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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-

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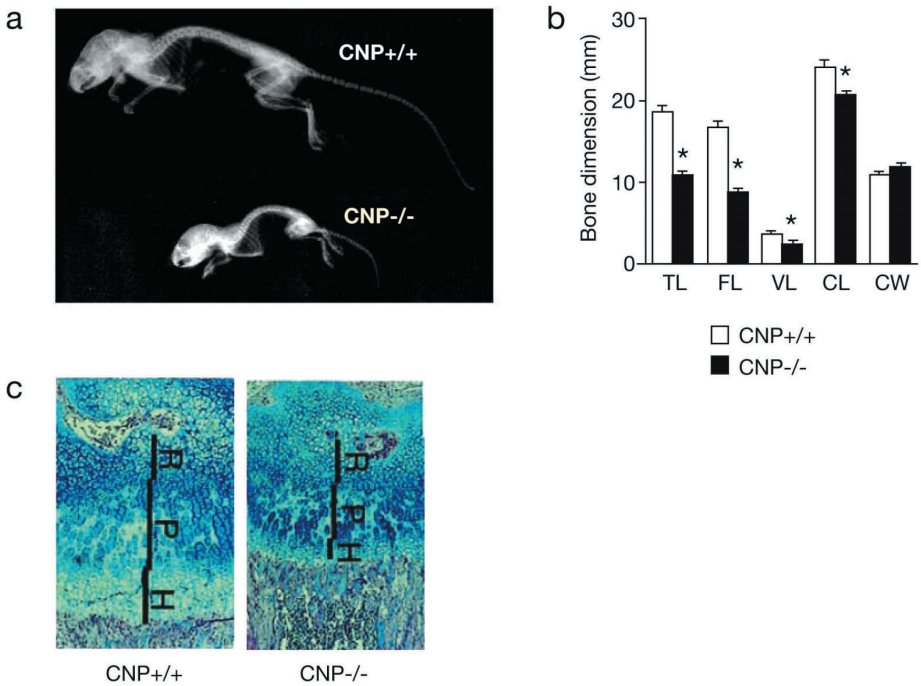

Fig. 1 Impaired skeletal growth observed in CNP-KO mouse. a. Soft x-ray picture of CNP-KO mouse (CNP-/-) compared by that of wild-type mouse (CNP+/+). b. The dimension of each bone from wild-type (CNP+/+) or CNP-KO (CNP-/-) mouse at the age of 10 weeks. TL: tibial length, FL: femoral length, VL: fifth 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

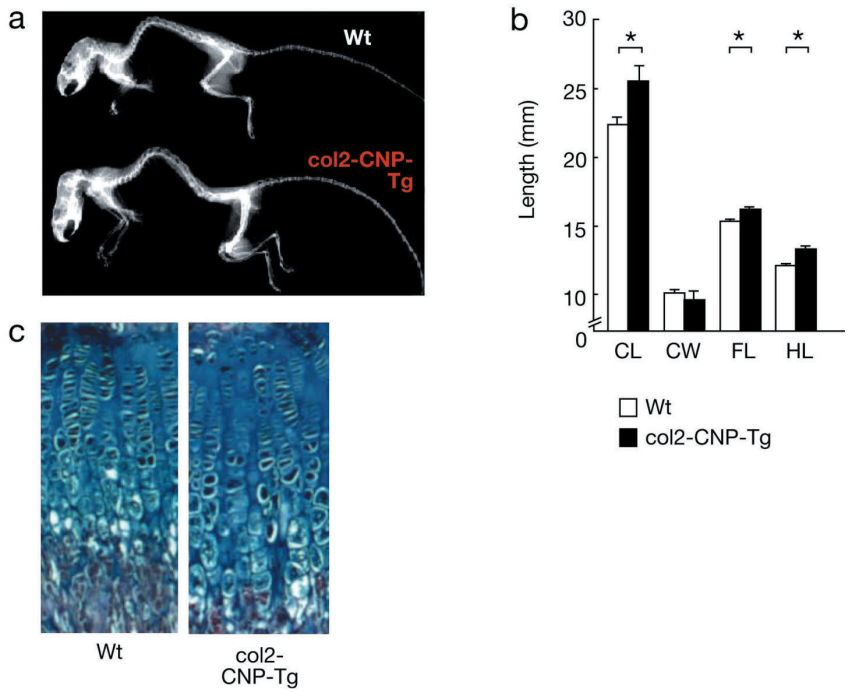

Fig. 2 Skeletal phenotype of col2-CNP-Tg mouse. a. Soft x-ray picture of wild-type (Wt) and col2-CNP-Tg mice. b. The length of each bone from wild-type (Wt) or col2-CNP-Tg mouse. CL: naso-occipital length of the calvarium, CW: maximal interparietal distance of the calvarium. FL: femoral length, HL: Humeral 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.

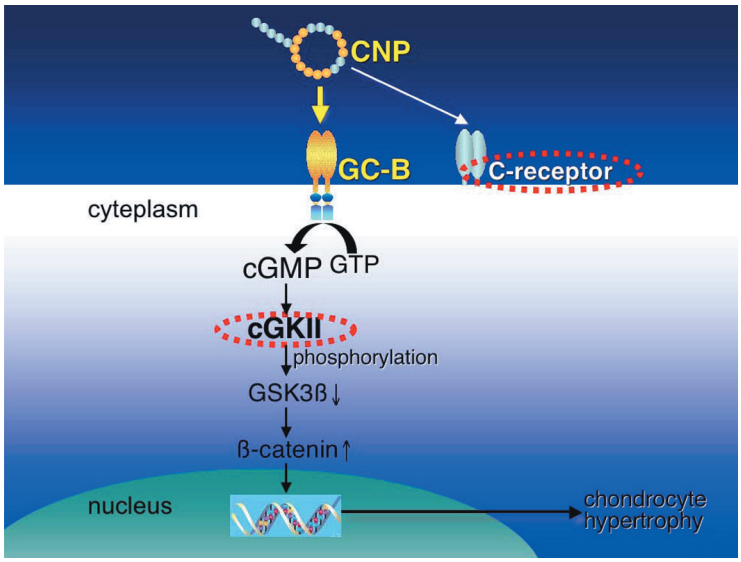

Fig. 3 Schematic representation of the pathway of the CNP/GC-B system.

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-

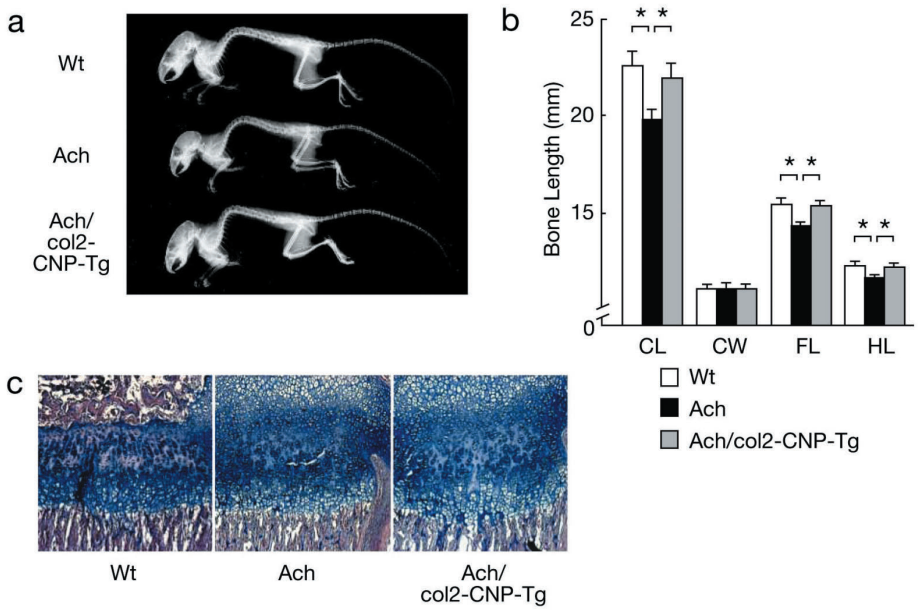

Fig. 4 Skeletal rescue of Ach mouse by targeted overexpression of CNP in cartilage. a. Soft x-ray picture of wild-type mouse (Wt), Ach mouse, and Ach mouse with targeted overexpression of CNP in cartilage (Ach/col2-CNP-Tg). b. The length of each bone from wild-type, Ach, and Ach/col2-CNP-Tg mouse at 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.

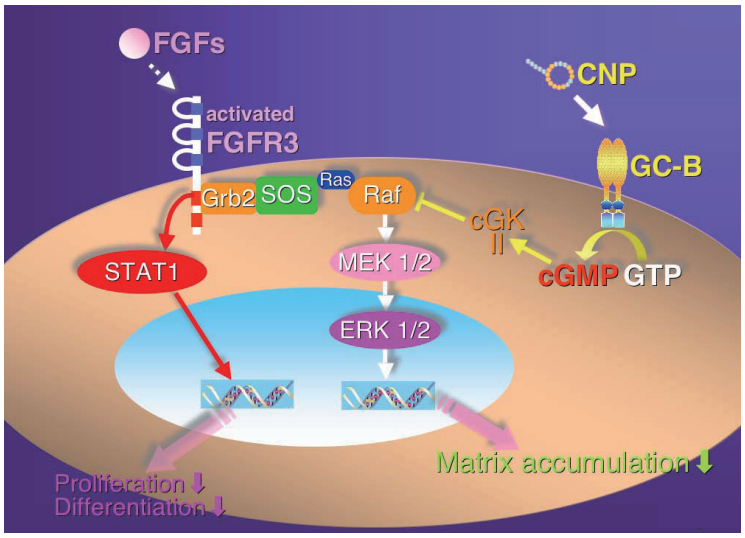

Fig. 5 Schema of the intracellular interaction between the FGF signaling and the CNP/GC-B system.

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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