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## Skeletal Analysis of the Long Bone Abnormality (*lbab/lbab*) Mouse, A Novel Chondrodysplastic C-Type Natriuretic Peptide 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 growth plate in *lbab/lbab* mice, especially 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 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.

**Keywords** C-type natriuretic peptide · Long bone abnormality (*lbab*) · Chondrodysplasia · Endochondral bone growth · Organ culture

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 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 in two lines of spontaneous mutant mice, *cn/cn* and *slw/slw*, disproportionate dwarfism is caused by loss-of-function mutations in the murine GC-B gene [7, 8].

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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 dysplasia with a 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*, and GC-B knockout mice.

The long bone abnormality (*lbab/lbab*) mouse was first identified in The Jackson Laboratory (Bar Harbor, ME) as a spontaneous autosomal recessive mutant characterized by impaired growth of the long bones [10]. Recent studies have elucidated that the impaired growth of *lbab/lbab* mice is caused by a hypomorphic mutation in the CNP gene; Jiao et al. [11] found that its impaired growth phenotype is associated with a single point mutation in the mouse CNP gene, and we showed that this phenotype is completely recovered by CNP overexpression [12]. Yoder et al. [13] characterized the mutant CNP in *lbab/lbab* mice and demonstrated 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 compared to authentic CNP. We also confirmed that the mutant CNP in *lbab/lbab* mice retains only about 10% activity to induce cyclic GMP production through GC-B compared to authentic CNP in an in vitro transfection assay using COS-7 cells [12]. Collectively, *lbab/lbab* is a novel chondrodysplastic mouse model with insufficient CNP action on endochondral bone growth. Nevertheless, the skeletal phenotypes of *lbab/lbab* mice 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* mice.

## Materials and Methods

### Mice

Heterozygous (*lbab/+*) mice (C57BL/6 J 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 F<sub>1</sub> offspring heterozygous for the transgene and for the *lbab* allele were mated with those with only the *lbab* allele

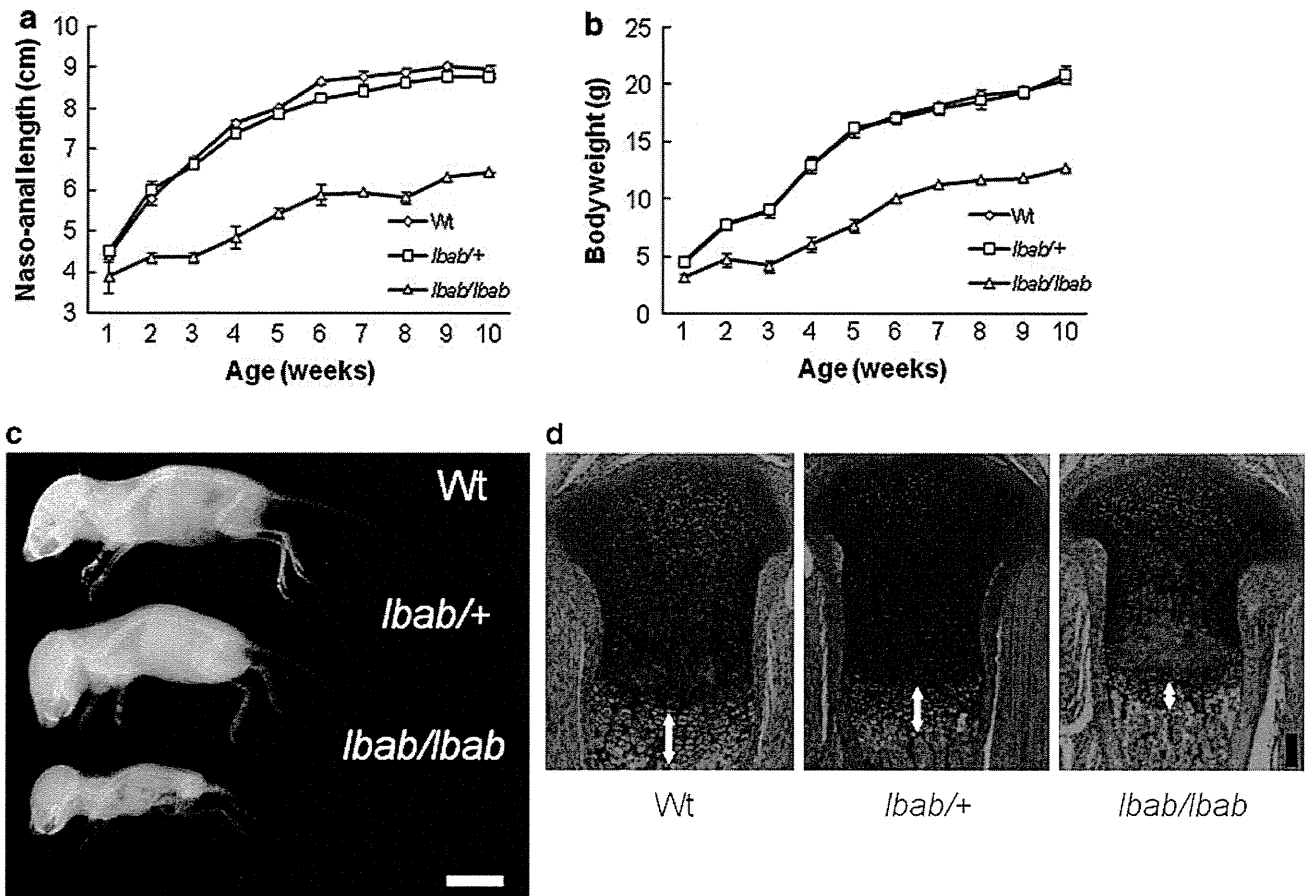
to generate *lbab/lbab* mice with the transgene expression (*lbab/lbab*-CNP-Tg/+ mice) [12]. Genotypes for the CNP transgene and the *lbab* allele were determined by PCR analysis using mouse genomic DNAs extracted from tails. Because there was no tendency of gender differences in the growth of each genotype (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 (nasoanal length) or that from the nose to the tip of the tail (nose–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 (Comscantechno, Yokohama, Japan). Three-dimensional microstructural image data were reconstructed and structural indices calculated using TRI/3D-BON software (RATOC System Engineering, Tokyo, Japan).

### Histological Examination

Tibiae were fixed in 10% formalin neutral buffer, decalcified in 10% EDTA, and embedded in paraffin. Sections (5  $\mu$ m thick) 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, Santa Cruz, CA), mouse anti-matrix metalloproteinase 13 (MMP-13) antibody (Thermo Fisher Scientific, Waltham, MA), and mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Copenhagen, Denmark). Immunostaining was performed using the Histofine Mousestain Kit (Nichirei Biosciences, Tokyo, Japan) according to the manufacturer's instruction. Peroxidase activity was visualized using diaminobenzidine. Sections were counterstained with hematoxylin, dehydrated, and then mounted with malinol (Muto Pure Chemicals, Tokyo, Japan). To confirm antibody specificity, normal rabbit serum (Sigma-Aldrich, St. Louis, MO), normal goat IgG (Santa Cruz Biotechnology), and mouse IgG (Dako) were used as first antibodies for negative controls.



**Fig. 1** Growth and skeletal phenotype of *lbab*<sup>+/+</sup> and *lbab*<sup>l/bab</sup> mice. Nasoanal lengths (a) and body weights (b) of female wild-type (Wt, open diamond), *lbab*<sup>+/+</sup> (open square), and *lbab*<sup>l/bab</sup> (open triangle) mice ( $n = 2-8$ ). c Whole skeletons of wild-type, *lbab*<sup>+/+</sup>, and *lbab*<sup>l/bab</sup>

*lbab* mice at 2 weeks of age. 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  $\mu$ m

## Organ Culture

Organ culture of fetal mouse tibiae or third metatarsi was performed as described previously [15]. Tibial or metatarsal explants from *lbab*<sup>+/+</sup> mice and their *lbab*<sup>l/bab</sup> littermates at 16.5 days postcoitus were cultured for 4 days with vehicle or  $10^{-7}$  M CNP (Peptide Institute, 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 of the lengths of proximal and distal cartilaginous primordia (CP), and the length of the osteogenic center (OC), 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. Sections (5  $\mu$ m thick) 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'-deoxyuridine labeling and detection kit II (Roche Applied Science,

Indianapolis, IN) according to the manufacturer's protocol.

## Statistical Analysis

Data were expressed as the mean  $\pm$  SEM. The statistical significance of differences between mean values was assessed using Student's *t*-test.

## Results

### Analyses of Skeletal Growth of *lbab*<sup>l/bab</sup> and *lbab*<sup>+/+</sup> Mice

As previously reported, *lbab*<sup>l/bab</sup> mice developed severe dwarfism characterized by short tails and extremities [11, 12]. At birth, *lbab*<sup>l/bab</sup> pups were slightly shorter than their wild-type littermates: the nasoanal and nose-tail lengths of *lbab*<sup>l/bab</sup> mice were 88 and 83% of those of

their wild-type littermates, respectively (Fig. 1a, Supplemental Fig. 1). The ratios of nasoanal and nose–tail lengths of *lhab/lhab* mice to those of wild-type mice sharply decreased to 65% and 55%, respectively, by the age of 3 weeks. After 5 weeks of age, these ratios stabilized at 66–72% and 57–62%, respectively (Fig. 1a, Supplemental Fig. 1). The body weight of *lhab/lhab* mice was 68% of that of their wild-type littermates at birth and decreased to 46% by the age of 3 weeks. The ratio did not increase until 5 weeks of age, becoming ~60% after 7 weeks (Fig. 1b). On the other hand, *lhab/+* mice were indistinguishable from their wild-type littermates at birth and grew almost similarly (Fig. 1a,b, Supplemental Fig. 1). Soft X-ray analysis revealed that longitudinal growth of the vertebrae, tail, and extremities was affected in *lhab/lhab* mice at the age of 2 weeks but was not affected in *lhab/+* mice (Fig. 1c). Histological analysis revealed that at the age of 3 days the tibial growth plate, especially the hypertrophic chondrocyte layer, of *lhab/lhab* mice was apparently thinner than that of wild-type mice (Fig. 1d). On the other hand, the thickness of the tibial growth plate of *lhab/+* mice was not different from that of wild-type mice (Fig. 1d).

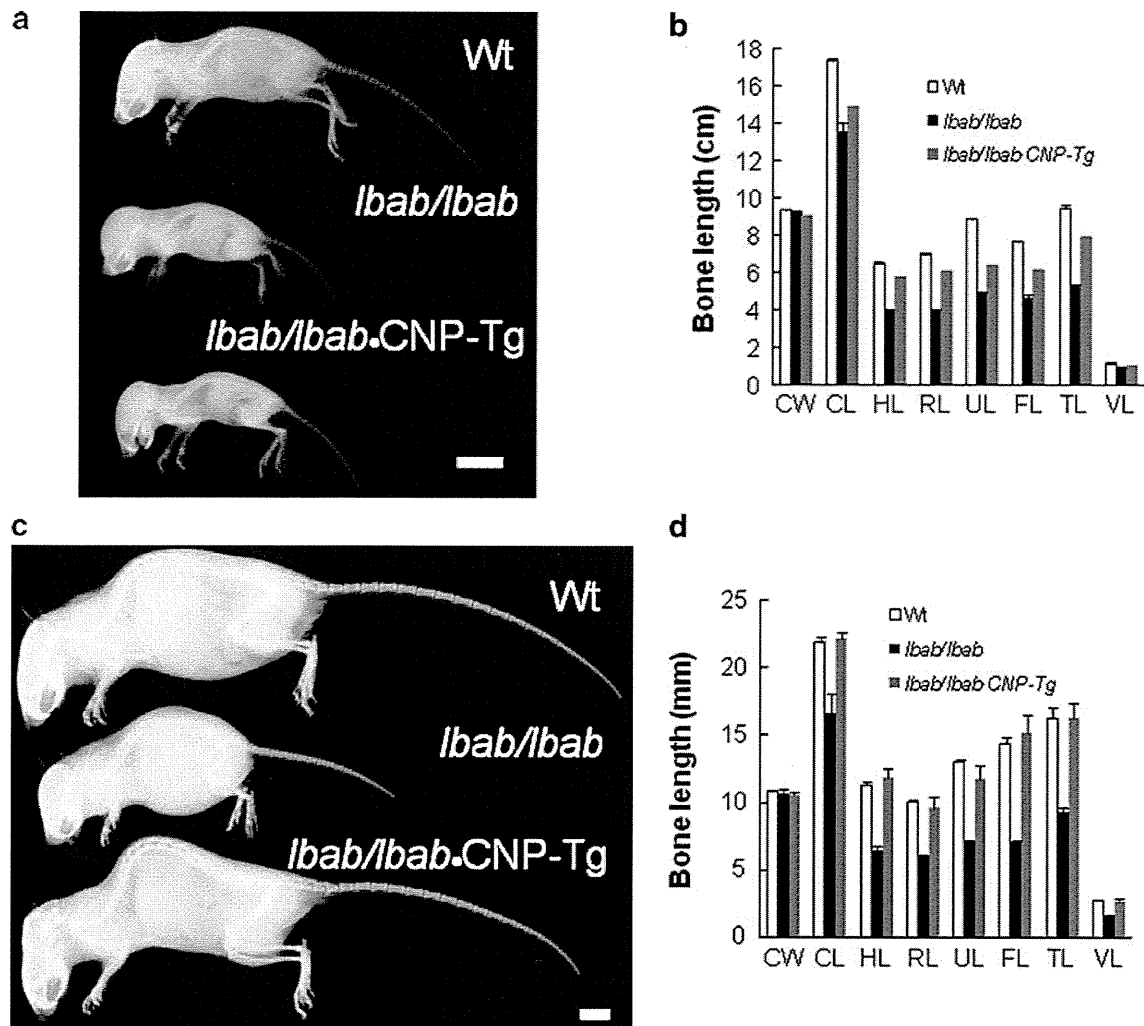
#### Effect of CNP Overexpression on Impaired Endochondral Bone Growth of *lhab/lhab* Mice

In order to further characterize the impaired skeletal growth of *lhab/lhab* 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 *lhab/lhab* mice with cartilage-specific CNP transgenic mice under the control of type II collagen promoter (CNP-Tg mice) and obtained *lhab/lhab* mice with transgenic expression of CNP in cartilage (*lhab/lhab*-CNP-Tg mice) [12]. At the first week after birth, the nasoanal length of *lhab/lhab*-CNP-Tg mice was almost the same as that of *lhab/lhab* mice and considerably smaller than that of wild-type mice: nasoanal lengths of wild-type, *lhab/lhab*, and *lhab/lhab*-CNP-Tg mice were  $4.38 \pm 0.06$ ,  $3.87 \pm 0.37$ , and  $4.00 \pm 0.12$  cm, respectively. Subsequently, *lhab/lhab*-CNP-Tg mice began to grow larger than *lhab/lhab* mice and promptly caught up with wild-type mice; although the nasoanal length of *lhab/lhab*-CNP-Tg mice was still considerably smaller than that of wild-type mice until 3 weeks of age ( $5.70 \pm 0.57$  and  $6.71 \pm 0.10$  cm, respectively, at age 3 weeks), it became almost comparable to that of wild-type mice after 4 weeks ( $7.38 \pm 0.48$  and  $7.61 \pm 0.10$  cm, respectively, at age 4 weeks). Further, the body weight of *lhab/lhab*-CNP-Tg mice was almost the same as that of *lhab/lhab* mice and smaller than that of wild-type mice until the age of 3 weeks but 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 *lhab/lhab* mice was partially recovered by targeted overexpression of CNP in cartilage in *lhab/lhab*-CNP-Tg mice (Fig. 2a): the recoveries in the longitudinal length of cranium and the lengths of the humerus, radius, ulna, femur, tibia, and vertebrae 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 *lhab/lhab* mice was almost completely recovered by targeted overexpression of CNP in cartilage, as observed in *lhab/lhab*-CNP-Tg mice (Fig. 2c, d). On the other hand, there were no significant differences in the width of the cranium, which is formed via intramembranous ossification, among the three genotypes at either 2 or 10 weeks (Fig. 2b, d).

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



**Fig. 2** 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. **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**) (Color figure online)

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 completely recovered by overexpression of CNP in cartilage (Fig. 4).

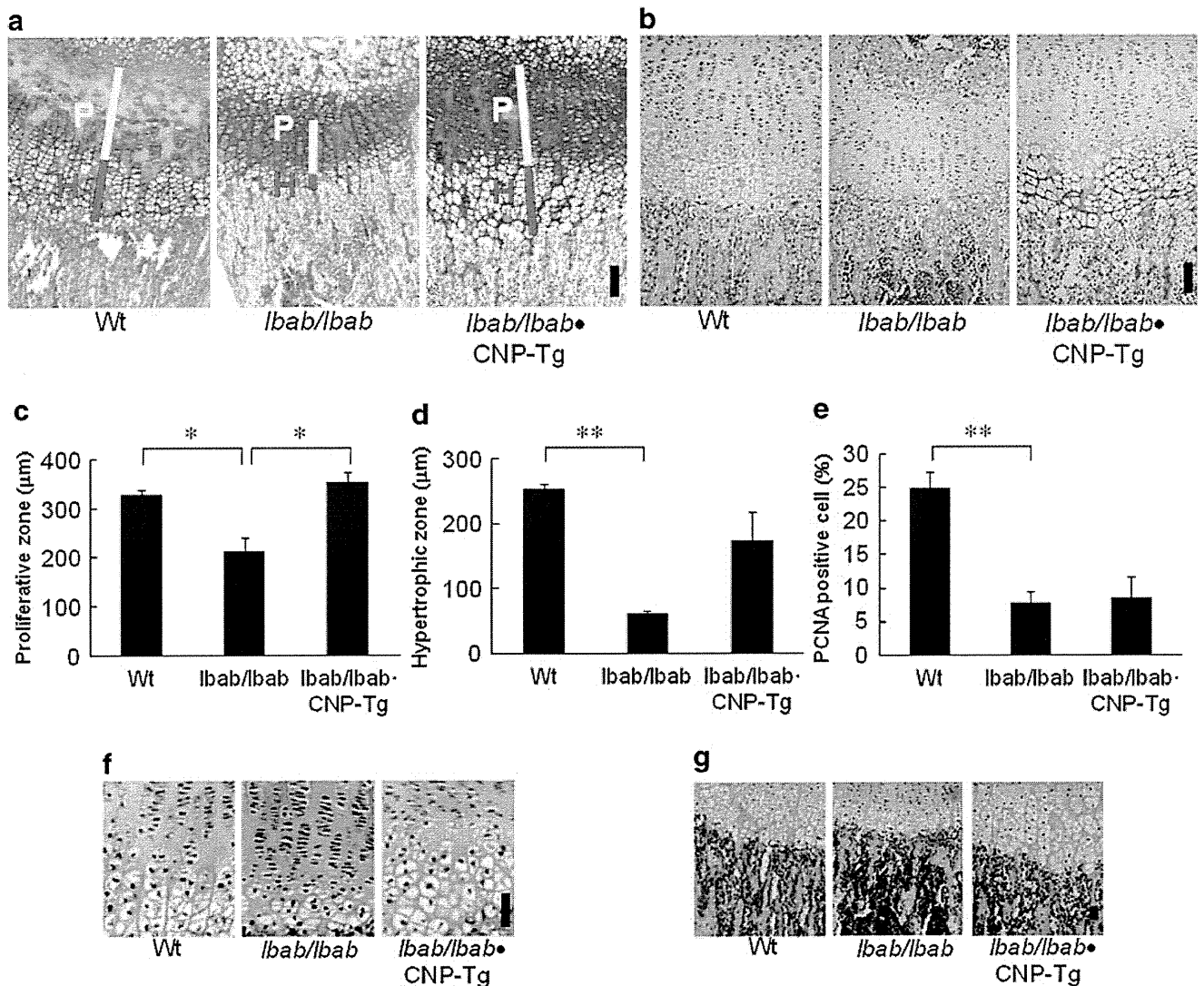
#### Recovery of Decreased Bone Volume in *lbab/lbab* Mouse by CNP Overexpression

Three-dimensional CT analysis manifested a marked reduction in bone volume of the humerus in *lbab/lbab* mice and considerable recovery in *lbab/lbab*-CNP-Tg mice (Fig. 5). At the age of 10 weeks, the quantified bone volume (BV/TV) and trabecular thickness (Tb.Th) of the humerus in *lbab/lbab* mice were 2.4% and 34.5  $\mu\text{m}$ , whereas those in wild-type mice were 4.1% and 40.3  $\mu\text{m}$ , respectively. The decreased BV/TV and Tb.Th in *lbab/lbab*

mice were increased to 5.4% and 37.0  $\mu\text{m}$ , respectively, in *lbab/lbab*-CNP-Tg mice.

#### Organ Culture Experiments of Tibiae from *lbab/lbab* Mice

In order to further analyze the impaired endochondral ossification of *lbab/lbab* mice, we performed 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 the CP of *lbab/lbab* tibiae were significantly smaller than those of *lbab/+* tibiae ( $3.80 \pm 0.04$



**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** and **b** = 100 µm. 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.  $n = 3-4$ .  $**P < 0.01$ . Immunohistochemical staining of *Ihh* (**f**) and *MMP-13* (**g**). Scale bar in **f** and **g** = 50 µm

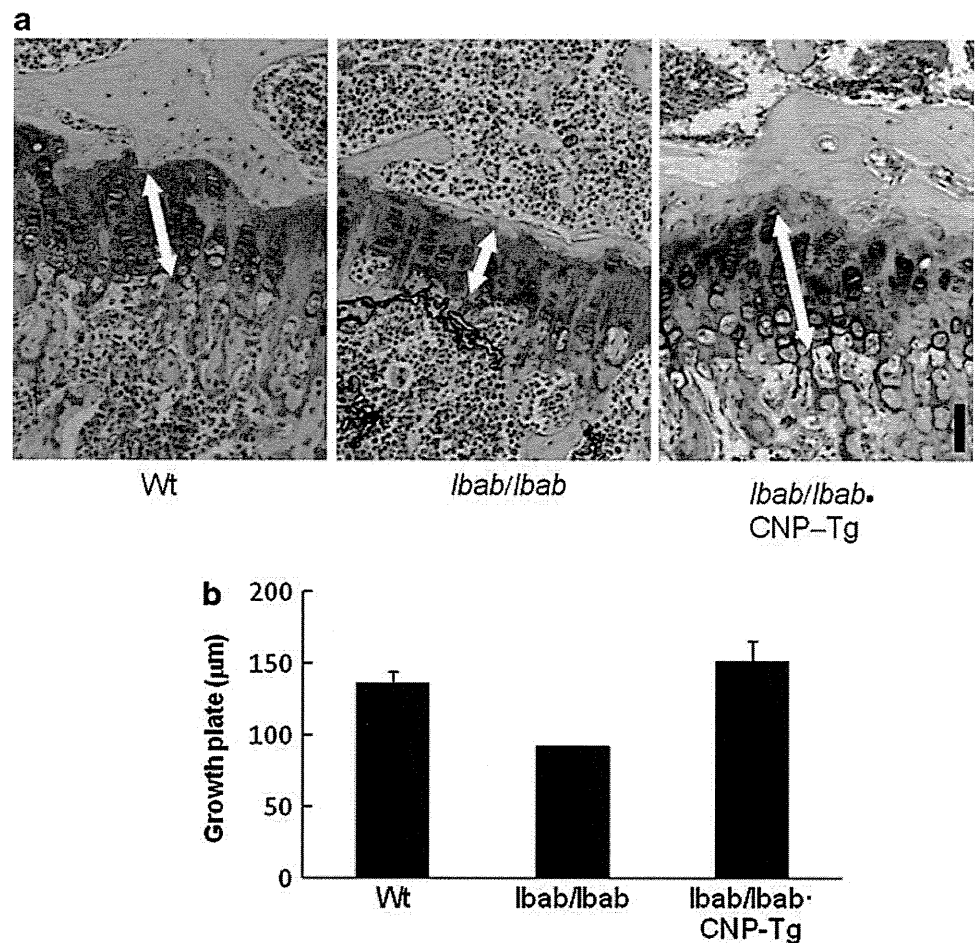
vs.  $4.25 \pm 0.03$  and  $2.19 \pm 0.02$  vs.  $2.43 \pm 0.01$  mm, respectively,  $n = 8-12$  each) (Fig. 6b, c). Tibial explants from *lbab/lbab* mice grew to the same extent as those from *lbab/+* mice during a 4-day culture period; the difference in the total length or in the length of the CP between *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 OC between the 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 the OC was not stimulated by CNP in either *lbab/lbab* or *lbab/+* explants (data not shown).

Histological examination at the end of the 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

**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  $\mu\text{m}$ . **b** Total heights of the growth plates.  $n = 2\text{--}5$  each



*lbab/+* explants. The area positive for immunostaining for *Ihh*, one of the markers for chondrogenic differentiation [16], tended to be a little decreased in *lbab/lbab* explants compared to that in *lbab/+* explants, although the intensity of the immunostaining was not different between the 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 the primordial growth plate and its hypertrophic chondrocyte layer of *lbab/lbab* explants treated with  $10^{-7}$  M CNP became comparable to those of *lbab/+* explants treated with the same dose of CNP (Fig. 7a–c). CNP increased the areas positive for *Ihh* immunostaining in both *lbab/+* and *lbab/lbab* explants. By addition of CNP, the sizes of the areas positive for, and the intensities of, *Ihh* immunostaining were not different between *lbab/+* and *lbab/lbab* explants (Supplemental Fig. 3). CNP did not

increase BrdU-positive chondrocytes in *lbab/lbab* explants (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 is delayed in the metatarsus compared to that in the tibia in an individual, we performed organ culture of metatarsi as well as tibiae from fetal mice at 16.5-days postcoitus and examined the expression of type X collagen and *Ihh*. In the case of *lbab/+* 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 the metatarsal growth plate represents an earlier stage of endochondral ossification than the 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





**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

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).

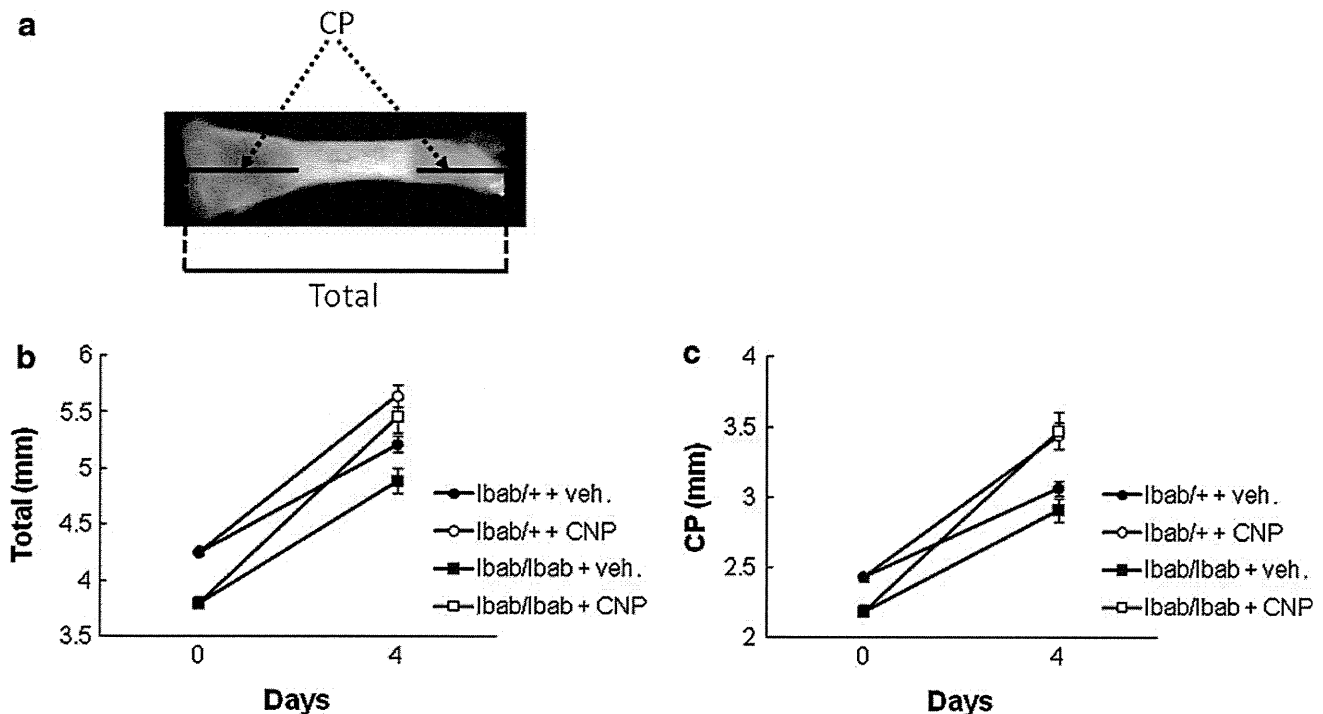
## Discussion

Previously, we and other groups had reported in brief communications that the short stature phenotype of *lbab/lbab* mice is caused by a mutation in the CNP gene [11–13]. Here, we further analyzed the skeletal phenotypes of *lbab/lbab* mice and report the results in this full-length article.

Analysis of the growth curves of nasoanal and nose–tail lengths revealed that the shortness of *lbab/lbab* mice is mild at birth but rapidly progresses by the age of 3 weeks, and then, after 4 weeks, the ratio of the length of *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 potentially when the volume of growth plate cartilage is relatively abundant.

We confirmed the thinness of the growth plate of *lbab/lbab* mice, especially in its hypertrophic chondrocyte layer, followed by the impaired growth of long bones. The thinness of the growth plate of *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 *lbab/lbab* bones by CNP was only partial at 2 weeks, 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 chondrocytes is decreased in *lbab/lbab* mice and that the decreased proliferation is not rescued by CNP overexpression, even though the thickness of the growth plate does fully recover.



**Fig. 6** Effect of CNP on cultured tibiae from fetal *lbab/+* and *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. Graphs of total (**b**) and CP (**c**) lengths of cultured tibiae from *lbab/+* and *lbab/lbab* mice treated

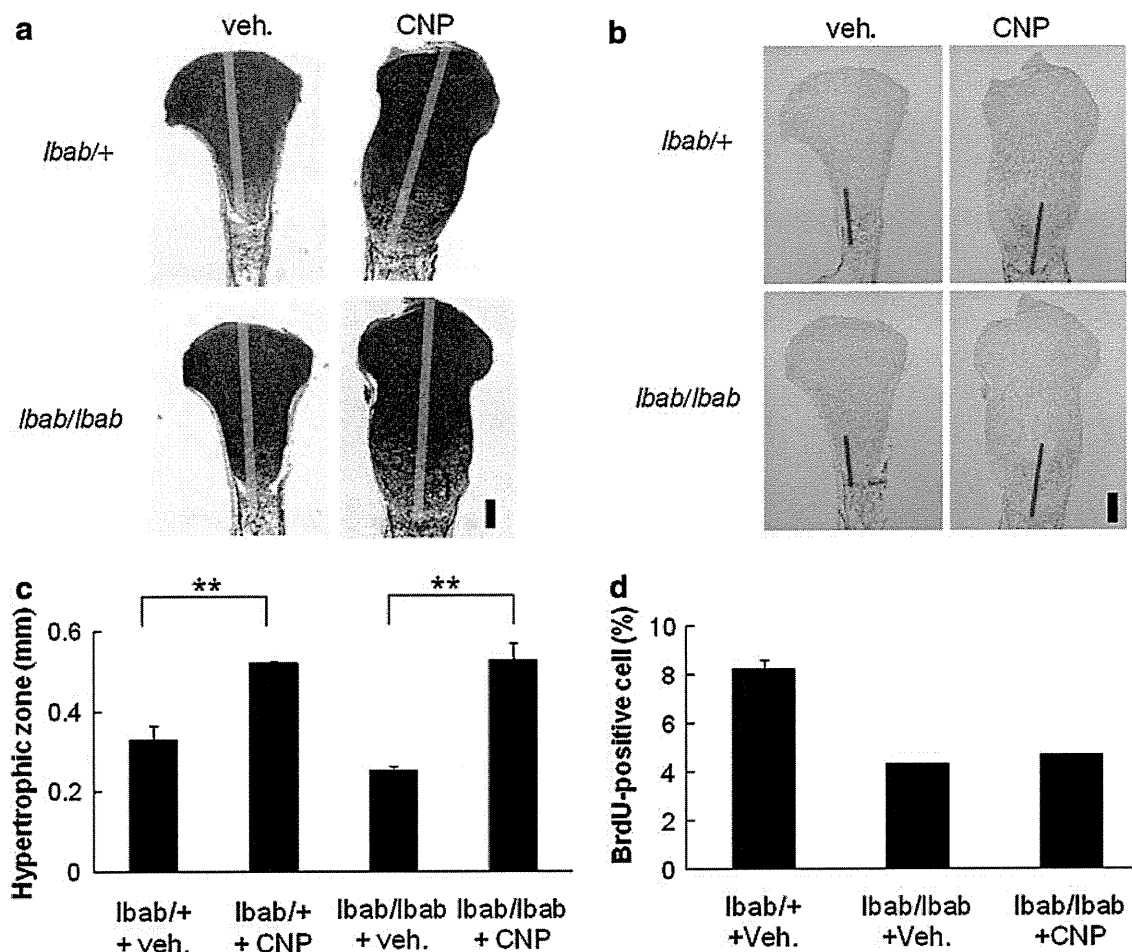
with vehicle (*veh.*) or  $10^{-7}$  M CNP (*CNP*) for 4 days. *Circles* indicate *lbab/+* tibiae, and *squares* indicate *lbab/lbab* tibiae. At the end of culture, *closed symbols* indicate tibiae treated with vehicle and *open symbols* indicate those treated with CNP.  $n = 8-12$  each

The reason the decreased proliferation of chondrocytes in the *lbab/lbab* growth plate was not rescued by CNP overexpression in chondrocytes is not clear, but it may be 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 *lbab/lbab* explants in organ culture experiments 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], might proceed to recover the thinned growth plate of *lbab/lbab* mice. 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 *lbab/lbab* growth plate and recovered by addition of CNP. In addition to the result that the expression of MMP-13 was not different between the terminal hypertrophic chondrocytes of wild-type, *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 *lbab/lbab* mice using three-dimensional CT analysis: the bone volume of *lbab/lbab* mice was substantially decreased compared to that of wild-type mice and recovered by cartilage-specific CNP overexpression. The mechanism of decrease in bone volume of *lbab/lbab* mice is still unknown, but CNP may be expressed in and affect 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 CNP-Tg expression from the CP 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 in vitro effects of CNP on osteoblastic cell lineages or osteoclasts have been reported [18–28], the in vivo effects of CNP on bone metabolism remain elusive; and further experiments are now ongoing in our laboratory.

We previously discovered that in two strains of mice, *cn/cn* and *slw/slw*, dwarfism is caused by spontaneous



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

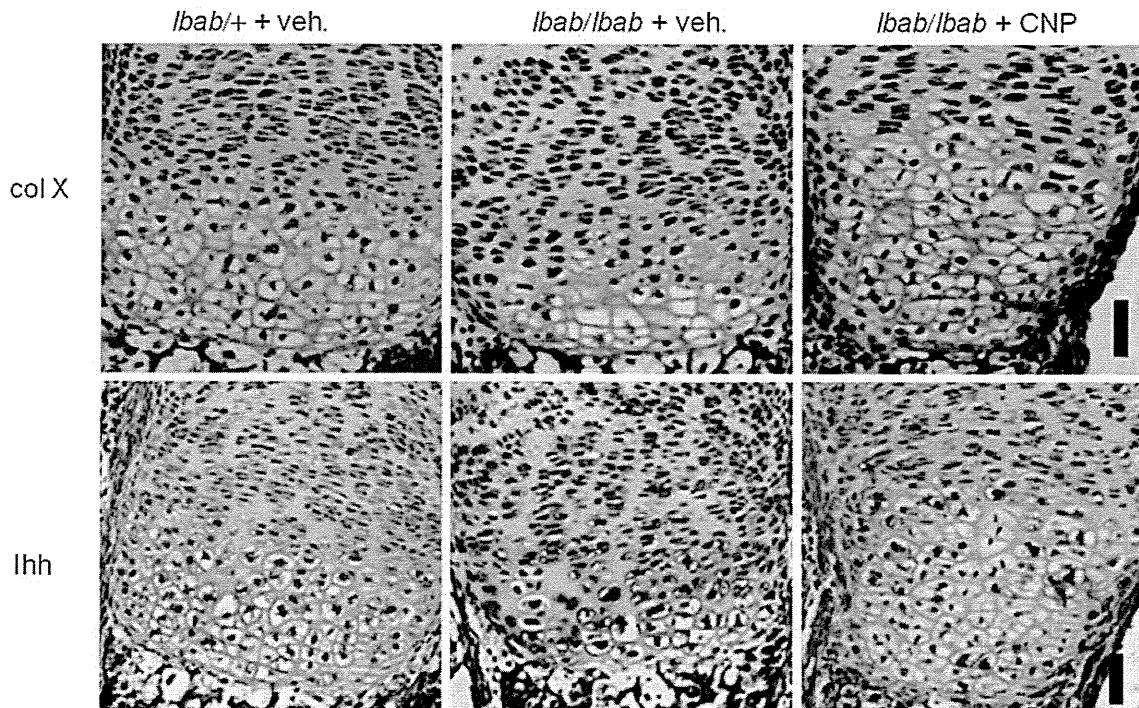
Scale bars 200  $\mu$ m. Height of hypertrophic chondrocyte layer (**c**) and proportion of BrdU-positive cells (**d**) of the growth plate of tibial explant from fetal *lbab/+* or *lbab/lbab* mice treated with  $10^{-7}$  M CNP or vehicle at the end of the 4-day culture period.  $n = 3$  each.  $***P < 0.01$  in **c** and  $n = 2-3$  each in **d** (Color figure online)

mutations in the GC-B gene [7, 8]. In humans, it has been identified that AMDM is caused by spontaneous loss-of-function mutations in the GC-B gene [9, 29]. The *lbab/lbab* mouse, the skeletal phenotype of which we have closely analyzed in the present report, has a spontaneous loss-of-function mutation in the CNP gene; by analogy to the GC-B gene, some forms of human skeletal dysplasia might be caused by mutations in the CNP gene. 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 model of a form of human skeletal dysplasia 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 it may have to do with differences between species or some other unknown mechanism(s). 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



**Fig. 8** Immunohistochemical staining of type X collagen (*upper panels*) and Ihh (*lower panels*) of the growth plates of metatarsal explants from fetal *lba+/+* and *lba/lba* mice treated with vehicle (*veh.*) or  $10^{-7}$  M CNP for 4 days. Scale bar 50  $\mu$ m

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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## Forkhead Box A1 (FOXA1) and A2 (FOXA2) Oppositely Regulate Human Type 1 Iodothyronine Deiodinase Gene in Liver

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Type 1 iodothyronine deiodinase (D1), a selenoenzyme that catalyzes the bioactivation of thyroid hormone, is expressed mainly in the liver. Its expression and activity are modulated by several factors, but the precise mechanism of its transcriptional regulation remains unclear. In the present study, we have analyzed the promoter of human D1 gene (*hDIO1*) to identify factors that prevalently increase D1 activity in the human liver. Deletion and mutation analyses demonstrated that a forkhead box (FOX)A binding site and an E-box site within the region between nucleotides –187 and –132 are important for *hDIO1* promoter activity in the liver. EMSA demonstrated that FOXA1 and FOXA2 specifically bind to the FOXA binding site and that upstream stimulatory factor (USF) specifically binds to the E-box element. Overexpression of FOXA2 decreased *hDIO1* promoter activity, and short interfering RNA-mediated knockdown of FOXA2 increased the expression of *hDIO1* mRNA. In contrast, overexpression of USF1/2 increased *hDIO1* promoter activity. Short interfering RNA-mediated knockdown of FOXA1 decreased the expression of *hDIO1* mRNA, but knockdown of both FOXA1 and FOXA2 restored it. The response of the *hDIO1* promoter to USF was greatly attenuated in the absence of FOXA1. Taken together, these results indicate that a balance of FOXA1 and FOXA2 expression modulates *hDIO1* expression in the liver. (*Endocrinology* 153: 492–500, 2012)

Thyroid hormone activation and inactivation are mediated by three selenoenzymes, type 1 iodothyronine deiodinase (D1), D2, and D3. D1 and D2 catalyze the conversion of T<sub>4</sub> to T<sub>3</sub> via removal of outerring iodine (1). The human D1 gene (*hDIO1*) is expressed in the liver, kidney, thyroid, and pituitary (2). The D2 gene is expressed in the central nervous system, pituitary, heart, and skeletal muscle, but it is absent in the liver (1). The D3 gene is expressed in the central nervous system and placenta, and it is involved in thyroid hormone inactivation by mediating the removal of innerring iodine. Unlike D2, D1 activity is considered to be regulated predominantly at the pretranslational level. The expression and activity of D1

are modulated by a variety of factors. T<sub>3</sub> induces the expression of *hDIO1* via two thyroid hormone responsive elements within its promoter (3), and nuclear factor  $\kappa$ B induced by TNF $\alpha$  inhibits the T<sub>3</sub>-dependent induction of D1 (4). However, the precise mechanism of the transcriptional regulation of *hDIO1* expression remains unclear.

In this study, we sought to identify factors that increased D1 activity in the liver, a main organ that expresses *hDIO1*. We assessed the promoter activity of the 5-kb 5'-flanking region of *hDIO1* and characterized regulatory element-binding proteins within this region. In this study, we identify responsive elements for the forkhead box (FOX) transcription factors FOXA1/FOXA2 and the ba-

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Abbreviations: D1, Type 1 iodothyronine deiodinase; FOX, forkhead box; *hDIO1*, human D1 gene; HNF, hepatocyte nuclear factor; MUT, mutated oligonucleotide; siRNA, short interfering RNA; USF, upstream stimulatory factor; WT, wild type.

sis/helix-loop-helix-leucine zipper transcription factor upstream stimulatory factor (USF), and we show that FOXA1, FOXA2, and USF all participate in the regulation of *hDIO1*. We also show that FOXA1 is required for the activation of the *hDIO1* promoter by USF and that FOXA2 represses the transcription of *hDIO1* and disrupts the interaction of USF with FOXA1 by occupying the FOXA binding site. Collectively, these results demonstrate that FOXA1 and FOXA2 display opposing activity in the regulation of *hDIO1* expression in the liver.

## Materials and Methods

### Cell culture

The human liver carcinoma cell line HepG2 was obtained from American Type Culture Collection (Manassas, VA) and cultured in MEM (Life Technologies, Carlsbad, CA) with 0.1 mM nonessential amino acid solution (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) at 37 C in a humidified atmosphere containing 5% CO<sub>2</sub>. TSA 201 cells, a clone of human embryonic kidney 293 cells (5), were cultured in DMEM (Life Technologies) with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B supplemented with 10% fetal bovine serum at 37 C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Plasmid construction

Deletion mutants of the 5'-flanking regions of *hDIO1* (–4949, –2023, –343, –187, –150, –131, and –103/–4, the translational start site was set at +1) were prepared by PCR using human genomic DNA from leukocytes as a template. The resulting PCR products were subcloned into *EcoRV* or *KpnI/HindIII*-digested pGL4.10 (Promega, Madison, WI) to create a fusion with the luciferase gene (–4949, –2023, –343, –187, –150, –131, and –103/–4 hDIO1-Luc). The PCR primers, containing *EcoRV*, *KpnI*, or *HindIII* linker, are listed in Table 1. The correct orientation of these deletion mutant constructs was confirmed by sequencing.

Mutations were created using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instruction; –187/–4 and –150/–4 hDIO1-

Luc were used as templates. For mutagenesis, the sequences of the FOXA binding element and E-box were specified in figure 3 below. Mutated constructs were isolated from each reaction and verified by sequencing.

Plasmids expressing cDNA for FOXA2 and USF1, pF1KB7038 and pF1KB8339, respectively, were generated by Kazusa DNA Research Institute (Chiba, Japan) and purchased from Promega. These plasmids were digested with *SgfI* and *PmeI*, and cDNA for FOXA2 and USF1 were ligated into the *SgfI/PmeI*-digested pF4A CMV Flexi vector (Promega), which uses the human cytomegalovirus intermediate-early enhancer/promoter to allow constitutive protein expression at native levels in mammalian cells. The open reading frame of human USF2 was generated by PCR using HeLa cell cDNA as a template. The PCR primers containing *SgfI* or *PmeI* linker are listed in Table 1. The PCR product was digested with *SgfI* and *PmeI*, cloned into the *SgfI/PmeI*-digested pF4A CMV Flexi vector, and verified by sequencing.

### Transient transfection and luciferase assay

HepG2 and TSA 201 cells were plated at  $1.5\text{--}2 \times 10^5$  and  $0.5\text{--}1 \times 10^5$  cells/well in 24-well tissue culture plates, respectively. Cells were maintained in 0.5 ml of antibiotic-free medium for 1 d before transfection. Transient transfections were performed using the Lipofectamine LTX reagent (Life Technologies) for HepG2 cells and the Lipofectamine 2000 reagent (Life Technologies) for TSA 201 cells according to the manufacturer's instruction. In HepG2 cells, transfections included 500 ng of experimental reporter constructs and 25 ng of pGL4.74, which contained the cDNA encoding *Renilla* luciferase (Promega) as an internal control for transfection efficiency. In TSA 201 cells, transfections included 100 ng of experimental reporter constructs and 5 ng of pGL4.74. In the experiments with plasmids expressing FOXA2 and/or USF, total amount of plasmid DNA was kept constant by adding the corresponding amount of pF4A without a cDNA insert. After transfection, cells were grown in antibiotic-free medium and harvested after 48 h. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega), and luminescence was measured by a 2030 ARVOX multilabel reader (PerkinElmer, Waltham, MA). Firefly luciferase activity was normalized to *Renilla* luciferase activity in each well to control for transfection efficiency.

### Computational analysis of the putative transcription factor binding sites

The putative transcription factor binding sites on the 5'-flanking region of *hDIO1* were identified by computational

**TABLE 1.** Oligonucleotides used in plasmid construction and RT-PCR

	Forward primer (5'-3')	Reverse primer (5'-3')	Accession no.
Plasmid construction			
–4949/–4 hDIO1-Luc	G G G G A T A T C G C A G G T G C A G C C T A G A G A T G T A A C G	C C C A A G C T T G G C A A A G C C A G A G T A A G C T C	AL031427
–2023/–4 hDIO1-Luc	C G G G G T A C C A C A C T T C C A T T C C A G T T A C A G	C C C A A G C T T G G C A A A G C C A G A G T A A G C T C	AL031427
–343/–4 hDIO1-Luc	C G G G G T A C C G A G A G A G C A T C T A A C A G G T T C	C C C A A G C T T G G C A A A G C C A G A G T A A G C T C	AL031427
–187/–4 hDIO1-Luc	C G G G G T A C C G A C C T T T G T G C A C C T G G T T A G	C C C A A G C T T G G C A A A G C C A G A G T A A G C T C	AL031427
–150/–4 hDIO1-Luc	C G G G G T A C C G A C A G A A A G G C A A A C A T C T T C	C C C A A G C T T G G C A A A G C C A G A G T A A G C T C	AL031427
–131/–4 hDIO1-Luc	C G G G G T A C C T C T G A C C T G A C T C C T T C C C C T G	C C C A A G C T T G G C A A A G C C A G A G T A A G C T C	AL031427
–103/–4 hDIO1-Luc	C G G G G T A C C G G T T G G C T G C T C T A C C C T G C	C C C A A G C T T G G C A A A G C C A G A G T A A G C T C	AL031427
pF4A-USF2	A G C A G C G A T C G C C A T G G A C A T G C T G G A C C C G G G T C T G G A	C G A G G T T T A A C C T G C C G G G T G C C C T C G C C C A	NM_003367
RT-PCR			
hDIO1	C A G A G T C A A G C G G A A C A T C C	C C G T T G G T C A C T A G A A T T G	NM_000792
Cyclophilin A	G C A C T G G A G A G A A A G G A T T T G G	C A G C A A T G G T G A T C T T C T T G C	NM_021130

analysis using TFSEARCH databases (<http://www.cbrc.jp/research/db/TFSEARCHJ.html>), based on the TRANSFAC databases (6).

### RNA isolation, RT-PCR, and quantitative PCR

Total RNA was extracted from HepG2 cells using the RNeasy Plus Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed with random hexamers using a First-strand cDNA Synthesis kit (GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instruction. The resulting cDNA were diluted 1:10 and subjected to PCR amplification with 0.5 mM each of the sense and antisense primers and 0.5 U of AmpliTaq Gold DNA polymerase (Life Technologies). The PCR primers used for *hDIO1* and human cyclophilin A gene are indicated in Table 1. The PCR conditions were 40 cycles of denaturation for 1 min at 95 C, annealing for 1 min at 52 C, and extension for 1 min at 72 C. The PCR products were electrophoresed in 2% agarose gels.

Quantitative PCR reactions were performed, recorded, and analyzed using TaqMan Gene Expression Assays with StepOnePlus real-time PCR system (Life Technologies). The probe and primers were Hs00270129\_m1 (human *FOXA1*), Hs00232764\_m1 (human *FOXA2*), and Hs00174944\_m1 (*hDIO1*) and purchased from Life Technologies. Diluted cDNA were amplified using the following conditions: 50 C for 2 min, 95 C for 10 min, and 40 cycles of 95 C for 15 sec and 60 C for 1 min, followed by continuous incubation at 25 C. Expression levels of *FOXA1*, *FOXA2*, and *hDIO1* were normalized to cyclophilin A to compensate for variations in input RNA.

### Preparation of cell extracts and EMSA

Nuclear extracts were prepared from HepG2 cells using the Nuclear Extract kit (Active Motif, Carlsbad, CA), according to the manufacturer's instruction. EMSA were conducted using a LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific, Rockford, IL) with slight modifications of the original manufacturer's instruction. Oligonucleotides 3'-end labeled with biotin were synthesized (Life Technologies) and annealed to generate double-stranded oligonucleotide probes. Two hundred femtomoles of oligonucleotide probe were incubated with 10–15  $\mu$ g of nuclear protein and 0.5  $\mu$ g of poly (dI-dC) in the presence or absence of competing oligonucleotide in 10 $\times$  binding buffer [containing 100 mM Tris, 500 mM KCl, and 10 mM dithiothreitol (pH 7.5)] and 75 mM KCl, and 5% glycerol was added to solutions containing probes with an E-box element. After a 30-min incubation at room temperature, DNA-protein complexes were separated by electrophoresis on a 6% DNA retardation gel (Life Technologies) at 4 C in 0.5 $\times$  Tris-borate, EDTA buffer [containing 89 mM Tris-borate and 2 mM EDTA (pH 8.0)]. For supershift assays, binding reactions were incubated for 45 min at room temperature with antibodies before the addition of labeled probes. The antibodies used in the supershift assays were as follows: 1  $\mu$ l (200  $\mu$ g/0.1 ml) of USF1 (sc-8983X), USF2 (sc-861X), E47 (sc-763X), *FOXA1* (sc-6553X), *FOXA2* (sc-6554X), and *FOXA3* (sc-5361X) and 5  $\mu$ l (200  $\mu$ g/0.5 ml) of normal goat and normal rabbit IgG, and all were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After electrophoresis, samples were transferred onto nylon membranes and

fixed by UV irradiation. Biotinylated DNA was detected using a Fujix Lumino-image analyzer (LAS-1000; Fuji Photo Film Co., Ltd., Tokyo, Japan).

### Transfection of short interfering RNA (siRNA)

An aliquot of 6 pmol siRNA specific for *FOXA1* and/or *FOXA2* (Stealth Select RNAi, Life Technologies) or a negative control siRNA (Stealth RNAi Negative Control, Life Technologies) was transfected into HepG2 cells using the Lipofectamine RNAiMax reagent (Life Technologies) by reverse transfection according to the manufacturer's instruction. After transfection, HepG2 cells were plated at  $1.5\text{--}2 \times 10^5$  cells/well in 24-well tissue culture plates and maintained in 0.5 ml of antibiotic-free medium for 24–48 h. mRNA extraction and analysis were performed as described above.

### Statistics

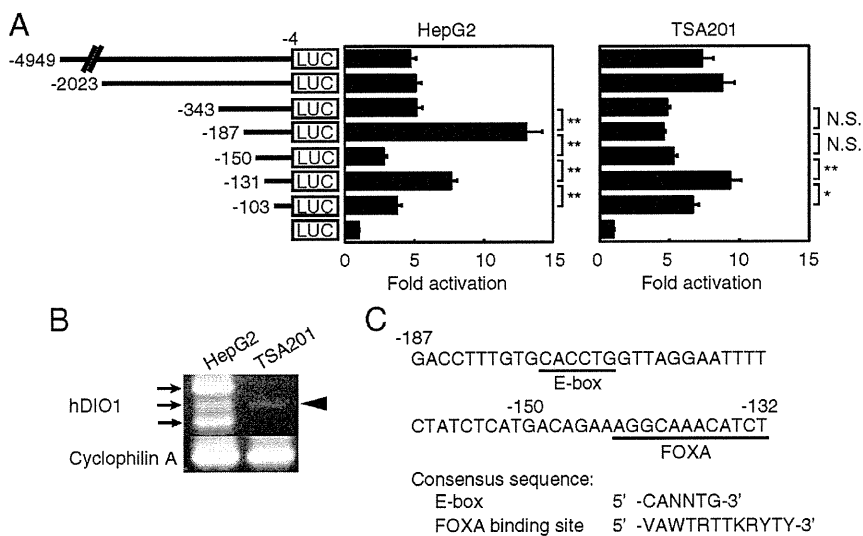
The data represent the mean  $\pm$  SEM and were obtained from at least three separate experiments, each performed in triplicate. Statistical analyses were performed to examine the significance of differences among the results using unpaired *t* test or ANOVA followed by Student-Newman-Keuls test or Dunnett's test.

## Results

### Functional analysis of the 5'-flanking region of the *hDIO1* gene

To identify regions within the promoter region of *hDIO1* important for regulating its expression, a series of 5'-deletion constructs was subcloned into the pGL4.10 vector and transiently transfected into HepG2 and TSA 201 cells (Fig. 1A). In both HepG2 and TSA 201 cells, luciferase activity increased by deletion of nucleotides –150 to –131 and decreased after deletion of –131 to –103. Among the tested constructs, the luciferase activity produced by transfection of –150/–4 *hDIO1*-Luc was specifically and markedly decreased in HepG2 cells. Additionally, more pronounced differences were seen between the activity of –150/–4 *hDIO1*-Luc and –131/–4 *hDIO1*-Luc in HepG2 cells compared with TSA 201 cells. In addition, luciferase activity was markedly increased by deleting the region from –343 to –187 and decreased after deletion of –187 to –150 only in HepG2 cells. Taken together, these results indicate that the region between nucleotides –187 and –132 is important for *hDIO1* promoter function in HepG2 cells. To confirm the expression of *hDIO1*, we performed RT-PCR using total RNA isolated from HepG2 and TSA 201 cells, a liver and kidney cell line, respectively. As shown in Fig. 1B, although there was a difference in the degree of gene expression, *hDIO1* was expressed in both cell lines; this was consistent with a previous report examining *hDIO1* tissue distribution (2). Additionally, multiple PCR products were detected, because there are several alternative splice variants of *hDIO1* (7). These results indicate that there may exist a sequence





**FIG. 1.** Liver-specific changes in *hDIO1* promoter activity. **A**, A series of 5'-deletion constructs of the *hDIO1* promoter were transiently transfected into HepG2 or TSA 201 cells. Promoter activity was normalized to *Renilla* luciferase activity and expressed as the relative activity to promoterless pGL4.10. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . N.S., Not significant. **B**, RT-PCR analysis of *hDIO1* expression. Electrophoretic analysis of RT-PCR products using total RNA from HepG2 and TSA 201 cells was performed. The arrows and arrowhead correspond to the RT-PCR products using total RNA from HepG2 and TSA 201 cells, respectively. Cyclophilin A was used as a positive control. **C**, The nucleotide sequences of the 5'-flanking region of *hDIO1* are shown. The translational start site was set at +1. Underlined sequences indicate putative binding sites for transcription factors. Consensus sequences of E-box site and FOXA binding site are shown at the bottom. Abbreviations for nucleotides: W (A or T), K (G or T), Y (C or T), R (A or G), V (A, C, or G), and N (A, C, G, or T). LUC, Luciferase.

essential for liver-specific expression of *hDIO1* within the  $-187$  to  $-132$  region of its promoter. A computational analysis of this region revealed the presence of a consensus E-box site between nucleotides  $-187$  and  $-151$  and a FOXA binding site between nucleotides  $-150$  and  $-132$  (Fig. 1C).

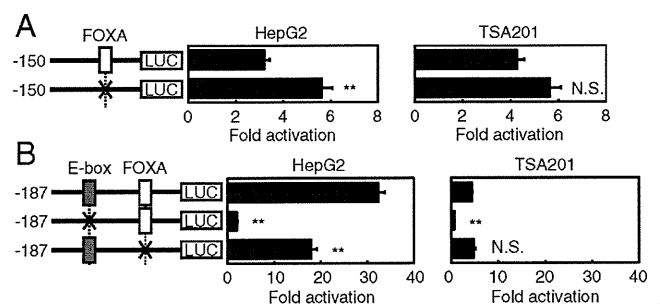
### Promoter activity associated with the FOXA binding site and the E-box

To better understand the contribution of the FOXA binding site and the E-box on the expression of *hDIO1* in liver-derived HepG2 cells, we examined luciferase activity in cells transfected with wild-type (WT) or mutated *hDIO1* promoter constructs. In HepG2 cells, luciferase activity was increased 2-fold by mutating the FOXA binding site when cells were transfected with a  $-150/-4$  *hDIO1*-Luc construct (Fig. 2A). In addition, when cells were transfected with a  $-187/-4$  *hDIO1*-Luc construct, luciferase activity was nearly completely lost by destruction of the E-box, and mutation of the FOXA binding site caused a decrease in luciferase activity by 50% (Fig. 2B). In TSA 201 cells transfected with  $-187/-4$  *hDIO1*-Luc, luciferase activity was almost completely abolished by mutation of the E-box, but mutation of the FOXA binding site in both  $-187/-4$  *hDIO1*-Luc and  $-150/-4$  *hDIO1*-Luc did not significantly affect luciferase activity (Fig. 2). Thus, the E-box present

within the *hDIO1* promoter is required for the enhancer activity in both liver- and kidney-derived cells, but the FOXA binding site exhibits liver-specific enhancer and repressor activity.

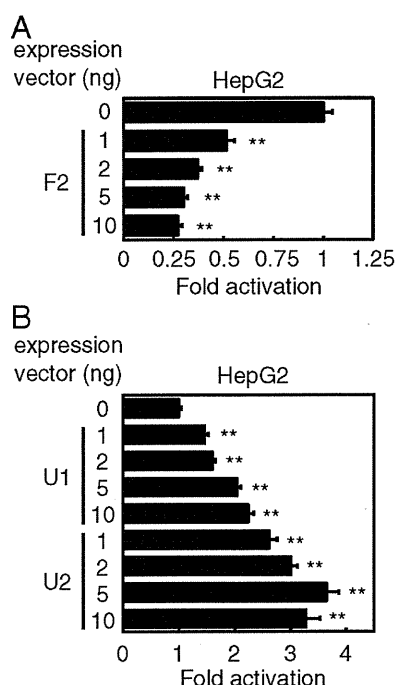
### Binding of FOXA1/FOXA2 to the FOXA binding site and USF to the E-box

To determine the transcription factors that bind to these elements in the promoter of *hDIO1* in HepG2 cells, we performed EMSA using oligonucleotides with the FOXA binding site and the E-box. Incubation of HepG2 cell extracts with oligonucleotides containing the FOXA binding site (Fig. 3A, WT-F) led to the formation of several DNA/protein complexes (Fig. 3B, lane 2). Formation of one of these complexes was inhibited by incubation with excess WT-F, but not mutated oligonucleotide (MUT)-F, demonstrating the specificity of this complex (Fig. 3B, lanes 3–6). Additionally, the complex was supershifted by addition of anti-FOXA1 and anti-FOXA2 antibodies (Fig. 3B, lanes 7 and 8). However, an antibody specific for FOXA3, which binds an identical sequence, or normal goat IgG did not disrupt complex formation (Fig. 3B, lanes 9 and 10). These results suggest that the putative FOXA binding site is specifically bound by FOXA1 or FOXA2. We next examined binding to the E-box sequence, and several complexes were formed by



**FIG. 2.** Changes in *hDIO1* promoter activity by FOXA binding site and E-box. Schematic diagram in the left of each figure representing WT and site-specific mutations of the *hDIO1* promoter, introduced into the upstream region of the luciferase gene. A cross represents the site-specific mutation of the putative FOXA binding site or E-box. Each construct was transiently transfected into HepG2 or TSA 201 cells. Promoter activity was normalized to *Renilla* luciferase activity and expressed as the relative activity to promoterless pGL4.10. Statistical significance was determined by unpaired *t* test (A) or ANOVA followed by Dunnett's test (B). \*\*,  $P < 0.01$ . N.S., Not significant.



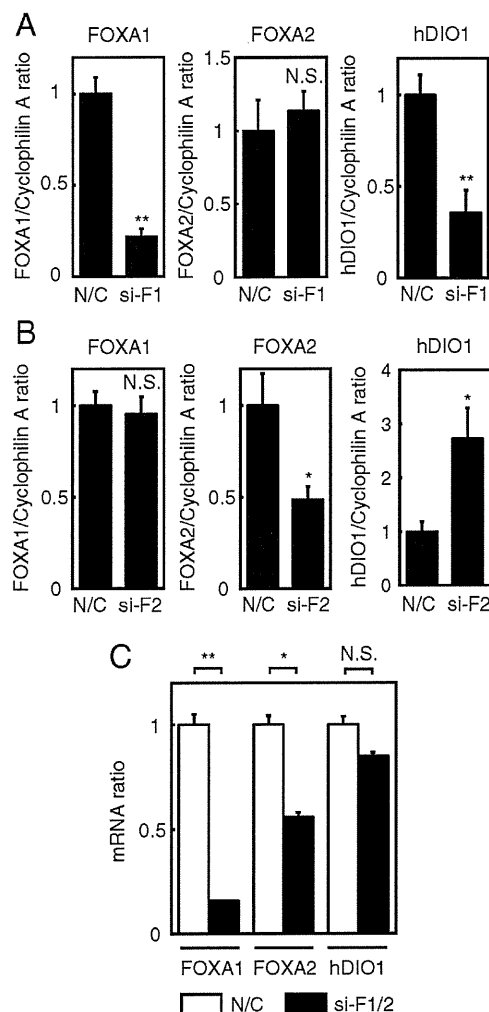


**FIG. 4.** Effect of overexpression of FOXA2 or USF on *hDIO1* promoter activity.  $-187/-4$  *hDIO1*-Luc was transiently transfected into HepG2 cells in the presence of increasing amounts of vectors expressing FOXA2 (F2) (A) or USF1 or USF2 (U1 and U2, respectively) (B). Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to  $-187/-4$  *hDIO1*-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Dunnett's test. \*\*,  $P < 0.01$ .

down of FOXA1 decreased the expression of *hDIO1* mRNA, and knockdown of FOXA2 increased the expression level of *hDIO1* mRNA. FOXA1 and FOXA2 did not affect each other's expression by knockdown of them (Fig. 5, A and B). In addition, when both FOXA1 and FOXA2 were knocked down simultaneously, no change in the expression of *hDIO1* mRNA was seen (Fig. 5C). Thus, *hDIO1* expression is positively regulated by FOXA1 and negatively regulated by FOXA2, and FOXA1 and FOXA2 interact with each other to regulate *hDIO1* expression.

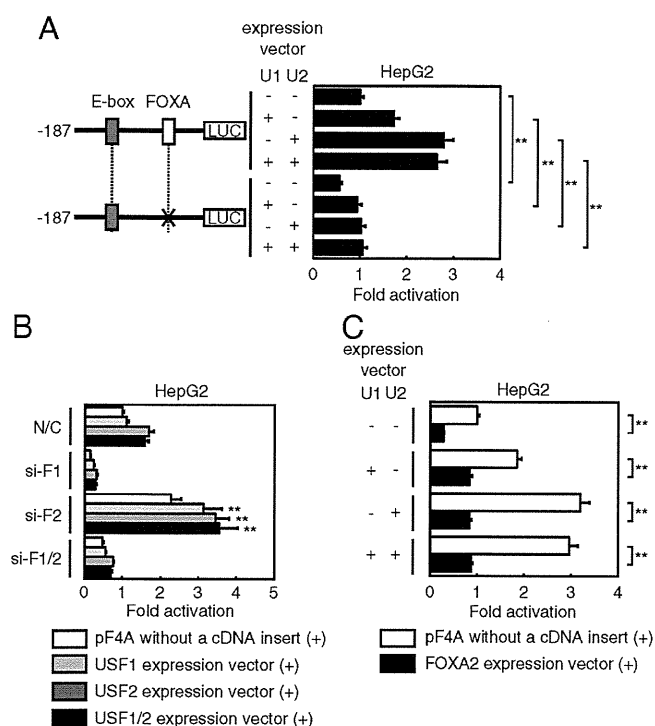
#### Interaction between FOXA and USF in the activation of the *hDIO1* promoter

Transcription factors frequently interact to coordinately regulate gene expression, and we first wished to determine whether the FOXA binding site and the E-box present in the *hDIO1* promoter interact. We cotransfected a WT or mutated  $-187/-4$  *hDIO1*-Luc construct and USF expression plasmids into HepG2 cells. When the FOXA binding site was mutated, the transcription activity of the *hDIO1* promoter in the presence of transfected USF was attenuated (Fig. 6A). Thus, activation of the *hDIO1* promoter by USF depends on the presence of a functional FOXA binding site. Next, we investigated the effects of FOXA on the response of the *hDIO1* promoter to USF.



**FIG. 5.** Knockdown of FOXA1 and/or FOXA2. Effect of transfection of FOXA1 (A) and FOXA2 (B) siRNA on the mRNA expression level of FOXA1, FOXA2, and D1. Simultaneous knockdown of FOXA1 and FOXA2 (C) was performed using siRNA that has identical sequences in both FOXA1 and FOXA2. N/C, Negative control siRNA; si-F1, siRNA specific for FOXA1; si-F2, siRNA specific for FOXA2; si-F1/2, siRNA specific for both FOXA1 and FOXA2. mRNA expression level was normalized to that of cyclophilin A in each sample and expressed as the relative activity to the basal expression (N/C). Statistical significance was determined by unpaired t test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . N.S., Not significant.

We knocked down the expression of FOXA and cotransfected a  $-187/-4$  *hDIO1*-Luc construct along with USF expression plasmids into HepG2 cells. As shown in Fig. 6B, the transcription activity of the *hDIO1* promoter was attenuated by knockdown of FOXA1 and enhanced by knockdown of FOXA2. The transcription activity of the *hDIO1* promoter was also attenuated by simultaneous knockdown of FOXA1 and FOXA2 to an extent similar to that seen for the knockdown of FOXA1. The suppressed activity by knockdown of FOXA1 was not restored by overexpression of USF, and the enhanced activity by knockdown of FOXA2 was further enhanced by overexpression of USF. Thus, the response of the *hDIO1* pro-



**FIG. 6.** Interaction between FOXA and USF in the activation of the *hDIO1* promoter. **A**, Dependence of the activation of *hDIO1* promoter by USF on the FOXA binding site. Schematic diagram in the *left* of figure representing WT and site-specific mutations of the *hDIO1* promoter, introduced into the upstream region of the luciferase gene. A cross represents the site-specific mutation of the putative FOXA binding site. Each construct was transiently cotransfected into HepG2 cells in the presence of 5 ng of vectors expressing USF1 (U1) and/or USF2 (U2). Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to  $-187/-4$  *hDIO1*-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. \*\*,  $P < 0.01$ . **B**, Effect of knockdown of FOXA on the activation of the *hDIO1* promoter by USF. Short interfering RNA specific for FOXA1 and/or FOXA2 or a negative control siRNA were transfected into HepG2 cells as described in *Materials and Methods*. One day after siRNA transfection,  $-187/-4$  *hDIO1*-Luc was transiently cotransfected in the presence of 5 ng of vectors expressing USF1 and/or USF2. N/C, Negative control siRNA; si-F1, siRNA specific for FOXA1; si-F2, siRNA specific for FOXA2; si-F1/2, siRNA specific for both FOXA1 and FOXA2. Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to  $-187/-4$  *hDIO1*-Luc cotransfected with pF4A without a cDNA insert after knockdown by siRNA specific for FOXA2. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. \*\*,  $P < 0.01$  relative to  $-187/-4$  *hDIO1*-Luc cotransfected with pF4A without a cDNA insert after knockdown by siRNA specific for FOXA2. **C**, Effect of overexpression of FOXA2 on the activation of the *hDIO1* promoter by USF.  $-187/-4$  *hDIO1*-Luc was transiently transfected into HepG2 cells in the presence of 5 ng of vectors expressing USF1 (U1), USF2 (U2), and/or FOXA2. Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to  $-187/-4$  *hDIO1*-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. \*\*,  $P < 0.01$ . LUC, Luciferase.

moter to USF was greatly attenuated by knockdown of FOXA1 and enhanced by knockdown of FOXA2. Furthermore, we cotransfected a  $-187/-4$  *hDIO1*-Luc con-

struct and USF expression plasmids with or without a FOXA2 expression plasmid into HepG2 cells. The transcription activity of the *hDIO1* promoter was enhanced in the presence of transfected USF (Fig. 6C, *white bar*), but the activity was greatly attenuated by cotransfection of the FOXA2 expression plasmid (Fig. 6C, *black bar*). Thus, the response of the *hDIO1* promoter to USF was attenuated by the coexpression of FOXA2. Collectively, these results indicate that FOXA1 is required for the activation of the *hDIO1* promoter by USF and that FOXA2 represses the transcription of *hDIO1* and disrupts the interaction of USF with FOXA1 by occupying the FOXA binding site.

## Discussion

In this study, we analyzed the 5'-upstream region of *hDIO1* to identify protein-DNA interactions within the *hDIO1* promoter. Our experiments demonstrated that the region between nucleotides  $-187$  and  $-132$  is important for *hDIO1* promoter activity in HepG2 cells. We identified functional elements for FOXA and USF within this region, and we showed that these sites are important for the transcriptional regulation of *hDIO1*. Recently, Ohguchi *et al.* (9) identified a proximal hepatocyte nuclear factor (HNF)4 $\alpha$  binding site in mice, and they demonstrated that the HNF4 $\alpha$  binding site is essential for the activation of the mouse D1 gene by HNF4 $\alpha$ . Deletion analyses of the 5'-flanking region of *hDIO1* were performed by Jakobs *et al.* (10) by transfecting 1.5- and 0.1-kb constructs into HepG2 cells, and they found that both constructs substantially increased luciferase activity compared with a promoterless vector. However, they did not perform a higher resolution promoter analysis, and we are the first to identify functional elements other than thyroid hormone responsive element in the *hDIO1* promoter.

The FOXA proteins were first identified as liver-enriched factors because of their ability to bind the transthyretin gene promoter, and they were originally termed HNF3 (11). There are three FOXA proteins, FOXA1 (HNF3 $\alpha$ ), FOXA2 (HNF3 $\beta$ ), and FOXA3 (HNF3 $\gamma$ ), which are encoded by different genes on different chromosomes (12). FOXA proteins play important roles in early embryonic development and organogenesis, and they are recognized as "pioneer factors" (13). In addition, the FOXA proteins control glucose metabolism through the regulation of multiple target genes in the liver, pancreas, and adipose tissue after birth (13). Our EMSA experiments demonstrated that FOXA1 and FOXA2 specifically bound the identical FOXA binding site of the *hDIO1* promoter. Although all three FOXA proteins exist relatively abundant in HepG2 cells (14) and recognize the same DNA sequences, slight differences in the binding affin-