These results indicate a beneficial effect of leptin therapy for type 2 diabetes mellitus with increased adiposity, which corresponds to a BMI in the range of 25–30 kg/m^2 [126].

Our previous and ongoing studies utilizing transgenic skinny mice and other animal models have demonstrated the pleiotropic actions of leptin in the regulation of energy homeostasis and food intake [98–101, 105, 108, 109] and its clinical usefulness as a therapy for multiple conditions, particularly diabetes mellitus [108, 118, 124, 125]. Tg skinny mouse may be a useful model to study the long-term effects of leptin therapy in vivo and to evaluate the clinical implications of leptin therapy.

Conclusions

Currently, the primary targets of our ongoing translational research of CNP and leptin are achondroplasia and lipoatrophic diabetes, respectively. Demonstration of the efficacy of CNP therapy for achondroplasia and leptin replacement therapy for lipoatrophic diabetes has relied heavily on basic and preclinical studies using excellent animal models. Although lipoatrophic diabetes is a rare disease in humans, the safety and efficacy of leptin replacement therapy for patients with lipoatrophic diabetes have been well established. Achondroplasia, while also a rare disease in humans, may be effectively managed with CNP therapy.

It has been possible to establish the safety and efficacy of these hormones in rare human diseases through studies that began with excellent animal models. These studies provided us with novel treatments for common human diseases, which were explored as adjacent to or in extension of these rare human diseases, as seen in the study of hypertension. Research on the SHR animal model and study of a relatively rare cause of hypertension, renovascular hypertension, led to more detailed studies on the blockade of renin–angiotensin system, bringing research forward to the current widespread field of cardiovascular disorders in translational research. These lessons teach us the importance of the breakthroughs using animal models and rare human diseases.

Conflict of interest statement The authors declare that they have no conflict of interests.

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Circulating C-Type Natriuretic Peptide (CNP) Rescues Chondrodysplastic CNP Knockout Mice from Their Impaired Skeletal Growth and Early Death

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C-type natriuretic peptide (CNP) is a potent stimulator of endochondral bone growth through a subtype of membranous guanylyl cyclase receptor, GC-B. Although its two cognate natriuretic peptides, ANP and BNP, are cardiac hormones produced from heart, CNP is thought to act as an autocrine/paracrine regulator. To elucidate whether systemic administration of CNP would be a novel medical treatment for chondrodysplasias, for which no drug therapy has yet been developed, we investigated the effect of circulating CNP by using the CNP transgenic mice with an increased circulating CNP under the control of human serum amyloid P component promoter (*SAP-Nppc-Tg* mice). *SAP-Nppc-Tg* mice developed prominent overgrowth of bones formed through endochondral ossification. In organ culture experiments, the growth of tibial explants of *SAP-Nppc-Tg* mice was not changed from that of their wild-type littermates, exhibiting that the stimulatory effect on endochondral bone growth observed in *SAP-Nppc-Tg* mice is humoral. Then we crossed chondrodysplastic CNP-depleted mice with *SAP-Nppc-Tg* mice. Impaired endochondral bone growth in CNP knockout mice were considerably and significantly recovered by increased circulating CNP, followed by the improvement in not only their longitudinal growth but also their body weight. In addition, the mortality of CNP knockout mice was greatly decreased by circulating CNP. Systemic administration of CNP might have therapeutic potential against not only impaired skeletal growth but also other aspects of impaired growth including impaired body weight gain in patients suffering from chondrodysplasias and might resultantly protect them from their early death. (***Endocrinology* 151: 4381–4388, 2010**)

Recent studies have elucidated that C-type natriuretic peptide (CNP) is a crucial regulator of endochondral bone growth (1, 2). The biological actions of CNP are thought to be mediated by the production of intracellular second-messenger cGMP through a subtype of membranous guanylyl cyclase receptor, guanylyl cyclase (GC)-B (3). We have exhibited that both CNP and GC-B are expressed in the proliferative and prehypertrophic chondrocyte layers of the growth plate (1) and that CNP or GC-B knockout mice develop severely short stature phenotype owing to their impaired endochondral bone growth (1, 4). On the contrary, mice with targeted overexpression of CNP in the growth plate by using type II collagen promoter exhibit prominent skeletal overgrowth (5, 6).

After these discoveries, we planned to translate this strong stimulatory effect of the CNP/GC-B system on bone growth into clinical treatment for patients suffering from diseases with impaired skeletal growth. Chondrodysplasias are a group of genetic disorders characterized by impaired skeletal growth. The many different forms of chondrodysplasias add to produce a significant number of affected individuals with significant morbidity and mortality (7). Nevertheless, no efficient drug therapy has been developed to date for the treatment of chondrodysplasias.

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Abbreviations: CNP, C-type natriuretic peptide; DIG, digoxigenin; GC, guanylyl cyclase; HE, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; SAP, serum amyloid P.

In our previous report, we achieved targeted overexpression of CNP in the growth plate of a mice model of achondroplasia (8), the most common form of chondrodysplasias with a constitutive active mutation in the fibroblast growth factor receptor 3 gene (9), and successfully treated its impaired skeletal growth and short stature phenotype (5).

In contrast to atrial natriuretic peptide and brain natriuretic peptide, the two cognate natriuretic peptides of CNP that act as cardiac hormones produced predominantly from atrium and ventricle of heart, respectively (10, 11), CNP is thought to be an autocrine/paracrine regulator, rather than an endocrine regulator (12, 13). Because we have to evaluate the effect of circulating CNP on endochondral bone growth in case we use CNP as a drug for chondrodysplasias via systemic administration, we generated CNP transgenic mice with increased circulating CNP as a model of systemic administration of CNP (14): these transgenic mice carried the human serum amyloid P (SAP) component promoter/mouse CNP fusion gene (*SAP-Nppc-Tg*), and the expression of the transgene was targeted to the liver (15). *SAP-Nppc-Tg* mice exhibited prominent overgrowth of bones formed through endochondral ossification (14), and furthermore, we successfully rescued achondroplastic model mice from their impaired bone growth by crossing them with *SAP-Nppc-Tg* mice (16).

In the present study, we further investigated the effect of circulating CNP by using *SAP-Nppc-Tg* mice. At first, to certify the humoral effect of the overexpressed CNP in *SAP-Nppc-Tg* mice on endochondral bone growth, we performed organ culture experiments by using tibial explants from *SAP-Nppc-Tg* mice and compared them with those from cartilage-targeted CNP transgenic mice under the control of type II collagen promoter (*Col2-Nppc-Tg* mice) (5). Then we studied the effects of circulating CNP on the chondrodysplastic CNP knockout ($Nppc^{-/-}$) mice by crossing them with *SAP-Nppc-Tg* mice.

Materials and Methods

Animals

Generation of CNP transgenic mice under the control of human SAP component promoter (*SAP-Nppc-Tg* mice) was reported previously (14). These mice carried the human SAP/mouse CNP fusion gene, and CNP overexpression in these mice was targeted to the liver (15). *SAP-Nppc-Tg* mice were intended to have increased circulating CNP levels, and plasma CNP concentrations measured by RIA were 84% higher in *SAP-Nppc-Tg* mice than in wild-type mice (14). Generation of CNP transgenic mice under the control of mouse type II collagen promoter (*Col2-Nppc-Tg* mice) (5) and CNP knockout mice ($Nppc^{-/-}$ mice) (1) was also described previously.

To generate $Nppc^{-/-}$ mice carrying *SAP-Nppc* transgene, male $Nppc^{+/-}$ mice were mated with female *SAP-Nppc-Tg* mice, and female F1 offspring heterozygous for both the transgene and the *Nppc* allele ablation were mated with male F1 offspring heterozygous only for the *Nppc* allele ablation to generate $Nppc^{-/-}$ mice with the transgene expression ($Nppc^{-/-}$/*SAP-Nppc-Tg* mice). For generation of homozygous *SAP-Nppc-Tg* mice, male and female heterozygous *SAP-Nppc-Tg* mice were mated, and the genotype of the resultant transgenic mice was determined by quantifying *SAP-Nppc* transgene using StepOnePlus real-time PCR systems (Applied Biosystems Inc., Foster City, CA).

The care of the animals and all experiments were conducted in accordance with the institutional guidelines of Kyoto University Graduate School of Medicine.

Organ culture

Tibias from fetal *SAP-Nppc-Tg* mice and their wild-type littermates (on d 16 of pregnancy), newborn *Col2-Nppc-Tg* mice and their wild-type littermates, and newborn $Nppc^{-/-}$/*SAP-Nppc-Tg* mice and their $Nppc^{-/-}$ littermates were dissected out and cultured for 4 d in Biggers, Gwatkin, Judah tissue culture medium for bone and cartilage (Invitrogen, Carlsbad, CA) with BSA (6 mg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan), ascorbic acid (150 μg/ml; Wako), and penicillin/streptomycin (10,000 U/ml; Wako) in 12-well plates. Tibias from newborn $Nppc^{-/-}$ mice were incubated with vehicle or CNP at the dose of 10^{-9}, 10^{-8}, or 10^{-7} M for 4 d. At the end of the culture period, the longitudinal length of tibial explants was measured using a linear ocular scale mounted on a dissecting microscope at ×10 magnification.

Skeletal analysis

Mice were subjected to soft x-ray analysis (30 kVp, 5 mA for 1 min; Softron type SRO-M5; Softron, Tokyo, Japan), and the lengths of bones were measured on the soft x-ray film.

Histological analysis

For light microscopy, sections were cut from paraffin-embedded specimens. For Alcian Blue-hematoxylin and eosin (HE) staining, sections were deparaffinized with xylene and rehydrated through an ethanol series and distilled water. The sections were treated with 3% acetic acid for 3 min and Alcian Blue (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 20 min. Then they were treated with hematoxylin (Muto) for 2 min, eosin alcohol (Muto) for 1 min, dehydrated, and then mounted with malinol (Muto).

As for *in situ* hybridization analyses for type II and type X collagens, 414- and 658-bp DNA fragments corresponding to the nucleotide positions 138-551 and 2893-3550 of mouse *Col2a1* and *Col10a1* cDNA (GenBank accession no. NM_031163 and 009925), respectively, were subcloned into pGEMT-Easy vector (Promega, Madison, WI) and were used for the generation of sense or antisense RNA probes. Digoxigenin (DIG)-labeled RNA probes were prepared with DIG RNA labeling mix (Roche, Stockholm, Sweden). Paraffin-embedded sections were hybridized with DIG-labeled RNA probes at 60 C for 16 h. The bound label was detected using 4-nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt, an alkaline phosphate color substrate. The sections were counterstained with Kernechtrot (Muto).

For immunohistochemical detection of proliferating cell nuclear antigen (PCNA), tissue sections were incubated with mouse

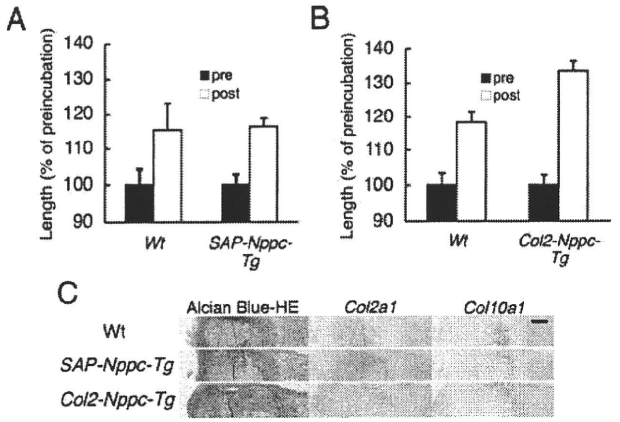

FIG. 1. Organ culture experiments using tibial explants from two different CNP transgenic mice. A and B, Graphs indicating percent of the longitudinal lengths of tibial explants at the end of incubation (*white bars*) compared by those of tibial explants at the beginning of incubation (*black bars*). Fetal (GD16) wild-type (Wt) *vs. SAP-Nppc-Tg* explants (A), and neonatal wild-type (Wt) *vs. Col2-Nppc-Tg* explants (B) are shown. C, Histological pictures of the growth plates of tibial explants at the end of 4-d culture period. From *top* to *bottom*, pictures of wild-type (Wt), *SAP-Nppc-Tg*, and *Col2-Nppc-Tg* explants are shown. *Left three panels* exhibit Alcian Blue-hematoxylin and eosin (HE) staining, and *middle three* and *right three panels* show *in situ* hybridization analyses for type II collagen (*Col2a1*) and type X collagen (*Col10a1*), respectively. *Scale bar*, 100 μm.

monoclonal anti-PCNA antibody (Dako, Glostrup, Denmark), and immunostaining was performed using Histofine mouse stain kit (Nichirei Corp., Tokyo, Japan) according to the manufacturer's instructions. Under the microscope (×400), three visual fields in the proliferative chondrocyte zone of the growth plate were randomly selected, and all cells and PCNA-positive cells in each field were counted. Then labeling index was calculated as the mean of these three values. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling staining was performed using *in situ* apoptosis detection kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instruction.

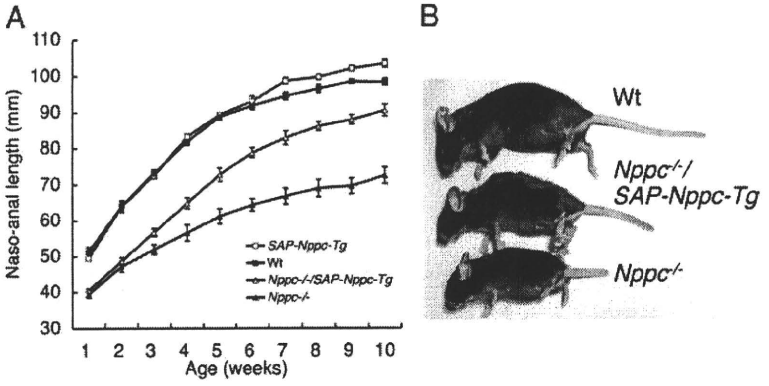

FIG. 2. Effect of circulating CNP on the longitudinal growth of $Nppc^{-/-}$ mice. A, Growth curves of nasoanal length of *SAP-Nppc-Tg* (*open square*), wild-type (*closed square*), $Nppc^{-/-}$/*SAP-Nppc-Tg* (*open triangle*), and $Nppc^{-/-}$ (*closed triangle*) mice. B, Gross appearance of wild-type (Wt), $Nppc^{-/-}$/*SAP-Nppc-Tg*, and $Nppc^{-/-}$ mice at the age of 15 wk.

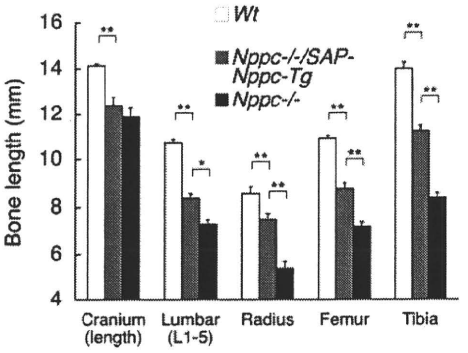

FIG. 3. Bone lengths of mice at the age of 3 wk measured on soft x-ray films. *White bars*, Wild-type mice; *gray bars*, $Nppc^{-/-}$/*SAP-Nppc-Tg* mice; *black bars*, $Nppc^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$.

Statistical analysis

Data are expressed as means ± SE. The statistical significance of differences in mean values was assessed by Student's *t* test. The difference in survival rates among genotypes was assessed by Kaplan-Meier analysis.

Results

Organ culture experiments using tibial explants from *SAP-Nppc-Tg mice*

We generated two lines of CNP transgenic mice under the control of an SAP promoter, and both of them exhibited prominent skeletal overgrowth phenotype (14). We used one of them with milder skeletal phenotype as the *SAP-Nppc-Tg* mice for further experiments. To confirm whether the effect of *SAP-Nppc*-transgene on skeletal growth is humoral, we performed organ culture experiments by using tibias from *SAP-Nppc-Tg* mice and compared them with those from CNP transgenic mice with targeted overexpression of CNP in the cartilage by using mouse type II collagen promoter (*Col2-Nppc-Tg* mice) (*5*).

At the end of the 4-d culture period, the length of tibial explants from *SAP-Nppc-Tg* mice was not changed from that from their wild-type littermates, whereas the length of tibial explants from *Col2-Nppc-Tg* mice was about 13% larger than that from their wild-type littermates (Fig. 1, A and B). Histological analyses revealed that the widths of both nonhypertrophic and hypertrophic chondrocyte layers of the growth plates in *SAP-Nppc-Tg* explants, shown to express type II and type X collagens by *in situ* hybridization analyses, respectively, were not changed from those in wild-type explants, whereas they were larger in *Col2-Nppc-Tg* explants (Fig. 1C).

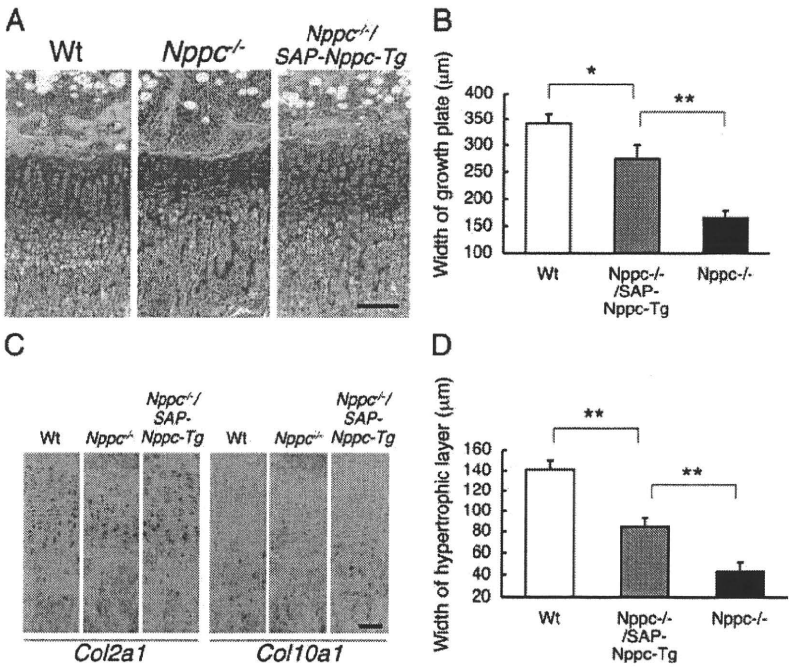

FIG. 4. Histological analyses of tibial growth plates of wild-type (Wt), $Nppc^{-/-}$, and $Nppc^{-/-}$/*SAP-Nppc-Tg* mice at the age of 3 wk. A, Histological pictures stained with Alcian Blue-hematoxylin and eosin (HE). *Scale bar*, 100 μm. B, Width of growth plates of tibias from wild-type (*white bar*), $Nppc^{-/-}$/*SAP-Nppc-Tg* (*gray bar*), and $Nppc^{-/-}$ (*black bar*) mice. *, $P < 0.05$; **, $P < 0.01$. C, Pictures of *in situ* hybridization analyses for type II collagen (*Col2a1*, *left three panels*) and type X collagen (*Col10a1*, *right three panels*). *Scale bar*, 50 μm. D, Width of hypertrophic chondrocyte layers of the growth plates of tibias from wild-type (*white bar*), $Nppc^{-/-}$/*SAP-Nppc-Tg* (*gray bar*), and $Nppc^{-/-}$ (*black bar*) mice. **, $P < 0.01$.

In addition, *in situ* hybridization analyses exhibited that the patterns and intensities of the staining for type II and type X collagens as the differentiation markers for nonhypertrophic and hypertrophic chondrocytes, respectively, were not different between in *SAP-Nppc-Tg* and wild-type explants. Furthermore, the proliferation of the growth plate chondrocytes in *SAP-Nppc-Tg* explants, estimated by immunohistochemical staining for PCNA, was almost the same as that in wild-type explants (labeling index: 60.4 ± 3.4 *vs.* 60.0 ± 2.4%). These results exhibit that CNP generated by *SAP-Nppc*-transgene affects endochondral bone growth in an endocrine manner.

The impaired endochondral bone growth of *Nppc*$^{-/-}$ mice was recovered by circulating CNP

Next we investigated the effect of circulating CNP on the chondrodysplastic phenotype of CNP knockout mice by crossing them with *SAP-Nppc-Tg* mice. Because $Nppc^{-/-}$ mice are thought to be infertile, we crossed $Nppc^{+/-}$ mice with *SAP-Nppc-Tg* mice and obtained $Nppc^{+/-}$/*SAP-Nppc-Tg* mice. Then these $Nppc^{+/-}$/*SAP-Nppc-Tg* mice were crossed with $Nppc^{+/-}$ mice to generate $Nppc^{-/-}$/*SAP-Nppc-Tg* mice.

At the first week after birth, $Nppc^{-/-}$/*SAP-Nppc-Tg* mice were smaller than their wild-type littermates, and the nasoanal length of $Nppc^{-/-}$/*SAP-Nppc-Tg* mice was almost the same as that of $Nppc^{-/-}$ mice (Fig. 2A). But they gradually became larger than $Nppc^{-/-}$ mice and became close to their wild-type littermates (Fig. 2, A and B). The nasoanal length of $Nppc^{-/-}$/*SAP-Nppc-Tg* mice was significantly larger than that of $Nppc^{-/-}$ mice at the age of 3 wk in male and at the age of 4 wk in female (male: 56.6 ± 1.1 mm and 51.9 ± 1.3 mm, respectively, n = 15 and 11 each, $P < 0.01$, and female: 63.3 ± 1.2 mm and 53.8 ± 0.7 mm, respectively, n = 10 and 10 each, $P < 0.01$). In accordance with the above observation, most bones formed through endochondral ossification in $Nppc^{-/-}$/*SAP-Nppc-Tg* mice grew longer than those in $Nppc^{-/-}$ mice. At the age of 3 wk, lumbar spine, radius, femur, and tibia of $Nppc^{-/-}$/*SAP-Nppc-Tg* mice were significantly longer than those of $Nppc^{-/-}$ mice, although they were still significantly shorter than those of their wild-type littermates (Fig. 3).

Histological analysis revealed that the width of the growth plate of tibias from $Nppc^{-/-}$/*SAP-Nppc-Tg* mice was significantly larger than that from $Nppc^{-/-}$ mice and was comparable with that from wild-type mice (Fig. 4, A and B). Width of every zone of the growth plate, especially that of hypertrophic chondrocyte zone expressing type X collagen as shown by *in situ* hybridization analysis, was significantly larger in $Nppc^{-/-}$/*SAP-Nppc-Tg* tibia than that in $Nppc^{-/-}$ tibia and was comparable with that in wild-type tibia (Fig. 4, A, C, and D).

The intensities or patterns of the staining for both type II and type X collagens by *in situ* hybridization were not different between that in $Nppc^{-/-}$/*SAP-Nppc-Tg* and that in $Nppc^{-/-}$ tibias, indicating that the differentiation for nonhypertrophic and hypertrophic chondrocytes in $Nppc^{-/-}$ growth plate was not affected by circulating CNP (Fig. 4C). Furthermore, immunohistochemical detection of PCNA revealed that the rate of PCNA-positive chondrocytes in $Nppc^{-/-}$/*SAP-Nppc-Tg* growth plate was not changed from that in $Nppc^{-/-}$ growth plate (labeling index: 23.0 ± 7.3 *vs.* 25.4 ± 1.4%), exhibiting that the proliferation of the chondrocytes in $Nppc^{-/-}$ growth plate was not altered by circulating CNP. In addition, we

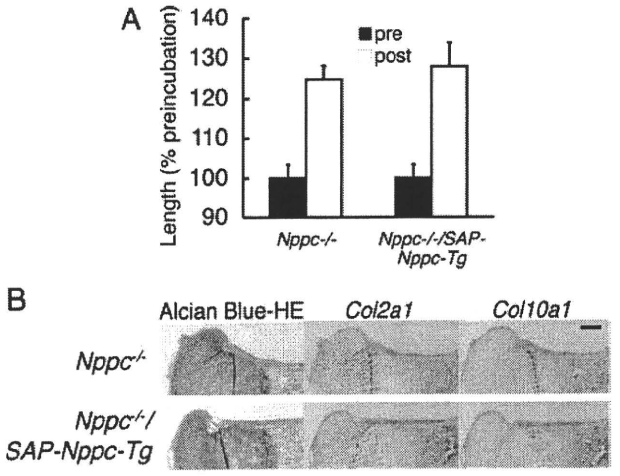

FIG. 5. Organ culture experiments using tibial explants from neonatal $Nppc^{-/-}$ and $Nppc^{-/-}$/*SAP-Nppc-Tg* mice. A, The graph indicating percent of the longitudinal length of tibial explants at the end of incubation (*white bars*) compared with that of tibial explants at the beginning of incubation (*black bars*). B, Histological analyses of the tibial explants at the end of the 4-d culture period. *Upper panels* show histological pictures of the growth plates of $Nppc^{-/-}$ explants, and *lower panels* show those of $Nppc^{-/-}$/*SAP-Nppc-Tg* explants. *Left panels* exhibit Alcian Blue-hematoxylin and eosin (HE) staining, and *middle* and *right panels* show *in situ* hybridization analyses for type II collagen (*Col2a1*) and type X collagen (*Col10a1*), respectively. *Scale bar*, 100 μm.

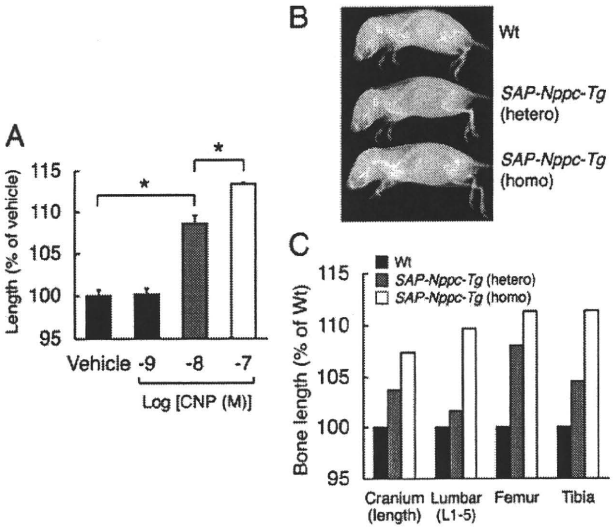

FIG. 6. Dose-dependent effect of circulating CNP on endochondral bone growth. A, Dose-dependent effect of addition of CNP on the growth of tibial explants from $Nppc^{-/-}$ mice in organ culture. The graph indicates percent of the longitudinal length of tibial explants incubated with indicated doses of CNP compared with that with vehicle, at the end of the 4-d culture period. *, $P < 0.05$. B, Soft x-ray picture of 3-wk-old wild-type (Wt) and *SAP-Nppc-Tg* mice with heterozygous (hetero) and homozygous (homo) *SAP-Nppc-Tg* transgene. Note that the nasoanal length is increased in accordance with the copy number of the transgene. C, The graph indicating percent of the length of each bone of heterozygous (*gray bar*) or homozygous (*white bar*) *SAP-Nppc-Tg* mice compared with that of wild-type mice (*black bar*) [n = 2 (Wt), four (hetero), and four (homo), each].

could scarcely find out the difference in the state of apoptosis of the growth plate chondrocytes between that in $Nppc^{-/-}$/*SAP-Nppc-Tg* and that in $Nppc^{-/-}$ tibias by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling staining (data not shown).

To further confirm whether the *SAP-Nppc*-transgene product humorally affects the endochondral bone growth in $Nppc^{-/-}$ mice, organ culture experiments using tibial explants from neonatal $Nppc^{-/-}$/*SAP-Nppc-Tg* and $Nppc^{-/-}$ mice were performed. At the end of the 4-d culture period, longitudinal length of tibial explants from $Nppc^{-/-}$/*SAP-Nppc-Tg* mice was not changed from that from $Nppc^{-/-}$ mice (Fig. 5A). Histological analyses revealed that the widths of both nonhypertrophic and hypertrophic chondrocyte layers of the growth plate, expressing type II and type X collagens, respectively, were not different between in $Nppc^{-/-}$/*SAP-Nppc-Tg* and $Nppc^{-/-}$ explants (Fig. 5B). Neither the differentiation (estimated by *in situ* hybridization analyses for type II and type X collagens, Fig. 5B) nor the proliferation (evaluated by PCNA analysis, labeling index: 41.0 ± 3.3 *vs.* 44.9 ± 3.0%) of the growth plate chondrocytes was different between that in $Nppc^{-/-}$/*SAP-Nppc-Tg* and that in $Nppc^{-/-}$ explants.

To investigate whether the stimulatory effect of circulating CNP on the endochondral bone growth of $Nppc^{-/-}$ mice is dose dependent, we studied the effect of CNP on the growth of tibial explants from neonatal $Nppc^{-/-}$ mice in organ culture experiment. As shown in Fig. 6A, the growth of tibial explants from $Nppc^{-/-}$ mice was stimulated by addition of CNP in a dose-dependent manner. Furthermore, we generated *SAP-Nppc-Tg* mice with homozygous *SAP-Nppc* transgene to confirm a dose-dependent effect of circulating CNP on endochondral bone growth *in vivo*.

At the age of 3 wk, soft x-ray analyses revealed that the longitudinal body length and the growth of every bone formed through endochondral bone growth were promoted in accordance with the copy number of *SAP-Nppc* transgene, indicating the dose-dependent effect of circulating CNP on endochondral bone growth *in vivo* (Fig. 6B). Collectively, these results suggest that circulating CNP would cure the impaired skeletal growth of $Nppc^{-/-}$ mice in a dose-dependent manner *in vivo*.

Effects of increased circulating CNP on the body weight gain and the survival rate of $Nppc^{-/-}$ mice

We also investigated the effects of circulating CNP on other aspects of the impaired growth of chondrodysplastic $Nppc^{-/-}$ mice. The body weight of $Nppc^{-/-}$/*SAP-Nppc-Tg* mice was smaller than that of their wild-type littermates and was comparable with that of their $Nppc^{-/-}$ littermates at the age of 1 wk (Fig. 7A). However, $Nppc^{-/-}$/*SAP-Nppc-Tg* mice gradually became heavier

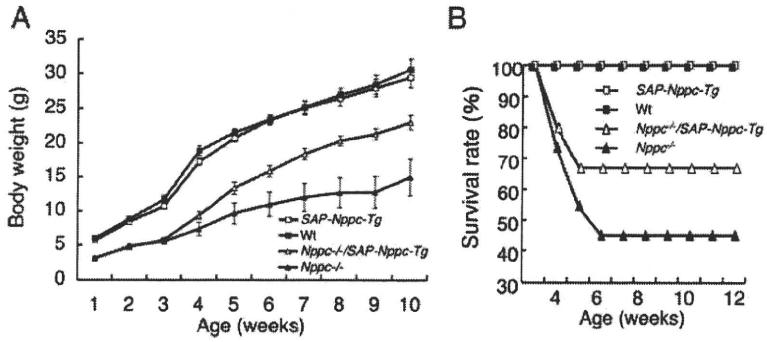

FIG. 7. Effect of circulating CNP on the body weight and the survival rate of $Nppc^{-/-}$ mice. A, Growth curves of body weight of *SAP-Nppc-Tg* (*open square*), wild-type (Wt; *closed square*), $Nppc^{-/-}$*/SAP-Nppc-Tg* (*open triangle*), and $Nppc^{-/-}$ (*closed triangle*) mice. B, Survival curves of *SAP-Nppc-Tg* (*open square*), wild-type (*closed square*), $Nppc^{-/-}$*/SAP-Nppc-Tg* (*open triangle*), and $Nppc^{-/-}$ (*closed triangle*) mice.

than their $Nppc^{-/-}$ littermates (Fig. 7A), and the body weight of $Nppc^{-/-}$*/SAP-Nppc-Tg* mice was significantly larger than their wild-type littermates at the age of 4 wk in males and 3 wk in females (males: 9.3 ± 0.5 g and 7.3 ± 0.9 g, respectively, n = 12 and 7 each, $P < 0.05$, and females: 5.4 ± 0.1 g and 4.7 ± 0.2 g, respectively, n = 11 and 12 each, $P < 0.05$). On the other hand, there was no difference in body weight between the *SAP-Nppc-Tg* and wild-type mice, albeit *SAP-Nppc-Tg* mice became larger than the wild-type mice in nasoanal length (Figs. 2A and 6).

We have previously reported that the survival rate of $Nppc^{-/-}$ mice greatly drops before adulthood, albeit the genotype ratio of $Nppc^{-/-}$ mice on d 16.5 of pregnancy is in accord with Mendelian proportion (1). In this study, analysis of intercrosses between $Nppc^{+/-}$*/SAP-Nppc-Tg* mice and $Nppc^{+/-}$ mice revealed that the genotype ratios of wild type to $Nppc^{+/-}$ to $Nppc^{-/-}$ and *SAP-Nppc-Tg* to $Nppc^{+/-}$*/SAP-Nppc-Tg* to $Nppc^{-/-}$*/SAP-Nppc-Tg* at weaning (3 wk of age) are 1:2.78:1 and 1:2.71:1.24 (total n = 104 and 110), respectively, indicating expected Mendelian proportions. As have we previously reported, the survival rate of $Nppc^{-/-}$ mice dropped to about 40% before adulthood (Fig. 7B). However, the survival rate of $Nppc^{-/-}$*/SAP-Nppc-Tg* mice was greatly improved compared with that of $Nppc^{-/-}$ mice (Fig. 7B).

Discussion

In the present study, we investigated the endocrine effects of CNP on chondrodysplastic CNP knockout mice by using *SAP-Nppc-Tg* mice.

In the organ culture experiments, the growth of *SAP-Nppc-Tg* tibias was not changed from that of wild-type tibias, whereas the growth of *Col2-Nppc-Tg* tibias was strongly promoted compared with that of wild-type tibias. This result confirms that the growth stimulating effect of bones formed through endochondral ossification in *SAP-Nppc-Tg* mice is not autocrine/paracrine but endocrine effect of CNP, which is produced by the *SAP-Nppc* transgene. Because we expected that we would observe the effect of circulating CNP on endochondral bone growth clearly in a state without basal CNP effect, we then investigated whether or not elevation of circulating CNP could recover the impaired endochondral bone growth caused by depletion of CNP in mice *in vivo*. Decreased width of the growth plate observed in $Nppc^{-/-}$ mice was recovered in $Nppc^{-/-}$*/SAP-Nppc-Tg* mice, and accordingly, impaired endochondral bone growth observed in $Nppc^{-/-}$ mice was considerably and significantly recovered in $Nppc^{-/-}$*/SAP-Nppc-Tg* mice.

The endocrine effect of CNP produced by the *SAP-Nppc* transgene in $Nppc^{-/-}$*/SAP-Nppc-Tg* mice was further confirmed by the organ culture experiments in that the growth of $Nppc^{-/-}$*/SAP-Nppc-Tg* tibias was not changed from that of $Nppc^{-/-}$ tibias. These results clearly indicate that CNP can humorally affect endochondral bone growth. Furthermore, the result of the organ culture experiment using $Nppc^{-/-}$ bones (Fig. 6A) and the gene-dose effect of *SAP-Nppc* transgene on bone growth *in vivo* (Fig. 6B, C) suggest that the endocrine effect of CNP on endochondral bone growth is dose dependent.

Chondrodysplasia is composed of many different forms of genetic disorders characterized by impaired endochondral bone growth (7, 17). Because the CNP/GC-B system plays a crucial role in endochondral bone growth, loss of function mutations in the genes coding for molecules related to the CNP/GC-B system could cause chondrodysplasia. In fact, recent studies have revealed that mutations in the gene encoding human GC-B cause one form of chondrodysplasia, acromesomelic dysplasia type Maroteaux (18, 19).

In mice, loss of function mutations in the GC-B gene cause impaired skeletal growth in spontaneous mutant *cn/cn* and short-limbed dwarfism (*slw/slw*) mice (20, 21). As for spontaneous mutations in other genes related to the CNP/GC-B system, a mutation in the gene coding for cGMP-dependent protein kinase type II, an important downstream mediator of the CNP/GC-B system, causes impaired endochondral bone growth in Komeda miniature rat Ishikawa (22, 23). Furthermore, recent studies have elucidated that a spontaneous loss of function mutation in the murine CNP gene causes impaired skeletal growth observed in the long bone abnormality (*lbab/lbab*) mice (24–26).

Just as in the case with rodents, any forms of human chondrodysplasia might be caused by mutations in the cGMP-dependent protein kinase type II or CNP gene, albeit they are not yet discovered. In case a form of human chondrodysplasia caused by a mutation in the CNP gene is discovered in future, CNP knockout mice would be a novel mice model of human chondrodysplasia. On the other hand, spontaneous GC-B mutant (*cn/cn* and *slw/slw*) mice and GC-B knockout mice are regarded as mice models of acromesomelic dysplasia type Maroteaux, and impaired skeletal growth of these mice would not be recovered by crossing them with *SAP-Nppc-Tg* mice. This notion is supported by the result of the organ culture experiment, in which tibial explants from fetal GC-B knockout mice are not increased in length by addition of CNP (4).

We previously reported that the impaired skeletal growth of achondroplastic model mice was almost completely recovered by crossing them with *SAP-Nppc-Tg* mice (16). The impairment of skeletal growth of the achondroplastic model mice that we used in our previous study was considerably mild compared with that of $Nppc^{-/-}$ mice: the nasoanal length of the achondroplastic model mice was about 10% shorter than that of wild-type mice at the age of 10 wk (14), whereas the nasoanal length of $Nppc^{-/-}$ mice was about 30% shorter than that of wild-type mice. The reason that the impaired skeletal growth of $Nppc^{-/-}$ mice was not completely rescued in $Nppc^{-/-}$/*SAP-Nppc-Tg* mice in our present study might be because the low graded elevation of the plasma CNP concentrations in *SAP-Nppc-Tg* mice (about 1.8 times higher than those in wild-type mice) was not sufficient for the complete rescue of severe skeletal phenotype of $Nppc^{-/-}$ mice, whereas it was enough to cure the mild skeletal impairment of the achondroplastic model mice. Although about 2 times of elevation of plasma CNP concentrations can stimulate bone growth in *SAP-Nppc-Tg* mice (14) or human with a chromosomal translocation (27), higher plasma concentration of CNP might be needed for the complete treatment of impaired bone growth in chondrodysplasia.

As for the mechanism of the skeletal rescue of CNP knockout mice by circulating CNP, the differentiation and the proliferation of the growth plate chondrocytes of $Nppc^{-/-}$/*SAP-Nppc-Tg* mice were not changed from those of $Nppc^{-/-}$ mice. This result coincides with our previous observation that CNP does not so strongly affect differentiation and proliferation of the growth plate chondrocytes *in vivo* (5, 14). On the other hand, proteoglycan synthesis is greatly increased in the growth plate of *SAP-Nppc-Tg* mice (14), so we speculate that the shortened $Nppc^{-/-}$ growth plate is restored by circulating CNP in $Nppc^{-/-}$/*SAP-Nppc-Tg* mice through the recovery of matrix synthesis, resulting in the recovery of endochondral bone growth.

The impaired growth of $Nppc^{-/-}$ mice was recovered in not only longitudinal length but also body weight, and furthermore, the mortality of $Nppc^{-/-}$ mice was greatly decreased, by circulating CNP. Together with our previous results that targeted overexpression of CNP in the cartilage of $Nppc^{-/-}$ mice improved not only their impaired longitudinal growth but also their impaired body weight gain and that prolonged their survival (1), we consider that the recovery from the impaired endochondral bone growth in $Nppc^{-/-}$ mice by circulating CNP resulted in the recovery of overall growth and also in longevity. The mechanisms through which recovery in skeletal growth results in the recovery of overall growth and the prolonged survival are not yet elucidated. One of the possibilities is that the malformation in the maxillofacial region of $Nppc^{-/-}$ mice, which is caused by impaired endochondral ossification, may disturb their teeth coming together correctly: this condition may prevent them from eating enough and lead them to malnutrition. Further investigation of the craniofacial phenotype of $Nppc^{-/-}$ mice is now ongoing in our laboratory (Nakao, K., Y. Okubo, N. Koyama, K. Osawa, M. Miura, A. Yasoda, K. Nakao, and K. Bessho, manuscript in preparation).

In conclusion, we have revealed that circulating CNP rescues the impaired growth and early death of chondrodysplastic CNP knockout mice through the recovery of endochondral bone growth. We have started to apply the strong stimulatory effect of the CNP/GC-B system on endochondral bone growth to the treatment of chondrodysplasias (16) for those no effective drug therapy is available to date. The results of our present paper suggest that systemic administration of CNP or its analog, which would stimulate GC-B, might have therapeutic potential against not only impaired skeletal growth but also other aspects of impaired growth including impaired body weight gain in patients suffering from chondrodysplasias and might resultantly protect them from their early death.

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REVIEW

Translational research of C-type natriuretic peptide (CNP) into skeletal dysplasias

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Abstract. By using transgenic and knockout mice, we have elucidated that C-type natriuretic peptide (CNP) is a potent stimulator of endochondral bone growth. In humans, loss-of-function mutations in the gene coding for guanylyl cyclase-B (GC-B), the specific receptor for CNP, have been proved to be the cause of acromesomelic dysplasia, type Maroteaux, one form of human skeletal dysplasias. Following these results, we have started to translate the stimulatory effect of CNP on endochondral bone growth into the therapy for patients with skeletal dysplasias. We have shown that targeted overexpression of CNP in cartilage or systemic administration of CNP reverses the impaired skeletal growth of mice model of achondroplasia, the most common form of human skeletal dysplasias.

Key words: C-type natriuretic peptide (CNP), Guanylyl cyclase-B (GC-B), Skeletal dysplasia, Achondroplasia, Translational research

THE NATRIURETIC peptide family consists of three structurally related peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [1]. The biological actions of natriuretic peptides are mediated by activation of two subtypes of membranous guanylyl cyclase (GC), GC-A and GC-B, followed by intracellular accumulation of cyclic GMP (cGMP) [2]. The rank order of potency to induce cGMP production via GC-A is ANP ≥ BNP >> CNP, while that via GC-B is CNP > ANP ≥ BNP [3]. Therefore, ANP and BNP serve as endogenous ligands for GC-A, whereas CNP is specific for GC-B. A third natriuretic peptide receptor with no intracellular guanylyl cyclase domain, dubbed the clearance receptor (C-receptor), is thought to be engaged in the receptor-mediated degradation of natriuretic peptides [2]. The ANP, BNP/GC-A system plays a pivotal role in the regulation of cardiovascular homeostasis, as demonstrated by their augmentation in various pathophysiological states such as heart failure [4-8], myocardial infarction [9, 10], cardiac hypertrophy [11, 12], and hypertension [13-15]. In fact, ANP and BNP are cardiac hormones secreted primarily by the atrium and ventricle of the heart, respectively [8, 15], with strong diuretic, natriuretic, and vasodilatory activities [4, 5, 8]. ANP and BNP are used in the treatment of heart failure [16, 17] and serve as sensitive biochemical markers for heart failure and cardiac hypertrophy [6-8].

CNP, the third member of natriuretic peptide family, was first purified from porcine brain [18]. While CNP is the primary natriuretic peptide in the human brain [19], it is also produced by vascular endothelial cells [20-22] and macrophages [23], and is thought to act as an autocrine/paracrine regulator and as a neuropeptide [19]. Furthermore, analysis of genetically engineered mice of the CNP/GC-B system revealed that CNP and GC-B play a pivotal role in the regulation of endochondral bone growth.

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I. The growth promoting effect of the CNP/GC-B system on endochondral bone growth

I-1. Skeletal phenotypes of genetically engineered mice of the CNP/GC-B system

We generated mice with a targeted disruption of the CNP gene (*Nppc*); the resultant CNP-KO mice ex-

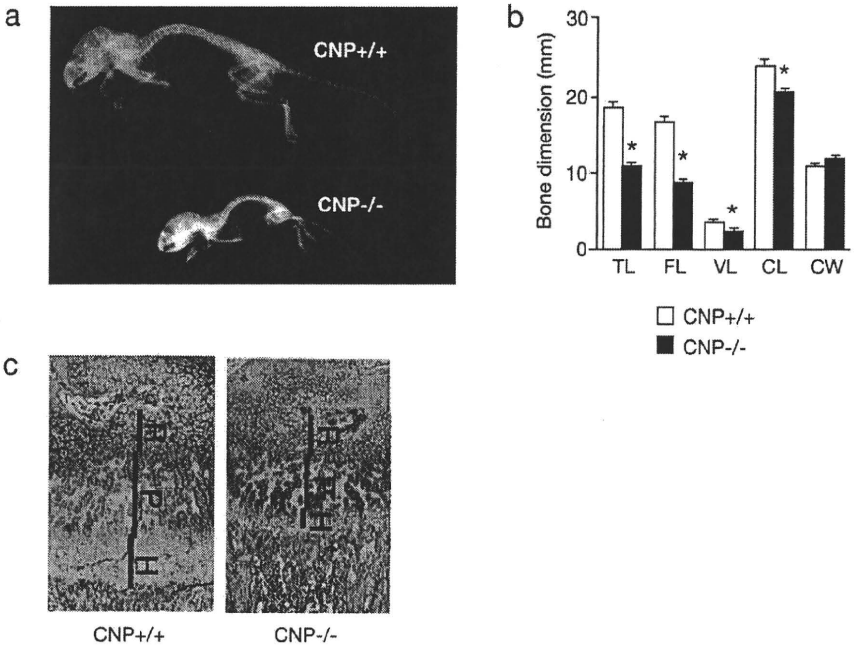

Fig. 1 Impaired skeletal growth observed in CNP-KO mouse. a. Soft x-ray picture of CNP-KO mouse (CNP-/-) compared by that of wild-type mouse (CNP+/+). b. The dimension of each bone from wild-type (CNP+/+) or CNP-KO (CNP-/-) mouse at the age of 10 weeks. TL: tibial length, FL: femoral length, VL: fifth lumber vertebral length, CL: naso-occipital length of the calvarium, CW: maximal interparietal distance of the calvarium. *, $P < 0.05$ *vs.* wild-type mouse. c. Histological analysis of the tibial growth plates from 7-day-old wild-type (CNP+/+) and CNP-KO (CNP-/-) mice. R: resting chondrocyte zone, P: proliferative chondrocyte zone, H: hypertrophic chondrocyte zone.

hibited markedly short stature due to impaired bone growth (Fig. 1) [24]. Mammalian bones are formed through two different mechanisms, endochondral ossification and membranous ossification. Most mammalian bones are formed through endochondral ossification, a process during which chondrocytes in the growth plate undergo proliferation, hypertrophy, cell death, and osteoblastic replacement [25]. The short stature phenotype of CNP-KO mice resulted from impaired bone growth through endochondral ossification [24]. Histological analysis of the growth plate of CNP-KO mice revealed that every chondrocyte layer of the growth plate is narrower in CNP-KO mice than in wild-type mice. Furthermore, mice depleted with the GC-B gene (*Npr2*) exhibit the same short stature phenotype as observed in CNP-KO mice [26], demonstrating that the CNP/GC-B system is a physiologically important stimulator of endochondral bone growth. On the contrary, cartilage specific CNP-transgenic mice under the control of type II collagen promoter (col2-CNP-Tg mice) exhibited prominent overgrowth of bones formed through endochondral ossification (Fig. 2) [27]. In contrast to CNP- or GC-B-KO mice, every chondrocyte layer of the growth plate of col2-CNP-Tg mice was wider than that of wild-type mice. Collectively, the CNP/GC-B system is a potent stimulator of endochondral bone growth.

I-2. The role of other molecules related to the CNP/GC-B system on endochondral bone growth (Fig. 3)

cGMP-dependent protein kinase (cGK) has been identified as a molecule activated downstream of the natriuretic peptide family and guanylyl cyclase system [28]. Mice depleted of one subtype of the cGK gene, cGKII (cGKII-KO mice), exhibit a short stature phenotype secondary to impaired endochondral bone growth [29], similar to that observed in CNP-KO mice [24]. We demonstrated that cGKII affected endochondral bone growth by functioning downstream of the CNP/GC-B system by showing that the impaired endochondral bone growth observed in cGKII-KO mice could not be rescued by targeted overexpression of

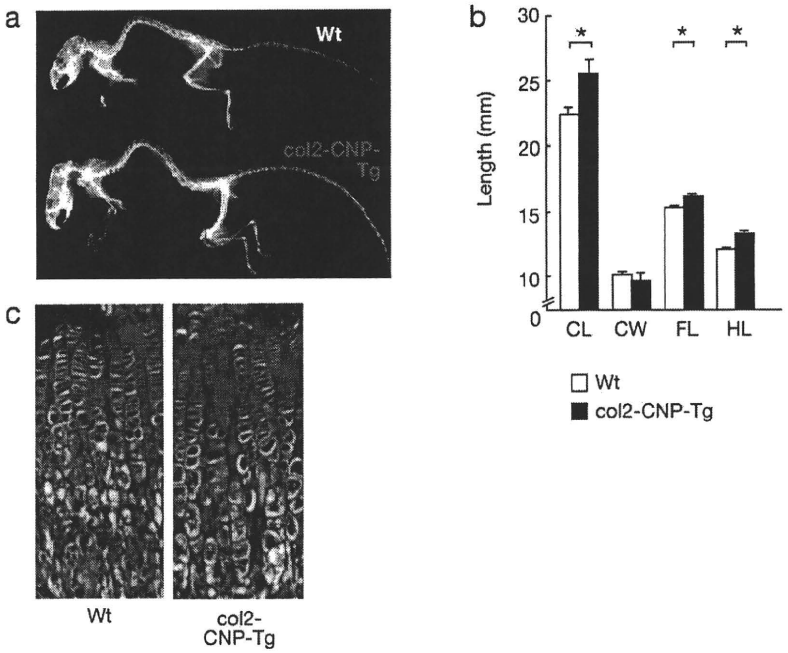

Fig. 2 Skeletal phenotype of col2-CNP-Tg mouse. a. Soft x-ray picture of wild-type (Wt) and col2-CNP-Tg mice. b. The length of each bone from wild-type (Wt) or col2-CNP-Tg mouse. CL: naso-occipital length of the calvarium, CW: maximal interparietal distance of the calvarium. FL: femoral length, HL: Humeral length. *, $P < 0.05$ *vs.* wild-type mouse. c. Histological analysis of the tibial growth plates from 7-day-old wild-type and CNP-KO (CNP-/-) mice.

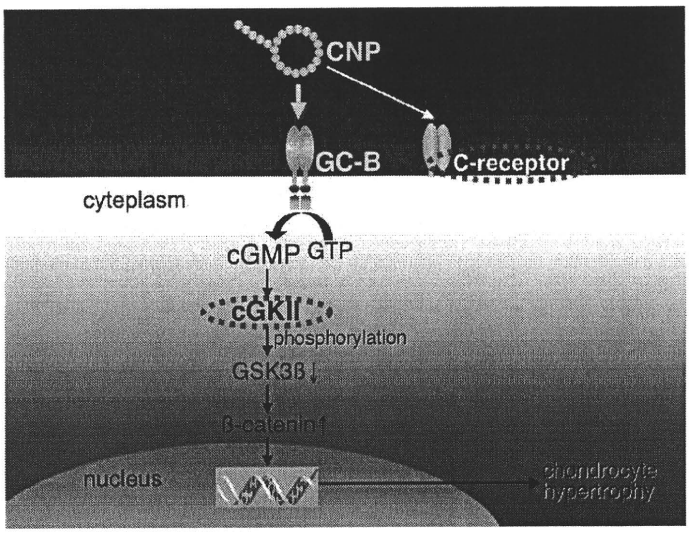

Fig. 3 Schematic representation of the pathway of the CNP/GC-B system.

CNP in the growth plate cartilage [30]. cGKII is reported to phosphorylate and inactivate GSK3β as the downstream molecule, resulting in the increased accumulation and transactivation function of β-catenin followed by hypertrophic differentiation of the growth plate chondrocyte [31].

As previously mentioned, C-receptor is thought to be engaged in the clearance of natriuretic peptide ligands, and mice depleted with C-receptor exhibit skeletal overgrowth phenotype like col2-CNP-Tg mice

[32]. Transgenic mice with overexpression of osteocrin, which is thought to be an endogenous ligand for C-receptor, also show skeletal overgrowth phenotype [33]. These results exhibit that the decreased clearance of CNP increases the concentration of CNP in the growth plate, followed by the stimulation of endochondral bone growth by the increased CNP there.

I-3. Skeletal phenotypes of spontaneous mutant animals of the CNP/GC-B system and its related molecules

Many lines of spontaneous mutant mice of the CNP/GC-B system have been identified so far [34-36]. Two strains of dwarf mice, one with an autosomal recessive mutant gene, named cn/cn [34], and short-limbed dwarfism (SLW) mice [35], possess spontaneous loss of function mutations in the GC-B gene. Another strain of dwarf mice, named long bone abnormality (Lbab) mice, displays a loss of function mutation in the CNP gene [37]; the resulting short stature phenotype and impaired endochondral bone growth could be abrogated by targeted overexpression of CNP in the growth plate cartilage [36].

As for spontaneous mutations in the genes coding for related molecules of the CNP/GC-B system, a spontaneous mutation in the cGKII gene (*Prkg2*) causes impaired endochondral bone growth phenotype in Komeda miniature rats Ishikawa (KMI), which coincides with that of cGKII-KO mice [38]. There exist several lines of mice with mutations in the C-receptor gene (*Npr3*), and all of these mutant mice exhibit skeletal over growth phenotype just like C-receptor-KO mice or osteocrin-transgenic mice [39].

II. Clinical application of CNP for skeletal dysplasias

II-1. The importance of the CNP/GC-B system on human endochondral bone growth

In 2004, Bartels *et al.* reported that one form of human skeletal dysplasias, acromesomelic dysplasia type Maroteaux, is caused by loss of function mutations in the GC-B gene (*NPR2*) [40]. Furthermore, they showed that heterozygous mutations in the human GC-B gene are associated with short stature. Assuming that one in 700 people unknowingly carries an *NPR2* mutation, approximately one in 30 individuals with idiopathic short stature would be a carrier of an *NPR2* mutation [41]. These implicate the CNP/GC-B system as a physiologically important enhancer of endochondral bone growth in humans. On the contrary, three patients carrying balanced translocations involving 2q37.1 chromosome band, in which the human CNP gene (*NPPC*) is located, were reported to have CNP overexpression and exhibit skeletal overgrowth phenotype [42, 43]. These reports further indicate that CNP is a potent stimulator of endochondral bone growth in humans, suggesting a clinical application of CNP or CNP analogues to diseases characterized by impaired skeletal growth.

II-2. The therapeutic effect of CNP on the impaired skeletal growth of mice model of achondroplasia

Following the discovery that the CNP/GC-B system is a potent stimulator of endochondral bone growth in rodents and in humans, we attempted to apply this effect of the CNP/GC-B system to the treatment of skeletal dysplasias, a group of genetic disorders characterized by severely impaired bone growth [44]. Achondroplasia, the most common form of skeletal dysplasias characterized by short-limbed dwarfism, is caused by a constitutive active mutation in the FGF receptor 3 (FGFR3) gene (*FGFR3*) [45]. The current therapy for achondroplasia is limited to distraction osteogenesis [46], an orthopedic procedure; no efficient medical therapies have been developed as yet. We demonstrated that targeted overexpression of a CNP transgene in the growth plate cartilage of a mouse model of achondroplasia [47] (Ach mouse) rescues its impaired bone growth and short stature phenotype [27] (Fig. 4). To elucidate the molecular mechanism by which CNP ameliorates achondroplasia, we examined the effect of CNP on ERK signaling that mediates biological actions of FGFR3. CNP inhibited FGF2-stimulated phosphorylation of ERK in a dose-dependent manner through cGMP activation via GC-B ligation, ultimately increasing matrix synthesis by chondrocytes [27]. Further *in vitro* study using rat chondrosarcoma chondrocytes from another laboratory revealed that CNP inhibited ERK pathway of FGF signaling at the level of Raf-1 through the activation of cGKII (Fig. 5) [48].

Distinct from ANP and BNP, CNP had been thought to be an autocrine/paracrine regulator. In order to elucidate whether CNP could work in an endocrine manner and be used as a drug via systemic administration or not, we generated transgenic mice with an elevated plasma concentration of CNP under the control of serum amyloid P component (SAP) promoter, which en-

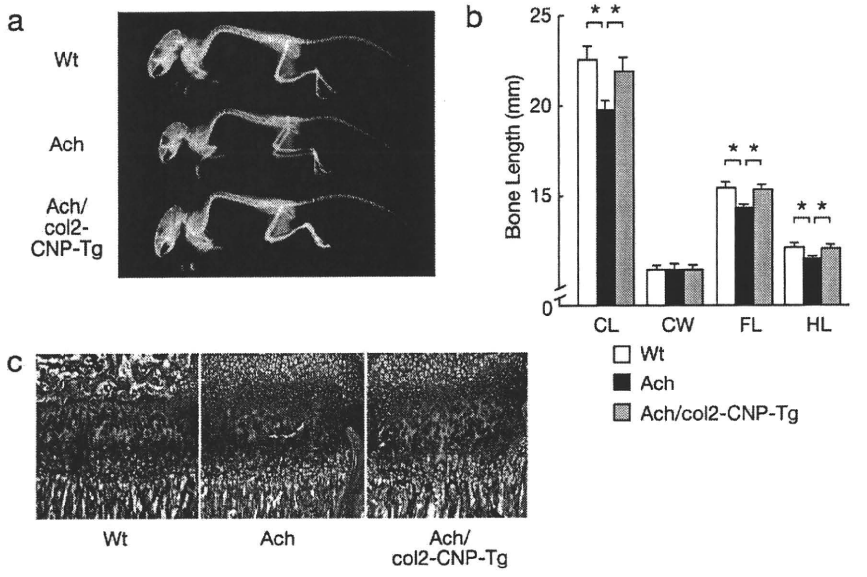

Fig. 4 Skeletal rescue of Ach mouse by targeted overexpression of CNP in cartilage. a. Soft x-ray picture of wild-type mouse (Wt), Ach mouse, and Ach mouse with targeted overexpression of CNP in cartilage (Ach/col2-CNP-Tg). b. The length of each bone from wild-type, Ach, and Ach/col2-CNP-Tg mouse at the age of 10 weeks. CL: naso-occipital length of the calvarium, CW: maximal interparietal distance of the calvarium, FL: femoral length, HL: humeral length. *, $P < 0.05$ *vs.* Ach mouse. c. Histological analysis of the tibial growth plates from 7-day-old wild-type, Ach, and Ach/col2-CNP-Tg mice.

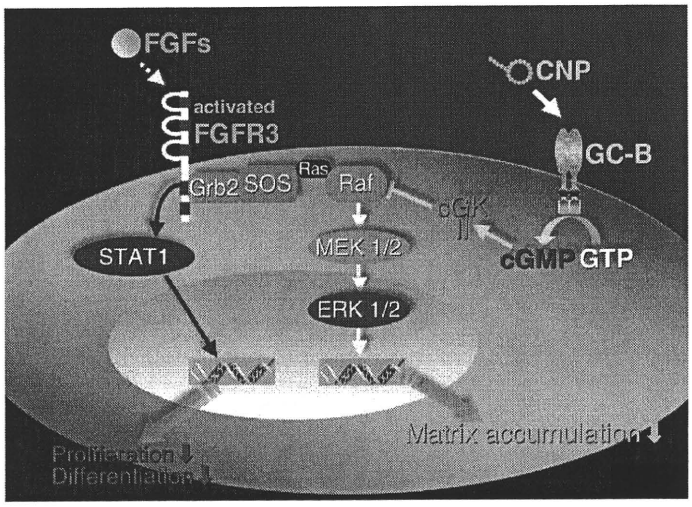

Fig. 5 Schema of the intracellular interaction between the FGF signaling and the CNP/GC-B system.

ables targeted overexpression of the transgene in the liver followed by the increased circulation of the gene product. The resultant transgenic mice (SAP-CNP-Tg mice) exhibited skeletal overgrowth phenotype just like that of col2-CNP-Tg mice [49]. Moreover, the impaired endochondral bone growth of Ach mice was almost completely recovered by crossing them with SAP-CNP-Tg mice [50]. These results indicate that CNP can work in an endocrine manner and be used as a drug for achondroplasia via systemic administration. Further we demonstrated that systemic and continuous administration of synthetic CNP-22 is effective to re-

verse the impaired bone growth observed in Ach mice [50]. The safety of systemic CNP administration in preclinical studies with the observation that CNP has only a minimal effect of blood pressure in humans [51] suggests that systemic administration of CNP provides a novel therapeutic strategy for the treatment of human skeletal dysplasias including achondroplasia.

III. Future direction of CNP therapy for skeletal dysplasias

Currently, the primary target of the translational research of CNP is achondroplasia. The efficacy of CNP therapy for achondroplasia has been demonstrated by preclinical studies using its mice model. As CNP has a strong stimulatory effect on the growth of bones of wild-type mice [49], it is expected that CNP therapy is effective for the treatment of skeletal dysplasias other than achondroplasia. Nevertheless, it is supposed that CNP therapy is not effective for acromesomelic dysplasia type Maroteaux, which is caused by loss-of-function mutations in the GC-B gene. In order to select forms of skeletal dysplasias which will be applied for CNP therapy, we must further establish the proof-of-concept of CNP therapy for skeletal dysplasias other than achondroplasia.

Because CNP is an intrinsic peptide, the possibility of the safety of CNP therapy might be considerably high. On the other hand, as is in common for peptide drugs, CNP may easily be degraded by endopeptidases. Accordingly, we will have to try to explore various strategies to activate the CNP/GC-B system: for example, inhibition of endopeptidases or blockade of C-receptor, specifically in the growth plate, might be included.

In conclusion, we have discovered that the CNP/GC-B system is a potent stimulator of endochondral bone growth by using transgenic and knockout mice. The translational research of this effect into skeletal dysplasias, including achondroplasia, is now ongoing in our laboratory.

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