following transfer to nitrocellulose membranes, the membranes were incubated with anti-CNP antibody (Santa Cruz, CA, USA), diluted 1:5000 overnight at 4 °C. Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz), which was used as the secondary antibody, was applied for 1 h. Immunoreactivity was visualized using the ECL Advance Western blotting detection kit (GE Healthcare, Amersham, UK).

Histological examination. lbab/*lbab* mice and their normal littermates were anesthetized and sacrificed 5 days after birth. Tibias were fixed in 4% paraformaldehyde overnight at 4 °C. Tibias were decalcified in 10% EDTA for 4 days. Specimens were dehydrated in ethanol, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. For immunohistochemistry, tibial sections were incubated with 10% bovine serum albumin (BSA) in PBS for 30 min, and sections for the detection of type II and X collagen were pretreated with hyaluronidase (25 mg/ml) for 30 min. Specific antibodies diluted with 3% BSA in PBS were then applied to the sections and incubated for 2 h at room temperature or overnight at 4 °C. Dilution ratios of antibodies were 1:5000 for type II collagen (LSL, Tokyo, Japan), 1:3000 for type X collagen (LSL), 1:200 for Indian hedgehog (IHH) (Santa Cruz) and 1:200 for parathyroid hormone-related protein (PTHrP) (Santa Cruz). The sections were washed with PBS and incubated with secondary antibodies for 30 min. Immune complexes were visualized by staining with diaminobenzidine and the sections were counterstained with hematoxylin.

Results

Rescue experiments

We examined whether the transgene that overexpresses the *Nppc* gene under the control of the chondrocyte-specific *Col2a1* promoter rescues the retarded longitudinal bone growth of *lbab*/*lbab* mice. *lbab*/*lbab* mice showed significantly shorter body length than wild-type littermates at 2 weeks of age, and these differences progressively became more obvious as the mice aged. The average body length of *lbab*/*lbab* mice was 65–75% of that of wild-type littermates at 2–10 weeks of age. On the other hand, there was no significant difference between the body lengths of *lbab*/*lbab* mice with the *Nppc* transgene and wild-type littermates at 2–10 weeks of age. Thus, CNP overexpressed in chondrocytes rescued skeletal abnormalities of *lbab*/*lbab* mice (Fig. 1A and B).

Effect of the mutation in the Nppc gene on CNP activity

In response to CNP binding, the guanylyl cyclase catalytic domain of NPRB is activated and stimulates cGMP synthesis. To analyze whether CNP derived from the *lbab* allele stimulates cGMP production through NPRB, we measured cGMP concentrations in COS-7 cells co-transfected with expression vectors containing a wild- or mutant-type *Nppc* gene and the *Npr2* gene, a gene for NPRB. In cells transfected with the wild-type *Nppc* gene (pcDNA-*Nppc* Arg117) and *Npr2* gene (pcDNA-*Npr2*), a considerable amount of cGMP was produced (3407 fmol/well). On the other hand, although the amount of cGMP in cells transfected with the mutant-type *Nppc* gene (pcDNA-*Nppc* Gly117) and the *Npr2* gene was significantly increased in comparison with that in cells transfected with the *Npr2* gene only, it was approximately one-ninth of the cGMP level (389 fmol/well) in cells transfected with the wild-type *Nppc* gene. No significant difference was observed between cells transfected with the wild- and mutant-type *Nppc* gene only (Fig. 2). Western blotting revealed that the amount of

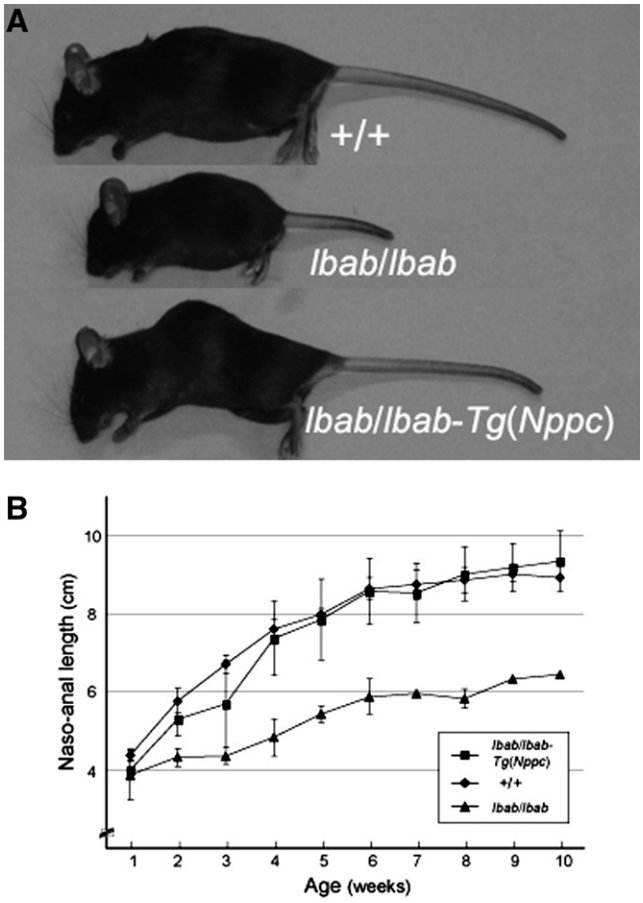

Fig. 1. Genetic rescue of *lbab*/*lbab* mice by crossing with mice overexpressing CNP. (A) Appearances of wild-type, *lbab*/*lbab* and *lbab*/*lbab*-Tg (*Nppc*) mice at 10 weeks of age. (B) Body lengths of wild-type, *lbab*/*lbab* and *lbab*/*lbab*-Tg (*Nppc*) mice. Body length changes of mice were measured from 1 to 10 weeks after birth. Data are shown as means ± SD. Sample size: wild-type females, 5–6; *lbab*/*lbab* females, 2–3; *lbab*/*lbab*-Tg (*Nppc*) females, 3–4.

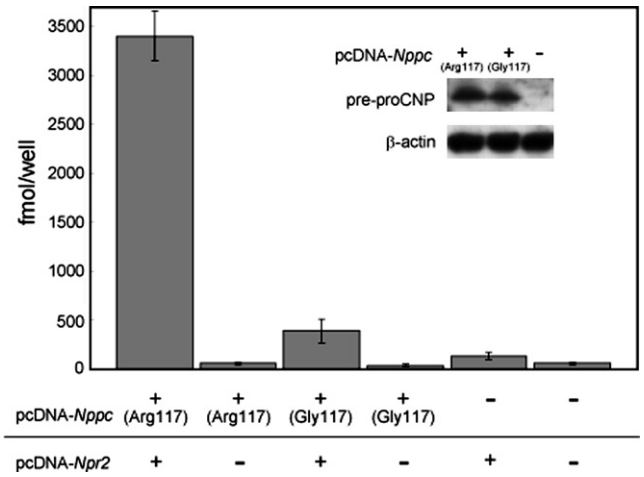

Fig. 2. Effect of the mutation in *Nppc* gene on CNP activity. The amount of cGMP was measured in COS-7 cells co-transfected with expression vectors containing the wild-type (pcDNA-*Nppc* Arg117) or mutant-type (pcDNA-*Nppc* Gly117) *Nppc* gene and the *Npr2* gene (pcDNA-*Npr2*) 48 h later after the transfection. Data are shown as means ± SD of 10 wells. Inset shows the expressions of pre-pro CNP and β-actin in transfected cells with wild- or mutant-type *Nppc* gene by Western blotting.

pre-pro-CNP proteins produced in cells transfected with the wild-type *Nppc* gene was equivalent to that with mutant-type *Nppc* gene (Fig. 2). These results demonstrate that the CNP produced in *lbab*/*lbab* mice lacks most of the ability to activate the guanylyl cyclase catalytic domain of NPRB.

Histological analysis of growth plates in lbab/lbab mice

During endochondral ossification, chondrocytes in the growth plate progress through resting, proliferative and hypertrophic stages. These sequential and synchronous processes are essential for longitudinal bone growth. We analyzed the tibial growth plates of *lbab*/*lbab* mice by histological analysis to determine the basis of their shorter long bones. At postnatal day 5, the epiphyseal growth plates had a reduced thickness in comparison with those of normal mice. Each zone of resting, proliferative and hypertrophic chondrocytes in the growth plates of *lbab*/*lbab* mice was distinguishable, as seen in normal mice; however, the proliferative zones of *lbab*/*lbab* mice were narrower than those of normal mice. Moreover, the most noticeable alteration in the growth plates of *lbab*/*lbab* mice was a markedly decreased number of hypertrophic chondrocytes. In addition, the size of hypertrophic chondrocytes was obviously reduced (Fig. 3A and B). These histological features are almost identical to those of CNP-deficient mice [5]. These results suggest that the retarded longitudinal bone growth of *lbab*/*lbab* mice is the result of impaired endochondral ossification caused by the mutation of the *Nppc* gene. Next, we immunohistochemically analyzed the localization of type II collagen, type X collagen, IHH and PTHrP, which are typical markers of chondrocyte phenotypes. The immunoreactive specificity and intensity of type II collagen, which is most abundantly expressed in the chondrocytes of the proliferative zone, were similar in *lbab*/*lbab* mice and normal mice (Fig. 3C and D). Type X collagen was detected in hypertrophic chondrocytes of *lbab*/*lbab* mice as well as normal mice, while the depth of the positive cell zone for type X collagen in *lbab*/*lbab* mice was significantly reduced, according to the reduced cell number, as compared with normal mice (Fig. 3E and F). Furthermore, signal intensity and specificity of IHH and PTHrP, which play crucial roles in controlling the pace of chondrocyte proliferation and differentiation, were almost the same in *lbab*/*lbab* mice and in normal mice (Fig. 3G–J). In conclusion, the expression patterns of the marker proteins for each chondrocyte layer were basically normal in *lbab*/*lbab* mice, and their chondrocytes in the growth plate seem to possess the ability to progress a series of cell states through proliferative, prehypertrophic and hypertrophic chondrocytes.

Discussion

A missense mutation in the *Nppc* gene of *lbab*/*lbab* mice was recently found by screening 122 genes and ESTs in the *lbab* candidate region, and no other specific mutation in those genes has been identified in *lbab*/*lbab* mice [7]. Although association of the mutation with the dwarf phenotype in *lbab*/*lbab* mice was shown, confirmative evidence showing that the missense mutation results in functional change of CNP has still not been provided. In the present study, we showed that growth retardation of the long bones of *lbab*/*lbab* mice was compensated for by the targeted expression of CNP in chondrocytes. A similar result was obtained as for CNP-deficient mice [5]. Moreover, in the transfection study, we found that CNP from the *lbab* allele lacked most of the ability to activate cGMP synthesis through NPRB. These results clearly demonstrate that the missense mutation causes a functional defect of CNP, and its alteration results in the dwarf phenotype with shorter long bones. Furthermore, we found that the tibial growth plates of *lbab*/*lbab* mice have reduced thickness due to narrowed zones of proliferative and hypertrophic chondrocytes, whereas no significant differences were observed between *lbab*/*lbab* mice and normal mice in the expression pattern of marker proteins in each chondrocyte layer. This chondrocytic phenotype and the dwarf phenotype of *lbab*/*lbab* mice are remarkably similar to those of CNP-deficient mice [5]. Thus, our results strongly support the notion that the skeletal defect in *lbab*/*lbab* mice is caused by the mutation in the *Nppc* gene.

The natriuretic peptide family shares a common ring structure that is formed by 17 amino acids with conserved residues (CFGXXXDRIXXXSGLGC) necessary for biological activity [1,9]. CNP elicits its physiological responses through cGMP synthesis by binding to a specific receptor, NPRB. The mutation identified in the *Nppc* gene of *lbab*/*lbab* mice is a C to G transversion leading to an amino acid substitution from Arg to Gly at codon 117 [7]. Arg at codon 117 of CNP is one of the common amino acids of the ring structure in the natriuretic peptide family [1] and is conserved among mammals, frogs and fishes [10]. The chemical properties of Arg and Gly are very different [11], and the amino acid substitution from Arg to Gly is a replacement of positively charged and hydrophilic Arg with hydrophobic Gly, which would cause a decrease in the net positive charge of protein. Thus, the Arg at codon 117 is likely a critical amino acid for the ring structure of CNP; therefore, CNP produced in *lbab*/*lbab* mice might have decreased binding affinity to NPRB. We revealed that CNP produced from the mutated *Nppc* gene has only about 10% of the activity of normal CNP. These results indicate that the missense mutation in *Nppc* gene of *lbab*/*lbab* mice is a hypomorphic mutation.

During endochondral ossification, mesenchymal cells initially differentiate into chondrocytes, and progress through proliferative, prehypertrophic and hypertrophic stages. These processes are critical for longitudinal bone growth and are controlled by a large number of genes [12]. CNP has been elucidated to be an essential factor for bone growth formed through endochondral ossification [5]. The expressions of *Nppc* gene and *Npr2* gene are overlapped in prehypertrophic chondrocytes [5], and CNP has shown a large effect on the expansion of hypertrophic zone in the tibial organ culture system [13]. These findings have suggested that CNP exerts its effect on prehypertrophic chondrocytes for the promotion of hypertrophic differentiation. This proposed role of CNP is consistent with our result that the mutated CNP, which has only slight activity, led to a considerably decreased number of hypertrophic

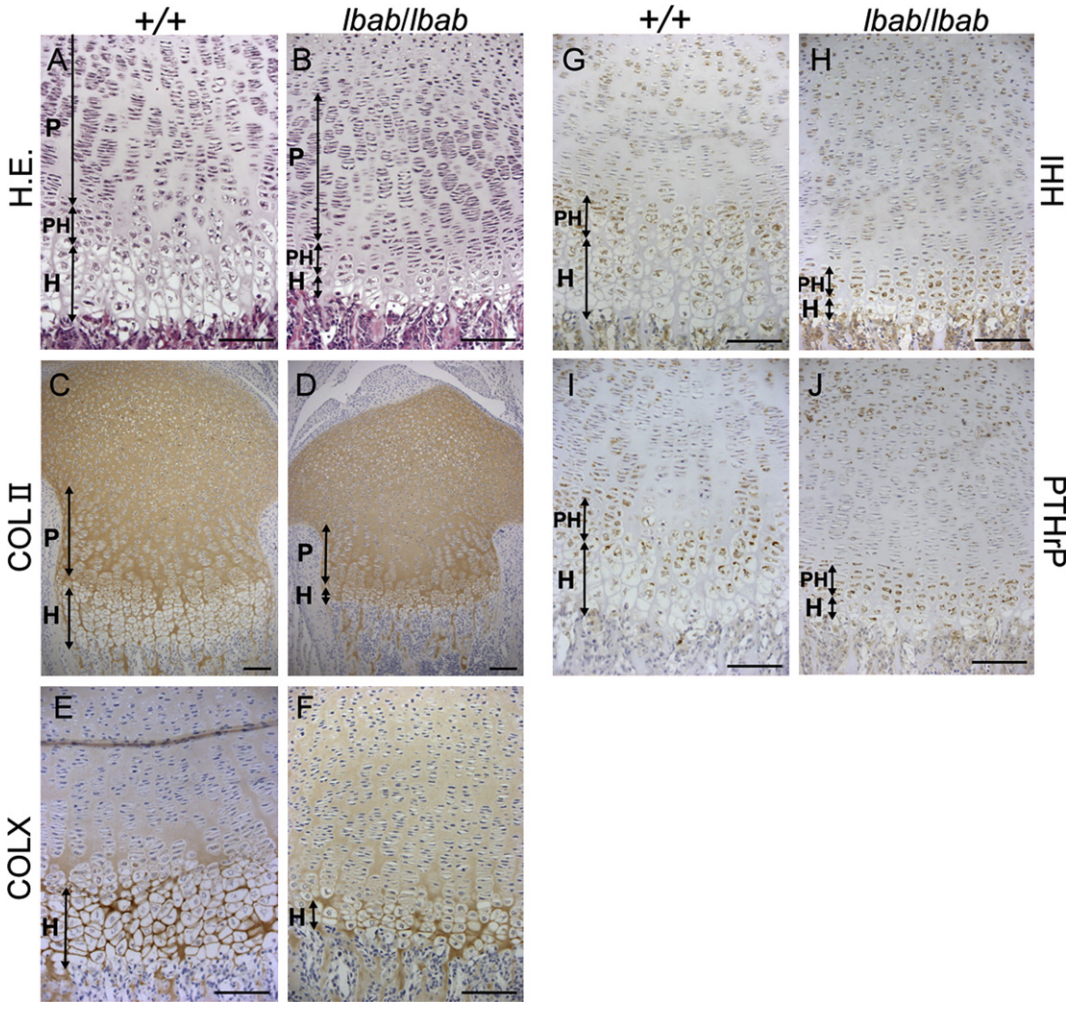

Fig. 3. Histological analysis of tibial growth plates of *lbab*/*lbab* and normal mice. The tibial epiphyseal growth plates of normal (A, C, E, G, and I) and *lbab*/*lbab* (B, D, F, H, and J) mice at 5 days of age. Sections stained with hematoxylin and eosin are (A) and (B). Immunohistological analysis shows the localization of type II collagen (C,D), type X collagen (E,F), IHH (G,H) and PTHrP (I,J). *Abbreviations:* P, zone of proliferative chondrocytes; PH, zone of prehypertrophic chondrocytes; H, zone of hypertrophic chondrocytes. Scale bar in each panel indicates 100 μm.

chondrocytes in the growth plate; however, it is still not understood how CNP contributes to this process. IHH, PTHrP and fibroblast growth factor receptor-3 (FGFR3) play crucial roles in controlling the longitudinal bone growth, and mice lacking one of the genes for these factors show skeletal disorders caused by a disruption of endochondral ossification [12]. IHH and PTHrP are presented mainly in prehypertrophic chondrocytes of the postnatal growth plate [14] and a negative feedback loop between IHH and PTHrP plays crucial roles in controlling the pace of chondrocyte proliferation and differentiation to hypertrophic chondrocytes [15]. Because the localization patterns of IHH and PTHrP overlapped with NPRB in prehypertrophic chondrocytes, we assumed that CNP may be correlated with these factors to coordinate chondrocyte proliferation and differentiation; however, the expressions of IHH and PTHrP were detected in *lbab*/*lbab* mice as seen in normal mice, and it seems unlikely that CNP affects chondrocyte proliferation and differentiation by regulating the expression of IHH and PTHrP. FGFR3 has been shown to play crucial roles in controlling the pace of differentiation through MEK1, an upstream kinase activator of ERK1/2 in the MAPK pathway [16]. Recently, an inhibitory effect of CNP on activation of this signal pathway through FGFs-FGFR3 has been elucidated in cultured long bone and chondrogenic cell lines [13,17]. From these findings, the ERK1/2 MAPK pathway through FGFR3 may be kept in activated condition in the chondrocytes of *lbab*/*lbab* mice, and it may cause the disturbed differentiation of chondrocytes. This hypothesis is supported by the fact that some histological features, such as a narrowed hypertrophic zone, in the tibial growth plate of *lbab*/*lbab* mice are observed in mice expressing a constitutively active mutant of FGFR3 or MEK1 [16,18]. However, inconsistent with this hypothesis, we did not observe an obvious difference in the expression of IHH between *lbab*/*lbab* mice and normal mice in spite of the fact that constitutively active FGFR3 inhibits the expression of *Ihh* gene in the growth plate [18]. Thus, in addition to the FGFR3-MAPK pathway, another signal pathways interacting with the CNP signal may exist to regulate chondrocyte differentiation. Further analyses using *lbab*/*lbab* mice as well as CNP-deficient mice will help to clarify the signal pathways, including FGFR3 signal cascade, that mediate the effects of the CNP signal.

The pathological features of *lbab*/*lbab* mice are observed not only in other mice with null or loss-of-function mutation in the *Nppc* gene or *Npr2* gene in common [5,19,8], but also in some types of human skeletal dysplasias [20]. Genetic abnormalities of CNP and NPRB in humans have been identified in a particular type of heritable skeletal dysplasias. Mutations in the *NPR2* gene have been identified in patients with Acromesomelic dysplasia Maroteaux type, a hereditary disorder characterized by disproportionate dwarfism [21]. Although no loss-of-function mutation in human *NPPC* gene has been reported, overexpression of CNP was recently found in patients with skeletal overgrowth due to a balanced trans-

location that has a breakpoint around the *NPPC* gene [22,23]. In addition, new therapeutic approaches via the activation of CNP/NPRB have been suggested for the treatment of human achondroplasia [17]; therefore, the evidence that implicates the CNP/NPRB system in human skeletal disorders has increased progressively, and it will be necessary to clarify the physiological roles of the CNP/NPRB system, which remain largely unknown. Thus, for the investigation of human diseases involving the CNP/NPRB system, *lbab*/*lbab* mice would be useful tools as hypomorphic mutant models of the CNP/NPRB system.

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