their wild-type littermates, respectively (Fig. 1a, Supplemental Fig. 1).The ratios of nasoanal and nose–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 weeks of age, these ratios stabilized at 66–72% and 57–62%, respectively (Fig. 1a, Supplemental Fig. 1). The body weight of *lbab/lbab* 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, *lbab/+* 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 *lbab/lbab* mice at the age of 2 weeks but was not affected in *lbab/+* mice (Fig. 1c). Histological analysis revealed that at the age of 3 days the tibial growth plate, especially 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 the tibial growth plate of *lbab/+* mice was not different from that of wild-type mice (Fig. 1d).

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 nasoanal 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: nasoanal 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 nasoanal 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 age 3 weeks), it became almost comparable to that of wild-type mice after 4 weeks (7.38 ± 0.48 and 7.61 ± 0.10 cm, respectively, at age 4 weeks). Further, the body weight of *lbab/lbab*·CNP-Tg mice was almost the same as that of *lbab/lbab* 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 *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 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 *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 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 *lbab/lbab* 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 *lbab/lbab* growth plate was completely recovered by targeted overexpression of CNP as observed in the *lbab/lbab*·CNP-Tg growth plate (Fig. 3c). The thinner hypertrophic chondrocyte layer in the *lbab/lbab* growth plate was also considerably recovered in the *lbab/lbab*·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 *lbab/lbab* growth plate (Fig. 3e). The number of PCNA-positive cells did not recover in the proliferative chondrocyte layer of the *lbab/lbab*·CNP-Tg growth plate, whereas the thinner proliferative chondrocyte layer in the *lbab/lbab* growth plate was almost completely recovered in the *lbab/lbab*·CNP-Tg growth plate (Fig. 3c). The area positive for immunostaining of Ihh, one of the markers of hypertrophic differentiation, was decreased in the *lbab/lbab* growth plate compared to the wild-type growth plate (Fig. 3f). The smaller size of the area positive for Ihh in the *lbab/lbab* growth plate was almost completely recovered in the *lbab/lbab*·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 *lbab/lbab* growth plate (Fig. 3g).

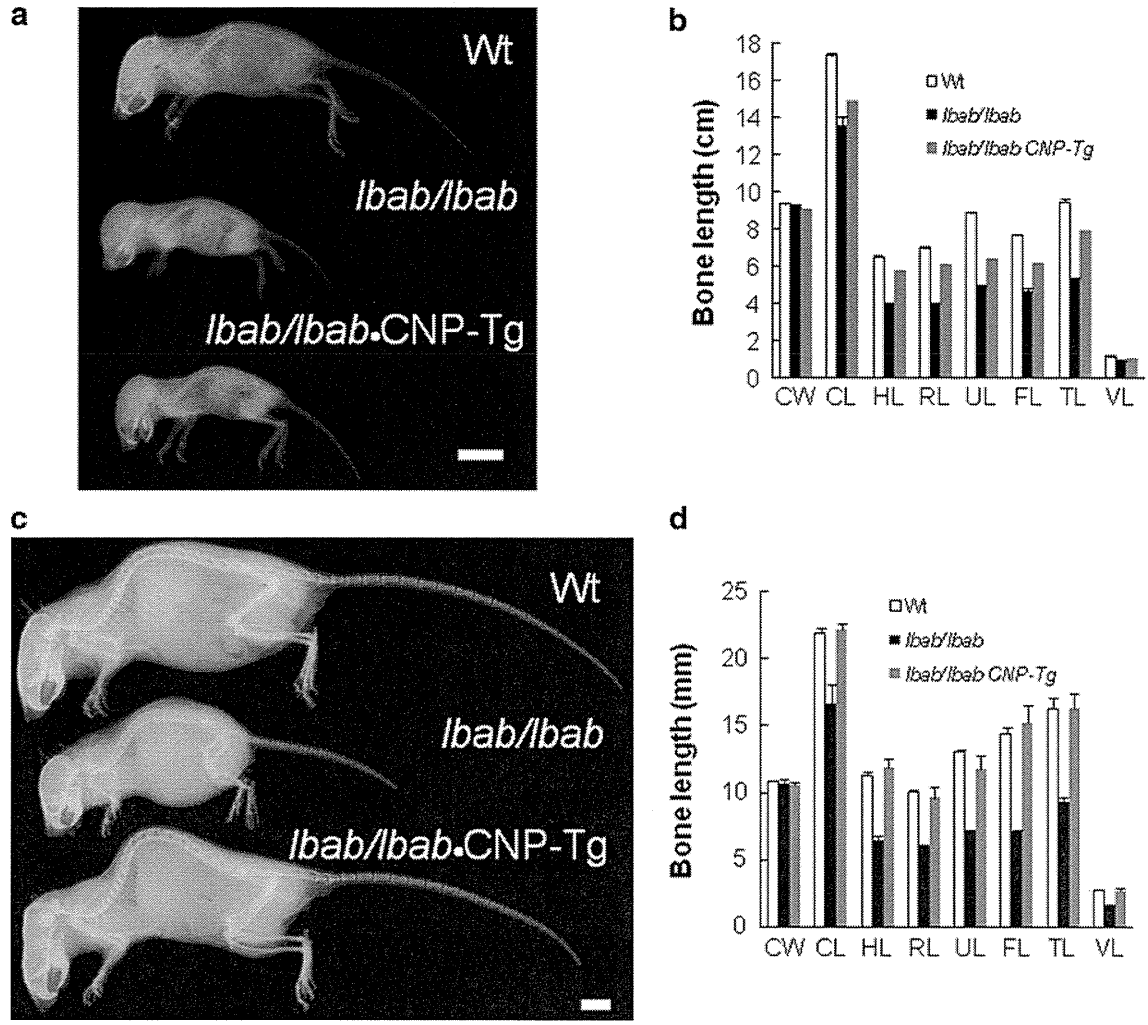

Fig. 2 Effect of CNP overexpression on impaired endochondral bone growth of *lbab/lbab* mice. Whole skeletons (**a**, **c**) and bone lengths measured on soft X-ray films (**b**, **d**) of female wild-type (*Wt*), *lbab/lbab*, and *lbab/lbab*·CNP-Tg mice at the age of 2 weeks (**a**, **b**) and 10 weeks (**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 μm, whereas those in wild-type mice were 4.1% and 40.3 μm, respectively. The decreased BV/TV and Tb.Th in *lbab/lbab* mice were increased to 5.4% and 37.0 μm, 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 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 the CP of *lbab/lbab* tibiae were significantly smaller than those of *lbab/+* tibiae (3.80 ± 0.04

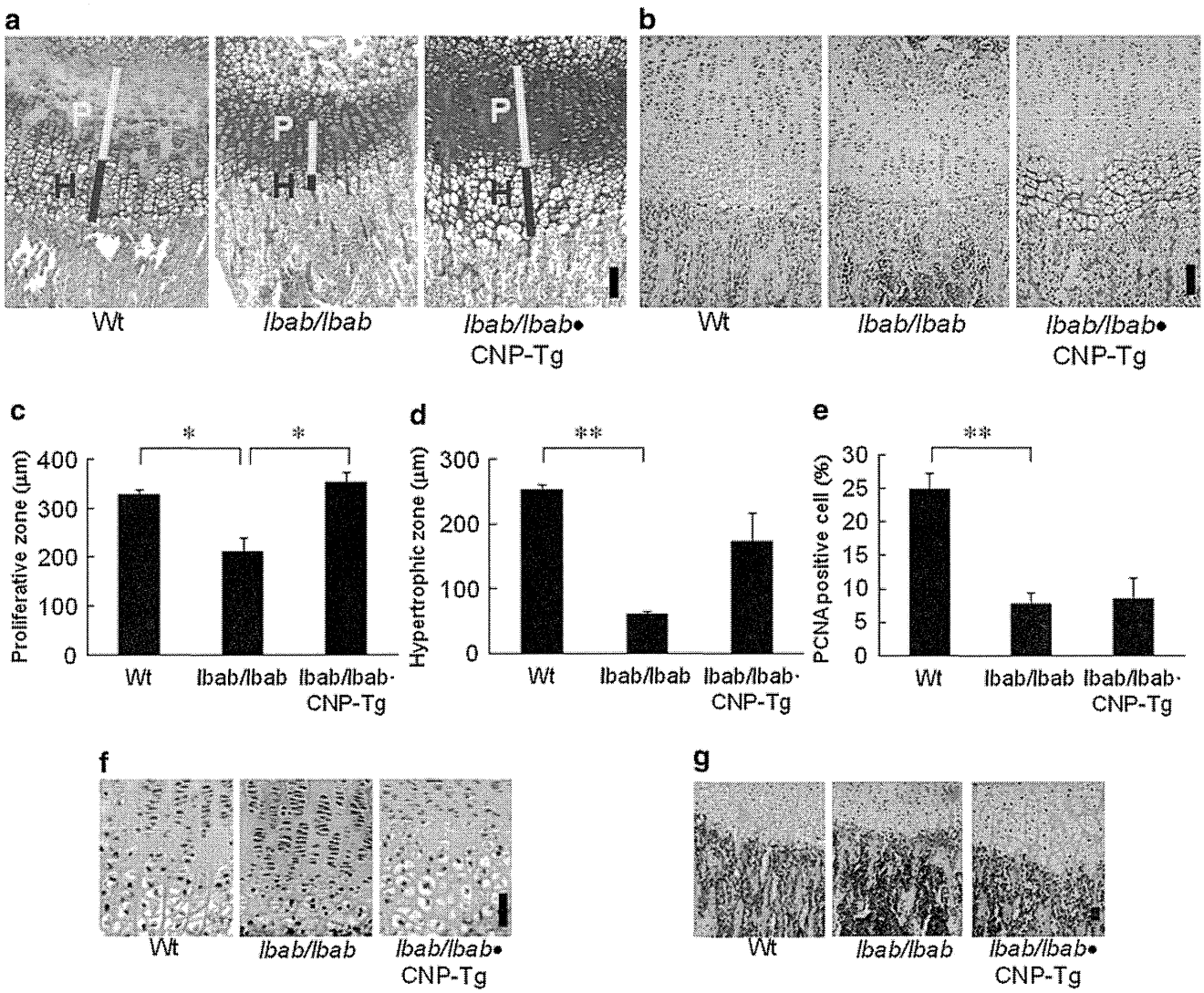

Fig. 3 Histological analysis of tibial growth plates from 2-week-old wild-type (*Wt*), *lbab/lbab*, and *lbab/lbab*·CNP-Tg mice. **a** Alcian blue and hematoxylin–eosin staining. *Yellow bars* (depicted as *P*) indicate proliferative chondrocyte layers, and *red bars* (depicted as *H*) indicate hypertrophic 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 ± 0.03 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 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

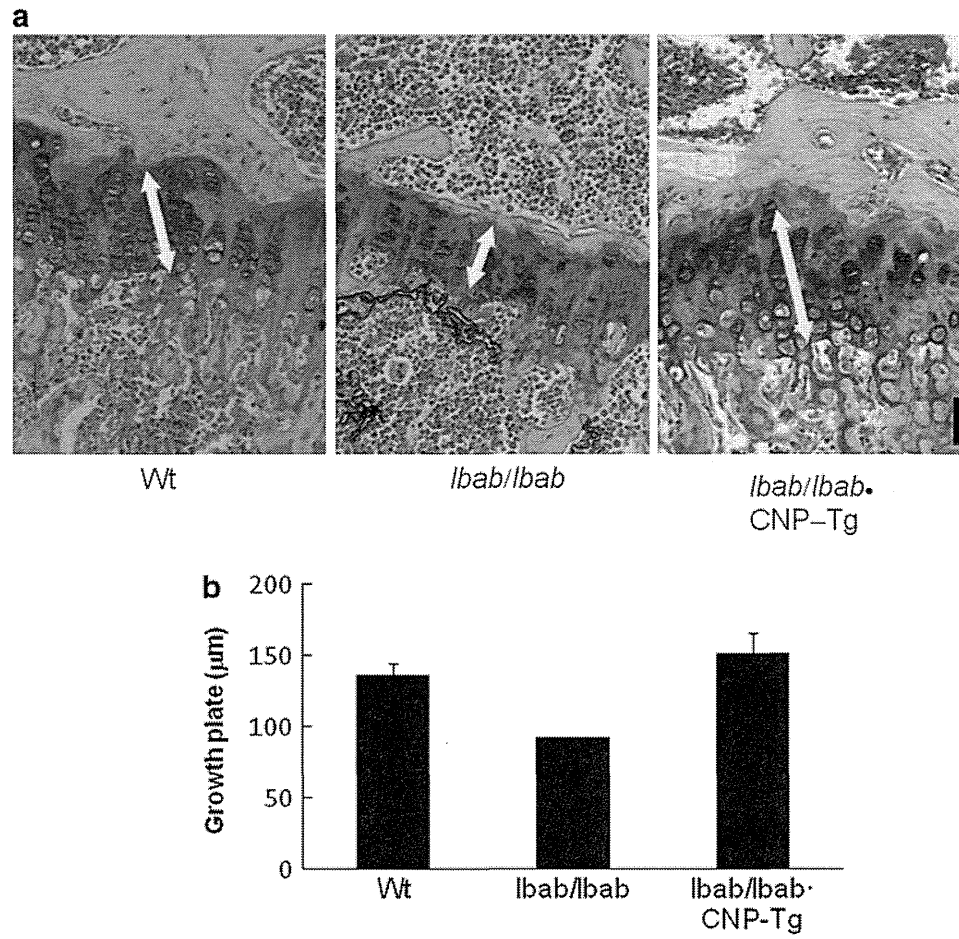

Fig. 4 Histological analysis of tibial growth plate from female 10-week-old wild-type (*Wt*), *lbab/lbab*, and *lbab/lbab*·CNP-Tg mice. **a** Alcian blue and hematoxylin–eosin staining. *Arrows* indicate the width of growth plates. *Scale bar* 50 μm. **b** Total heights of the growth plates. $n = 2$–5 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 potently 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.

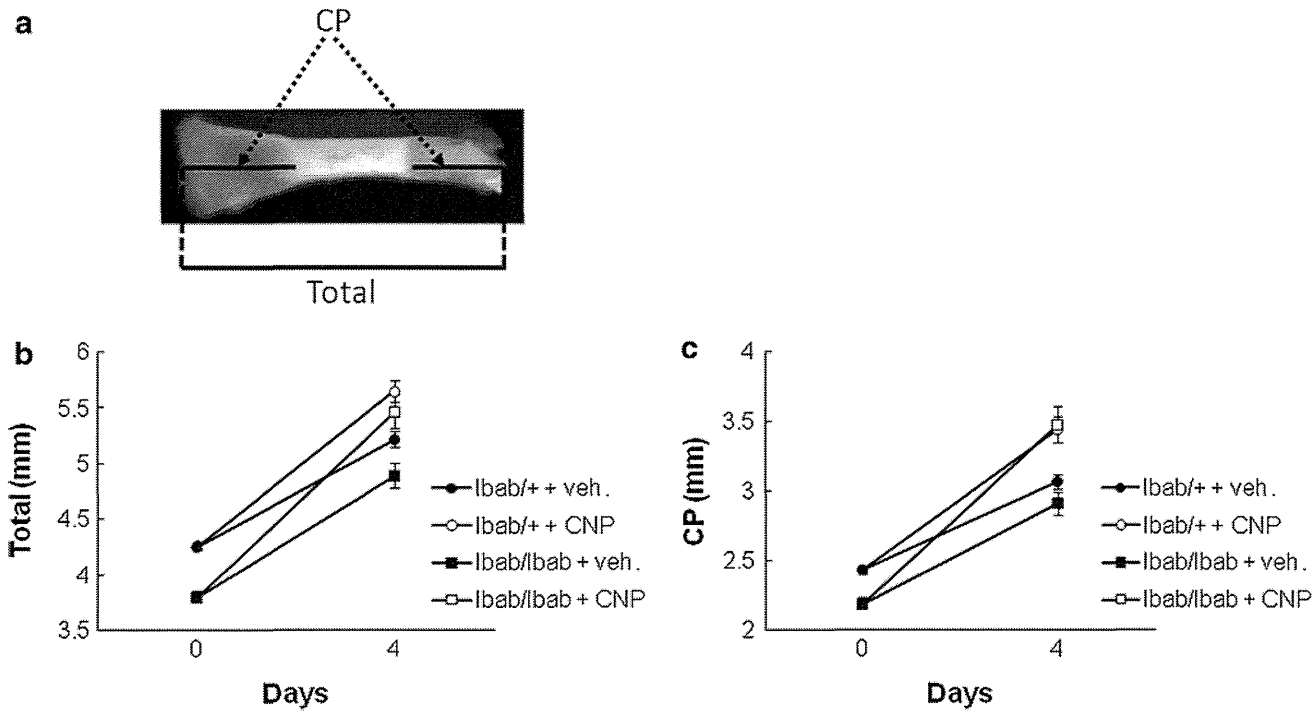

Fig. 6 Effect of CNP on cultured tibiae from fetal *lbab/+* and *lbab/lbab* mice. **a** A representative picture of a tibial explant from a fetal mouse. Total longitudinal length (*Total*) and the sum lengths of cartilaginous primordia (*CP*) are indicated. Graphs of total (**b**) and CP (**c**) lengths 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

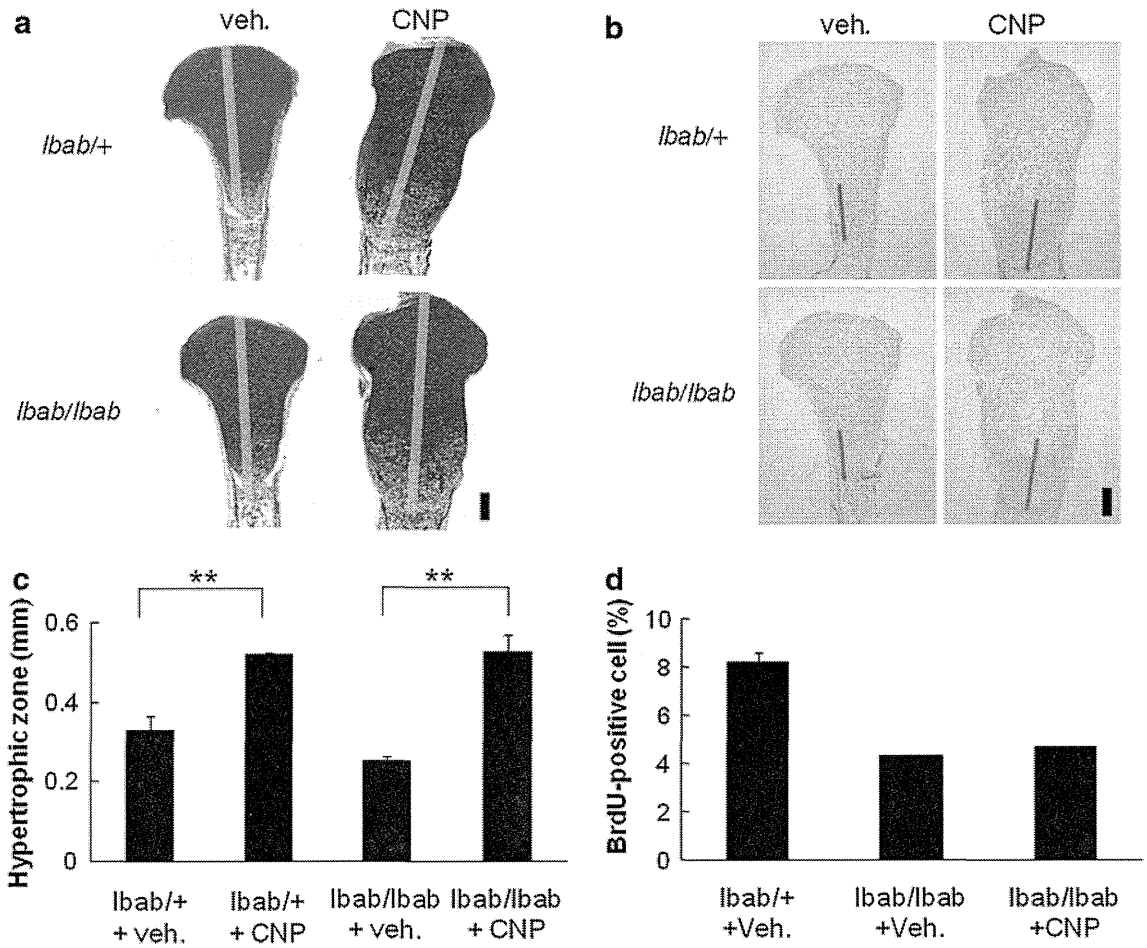

Fig. 7 Histological analyses of the growth plates of tibial explants from fetal *lbab/+* and *lbab/lbab* mice treated with vehicle (*veh.*) or 10^{-7} M CNP (*CNP*) for 4 days. Alcian blue and hematoxylin–eosin staining (**a**) and immunohistochemical staining for type X collagen (**b**). *Yellow bars* in **a** indicate lengths of cartilaginous primordial, and *red bars* in **b** indicate heights of hypertrophic chondrocyte layers. *Scale bars* 200 μm. 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

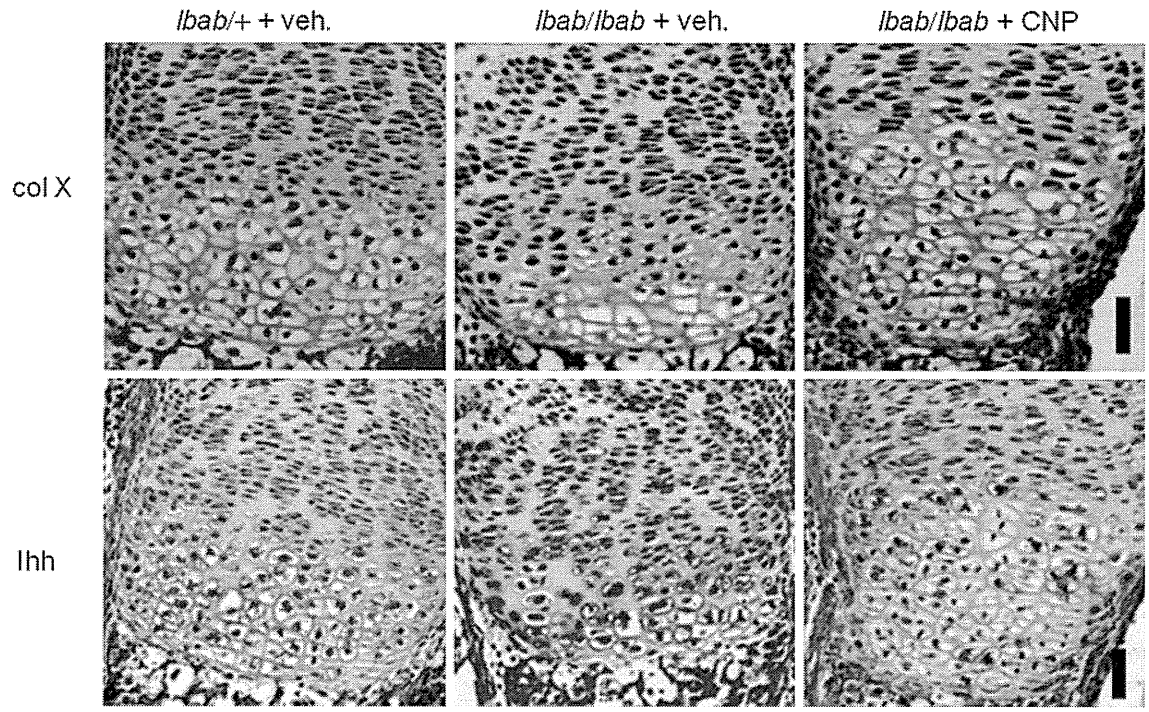

Fig. 8 Immunohistochemical staining of type X collagen (*upper panels*) and Ihh (*lower panels*) of the growth plates of metatarsal explants from fetal *lbab/+* and *lbab/lbab* mice treated with vehicle (*veh.*) or 10^{-7} M CNP for 4 days. *Scale bar* 50 μm

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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