

Full Length Article

C-type natriuretic peptide restores impaired skeletal growth in a murine model of glucocorticoid-induced growth retardation



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ARTICLE INFO

Article history:

Received 17 May 2016

Revised 17 August 2016

Accepted 31 August 2016

Available online 2 September 2016

Keywords:

C-type natriuretic peptide (CNP)

Glucocorticoid

Growth retardation

Skeletal growth

ABSTRACT

Glucocorticoids are widely used for treating autoimmune conditions or inflammatory disorders. Long-term use of glucocorticoids causes impaired skeletal growth, a serious side effect when they are used in children. We have previously demonstrated that C-type natriuretic peptide (CNP) is a potent stimulator of endochondral bone growth. In this study, we investigated the effect of CNP on impaired bone growth caused by glucocorticoids by using a transgenic mouse model with an increased circulating CNP level. Daily administration of a high dose of dexamethasone (DEX) to 4-week-old male wild-type mice for 4 weeks significantly shortened their naso-anal length, which was restored completely in DEX-treated CNP transgenic mice. Impaired growth of the long bones and vertebrae by DEX was restored to a large extent in the CNP transgenic background, with recovery in the narrowed growth plate by increased cell volume, whereas the decreased proliferation and increased apoptosis of the growth plate chondrocytes were unaffected. Trabecular bone volume was not changed by DEX treatment, but decreased significantly in a CNP transgenic background. In young male rats, the administration of high doses of DEX greatly decreased N-terminal proCNP concentrations, a marker of CNP production. In organ culture experiments using fetal wild-type murine tibias, longitudinal growth of tibial explants was inhibited by DEX but reversed by CNP. These findings now warrant further study of the therapeutic potency of CNP in glucocorticoid-induced bone growth impairment.

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1. Introduction

Glucocorticoid (GC)-based drugs are widely used to treat various diseases because of their immunosuppressive and anti-inflammatory effects. However, the long-term use of these drugs causes many side effects on various tissues, including bone. The most notable deleterious effects on bone associated with GCs are osteoporosis and subsequent bone fractures [1]. These side effects on bone have been proposed to be caused by manifold functions of GCs, such as suppression of the proliferation and differentiation of osteoblasts, acceleration of the apoptosis of osteoblasts and osteocytes [2], and attenuation of the function of insulin-like growth factor-1 (IGF-1), which promotes bone formation [3,

4]. Moreover, it is well-known that long-term administration of GCs to children induces growth retardation. GC-induced growth impairment correlates with the dose of GCs [5]. Although it has been reported that GCs used for physiological replacement (0.075–0.125 mg/kg/day prednisone or 0.3–0.375 mg/kg/day hydrocortisone) can induce growth retardation [6], a recent study suggests that growth impairment is actualized when the GC dose exceeds 0.2 mg/kg/day prednisone equivalents [5]. Alternate-day treatment of boys with prednisone also involves growth impairment, and the impairment persists even after the treatment is discontinued, resulting in reduction of their adult height [7]. There are many studies concerning growth retardation due to GCs. Long-term, high-dose regimen of GCs induces apoptosis, impairs differentiation, prevents proliferation of growth plate chondrocytes, and inhibits bone growth [8,9]. GCs have direct effects on chondrocytes in growth plate and in addition, GCs impair the anabolic effects of growth hormone (GH)/IGF-1 axis on growth plate chondrocytes [10,11]. As for treatment of GC-induced growth retardation, GH therapy has been attempted but the effect was reported to be limited [12]. Ablation of Bax, the pro-apoptotic protein, was indicated to rescue GC-induced growth retardation [13], but effective therapy for GC-induced growth retardation has not been established.

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C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family along with atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) [14,15]. There is growing evidence that CNP is associated with endochondral bone formation and linear growth. Mice depleted of CNP or its specific receptor, natriuretic peptide receptor 2 (NPR2), develop a severe short stature phenotype owing to their impaired endochondral bone growth, indicating that CNP/NPR2 signaling is a pivotal and physiological stimulator of endochondral bone growth [16,17]. In contrast, transgenic mice subject to targeted overexpression of CNP in cartilage or increased levels of circulating CNP exhibit prominent skeletal overgrowth phenotype [18,19].

In humans, biallelic loss of function mutations in the NPR2 gene cause one form of short-limbed skeletal dysplasia, acromesomelic dysplasia–type Maroteaux [20,21]. Furthermore, monoallelic loss-of-function mutations in the NPR2 gene are reported to be related to short stature [22,23]. In contrast, monoallelic gain-of-function mutations in the NPR2 gene cause a prominent skeletal overgrowth phenotype [24,25]. Making use of this stimulatory effect of CNP/NPR2 signaling on skeletal growth, we are now undertaking translational research on the activation of CNP/NPR2 to restore impaired skeletal growth. Previously, we studied the stimulatory effect of CNP/NPR2 activation on impaired bone growth in achondroplasia, the most common form of skeletal dysplasia, using the relevant mouse model; we showed that either targeted overexpression of CNP or increasing the levels of circulating CNP using a transgenic approach could almost completely restore the impaired skeletal growth of achondroplastic model mice [18,26,27]. Furthermore, we showed that exogenous administration of synthetic CNP to these mice also restored the impaired skeletal growth [27]. We expect that CNP/NPR2 activation can be used to treat impaired skeletal growth under various conditions. In this study, we investigated the effect of CNP on GC-induced impaired skeletal growth using a mouse model subjected to high-dose GC treatment and transgenic mice with increased circulating CNP levels.

2. Materials and methods

2.1. Animals

All experimental procedures involving animals were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (Permit number: MedKyo07598). Care of animals and all animal experiments were conducted in accordance with the institutional guidelines of Kyoto University Graduate School of Medicine.

CNP transgenic mice under the control of human serum amyloid P component (SAP) promoter (*SAP-Nppc-Tg* mice) were generated in the C57BL/6J background by the method previously reported [19] and we used *SAP-Nppc-Tg* line 17 described in the report [19] for the following experiments. These mice harbor the human SAP/mouse CNP fusion gene and produce excessive CNP in their livers, resulting in higher plasma CNP levels than wild-type mice [19]. F344/Jcl rats were purchased from CLEA Japan, Inc. (Tokyo, Japan).

2.2. Experimental design

We used *SAP-Nppc-Tg* mice as our experimental model to be treated with CNP. Four groups of mice were established at the start of this study. The first group was composed of *SAP-Nppc-Tg* mice treated with saline at a dose of 10 ml/kg/day (CNP/vehicle group). The second was composed of wild-type C57BL/6J mice treated with saline at the same dose (WT/vehicle group). The third was composed of wild-type C57BL/6J mice treated with dexamethasone (DEX) at a dose of 2 mg/kg/day (WT/DEX group). DEX was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) as dexamethasone sodium phosphate (No. 040-30811, Wako), dissolved in saline, and injected subcutaneously at 200 µg/ml concentration so that the volume of the solution was equivalent to that of saline. The last group was composed of *SAP-Nppc-Tg* mice

treated with DEX at the same dose (CNP/DEX group). DEX and vehicle were injected daily for 4 weeks, from 4 weeks of age to 8 weeks. The naso-anal length and body weight were measured weekly from 3 weeks of age to 8 weeks under isoflurane-induced anesthesia. During the measurement of naso-anal length, the cranium of the target mouse was fixed and the body was stretched to its fullest extent. In preliminary trials, we had performed an inter-observer variation assay and confirmed the reproducibility of the measurement results between observers (Supplementary Fig. 1). The difference between observers was at most 4% of naso-anal length and the data were thought to be correct enough to be evaluated. Following length measurement was performed by the same observer throughout the whole experiment. This observer was not aware of the group to which each mouse belongs.

2.3. Skeletal analysis

At 4 weeks and at 8 weeks of age (the end of the experiment), the anteroposterior and transverse diameters of the cranial bone and the lengths of the humerus, radius, ulna, femur, tibia, fibula, and lumbar vertebrae of each mouse were measured on soft X-ray film. Lengths of the humerus, radius, ulna, femur, tibia, and fibula were averages of the right and the left. We measured the span from the first lumbar vertebra to the fifth as the length of lumbar vertebrae.

2.4. Microstructural analysis of bone

At the end of the experiment, microstructural analysis of bone was performed with micro-computed tomography (micro-CT, SMX-100CT-SV3, Shimadzu Co., Kyoto, Japan). The scan area was set as the 1.0 mm region from 0.2 mm proximal of the distal growth plate of femur. Micro-CT scan was used to determine the bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). These structural indices were calculated using a three-dimensional trabecular bone analysis software (TRI/3D-BON, Ratoc System Engineering Co., Tokyo, Japan).

2.5. Histological analysis

Mice were sacrificed at the end of the experiment and their tibias collected. These tibias were fixed and decalcified with 10% EDTA for 2 weeks before they were embedded in paraffin and cut longitudinally to analyze the growth plate. After the bone sections were deparaffinized and rehydrated, Alcian-blue staining and immunohistochemical staining for type II and type X collagens of tibial growth plates were performed using rabbit anti-type II collagen antibody (Novotech Pty Ltd., Sydney, Australia) and mouse anti-type X collagen antibody (Quartett Immunodiagnostika and Biotechnologie Vertriebs GmbH, Berlin, Germany), respectively, as previously described [28]. The width of the growth plate was measured for each specimen.

The analysis of apoptosis in growth plate chondrocytes was performed by TdT-mediated dUTP nick-end labeling (TUNEL) assay using Apop Tag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore No. S7100). TUNEL-positive cells were counted within three randomly chosen fields in the hypertrophic chondrocyte zone of the growth plate and expressed as the percentage of positive cells per field.

2.6. 5-Bromo-2'-deoxy-uridine (BrdU) assay

Four-week-old mice divided into WT/vehicle, WT/DEX, and CNP/DEX groups were given saline or DEX for a week. After the administration, we injected BrdU intraperitoneally at a dose of 30 µg/g an hour before sacrifice. The tibias were resected, fixed, decalcified, and longitudinally cut. The BrdU assay was performed on their growth plates using 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I (Roche No. 11296736001). BrdU-positive cells were counted in three randomly chosen fields in the proliferative chondrocyte zone of the

growth plate and the results were expressed as the percentage of positive cells per field.

2.7. Measurement of NT-proCNP

Thirty-three-day-old male rats were divided into two groups and treated with either saline or DEX for 2 days at the same dose as the mice described above. After administration (5-week-old), their blood was collected and blood levels of N-terminal proCNP (NT-proCNP), as the representative for CNP production, were measured using proCNP, N-terminal, EIA Kit (BI-20872, Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria). This kit is designed to measure human NT-proCNP by using sandwich assay. It is announced that cross reactivities of capture antibody and primary antibody with rat NT-proCNP are 88% and 95%, respectively.

2.8. Organ culture

Three ICR mice on the 16th day of pregnancy (Shimizu Experimental Supply, Co., Japan) were sacrificed and tibias collected from their fetal mice were cultured for 4 days in BGJb medium (No. 12591-038, Gibco) with 6 mg/ml of albumin from bovine serum (No. 010-23382, Wako), 150 µg/ml of ascorbic acid (No. 012-04802, Wako), and 10 µl/ml of penicillin-streptomycin solution (No. 168-23191, Wako). These tibias were incubated at 37 °C with vehicle, 1 µM of DEX, 1 µM of CNP, or both DEX and CNP at the same doses. CNP was purchased from Peptide Institute (Minoh, Japan) as CNP-22 (4229-v, Peptide Institute). The medium was exchanged every day with the addition of vehicle, DEX, CNP, or both DEX and CNP. The length of each tibia was measured by a light microscope with a linear ocular scale at the start and at the end of the culture period. Histological analysis of each tibia was performed at the end of the experimental period. Preparation of the specimen and Alcian-blue staining were performed by the method described above.

2.9. Statistical analysis

Statistical analysis of the data was performed using either Student's *t*-test or two-way factorial analysis of variance (ANOVA), followed by Turkey-Kramer test as a *post hoc* test. Data were expressed as means $\pm$ SE. The differences were considered significant when *P* values were <0.05 .

3. Results

3.1. The effects of increased circulating CNP on impaired growth of a GC-treated mouse model

CNP transgenic mice under the control of human serum amyloid P component (SAP) promoter (*SAP-Nppc-Tg* mice) have approximately twice as much circulating CNP compared with wild-type mice [19]. Therefore, we used these transgenic mice as an experimental model for treatment with CNP. As we had observed that there was no significant difference in the body length or weight between wild-type and *SAP-Nppc-Tg* mice at the age of 4 weeks, we planned to perform experiments using 4-week-old wild-type and *SAP-Nppc-Tg* mice. Because the CNP-transgene was targeted to be overexpressed in the liver under the control of SAP promoter in *SAP-Nppc-Tg* mice, we also checked the weight of the livers of *SAP-Nppc-Tg* mice at the age of 4 weeks and found not changed compared with that of wild-type mice (0.91 ± 0.08 g, $n = 5$ and 0.91 ± 0.05 g, $n = 7$ in wild-type and *SAP-Nppc-Tg* mice, respectively, $P = 0.93$). We divided male mice into four groups. The first group was composed of *SAP-Nppc-Tg* mice treated with saline as a vehicle (CNP/vehicle group); the second was composed of wild-type mice treated with saline (WT/vehicle group); the third was composed of wild-type mice treated with dexamethasone (DEX) at a dose

of 2 mg/kg/day (WT/DEX group); and the last was composed of *SAP-Nppc-Tg* mice treated with DEX at the same dose (CNP/DEX group). Either DEX or vehicle was injected daily from 4 weeks of age until 8 weeks (for 4 weeks). Gross appearance and soft X-ray pictures of the four groups of mice at the end of the experimental period (8 weeks of age) revealed that WT/DEX mice exhibited short lengths owing to their impaired skeletal growth, which is completely restored in CNP/DEX mice (Fig. 1A and B). CNP/vehicle mice displayed the overgrowth phenotype that we previously reported (Ref. [19] and Fig. 1A and B). As confirmed in the growth curves depicted in Fig. 1C, there were no significant differences in the naso-anal length between the four groups of mice at the start of this study (4-week-old). The naso-anal length of WT/DEX mice became significantly smaller than that of WT/vehicle mice, whereas that of CNP/DEX mice became significantly larger than that of WT/DEX mice after 5 weeks of age ($n = 10, 8$, and 10 in the WT/DEX, WT/vehicle, and CNP/DEX groups, respectively, $P < 0.05$, WT/DEX vs. WT/vehicle or CNP/DEX). The naso-anal length of CNP/DEX mice was almost the same as that of WT/vehicle mice at all the time points during the experimental period. The length of CNP/vehicle was significantly larger than that of either the WT/vehicle or CNP/DEX ($n = 11$ in CNP/vehicle group, $P < 0.05$, CNP/vehicle vs. WT/vehicle or CNP/DEX). As for the growth velocity during the administration period, DEX decreased the averaged growth velocity of wild-type mice by 39% whereas the decrease in *SAP-Nppc-Tg* mice was 58% (Fig. 1D). Furthermore, although CNP increased the growth velocity by 88% in vehicle-treated group, the increase in DEX-treated group was only 29% (Fig. 1D). The growth velocity of CNP/DEX mice was comparable to that of WT/vehicle mice (Fig. 1D). As for body weight, WT/DEX mice became significantly lighter than WT/vehicle mice after the start of DEX treatment and continued to be significantly lighter than WT/vehicle mice during the remainder of the experimental period. CNP/DEX mice were comparable to WT/DEX mice and significantly lighter than WT/vehicle mice after the start of the administration ($n = 8, 10$, and 10 in the WT/vehicle, WT/DEX, and CNP/DEX groups, respectively, $P < 0.05$, CNP/DEX or WT/DEX vs. WT/vehicle) (Fig. 1E). The weight of CNP/vehicle mice was comparable to that of WT/vehicle mice and significantly higher than that of CNP/DEX or WT/DEX mice ($n = 11$ in the CNP/vehicle group, $P < 0.05$, CNP/DEX or WT/DEX vs. CNP/vehicle) (Fig. 1E).

We performed the same series of experiments using female mice and obtained the same qualitative results as for male mice on the final linear growth at the end of the 4-week experimental period (Supplementary Fig. 2), so we chose to use male mice for the following experiments.

3.2. The effect of CNP on impaired bone growth of a GC-treated mouse model

We went on to measure the length of each bone in the four groups of male mice on soft X-ray film. We confirmed that there were no significant differences in the lengths of most bones we measured (anteroposterior and transversal diameters of cranium, humerus, radius, ulna, femur, tibia, fibula, and lumbar vertebrae) between 4-week-old wild-type and *SAP-Nppc-Tg* mice (Fig. 2A). Nevertheless, the peripheral bones of *SAP-Nppc-Tg* mice tended to be longer than those of wild-type mice, and only the ulnae of *SAP-Nppc-Tg* mice were significantly longer than those of wild-type mice. At the end of the experimental period for DEX treatment, WT/DEX mice had significantly shorter appendicular bones than the WT/vehicle group (Fig. 2B). On the other hand, most appendicular bones of CNP/DEX mice became comparable to the WT/vehicle group, while some appendicular bones of CNP/DEX mice became noticeably longer than those of the WT/vehicle group (Fig. 2B). The lumbar vertebrae of WT/DEX mice were significantly shorter than those of mice in both the WT/vehicle and CNP/DEX groups. The lumbar vertebrae of CNP/DEX mice were comparable to those of WT/vehicle mice (Fig. 2B). As for the cranium, skull length in the WT/DEX group tended to be shorter than the WT/vehicle group while skull length in

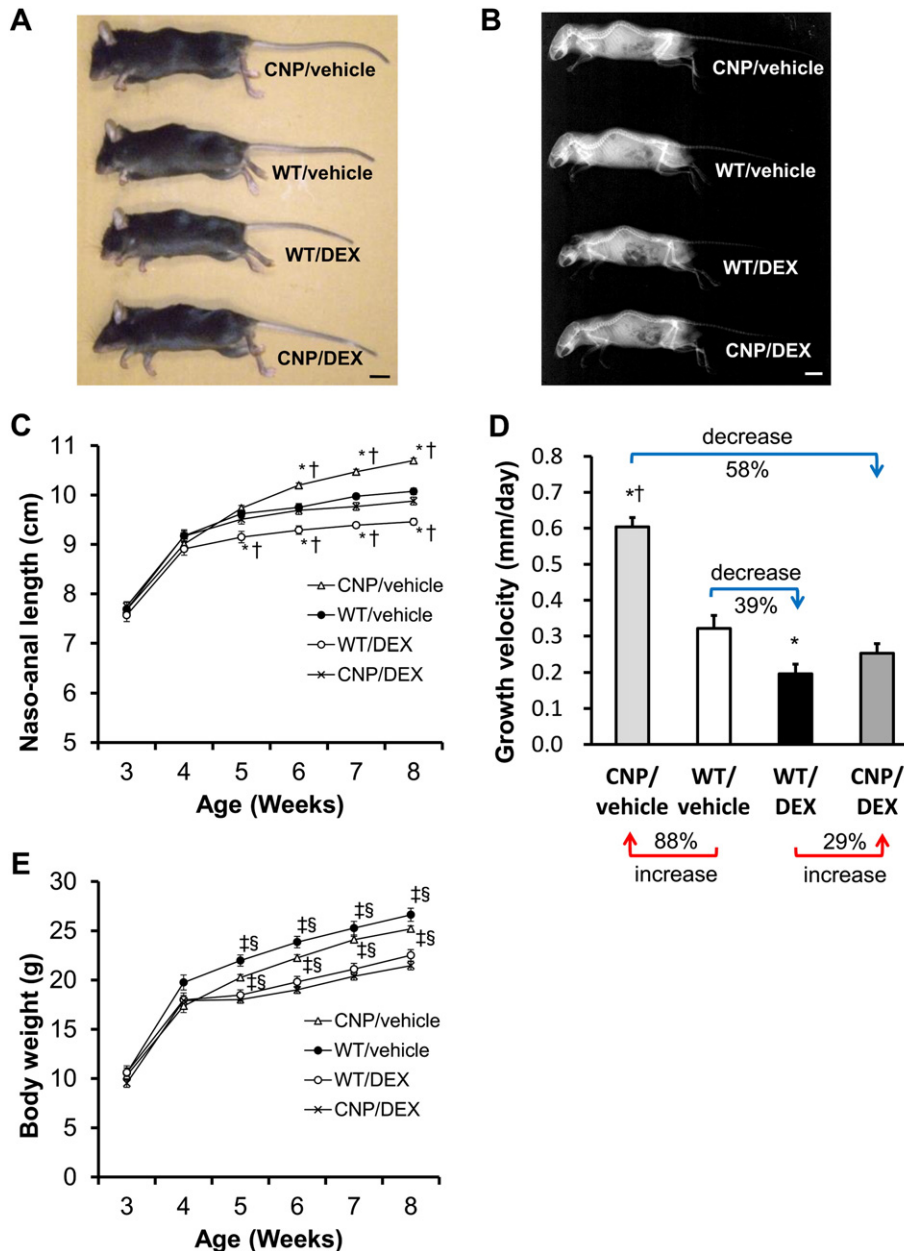


Fig. 1. Gross phenotypes of 8-week-old male wild-type (WT) and *SAP-Nppc-Tg* (CNP) mice treated with saline (vehicle) or DEX. (A) Gross appearances and (B) soft X-ray pictures of CNP/vehicle, WT/vehicle, WT/DEX, and CNP/DEX mice. Scale bar in each panel indicates 10 mm. (C) Growth curves of naso-anal length of CNP/vehicle (Δ), WT/vehicle (●), WT/DEX (○), and CNP/DEX (×). (D) Averaged growth velocity of naso-anal length from the age of 4 weeks to 8 weeks. (E) Growth curves of body weight of CNP/vehicle (Δ), WT/vehicle (●), WT/DEX (○), and CNP/DEX (×). CNP/vehicle, $n = 11$; WT/vehicle, $n = 8$; WT/DEX, $n = 10$; and CNP/DEX, $n = 10$. (C, D) *: $P < 0.05$, vs. WT/vehicle and †: $P < 0.05$, vs. CNP/DEX. (E) ‡: $P < 0.05$, vs. WT/DEX and §: $P < 0.05$, vs. CNP/DEX.

the CNP/DEX group tended to be longer than the WT/DEX group, but there were no significant differences. The skull width of WT/DEX mice became significantly smaller than the WT/vehicle group, and that of the CNP/DEX group was almost equal to the WT/DEX group (Fig. 2B). In CNP/vehicle mice, appendicular bones, lumbar vertebrae, and skull length were all significantly longer than those in WT/vehicle mice, as we have previously reported (Ref. [19] and Fig. 2B). There was no significant difference in skull width between CNP/vehicle and WT/vehicle mice (Ref. [19] and Fig. 2B).

3.3. Histological examination of the effect of CNP on the growth plate of GC-treated mouse model

We subsequently performed histological analyses of the tibial growth plates of the four groups of mice at the end of the experimental

period. As depicted in the histological pictures in Fig. 3A and as shown by the graph of the widths in Fig. 3B, the growth plates of WT/DEX mice were significantly thinner than those of WT/vehicle mice. Although the growth plates of CNP/DEX mice became significantly thinner than those of CNP/vehicle mice, they became significantly thicker than those of WT/DEX mice and furthermore, were thicker than those of WT/vehicle mice (Fig. 3B). Similar results were observed in the hypertrophic zones of their growth plates stained with type X collagen (Fig. 3C), although there were no significant differences between WT/vehicle and CNP/DEX mice (Fig. 3D). The hypertrophic zones of CNP/vehicle mice were not diminished in the CNP/DEX group, nor were they significantly larger than in the WT/vehicle group (Fig. 3D). There were no significant differences in the thickness of the proliferative zones stained type II collagen among the WT/vehicle, WT/DEX, and CNP/DEX groups, although that of the CNP/vehicle group was significantly thicker than

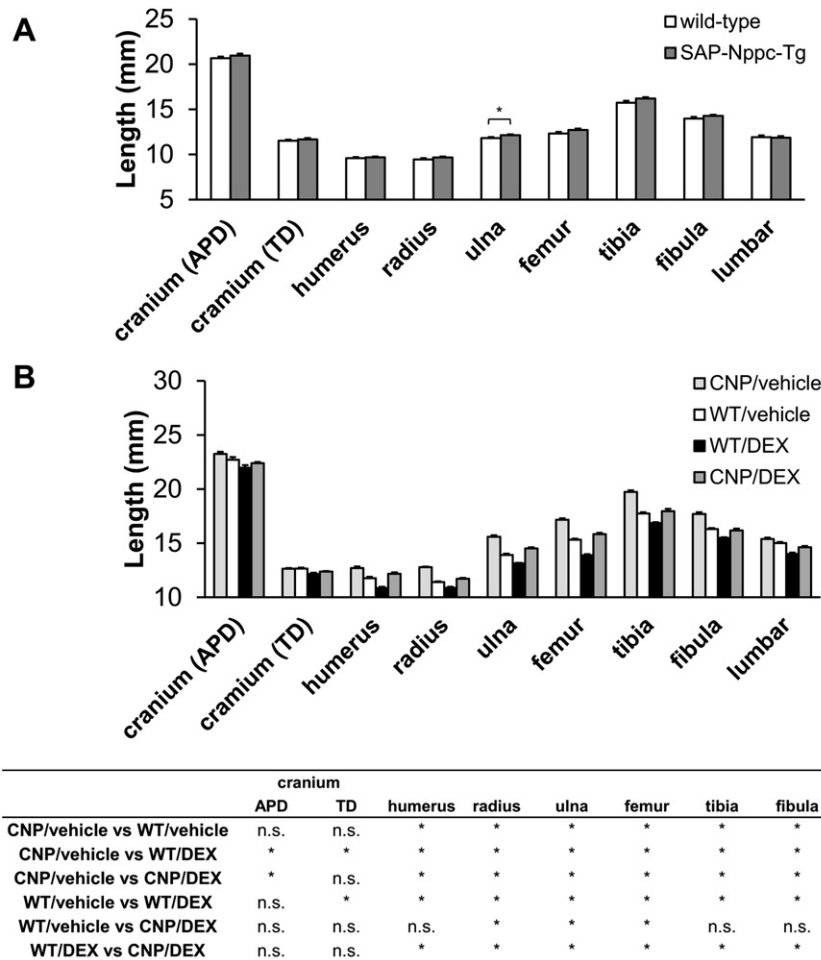


Fig. 2. Bone lengths of wild-type and *SAP-Nppc-Tg* mice at the start of the experiment (4-week-old) (A) and after the treatment of DEX or vehicle for 4 weeks (8-week-old) (B). APD and TD: anteroposterior and transverse diameters, respectively. $n = 6$, each, in each bone length in wild-type and *SAP-Nppc-Tg* mice (A), and $n = 11, 8, 10$, and 10 in the CNP/vehicle, WT/vehicle, WT/DEX, and CNP/DEX groups, respectively (B). Statistical differences between groups are summarized in the lower table in B. *: $P < 0.05$ and n.s.: not significant.

the other three groups (Fig. 3E and F). Histological images of Alcian-blue staining at higher magnification suggested that the size of cells in the growth plate is reduced by DEX and increased by CNP, especially in the hypertrophic zone (Fig. 3A, lower panels).

To further elucidate the mechanism underlying thinner growth plates in WT/DEX mice and the observed recovery in CNP/DEX mice, we examined the proliferation of growth plate chondrocytes using a BrdU assay. We could observe the significant reduction of cell proliferation in the growth plates of WT/DEX mice compared with WT/vehicle mice, but the reduction was not restored in the growth plates of CNP/DEX mice (Fig. 4A).

Next, we measured the apoptosis of hypertrophic chondrocytes using a TUNEL assay. As shown in Fig. 4B, TUNEL-positive cells in the hypertrophic zones were significantly increased in WT/DEX mice compared with WT/vehicle mice, indicating that apoptosis was increased in hypertrophic zones of WT/DEX mice. CNP/DEX mice also had increased numbers of TUNEL-positive cells in their hypertrophic zones, and there was no significant difference between CNP/DEX mice and WT/DEX mice (Fig. 4B).

3.4. The effect of CNP on bone microstructure of GC-treated mouse model

We went on to perform microstructural analysis of the distal metaphyses of femurs of these groups of mice at the end of the experiment using micro-CT. As we have performed and reported on the microstructural analysis of *SAP-Nppc-Tg* bone in detail in our previous report [28], herein we investigated and compared the microstructures of other

three groups of mice, namely, WT/vehicle, WT/DEX, and CNP/DEX. WT/DEX mice tended to have larger BV/TV and a significantly larger Tb.N than the WT/vehicle group (Fig. 5A and B). In the WT/DEX group, Tb.Th was almost equal to that in the WT/vehicle group and Tb.Sp was significantly smaller than in the WT/vehicle group (Fig. 5C and D). In the CNP/DEX group, BV/TV and Tb.N were significantly smaller than in the WT/DEX group (Fig. 5A and B), while Tb.Th was significantly smaller than that in the WT/vehicle group (Fig. 5C). In contrast, Tb.Sp was significantly larger than that in the WT/DEX group (Fig. 5D).

3.5. Change in blood NT-proCNP levels in GC-treated rat model

In order to verify the influence of GC on circulating levels of CNP in rodents, we determined the blood NT-proCNP levels of rats treated with DEX. NT-proCNP levels in rats treated with DEX for 2 days at a dose of 2 mg/kg/day were 3.8 ± 0.4 pmol/L whereas those in the vehicle-treated control group were 18.2 ± 1.9 pmol/L; NT-proCNP levels in DEX-treated rats were significantly lower than those in vehicle-treated rats ($P < 0.01$, $n = 4-5$, each).

3.6. The effect of CNP in an organ culture experiment

To elucidate the direct effect of DEX and CNP on endochondral bone growth, we performed an organ culture experiment using tibial explants from fetal mice. Growth of tibias incubated with DEX was significantly impaired but recovered by addition of CNP, which was consistent with the results of our *in vivo* experiments described above

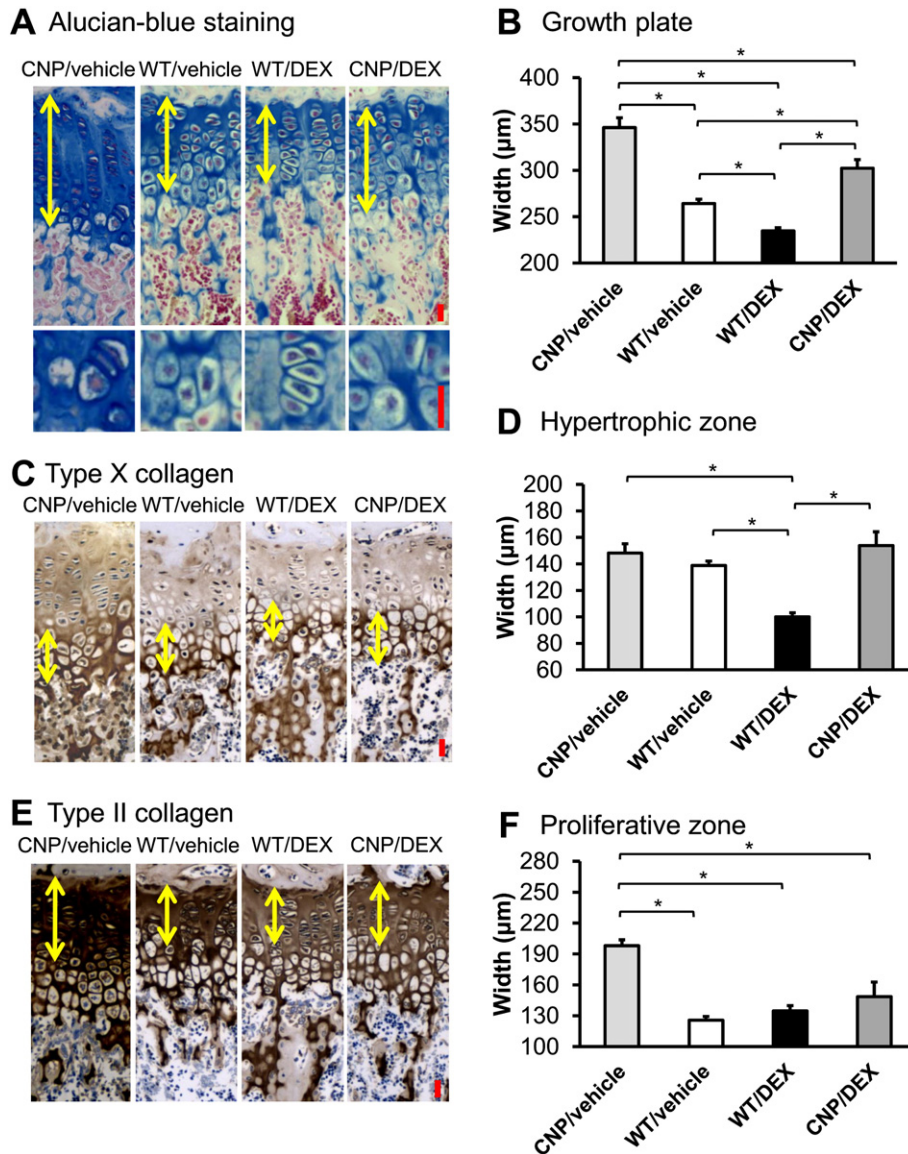


Fig. 3. Histological pictures of growth plates and the width of each chondrocyte layer measured on the pictures. (A, C, E) The histological pictures of growth plates of Alcian-blue staining (A) and immunohistochemical staining for type X (C) and type II (E) collagens. Lower panels in (A) exhibit a part of hypertrophic chondrocyte layers in the respective upper panel pictures with higher magnification. Yellow arrows indicate the widths of the growth plates (A), hypertrophic chondrocyte layers (C), and proliferative chondrocyte layers (E). All red scale bars in A, C, E: 50 μm . (B, D, F) Graphs of widths of growth plates (B), hypertrophic zones (D), and proliferative zones (F) measured on histological pictures of Alcian-blue staining and immunohistochemical staining for type X and type II collagens, respectively. $n = 6, 6, 8$, and 8 in the CNP/vehicle, WT/vehicle, WT/DEX, and CNP/DEX groups, respectively. *: $P < 0.05$.

(Fig. 6A, B). The results of histological analysis of cultured tibias were also consistent with those of *in vivo* experiments (Fig. 6C–E). Hypertrophic zones of the growth plates were thickened in CNP-treated, and both DEX and CNP-treated explants compared with vehicle-treated and DEX-treated explants, respectively. Hypertrophic zones of explants treated with both DEX and CNP were rather thicker than those treated with vehicle (Fig. 6C, D). There was no significant difference in the thickness of non-hypertrophic zones among these four groups (Fig. 6E).

4. Discussion

In the present study, we examined the effect of CNP on the impaired skeletal growth in a mouse model for GC-induced growth retardation. We used *SAP-Nppc-Tg* mice, which have increased circulating CNP levels [19]. We could successfully restore the impaired skeletal growth of a mouse model of GC-treatment with a higher level of circulating CNP. DEX impaired the growth of cranial and appendicular bones but CNP overexpression reversed the impairment of appendicular bone growth.

Skull length tended to be lengthened by CNP but skull width was not rescued. Whereas appendicular bones are formed through endochondral ossification, skeletogenesis of cranial bone consists of both endochondral and intramembranous ossifications and cranial bone is widened mainly by intramembranous ossification. CNP is a stimulator of bone growth based on endochondral ossification, and we have previously reported that CNP did not affect skull width using transgenic and knockout mice [29]. We therefore concluded that although DEX impairs both endochondral and intramembranous bone growth, CNP could restore only the endochondral bone growth impaired by DEX. As for the growth of bones formed through endochondral ossification, recovery from DEX-induced growth impairment induced by CNP was stronger in appendicular bones than in vertebrae. The reason for this is not known at present, but the shortness of the longitudinal length and the thinness of the growth plate in each vertebral body may affect the rescue effect of CNP on impaired endochondral bone growth by DEX.

The growth velocity of CNP/DEX mice was comparable to that of WT/vehicle mice. Elevated circulating CNP in *SAP-Nppc-Tg* mice would be

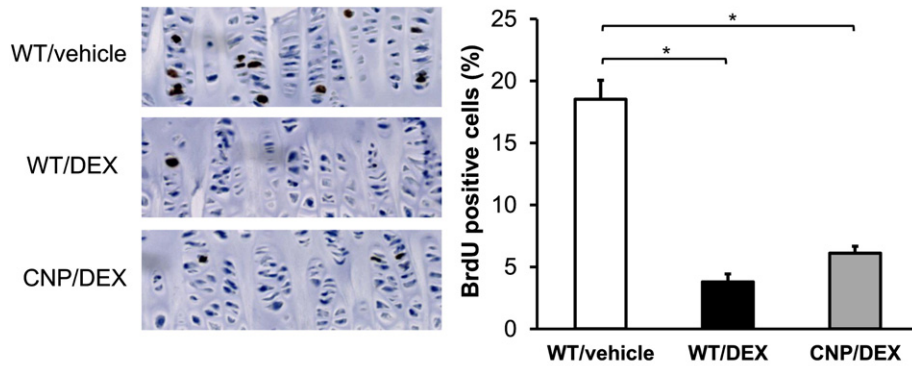
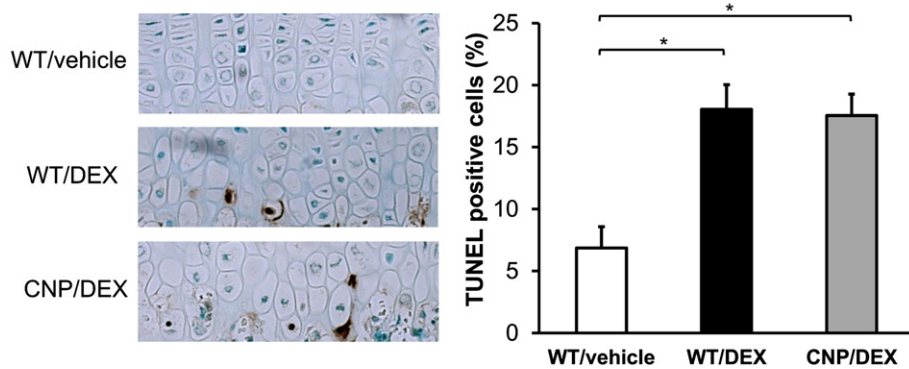
A BrdU assay**B** TUNEL assay

Fig. 4. Analysis of proliferation and apoptosis of growth plate chondrocytes. BrdU assay was performed on proliferative chondrocyte layers (A) and TUNEL assay on hypertrophic chondrocyte layers (B) at the end of the 4-week experimental period. (A) The representative picture of BrdU positive chondrocytes in each group (left) and the graph of the ratio of BrdU positive cells (right). $n = 4, 5$, and 3 in the WT/vehicle, WT/DEX, and CNP/DEX groups, respectively. *: $P < 0.05$. (B) A representative picture of TUNEL-positive chondrocytes in each group (left) and the graph of the ratio of TUNEL-positive cells (right). $n = 6, 8$, and 8 in the WT/vehicle, WT/DEX, and CNP/DEX groups, respectively. *: $P < 0.05$.

responsible for the restoration of GC-induced growth impairment because *SAP-Nppc-Tg* mice had a similar overgrowth phenotype to mice treated with intravenous administration of CNP-22 [27]. However,

other factors than circulating CNP should be considered. At first, any possible advantageous factors relevant to CNP/NPR2 signaling might be increased in *SAP-Nppc-Tg* mice. For example, we have reported that

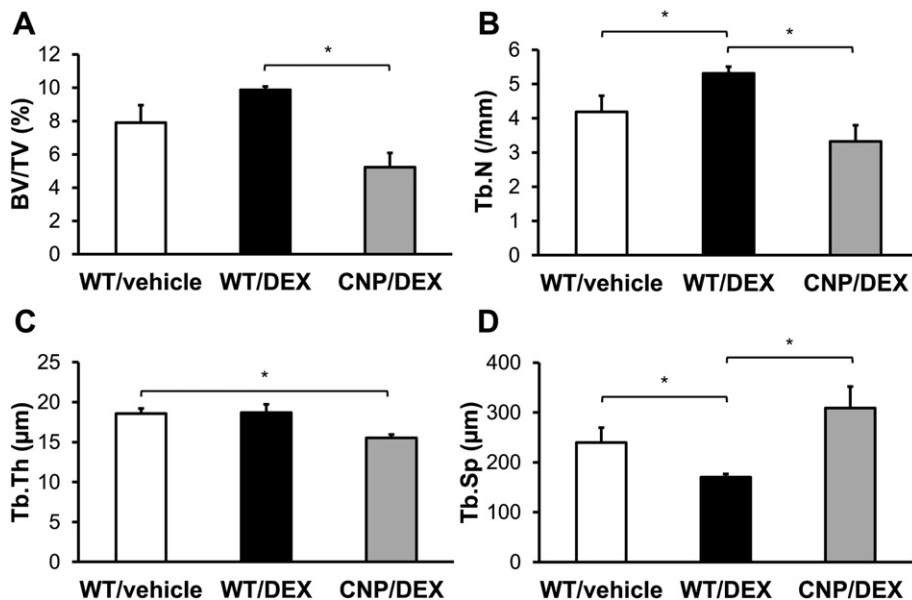


Fig. 5. Analyses of bone microstructure by micro-CT. Graphs of bone volume fraction (BV/TV) (%) (A), trabecular number (Tb.N) (/mm) (B), trabecular thickness (Tb.Th) (μm) (C), and trabecular separation (Tb.Sp) (μm) (D) at the end of experimental period are shown. $n = 7, 4$, and 5 in the WT/vehicle, WT/DEX, and CNP/DEX groups, respectively. *: $P < 0.05$.

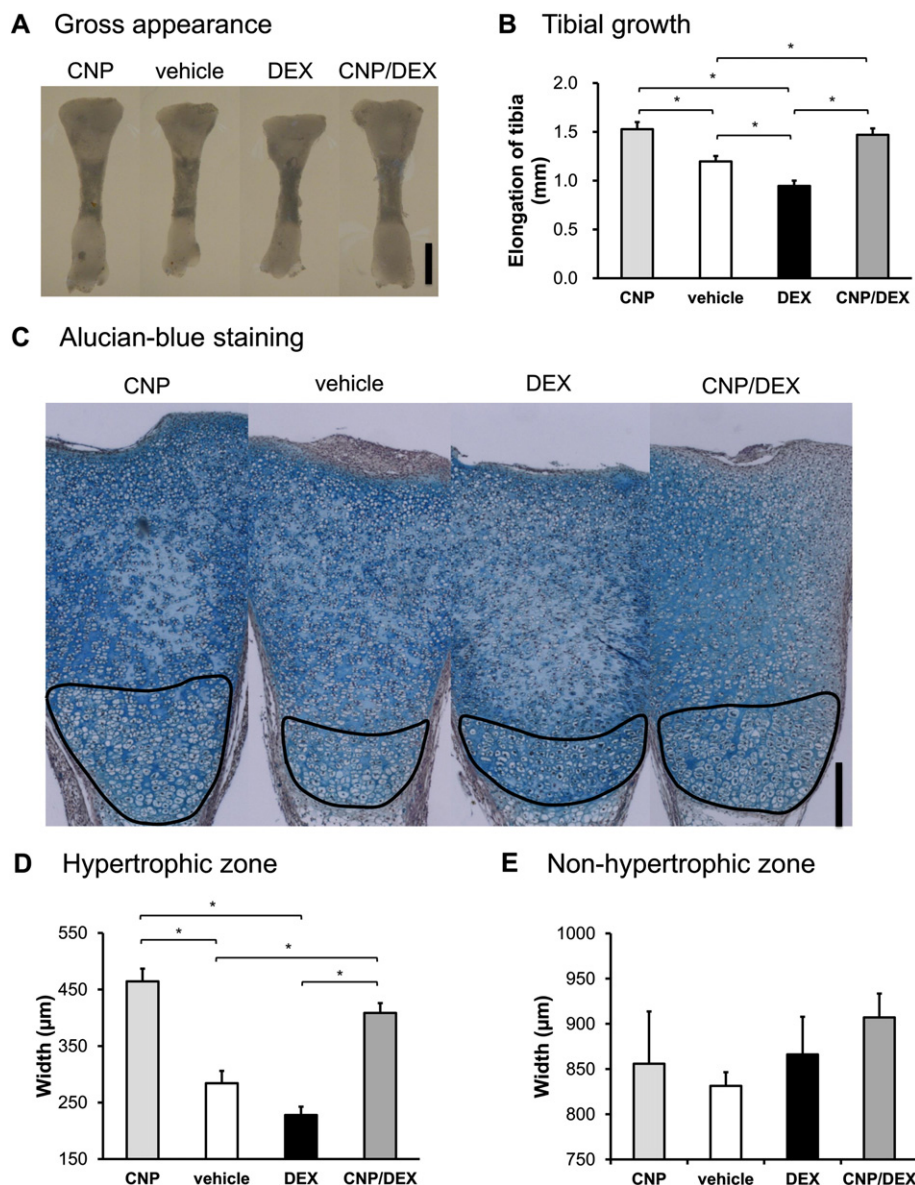


Fig. 6. The effects of DEX, CNP, or both on the growth of explanted fetal murine tibias in organ culture. (A, B) Gross appearance of tibial explants incubated with CNP, vehicle, DEX, or both CNP and DEX (CNP/DEX) for 4 days (A), and the graph of elongation of tibial explants shown by the difference from initial length in each treatment (B). (C–E) Histological evaluation of cultured tibias at the end of experimental period. (C) Histological pictures of proximal growth plates of Alucian-blue staining. Hypertrophic zones are surrounded by black curves. Graphs of widths of hypertrophic zones (D) and non-hypertrophic zones (E) measured on histological pictures of Alucian-blue staining are shown. Scales: 1.0 mm in (A) and 200 μm in (C). $n = 15, 15, 14,$ and 15 in the vehicle, DEX, CNP, and CNP/DEX-treated groups, respectively (B), and $n = 5$, each, in the vehicle, DEX, CNP, and CNP/DEX-treated groups (D–E). *: $P < 0.05$.

the mRNA expression of natriuretic peptide receptor 3, the clearance receptor of CNP, is down-regulated in costal cartilage of *SAP-Nppc-Tg* mice compared with in heart [19]. Thus, the effect of CNP on cartilage could be more enhanced and indeed, skeletal phenotype of *SAP-Nppc-Tg* mice is prominent compared with cardiovascular phenotype [19]. Next, overexpression of CNP in the liver might affect the clearance of DEX and then reduce the skeletal impairment by DEX. Nevertheless, there was no significant difference in the liver weight between wild-type and *SAP-Nppc-Tg* mice.

It is well-known that the body weight is reduced by DEX in mice. As GC is reported to have no influence on food intake in mice [30], DEX-induced body weight loss seems to be mainly caused by muscle atrophy because the body weight loss is completely restored by intervention against muscle atrophy [31]. As for the body weight of *SAP-Nppc-Tg* mice, we previously reported that body weights are not different between *SAP-Nppc-Tg* and wild-type mice [19]. The effect of CNP on metabolic status is controversial; one report suggests that CNP increases

food intake [32], whereas another indicates that CNP suppresses food intake [33]; the effect of CNP on appetite likely differs depending on the way of administration, etc. Recently, we reported that brain-specific NPR2 deleted mice neither have higher lipid deposition nor higher weight gain than wild-type mice, when fed a standard diet [34]. These mice did not exhibit altered energy homeostasis including body temperature, food intake, and oxygen consumption compared to wild-type mice. We think that CNP does not have clear effect on energy homeostasis, such as food intake or variation of adipose tissue, at least in this *SAP-Nppc-Tg* model at present. Therefore, *SAP-Nppc-Tg* mice in this experiment have no significant weight change compared with wild-type mice. The overgrowth in length in *SAP-Nppc-Tg* mice with no body weight change owes only to skeletal overgrowth, resulting in the development of “slender” mice.

As for the mechanism underlying the protective effect of CNP by DEX-induced impaired skeletal growth, we showed that CNP completely restored the hypertrophic zones of growth plates reduced by DEX by

histological analysis but did not affect either the increased apoptosis or the decreased proliferation of growth plate chondrocytes induced by DEX. As for the width of the hypertrophic zone, the width for the CNP/DEX group was comparable to that for the CNP/vehicle group, although that for the WT/DEX group was dramatically lower than the WT/vehicle group. This indicates that CNP protects against inhibition of hypertrophic differentiation by DEX. On the other hand, the width of the proliferative zone in the CNP/DEX group was much smaller than in the CNP/vehicle group, which concurs with the finding that CNP could not rescue the DEX-induced impairment of proliferation of growth plate chondrocytes. Collectively, CNP might mitigate the delayed hypertrophic differentiation of chondrocytes caused by DEX and restore DEX-induced impaired endochondral bone growth. Furthermore, we showed that the size of growth plate chondrocytes is lessened by DEX and restored by CNP, especially in the hypertrophic zone. In addition, we have demonstrated that CNP promotes extracellular matrix synthesis of growth plate chondrocytes in our previous report [18]. Recently, extracellular matrix deposition and cell volume enlargement were reported to be the main contributors of longitudinal growth of long bones through the observation of dynamic images of the growth plate cartilage [35]. CNP seems to lengthen long bones by stimulating these key factors.

We have previously demonstrated that genetic overexpression or systemic administration of CNP rescues the impaired skeletal growth observed in a mouse model for achondroplasia, the most common form of skeletal dysplasia caused by the constitutive activation of fibroblast growth factor receptor 3 (FGFR3), a potent negative regulator of endochondral bone growth [18,26,27]. We postulated that the mechanism of rescue involves inhibition of extracellular signal-regulated kinase (ERK) phosphorylation in the mitogen-activated protein kinase (MAPK) pathway of FGFR3 signaling by CNP/NPR2 signaling. Nevertheless, we think that the stimulatory effect of CNP/NPR2 signaling on endochondral bone growth is not restricted to the inhibition of the ERK MAPK pathway for FGFR3 signaling. As examples of other CNP/NPR-B signaling pathways stimulating endochondral ossification, the p38 MAPK-dependent pathway [36] and the AKT-GSK3 β -dependent pathway [37] have been reported to be involved. Our present study revealed that although CNP have a stimulatory effect on endochondral bone growth even under a high dose of GC, CNP did not reduce the inhibitory effect of DEX (DEX decreased the growth velocity of wild-type mice by 39% whereas the decrease in *SAP-Nppc-Tg* mice was 58%) and DEX reduced the stimulatory effect of CNP (the growth velocity of CNP/DEX mice was 29% larger than WT/DEX mice, whereas that of CNP/vehicle mice was 88% larger than WT/vehicle mice). The specific actions of DEX on CNP pathway activity clearly require further study.

There are some reports on the interaction between glucocorticoid and CNP. Subcutaneous injection of DEX is reported to decrease plasma CNP and NT-proCNP levels in ewes [38,39], whereas *in vitro* DEX has been proposed to stimulate the expression of CNP in chondrocytes [40]. In the present study, we tried to determine the effect of GC on CNP production in mice *in vivo* by measuring plasma NT-proCNP level, because plasma CNP level itself is so low that it is quite difficult to measure the value precisely. Although measurements of CNP (CNP-22 ultra-sensitive RIA kit; Phoenix Pharmaceuticals, Belmont, CA) have been used by us previously, this assay requires too much plasma to be collected from young mice we used in the present study [19]. Further, we could not measure plasma NT-proCNP levels in mice because there is no available antibody against murine NT-proCNP. We therefore investigated plasma NT-proCNP concentrations of rats as the representative for rodents, and observed a drastic reduction in NT-proCNP levels following DEX administration. While it is unclear if the reduction in CNP production is related to the impaired skeletal growth caused by DEX, plasma NT-proCNP levels are reported to be correlated with linear growth velocity in rats, lambs, and humans [41–43]; therefore, the reduced CNP levels caused by DEX might be at least a part of the cause of DEX-induced growth retardation. The mechanism by which GC reduces CNP

is not clear; as DEX is suggested to stimulate the expression of CNP in chondrocytes *in vitro* [40], DEX may affect the production of CNP indirectly *in vivo*. In fact, GC affects various systems that regulate skeletal growth, including growth hormone and IGF-I axis [4,44,45]. Further studies are needed to elucidate the regulation of CNP by GC, especially *in vivo*.

In this study, we used *SAP-Nppc-Tg* mice, which have approximately two times as much circulating CNP compared with wild-type mice, as a mouse model for systemic CNP administration [46]. Indeed, the SAP promoter has been reported to be activated just after birth [47]; thus, it might not be an ideal model for administration, in that circulating CNP levels would increase during the same time as the GC-administration period. Nevertheless, there was no significant difference in the naso-anal length between wild-type and *SAP-Nppc-Tg* mice at the age of 4 weeks, the start point of our experiments. Furthermore, we verified that the length of each bone that we measured was unchanged between wild-type and *SAP-Nppc-Tg* mice at the age of 4 weeks, except for the ulna. However, there remains the possibility that various differences in endochondral bone growth between wild-type and *SAP-Nppc-Tg* mice already existed at the age of 4 weeks. In order to support our present *in vivo* result, we further performed organ culture experiments to exclude the effect of increased circulating levels of CNP in *SAP-Nppc-Tg* mice before DEX administration and to elucidate the direct effect of CNP on DEX-induced impairment of endochondral bone growth. We obtained the same qualitative result as in our *in vivo* experiments. In addition, *SAP-Nppc-Tg* mice have the human SAP/mouse CNP fusion gene and whole length of CNP is produced in their livers. Thus, this mouse is indeed a model treated with proCNP and increased circulating mixture of proCNP, CNP-53, and CNP-22 might exist, unlike mice treated with single molecular form of bioactive CNP, such as CNP-22 or CNP-53. Little is known about processing of CNP molecule *in vivo*, thus it is quite difficult to predict whether *SAP-Nppc-Tg* mice and CNP-treated mice have different phenotype or not. Both groups of mice would have activated CNP/NPR2 signaling on one level or another and *SAP-Nppc-Tg* mice could imitate CNP-22-treated or CNP-53-treated mice in terms of longitudinal growth. However, further investigation into the difference of bioactive forms of CNP between mouse models including *SAP-Nppc-Tg* is desirable for precise evaluation. To use *SAP-Nppc-Tg* mice as a model for systemic CNP administration has technical merit, but we might have to perform CNP administration to a murine model for GC-induced growth retardation in order to obtain more accurate data. Our *SAP-Nppc-Tg* model is an approximation but we think we could assume much about the therapeutic potency of CNP for GC-induced impaired bone growth and analyze its mechanism.

It is well-known that systemic administration of GC reduces bone mineral density. However, our microstructural analysis with micro-CT revealed that the trabeculae of distal metaphyses of femurs were not diminished by DEX. Similar results with regard to the effect of DEX on trabeculae were reported in a study in which DEX was administered to young mice [48]. Because these microstructural analyses were only performed at the proximal region from the distal growth plates of femurs, the decreased longitudinal growth rate by DEX might affect the results and the bone volume of WT/DEX mice was maintained to the extent of that of WT/vehicle mice. On the other hand, CNP decreased the trabecular volume of DEX-treated mice. We recently reported that the trabecular volume was significantly decreased in *SAP-Nppc-Tg* mice compared with wild-type mice at the age of 8 weeks [28]. As the trabecular volume in WT/DEX mice was comparable with that in the WT/vehicle group, it is consistent that the trabecular volume in CNP/DEX mice was lower than that in WT/DEX mice. In clinical settings, we would treat the decrease in bone mass caused by CNP in any way. However, we believe that further study is required concerning the effect of DEX or CNP on bone strength when administered to young mice.

In conclusion, we have determined that CNP is efficient for restoring the impaired skeletal growth caused by high doses of GC in a mouse model. As the stimulatory effect of CNP on endochondral bone growth

is both potent and universal, we expect an increasing number of therapeutic targets in various situations involving impairment of endochondral bone growth.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bone.2016.08.026>.

Funding

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan; the Ministry of Education, Culture, Sports, Science and Technology of Japan (#21591176 and 26461381); and the Uehara Memorial Foundation, Tokyo, Japan.

Declaration of interest

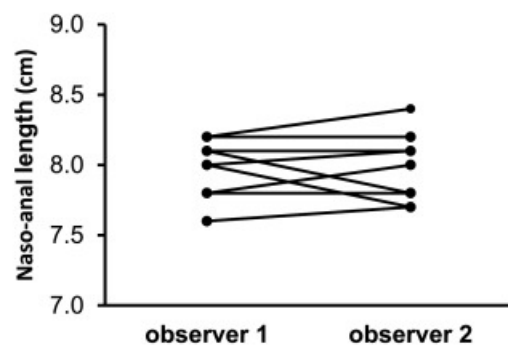
Conflicts of interest: None.

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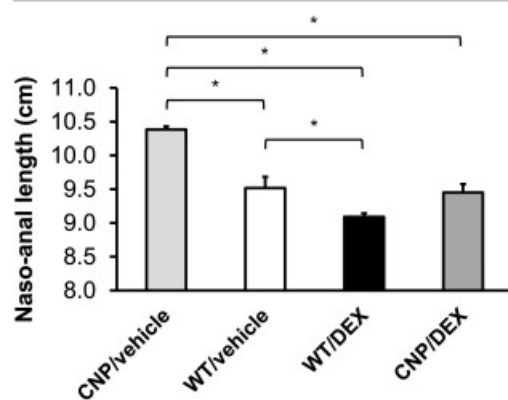
The following are the supplementary data related to this article.



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Supplementary Fig. 1. Naso-anal lengths of 3-week-old mice measured by 2 observers. $n = 9$, $P > 0.99$.



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Supplementary Fig. 2. Naso-anal lengths of female CNP/vehicle, WT/vehicle, WT/DEX, and CNP/DEX mice at the end of 4-week experimental period (at the age of 8 weeks). CNP/vehicle, $n = 7$; WT/vehicle, $n = 11$; WT/DEX, $n = 11$; and CNP/DEX, $n = 8$. *: $P < 0.05$.