Is C-type natriuretic peptide regulated by a feedback loop? A study on systemic and local autoregulatory effect

Yohei Ueda1*, Keisho Hirota1, Ichiro Yamauchi1, Takuro Hakata1, Takafumi Yamashita2, Toshihito Fujii1, Akihiro Yasoda3*, Nobuya Inagaki1

1 Department of Diabetes, Endocrinology and Nutrition, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto, Japan, 2 Department of Metabolism and Endocrinology, Kishiwada City Hospital, Kishiwada-shi, Osaka, Japan, 3 Clinical Research Center, National Hospital Organization Kyoto Medical Center, Fukakusa, Fushimi-ku, Kyoto, Japan

* yohueda@kuhp.kyoto-u.ac.jp (YU); ayasoda@kuhp.kyoto-u.ac.jp (AY)

Abstract

C-type natriuretic peptide (CNP) is a pivotal enhancer of endochondral bone growth and is expected to be a therapeutic reagent for impaired skeletal growth. Although we showed that CNP stimulates bone growth as a local regulator in the growth plate via the autocrine/paracrine system, CNP is abundantly produced in other various tissues and its blood concentration is reported to correlate positively with growth velocity. Therefore we investigated the systemic regulation of CNP levels using rodent models. In order to examine whether CNP undergoes systemic feedback regulation, we investigated blood CNP levels and local CNP expression in various tissues, including cartilage, of 4-week-old rats after systemic administration of sufficient amounts of exogenous CNP (0.5 mg/kg/day) for 3 days. This CNP administration did not alter blood NT-proCNP levels in male rats but decreased mRNA expression only in tissue that included cartilage. Decrease in expression and blood NT-proCNP were greater in female rats. To analyze the existence of direct autoregulation of CNP in the periphery as an autocrine/paracrine system, we estimated the effect of exogenous supplementation of CNP on the expression of endogenous CNP itself in the growth plate cartilage of extracted fetal murine tibias and in ATDC5, a chondrogenic cell line. We found no alteration of endogenous CNP expression after incubation with adequate concentrations of exogenous CNP for 4 and 24 hours, which were chosen to observe primary and later transcriptional effects, respectively. These results indicate that CNP is not directly autoregulated but indirectly autoregulated in cartilage tissue. A feedback system is crucial for homeostatic regulation and further studies are needed to elucidate the regulatory system of CNP production and function.

Introduction

C-type natriuretic peptide (CNP) is the third member of the natriuretic peptide family along with atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) [1, 2]. ProCNP, a
progenitor of CNP, is produced in various tissues such as the brain, blood vessels, cartilage, and bone [3–9]. ProCNP is cleaved by furin into amino-terminal proCNP (NT-proCNP) and CNP-53 and CNP-53 is cleaved again to yield CNP-22 [10]. CNP-53 and CNP-22 are bioactive forms of CNP, which increase intracellular cGMP levels by binding to natriuretic peptide receptor B (NPR-B). Past studies using knockout rodents [9, 11, 12] and transgenic mice [13, 14] have revealed that CNP stimulates endochondral ossification and promotes linear growth. In humans several genetic diseases with impaired skeletal growth [15–21] or skeletal overgrowth [22–27] are linked to the CNP/NPR-B system. Thus CNP is a pivotal physiological stimulator of endochondral bone growth in humans and is now greatly anticipated to be a therapeutic reagent for the impaired skeletal growth observed in genetic disorders such as achondroplasia [13, 28, 29] or caused by drugs such as glucocorticoids [30, 31].

Although systemic administration of CNP was an approach that raises plasma CNP concentration, which was thought to be effective in the treatment of impaired skeletal growth, we previously generated and studied cartilage-specific CNP or NPR-B knockout mice and showed that CNP was a local regulator of the growth plate cartilage, promoting skeletal growth through an autocrine/paracrine mechanism rather than an endocrine mechanism [32]. Intracellular CNP/NPR-B activity could be related to circulating CNP level. Patients with homozygous loss of function mutations in NPR2, a coding gene for NPR-B, have high blood CNP levels [16, 21]. These alterations of CNP levels suggest the presence of feedback system of CNP. However, blood CNP levels of patients with heterozygous loss-of-function mutations in NPR2 have been reported to be within the normal range even though they exhibit short stature [16]. As for gain-of-function mutations in NPR2, while one clinical case with an NPR-B activating mutation was reported to have low circulating NT-proCNP levels [26], another case was reported not to have low NT-proCNP levels [27]. Regulation of CNP production have been not fully studied.

We think that it is important to comprehend the systemic regulatory system of CNP in case we use it or an analogue as a remedy. In this study, we intended to clarify whether there exists systemic or endocrinological regulation of blood CNP levels, i.e., feedback regulation of CNP, in in vivo experiments using wild-type rats. A feedback loop is key to hormonal regulation and analyses to confirm its existence would be very meaningful. Next we examined the effect of CNP on CNP production in various tissues that could affect plasma CNP concentrations. Finally, because the local effect of CNP is supposed to be vitally important in stimulating endochondral bone growth, we analyzed the existence of CNP autoregulation in chondrocytes using growth plates of tibial explants from fetal rats and a chondrogenic cell line, ATDC5.

Materials and methods

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. The animals were housed in a humidity and temperature-controlled environment with an automatic 12-hour light/dark cycle. They were fed a standard pelleted lab chow diet (CRF-1; Oriental Yeast Co., Ltd., Japan) and tap water ad libitum. Surgical procedures were performed under isoflurane-induced anesthesia and carbon dioxide was used for euthanasia. All efforts were made to minimize suffering.

Animal experiments on CNP knockout rats and wild-type rats were performed using F344/Stm rats deposited with the National Bio Resource Project for Rats in Japan (www.anim.med...
CNP knockout rats were generated in the F344/Stm background by the method previously reported [12] and we used the homozygous Δ774 mutant rats described in the report.

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 and we used SAP-Nppc-Tg line 17 described in the report 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 [14].

We used male rodents for the following experiments unless otherwise noted because CNP mRNA expression is stimulated by GnRH [33] and therefore CNP production in female rats would be variable during the estrus cycle.

Organ culture
On day 16.5 of pregnancy, rats were sacrificed and humeruses, radiuses, ulnas, femurs, tibias, and fibulas were resected from fetal rats. These bones were cultured for 24 hours 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). The tibias were incubated at 37˚C in a humidified atmosphere of 5% CO₂.

Cell culture
A chondrogenic cell line of ATDC5 cells [34] was purchased from RIKEN CELL BANK (No. RCB0565, RIKEN CELL BANK, Tsukuba, Japan). The cell line was authenticated by RIKEN CELL BANK. Cells were maintained with Dulbecco’s modified Eagle’s Medium/Nutrient Mixture F-12 Ham (No. D6421, SIGMA) containing 5% fetal bovine serum (No. 10270–106, Thermo Fisher Scientific), 100 U/ml penicillin, and 100 μg/ml streptomycin (No. 26253–84, Nacalai) at 37˚C in a humidified atmosphere of 5% CO₂. The medium was replaced every other day.

Administration of CNP
CNP-53 was purchased from PEPTIDE INSTITUTE, INC. (Ibaraki, Japan) as human CNP-53 (No. 4241-s, PEPTIDE INSTITUTE) and dissolved in water to a concentration of 50 μg/ml.

Rats were treated with 10 ml/kg/day of water as a vehicle or 0.5 mg/kg/day (10 ml/kg/day) of CNP-53. Water and CNP-53 were administered using osmotic pumps (ALZET® osmotic pump 1007D, Durect Corporation, CA, USA). Osmotic pump 1007D is designed to continuously release drugs for 7 days. Pumps containing 7 days of vehicle or CNP were surgically implanted subcutaneously. The dose of 0.5 mg/kg/day was determined in accordance with our previous report in which this dose of CNP-53 restored the dwarfism of CNP knockout rats [35].

Measurement of NT-proCNP
Four-week-old rats were treated with vehicle or CNP-53 in the same manner described above. After 3 days of administration, blood was collected and serum NT-proCNP levels, as the marker of CNP production, were measured using proCNP, N-terminal, EIA Kit (BI-20812, Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria). This kit is designed to measure human NT-proCNP by using a sandwich assay. Rat NT-proCNP shares a 92% homology to human NT-proCNP and the rat serum samples were verified for this kit according to a spike
recovery test (97%) and a linearity test (87%). The treatment duration was determined in accordance with our previous report in which 3 days of glucocorticoid treatment suppressed the CNP production in wild-type rats [30]. As a control group, NT-proCNP levels in 4-week-old rats without interventions were also measured.

**Measurement of cGMP**

ATDC5 cells were plated at $1.0 \times 10^5$ cells/well in 6-well tissue culture plates. ATDC5 cells were differentiated into proliferative chondrocytes by incubation with 10 μg/ml bovine insulin (No. 10516, SIGMA) for 14 days. Differentiated ATDC5 cells were incubated with vehicle or $10^{-7}$ M CNP-22 for 30 minutes. Culture media were acetylated and their cGMP level were measured using the cGMP ELISA kit (No. 581021, Cayman Chemical, Michigan, USA).

**Quantitative RT-PCR analysis**

Four-week-old rats were treated for 3 days as described above, and were then sacrificed and their lumbar vertebrae, blood vessels, cerebrums, and tibial diaphyses were resected. Total RNA was extracted from the tissues using RNeasy Lipid Tissue Mini Kit (No. 74084, QIAGEN). One μg of total RNA was reverse-transcribed using ReverTra Ace (No. TRT-101, TOYOBO Life Science, Osaka, Japan). Quantitative PCR analysis was performed using THUNDERBIRD SYBR qPCR MIX (No. QPS-201, TOYOBO Life Science) with the StepOne-Plus™ Real-time PCR System (Thermo Fisher Scientific, Massachusetts, USA).

For the analysis of cerebrums, results were normalized using rat β-glucuronidase (Gusb) and rat 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (Ywhaz), as reference genes. The reference genes for blood vessels were Ywhaz and rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The reference genes for both lumbar vertebrae and tibial diaphyses were rat hypoxanthine phosphoribosyltransferase 1 (Hprt1) and rat peptidylprolyl isomerase A (Ppia). These reference genes were selected based on past reports [36–38].

Four-week-old wild-type mice and SAP-Nppc-Tg mice were also sacrificed and their lumbar vertebrae were resected. RNA extraction, reverse-transcription, and quantitative PCR analysis were performed as described above. Results were normalized using Hprt and Ppia as reference genes.

For **ex vivo** experiments, limb bones from rat fetuses were incubated in 12-well tissue culture plates with vehicle or $10^{-7}$ M CNP-22 for 24 hours. The dose of CNP-22 was reported to be sufficient to enhance longitudinal growth of fetal tibias in organ culture experiment [39]. CNP-22 was purchased from Peptide Institute (4229-v, PEPTIDE INSTITUTE, Ibaraki, Japan). The culture duration was determined in accordance with our previous report including the organ culture experiment using tibias from fetal rats [35]. RNA extraction, reverse-transcription, and quantitative PCR analysis were performed as described above. Results were normalized using Hprt1 and Ppia as reference genes.

For **in vitro** experiments, ATDC5 cells were plated at $1.0 \times 10^5$ cells/well in 6-well tissue culture plates. ATDC5 cells were differentiated into proliferative chondrocytes by incubation with 10 μg/ml bovine insulin (No. 10516, SIGMA) for 14 days. Differentiated ATDC5 cells were incubated with vehicle or $10^{-7}$ M CNP-22 for 4 hours or 24 hours. RNA extraction, reverse-transcription, and quantitative PCR analysis were performed as described above. Because ATDC5 is a cell line from murine teratoma, primers were designed to detect murine Nppc, a coding gene for CNP. Results were normalized using murine hypoxanthine guanine phosphoribosyl transferase (Hprt) and murine peptidylprolyl isomerase A (Ppia) as reference genes.
The primers used in this analysis are listed in S1 Table. Nppc is a highly conserved gene and the sequences of primers for rat Nppc and murine Nppc were the same.

**Statistical analysis**

Data are expressed as means ± SE in the text and depicted as dot and box plots with cross marks at means and bold lines at medians. Boxes represent interquartile ranges and whiskers represent minima and maxima with the exception of outliers (1.5 times out of interquartile range). Statistical analysis of the data was performed using either Student’s t-test or one-way factorial analysis of variance (ANOVA), followed by the Tukey–Kramer test as a post hoc test. The differences were considered significant when P values were less than 0.05.

**Results**

**The effect of CNP on circulating NT-proCNP**

To explore whether CNP had a feedback effect or not, we investigated the serum NT-proCNP level as a marker of CNP production in wild-type rats under treatment with vehicle or CNP. We administered vehicle or 0.5 mg/kg/day of CNP for 3 days to 4-week-old male rats via osmotic pump. As depicted in Fig 1, we measured NT-proCNP levels and found that those in vehicle- and CNP-treated rats were comparable to control rats (88.81 ± 9.99 pmol/L, 75.47 ± 5.95 pmol/L, and 73.30 ± 7.04 pmol/L in control, vehicle-, and CNP-treated rats, respectively; n = 3, 6 and 7, in control, vehicle-, and CNP-treated rats, respectively). To exclude
the cross-reaction between exogenous CNP and endogenous CNP products we measured NT-proCNP levels in CNP knockout rats treated with vehicle or CNP. Circulating NT-proCNP levels were below the limit of detection in both vehicle- and CNP-treated CNP knockout mice. These findings suggest that CNP is not regulated by positive or negative feedback mechanisms.

The local effect of CNP on CNP production in CNP abundant tissues

Next we focused on local CNP production in wild-type rats and measured CNP expression in each tissue abundant in CNP, i.e., central nervous tissue, cardiovascular tissue, and skeletal tissue including cartilage. Fig 2A and 2B show that the mRNA expression of Nppc, a coding gene for CNP, is not changed by exogenous CNP in cerebrums (1.03 ± 0.08-fold in CNP-treated rats, P = 0.21, n = 7 for each group) or blood vessels (0.85 ± 0.07-fold in CNP-treated rats, P = 0.21, n = 7 and 6, in vehicle- and CNP-treated rats, respectively). As shown in Fig 2C, Nppc expression in lumbar vertebrae, including cartilaginous tissues, was significantly decreased by CNP administration (0.71 ± 0.10-fold in CNP-treated rats, P = 0.034, n = 13 for each group). For further investigation into skeletal tissues, we analyzed the change of Nppc expression in tibial diaphyses without growth plates; as depicted in Fig 2D, there was no significant change in Nppc expression (0.82 ± 0.11-fold in CNP-treated rats, P = 0.38, n = 7 for each group). These results suggest that Nppc expression in growth plates in vivo is reduced by systemic CNP administration. Data shown in Fig 2A–2D were normalized to one reference gene (Gusb, Ywhaz, Hprt1, and Hprt1, respectively) and the same results were obtained when another reference gene (Ywhaz, Gapdh, Ppia, and Ppia, respectively) was used (S1 Fig).

To analyze long term effect of CNP, CNP expression in lumbar vertebrae of 4-week-old mice and SAP-Nppc-Tg mice were also measured. Nppc expression in SAP-Nppc-Tg mice tended to be lower than that in wild-type mice but the CNP effect did not seem to be clearly enhanced by the long term overexposure (0.71 ± 0.11-fold in CNP-treated rats, P = 0.13, n = 7 for each group). Data was normalized to Hprt and the same result was obtained when Ppia was used as a reference gene (0.77 ± 0.08-fold in CNP-treated rats, P = 0.20, n = 7 for each group).

The effect of CNP on female rats

Although CNP production in female rats might be variable during the estrus cycle, investigation of female rats would be useful for further understanding of CNP regulation. As we got important results in blood NT-proCNP levels and Nppc expression in lumbar vertebrae in male rats, we also measured them in female rats. As shown in Fig 3A, NT-proCNP levels in female rats were measured and similar results were obtained (61.55 ± 3.7 pmol/L vs. 52.81 ± 2.23 pmol/mg, P = 0.079, n = 7 and 6, in vehicle- and CNP-treated rats, respectively), although NT-proCNP levels in CNP-treated rats tended to slightly decrease. NT-proCNP levels in female rats treated with vehicle tend to be lower than those in male rats (88.81 ± 9.99 pmol/L vs. 61.55 ± 3.7 pmol/L, P = 0.065, n = 6 and 7, in male and female rats, respectively). As shown in Fig 3B, Nppc expression in female lumbar vertebrae also decreased by CNP administration (0.59 ± 0.05-fold in CNP-treated rats, P = 0.007, n = 9 for each group). Data was normalized to Hprt1 and the same result was obtained when Ppia was used as a reference gene (S2 Fig).

The effect of CNP on tibial growth plate

For further study on growth plates, we performed an ex vivo organ culture experiment by using appendicular growth plates isolated from fetal rats. As depicted in Fig 4, incubation for 24 hours with CNP did not significantly change Nppc expression (1.03 ± 0.21-fold in CNP-
Fig 2. The effect of CNP administration on local Nppc expression. Nppc expression in (A) cerebrums, (B) blood vessels, (C) lumbar vertebrae, and (D) tibial diaphyses after 3 days of vehicle or CNP treatment were measured. The mRNA levels of Nppc were normalized by using rat (A) Gusb, (B)
treated limbs, \( P = 0.90, n = 3 \) for each group). Data shown in Fig 4 were normalized to \( Hprt1 \) and the same result was obtained when \( Ppia \) was used as a reference gene (S3 Fig).

CNP regulation in differentiated ATDC5 cells

Finally, we performed experiments using the chondrogenic cell line ATDC5, which mimics a differentiating growth plate chondrocyte, in order to evaluate the CNP effect on a single layer of chondrocytes. We confirmed that incubation with insulin for 14 days sufficiently differentiated ATDC5 into CNP-producing chondrocytes (Fig 5A). Incubation with CNP for 30 minutes successfully elevated cGMP levels. cGMP concentration in culture medium was almost undetectable \( (0.01 \pm 0.03 \text{ pmol/ml}) \) when incubated with vehicle and markedly elevated \( (85.36 \pm 7.61 \text{ pmol/ml}) \) when incubated with CNP \( (P = 0.0004, n = 3 \) for each group). However, as shown in Fig 5B, incubation with CNP for 4 hours did not increase \( Nppc \) expression in differentiated chondrocytes \( (0.90 \pm 0.05 \text{-fold in CNP-treated cells, } P = 0.29, n = 6 \) for each group). Fig 5C shows the \( Nppc \) expression after incubation with vehicle or CNP for 24 hours. CNP did not change \( Nppc \) expression in differentiated chondrocytes even after incubation for

---

**Fig 3.** The effect of CNP administration on circulating NT-proCNP and local \( Nppc \) expression in female rats. (A) serum NT-proCNP levels and (B) \( Nppc \) expression in lumbar vertebrae after 3 days of vehicle or CNP treatment was measured in female rats. The mRNA levels of \( Nppc \) were normalized to rat \( Hprt1 \) as a reference gene. The data are represented as fold-change versus the values for vehicle-treated rats. (A) \( n = 7 \) and 6, in vehicle-and CNP-treated rats, respectively. (B) \( n = 9 \) for each of the vehicle- and CNP-treated rats. \( *: P < 0.05. \)

https://doi.org/10.1371/journal.pone.0240023.g003
24 hours (0.99 ± 0.06-fold in CNP-treated cells, P = 0.95, n = 6 for each group). Data shown in Fig 4 were normalized to Hprt and the same result was obtained when Ppia was used as a reference gene (S4 Fig).

Fig 4. Nppc expression in organ culture experiment using tibias of fetal rats incubated with vehicle or CNP for 24 hours. The mRNA levels of Nppc were normalized to rat Hprt1 as the reference gene. The data are represented as fold-change versus the values for vehicle-treated tibias; n = 3 for each of the vehicle- and CNP-treated tibias. https://doi.org/10.1371/journal.pone.0240023.g004

24 hours (0.99 ± 0.06-fold in CNP-treated cells, P = 0.95, n = 6 for each group). Data shown in Fig 4 were normalized to Hprt and the same result was obtained when Ppia was used as a reference gene (S4 Fig).

Fig 5. In vitro experiments using the ATDC5 cell line. (A) The change in Nppc mRNA levels of ATDC5 cells differentiated by incubation with bovine insulin. (B, C) The effect of CNP on Nppc mRNA expression in differentiated ATDC5 cells incubated with vehicle or CNP for 4 hours (B) and for 24 hours (C). The mRNA levels were normalized to murine Hprt as the reference gene. The data are represented as fold-change versus the values for cells at day 0 (A) and for vehicle-treated cells (B, C). (A) n = 3 for each time point. *: P < 0.05 vs. day 0. (B, C) n = 6, each, in the vehicle- and CNP-treated cells. https://doi.org/10.1371/journal.pone.0240023.g005
The effect of CNP on local factors involved in the activity of CNP

Next we focused on factors which affects the action, production, and degradation of CNP. As these factors, expression of NPR-B, natriuretic peptide receptor C (NPR-C), osteocrin (OSTN), furin, neutral endopeptidase (NEP) were measured. Their coding genes are *Npr2*, *Npr3*, *Ostn*, *Furin*, and *Mme*, respectively. As shown in Fig 6, CNP administration did not changed the mRNA expression of *Npr2* (0.91 ± 0.07-fold in CNP-treated rats, *P* = 0.44, n = 13 for each group), *Npr3* (1.15 ± 0.13-fold in CNP-treated rats, *P* = 0.43, n = 13 for each group), *Ostn* (0.90 ± 0.08-fold in CNP-treated rats, *P* = 0.33, n = 13 for each group), *Furin* (1.07 ± 0.10-fold in CNP-treated rats, *P* = 0.49, n = 13 for each group), or *Mme* (1.00 ± 0.07-fold in

![Fig 6. The effect of CNP administration on local factors regulating CNP effect. The expression of (A) Npr2, (B) Npr3, (C) Ostn, (D) Furin, and (E) Mme in lumbar vertebrae after 3 days of vehicle or CNP treatment were measured. The mRNA levels were normalized to rat Hprt1 as a reference gene. The data are represented as fold-change versus the values for vehicle-treated rats. n = 13 for each of the vehicle- and CNP-treated rats.](https://doi.org/10.1371/journal.pone.0240023.g006)
CNP-treated rats, $P = 0.97$, $n = 13$ for each group) in lumbar vertebrae. Data were normalized to $Hprt1$ and the same results were obtained when $Ppia$ was used as a reference gene (S5 Fig).

Discussion
In the present study, we investigated the regulation of CNP levels in rodent models. To begin, we studied whether autoregulation of CNP exists in biological systems, i.e., endocrinological feedback regulation. Past studies on circulating CNP levels include clinical studies [40–44] and in vivo studies using animals [30, 45–48]. Although there are a few clues about CNP feedback in previous clinical reports, it is controversial. Here, we used a rat injection model. As for the setting of CNP stimulation, we adopted continuous subcutaneous injection of CNP and decided to estimate the CNP levels three days after the start of administration. We administered exogenous CNP-53 at the same dose that previously restored the dwarfism of CNP knockout rats [35] and found that sufficient exogenous CNP injection had little if any effect on circulating NT-proCNP levels. The result in male rats is not supportive for the existence of feedback regulation of CNP, but local production and content of CNP would be important for understanding CNP action and regulation [32]. Indeed, CNP is produced in various tissues and not all circulating CNP is derived from the growth plate [49]. Thus circulating CNP levels would be reflected by the sum of secreted CNP from these tissues. Furthermore, It is important to keep in mind that differential responses of CNP signaling pathways likely occur across tissues, resulting in different regulatory effects by CNP; for example, CNP suppresses ERK phosphorylation in chondrocytes [13, 31], whereas it promotes ERK phosphorylation in pituitary cells [50]. Therefore, if CNP is positively regulated in some tissues and negatively regulated in other tissues, the sum of circulating NT-proCNP might not be changed.

Next we focused on local CNP production by elevating the levels of CNP and measured $Nppc$ mRNA in each tissue in wild-type rats. We evaluated local CNP production in each tissue known to be abundant in CNP, for example, nervous system tissue, blood vessels, and growth plate cartilage, and found that $Nppc$ expression was decreased only in growth plate cartilage. We used lumbar vertebrae as an approximation for growth plate tissue as it was difficult to obtain pure growth plate tissue in vivo. However, lumbar vertebrae consist of tissues other than the growth plate such as bone and bone marrow, and it is possible that a change in $Nppc$ expression in lumbar vertebrae is due to the change in these tissues other than growth plate tissue. Therefore, we performed additional experiments using tibial diaphyses, which is bone tissue without a growth plate, and found $Nppc$ expression was not changed in bone and bone marrow. These findings support the hypothesis that exogenous CNP decreases CNP expression only in growth plate cartilage.

In these experiments, both direct and indirect effect of CNP administration is observed. For further study in pure growth plate tissue as the main target site of the CNP/NPR-B signaling system, we performed an ex vivo study using fetal murine tibias to analyze direct effect of CNP on growth plate cartilage and showed that CNP production in the growth plate in explanted tibias are not changed by CNP treatment. However, the organ culture experiments include whole growth plates and might be inappropriate for the investigation into the direct effect of CNP on the CNP abundant layer of the growth plate. As an in vitro study, we investigated the direct effects of exogenous CNP on CNP expression in moderately differentiated ATDC5 cells, mimicking growth plate chondrocytes in the proliferative and prehypertrophic layers. Although in vitro studies on CNP regulation to date have been performed by using a pituitary cell line [33, 51] and granulosa cells [52], investigation into CNP regulation in cartilaginous cells is essential for the understanding of the growth effect of CNP. We examined two different treatment periods, 4 hours and 24 hours, targeting the first transcription and
secondary or later transcriptional effect, respectively. We failed to find significant alteration of CNP expression by CNP.

CNP is produced by cleavage of proCNP and furin is an essential enzyme for the processing of proCNP. [10]. Produced CNP binds to NPR-B and CNP/NPR-B signaling is activated through cGMP production. This signaling system is affected by NPR-C, a clearance receptor of CNP, and OSTN, a specific ligand of NPR-C. CNP coupled with NPR-C is degraded and OSTN increases CNP activity by acting as an intrinsic ligand of NPR-C [53]. NEP is also related to rapid degradation of CNP [54]. For further understanding of the regulation of the CNP/NPR-B signaling system, we performed investigation on the local expressions of these factors involved in the regulation of CNP production, action, and degradation, which resulted no change in any of Npr2, Npr3, Ostn, Furin, and Mme. Intracellular cGMP produced by CNP/NPR-B signaling is degraded by phosphodiesterase (PDE) [55]. Thus changes in PDE would be alter CNP/NPR2 activity. In ATDC5 cells, when Nppc expression is raised through differentiation, cGMP hydrolytic activities of PDE1 and PDE5 are enhanced [56] and CNP is reported to enhance PDE2 which degrades cGMP and suppress PDE3 which degrades cAMP mainly [57]. Alteration of PDE activity due to CNP might be related to regulation of CNP/NPR-B activity.

Because CNP is actually produced in various tissues, Nppc alteration only in growth plates might not clearly change blood NT-proCNP level. It is reported that about 40% of circulating CNP is produced in endothelial cells [49], but the value of 40% is the result at 16 weeks of age when growth plates would be thinned. In younger rats whose growth plates are thicker, Nppc expression in growth plates would have more impact on the circulating NT-proCNP. Actually in humans, NT-proCNP levels in children are about 2–3 times higher than those in adults [58, 59]. Thus our experiments using 4-week-old rats are likely to emphasize the effect of local Nppc change on circulating NT-proCNP. Nevertheless, decreased Nppc expression in lumbar vertebrae did not clearly reduce circulating NT-proCNP levels in our experiments. Although growth plates might be the major source of circulating NT-proCNP in young rats, 30–40% decrease in cartilaginous Nppc expression might not be sufficient to significantly change circulating NT-proCNP levels because of the NT-proCNP from other tissues. Thus it might be difficult to decrease blood NT-proCNP level by enhancing growth plate CNP/NPR-B activity and consequent negative feedback on CNP gene expression in growth plates.

As for the effect of elevated circulating CNP levels in genetic disorders of growth in humans, the report on the loss-of-function mutation in NPR3 is important. Deterioration of NPR3 causes the elevation of circulating CNP levels via the reduction of CNP degradation but it is not expected to affect NT-proCNP metabolism. Blood CNP and NT-proCNP levels are reported in two pediatric patients: CNP levels are elevated (1.6 SDS and 6.4 SDS, respectively) and blood NT-proCNP levels are slightly decreased (-0.7 SDS and -0.05 SDS, respectively) [60]. Drastic enhancement of CNP/NPR-B activity reduces NT-proCNP more clearly. Some patients with gain-of-function mutations of NPR2 are reported to have decreased NT-proCNP levels [23, 26] but other patients are reported to have normal NT-proCNP levels [23, 27]. In reference 23, a child patient has lower NT-proCNP level and adult patients have normal NT-proCNP levels. The data support our results that CNP suppresses CNP production mainly in growth plate. However, the data should be interpreted carefully because of the lack of age-matched reference range [23].

We found slight decrease in blood NT-proCNP level due to CNP administration to female mice. The change in female mice might be due to viability related to estrus cycle, or difference in susceptibility to CNP. Blood NT-proCNP levels in female rats tend to be lower than those in male rats. If endogenous CNP levels in female rats are lower than those of male rats, exogenous CNP administration might be more effective in female rats. While further studies are needed.
on sex differences in CNP production and efficacy, the greater reduction of Nppc expression in growth plate of females (which would reflect activation of CNP/NPR-B by exogenous CNP) may connect with the reduction of circulating NT-proCNP levels. From the clinical and experimental data raised above, strong and sufficient activation of CNP/NPR-B would be necessary to reduce circulating NT-proCNP levels significantly.

As for the suppression of CNP/NPR-B signaling in genetic disorders of growth, drastic change in CNP/NPR-B signaling, for example, ablation of NPR-B, and subsequent critical change in Nppc expression in growth plate could be expected to change the circulating NT-proCNP level. NT-proCNP is increased in patients with biallelic loss-of-function mutations in NPR2 [16, 21] while not increased in patients with monoallelic loss-of-function mutations in NPR2 [16]. However, it should be noted that SDS of plasma NT-proCNP in patients with monoallelic loss-of-function mutations in NPR2 have not been specifically addressed in children. As another condition with suppressed NPR-B activity, achondroplasia have been reported. Achondroplasia is a genetic disease which causes short stature due to a gain-of-function mutation in fibroblast growth factor receptor 3 (FGFR3). A recent study indicates that FGFR3 suppresses NPR-B activity through dephosphorylation of the juxtamembrane domain of NPR-B [61] and the impairment of NPR-B activity is related to the impaired skeletal growth shown in achondroplasia [62]. On the other hand circulating NT-proCNP levels are elevated in patients with achondroplasia [44], which suggests that FGFR-3 induced suppression of CNP/NPR-B signaling would increase Nppc expression.

In the present study, Nppc expression in growth plate cartilage was found to be decreased by CNP treatment through experiments in vivo but not decreased through experiments ex vivo and in vitro. These findings suggest that CNP is regulated by feedback system although surrounding tissues are required for the feedback regulation. For example, Indian hedgehog and parathyroid hormone-related protein (PTHrP), involved in hypertrophic differentiation of chondrocytes, form a negative feedback loop and PTHrp-positive chondrocytes in growth plate are reported to migrate to the bone tissue outside the growth plate [63]. As for the feedback regulation of CNP, cells outside the growth plate might secrete some factor that suppresses CNP production. The mechanism of regulating CNP is not well studied and this is an important theme in the future study. The result that Nppc expressions were not changed in tissues other than growth plate cartilage suggests the main site of CNP function would be cartilage tissue. Endothelial cells are abundant in CNP and its effect on endothelial cells are reported to be exerted through NPR-C instead of NPR-B [64]. Feedback regulation of CNP would require activation of NPR-B and its downstream. The exact pathway of feedback is unclear and further studies are needed.

In conclusion, we showed that exogenous CNP decreased endogenous CNP production in growth plate cartilage through in vivo experiments. The regulatory effect seems to be indirect because exogenous CNP did not change CNP production through ex vivo and in vitro experiments. Regulation of the CNP effect is enigmatic because CNP transcription is not well studied and the CNP effect would be controlled not only by CNP production but also by CNP clearance. More studies are required to further elucidate the regulatory system of CNP production and function.

Supporting information

S1 Fig. The effect of CNP administration on local Nppc expression normalized by another reference gene. Nppc expression in (A) cerebrums, (B) blood vessels, (C) lumbar vertebrae, and (D) tibial bones after 3 days of vehicle or CNP treatment were measured. The mRNA levels of Nppc were normalized to rat (A) Ywhaz, (B) Gapdh, and (C, D) Ppia as the reference.
genes, respectively. The data are represented as fold-change versus the values for vehicle-treated rats. (A, D) n = 7 for each of the vehicle- and CNP-treated rats. (B) n = 7 and 6, in vehicle- and CNP-treated rats, respectively. (C) n = 13 for each of the vehicle- and CNP-treated rats. *: P < 0.05.

S2 Fig. The effect of CNP administration on local Nppc expression in female rats normalized by another reference gene. Nppc expression in lumbar vertebrae after 3 days of vehicle or CNP treatment was measured in female rats. The mRNA levels of Nppc were normalized to rat Ppia as a reference gene. The data are represented as fold-change versus the values for vehicle-treated rats. n = 9 for each of the vehicle- and CNP-treated rats.

S3 Fig. Nppc expression in the organ culture experiment using tibias of fetal rats incubated with vehicle or CNP for 24 hours, normalized to another reference gene. The mRNA levels of Nppc were normalized to rat Ppia as the reference gene. The data are represented as fold-change versus the values for vehicle-treated tibias; n = 3 in each of the vehicle- and CNP-treated tibias.

S4 Fig. In vitro experiments of the ATDC5 cell line using another reference gene. (A) The change of Nppc mRNA levels of ATDC5 cells differentiated by incubation with bovine insulin. (B, C) The effect of CNP on Nppc mRNA expression in differentiated ATDC5 cells incubated with vehicle or CNP for 4 hours (B) and 24 hours (C). The mRNA levels were normalized to murine Ppia as the reference gene. The data are represented as fold-change versus the values for cells at day 0 (A) and for vehicle-treated cells (B, C). (A) n = 3 for each time point. *: P < 0.05 vs. day 0. (B, C) n = 6, in each of the vehicle- and CNP-treated cells.

S5 Fig. The effect of CNP administration on local factors regulating CNP effect normalized by another reference gene. The expression of (A) Npr2, (B) Npr3, (C) Ostn, (D) Furin, and (E) Mme in lumbar vertebrae after 3 days of vehicle or CNP treatment were measured. The mRNA levels were normalized to rat Ppia as a reference gene. The data are represented as fold-change versus the values for vehicle-treated rats. n = 13 for each of the vehicle- and CNP-treated rats.

S1 Table. Primer sequences used for quantitative RT-PCR analysis. Nppc is a highly conserved gene and the primer sequences of rat Nppc and murine Nppc were the same.

Author Contributions

Conceptualization: Yohei Ueda, Akihiro Yasoda.

Data curation: Yohei Ueda, Keisho Hirota, Ichiro Yamauchi, Takafumi Yamashita.

Formal analysis: Yohei Ueda, Keisho Hirota, Ichiro Yamauchi, Takafumi Yamashita.

Funding acquisition: Akihiro Yasoda, Nobuya Inagaki.

Investigation: Yohei Ueda, Ichiro Yamauchi, Takuro Hakata, Akihiro Yasoda.

Methodology: Yohei Ueda, Keisho Hirota, Ichiro Yamauchi, Toshihito Fujii.
Project administration: Akihiro Yasoda.
Resources: Toshihito Fujii.
Software: Yohei Ueda.
Supervision: Nobuya Inagaki.
Validation: Keisho Hirota, Ichiro Yamauchi, Takafumi Yamashita, Toshihito Fujii.
Visualization: Yohei Ueda.
Writing – original draft: Yohei Ueda, Ichiro Yamauchi, Akihiro Yasoda.
Writing – review & editing: Keisho Hirota, Takuro Hakata, Takafumi Yamashita, Toshihito Fujii, Akihiro Yasoda, Nobuya Inagaki.

References
1. Sudoh T, Minamino N, Kangawa K, Matsu H. C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. Biochem Biophys Res Commun. 1990; 168(2):863–70. https://doi.org/10.1016/0006-291x(90)92401-k PMID: 2139780
2. Nakao K, Ogawa Y, Suga S, Imura H. Molecular biology and biochemistry of the natriuretic peptide system. I: Natriuretic peptides. J Hypertens. 1992; 10(9):907–12. PMID: 1328371
3. Ueda S, Minamino N, Aburaya M, Kangawa K, Matsu H. Distribution and characterization of immunoreactive porcine C-type natriuretic peptide. Biochem Biophys Res Commun. 1991; 175(3):759–67. https://doi.org/10.1016/0006-291x(91)91631-l PMID: 1827257
4. Komatsu Y, Nakao K, Suga S, Ogawa Y, Mukoyama M, Arai H, et al. C-type natriuretic peptide (CNP) in rats and humans. Endocrinology. 1991; 129(2):1104–6. https://doi.org/10.1210/endo-129-2-1104 PMID: 1855454
5. Yandle TG, Fisher S, Charles C, Espiner EA, Richards AM. The ovine hypothalamus and pituitary have markedly different distribution of C-type natriuretic peptide forms. Peptides. 1993; 14(4):713–6. https://doi.org/10.1016/0196-9781(93)90102-m PMID: 8234014
6. Minamino N, Aburaya M, Kojima M, Miyamoto K, Kangawa K, Matsu H. Distribution of C-type natriuretic peptide and its messenger RNA in rat central nervous system and peripheral tissue. Biochem Biophys Res Commun. 1993; 197(1):326–35. https://doi.org/10.1016/bbrc.1993.2479 PMID: 8250942
7. Suga S, Itoh H, Komatsu Y, Ogawa Y, Hama N, Yoshimasa T, et al. Cytokine-induced C-type natriuretic peptide (CNP) secretion from vascular endothelial cells—evidence for CNP as a novel autocrine/paracrine regulator from endothelial cells. Endocrinology. 1993; 133(6):3038–41. https://doi.org/10.1210/endo.133.6.824333 PMID: 824333
8. Komatsu Y, Itoh H, Suga S, Ogawa Y, Kishimoto I, et al. Regulation of endothelial production of C-type natriuretic peptide in coculture with vascular smooth muscle cells. Role of the vascular natriuretic peptide system in vascular growth inhibition. Circ Res. 1996; 78(4):606–14. https://doi.org/10.1161/01.res.78.4.606 PMID: 8635218
9. Chusho H, Tamura N, Ogawa Y, Yasuda A, Suda M, Miyazawa T, et al. Dwarfism and early death in mice lacking C-type natriuretic peptide. Proc Natl Acad Sci U S A. 2001; 98(7):4016–21. https://doi.org/10.1073/pnas.071389098 PMID: 11259675
10. Wu C, Wu F, Pan J, Morser J, Wu Q. Furin-mediated processing of Pro-C-type natriuretic peptide. J Biol Chem. 2003; 278(28):25847–52. https://doi.org/10.1074/jbc.M301223200 PMID: 12736257
11. Tamura N, Dochittlet LK, Hammer RE, Shelton JM, Richardson JA, Garbers DL. Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. Proc Natl Acad Sci U S A. 2004; 101(49):17300–5. https://doi.org/10.1073/pnas.0407894101 PMID: 15572448
12. Fuji T, Hirota K, Yasoda A, Takizawa A, Morozumi N, Nakamura R, et al. Rats deficient C-type natriuretic peptide suffer from impaired skeletal growth without early death. PLoS One. 2018; 13(3): e0194812. https://doi.org/10.1371/journal.pone.0194812 PMID: 29566041
13. Yasoda A, Komatsu Y, Chusho H, Miyazawa T, Ozasa A, Miura M, et al. Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. Nat Med. 2004; 10(1):80–6. https://doi.org/10.1038/nm971 PMID: 14702637
14. Kake T, Kitamura H, Adachi Y, Yoshioka T, Watanabe T, Matsushita H, et al. Chronically elevated plasma C-type natriuretic peptide level stimulates skeletal growth in transgenic mice. Am J Physiol
32. Nakao K, Osawa K, Yasoda A, Yamanaka S, Fujii T, Kondo E, et al. The Local CNP/GC-B system in growth plate is responsible for physiological endochondral bone growth. Sci Rep. 2015; 5:10554. https://doi.org/10.1038/srep10554 PMID: 26014585

33. Thompson IR, Chand AN, Jonas KC, Burin JM, Steinhelper ME, Wheeler-Jones CP, et al. Molecular characterisation and functional interrogation of a local natriuretic peptide system in rodent pituitaries, alphaT3-1 and LbetaT2 gonadotroph cells. J Endocrinol. 2009; 203(2):215–29. https://doi.org/10.1677/JOE-09-0189 PMID: 1966697

34. Atsumi T, Miwa Y, Kimata K, Ikawa Y. A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Differ Dev. 1990; 30(2):109–16. https://doi.org/10.1016/0922-3371(90)90079-c PMID: 2201423

35. Hirota K, Furuya M, Morozumi N, Yoshikyo K, Yotsumoto T, Jindo T, et al. Exogenous C-type natriuretic peptide restores normal growth and prevents early growth plate closure in its deficient rats. PLoS One. 2018; 13(9):e0204172. https://doi.org/10.1371/journal.pone.0204172 PMID: 30235256

36. Xu D, Liu A, Wang X, Zhang M, Zhang Z, Tan Z, et al. Identifying suitable reference genes for developing and injured mouse CNS tissues. Dev Neurobiol. 2018; 78(1):39–50. https://doi.org/10.1002/dneu.22558 PMID: 29134774

37. Royer C, Begin AG, Plawinski L, Levesque L, Durrieu MC, Laroche G. Validation of reference genes for real-time PCR of cord blood mononuclear cells, differentiating endothelial progenitor cells, and mature endothelial cells. Exp Cell Res. 2018; 370(2):389–98. PMID: 31016063

38. Zhai Z, Yao Y, Wang Y. Importance of suitable reference gene selection for quantitative RT-PCR during ATDC5 cells chondrocyte differentiation. PLoS One. 2013; 8(5):e64786. https://doi.org/10.1371/journal.pone.0064786 PMID: 23705012

39. Yasoda A, Ogawa Y, Suda M, Tamura N, Mori K, Sakuma Y, et al. Natriuretic peptide regulation of endochondral ossification. Evidence for possible roles of the C-type natriuretic peptide/guanylyl cyclase-B pathway. J Biol Chem. 1998; 273(19):11695–700. https://doi.org/10.1074/jbc.273.19.11695 PMID: 9565590

40. Hama N, Itch H, Shirakami G, Suga S, Komatsu Y, Yoshimasa T, et al. Detection of C-type natriuretic peptide in human circulation and marked increase of plasma CNP level in septic shock patients. Biochem Biophys Res Commun. 1994; 198(3):1177–82. https://doi.org/10.1006/bbrc.1994.1165 PMID: 8117275

41. Bahrami S, Pelinka L, Khadem A, Maitzen S, Hawa G, van Griensven M, et al. Circulating NT-proCNP predicts sepsis in multiple-traumatized patients without traumatic brain injury. Crit Care Med. 2010; 38(1):161–6. https://doi.org/10.1097/CCM.0b013e3181b78a06 PMID: 19730251

42. Turkdogan KA, Zorlu A, Engin A, Guven FM, Polat MM, Turgut OO, et al. C-type natriuretic peptide is associated with the severity of Crimean-Congo hemorrhagic fever. Int J Infect Dis. 2012; 16(4):371–8. https://doi.org/10.1016/j.ijid.2012.04.009 PMID: 22695238

43. Espiner EA, Dalynmpie-Alford JC, Prickett TC, Alamri Y, Anderson TJ. C-type natriuretic peptide in Parkinson’s disease: reduced secretion and response to deprenyl. J Neural Transm (Vienna). 2014; 121(4):371–8. https://doi.org/10.1007/s00702-013-1123-9 PMID: 24306276

44. Olney RC, Prickett TC, Espiner EA, Mackenzie WG, Duker AL, Ditro C, et al. C-type natriuretic peptide plasma levels are elevated in subjects with achondroplasia, hypochondroplasia, and thanatophoric dysplasia. J Clin Endocrinol Metab. 2015; 100(2):E355–9. https://doi.org/10.1210/jc.2014-2814 PMID: 25387261

45. Prickett TC, Lynn AM, Barrell GK, Darlow BA, Cameron VA, Espiner EA, et al. Amino-terminal proCNP: a putative marker of cartilage activity in postnatal growth. Pediatr Res. 2005; 58(2):334–40. https://doi.org/10.1203/01.PDR.0000169964.66260.4B PMID: 1606435

46. Prickett TC, Barrell GK, Welbly M, Yandle TG, Richards AM, Espiner EA. Response of plasma CNP forms to acute anabolic and catabolic interventions in growing lambs. Am J Physiol Endocrinol Metab. 2007; 292(5):E1395–400. https://doi.org/10.1152/ajpendo.00469.2006 PMID: 17227962

47. Prickett TC, Barrell GK, Welbly M, Yandle TG, Richards AM, Espiner EA. Effect of sex steroids on plasma C-type natriuretic peptide forms: stimulation by oestadiol in lambs and adult sheep. J Endocrinol. 2008; 199(3):481–7. https://doi.org/10.1677/JOE-08-0267 PMID: 18784186

48. Prickett TC, Bothwell JC, Yandle TG, Richards AM, Espiner EA. Pharmacodynamic responses of plasma and tissue C-type natriuretic peptide to GH: correlation with linear growth in GH-deficient rats. J Endocrinol. 2012; 212(2):217–25. https://doi.org/10.1530/JOE-11-0387 PMID: 22087017

49. Nakao K, Kuwahara K, Nishiki T, Nakagawa Y, Kinoshita H, Minami T, et al. Endothelium-Derived C-Type Natriuretic Peptide Contributes to Blood Pressure Regulation by Maintaining Endothelial Integrity. Hypertension. 2017; 69(2):286–96. https://doi.org/10.1161/HYPERTENSIONAHA.116.08219 PMID: 28049696
50. Jonas KC, Melrose T, Thompson IR, Baxter GF, Lipscomb VJ, Niessen SJ, et al. Natriuretic peptide activation of extracellular regulated kinase 1/2 (ERK1/2) pathway by particulate guanylyl cyclases in GH3 somatolactotropes. Cell Tissue Res. 2017; 369(3):567–78. https://doi.org/10.1007/s00441-017-2624-x PMID: 28451751

51. Mirczuk SM, Lessey AJ, Catterick AR, Perrett RM, Scudder CJ, Read JE, et al. Regulation and Function of C-Type Natriuretic Peptide (CNP) in Gonadotrope-Derived Cell Lines. Cells. 2019; 8(9).

52. Dos Santos JT, De Cesaro MP, Ferst JG, Pereira Dau AM, da Rosa PRA, Pasqual BM, et al. Luteinizing hormone upregulates NPPC and downregulates NPR3 mRNA abundance in bovine granulosa cells through activation of the EGF receptor. Theriogenology. 2018; 119:28–34. https://doi.org/10.1016/j.theriogenology.2018.06.012 PMID: 29960164

53. Kanai Y, Yasoda A, Mori KP, Watanabe-Takano H, Nagai-Okatani C, Yamashita Y, et al. Circulating osteocrine stimulates bone growth by limiting C-type natriuretic peptide clearance. J Clin Invest. 2017; 127(11):4136–47. https://doi.org/10.1172/JCI94912 PMID: 28990933

54. Kenny AJ, Bourne A, Ingram J. Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. Biochem J. 1993; 291 (Pt 1):83–8.

55. Hamad AM, Range S, Holland E, Knox AJ. Regulation of cGMP by soluble and particulate guanylyl cyclases in cultured human airway smooth muscle. Am J Physiol. 1997; 273(4):L807–13. https://doi.org/10.1152/ajplung.1997.273.4.L807 PMID: 9357856

56. Fujishige K, Koteru J, Yanaka N, Akatsuka H, Omori K. Alteration of cGMP metabolism during chondrogenic differentiation of chondroprogenitor-like EC cells, ATDC5. Biochim Biophys Acta. 1999; 1452 (3):219–27. https://doi.org/10.1016/s0167-4889(99)00141-x PMID: 10590311

57. Vandecasteele G, Verde I, Rücker-Martin C, Donzeau-Gouge P, Fischmeister R. Cyclic GMP regulation of the L-type Ca(2+) channel current in human atrial myocytes. J Physiol. 2001; 533(Pt 2):329–40. https://doi.org/10.1111/j.1469-7793.2001.0329a.x PMID: 11389195

58. Prickett TC, Charles CJ, Yandle TG, Richards AM, Espiner EA. Skeletal contributions to plasma CNP forms: evidence from regional sampling in growing lambs. Peptides. 2009; 30(12):2343–7. https://doi.org/10.1016/j.peptides.2009.07.023 PMID: 19664666

59. Espiner E, Prickett T, Olney R. Plasma C-Type Natriuretic Peptide: Emerging Applications in Disorders of Skeletal Growth. Hormone research in paediatrics. 2018; 90(6):345–57. https://doi.org/10.1159/000496544 PMID: 30844819

60. Boudin E, de Jong TR, Prickett TCR, Lapauw B, Toye K, Van Hoof V, et al. Bi-allelic Loss-of-Function Mutations in the NPR-C Receptor Result in Enhanced Growth and Connective Tissue Abnormalities. Am J Hum Genet. 2018; 103(2):288–95. https://doi.org/10.1016/j.ajhg.2018.06.007 PMID: 30032985

61. Robinson JW, Egbert JR, Davydova J, Schmidt H, Jaffe LA, Potter LR. Dephosphorylation is the mechanism of fibroblast growth factor inhibition of guanylyl cyclase-B. Cell Signal. 2017; 40:222–9. https://doi.org/10.1016/j.cellsig.2017.09.021 PMID: 28964968

62. Shuhailbar LC, Robinson JW, Vigne G, Shuhailbar NP, Egbert JR, Baena V, et al. Dephosphorylation of the NPR2 guanylyl cyclase contributes to inhibition of bone growth by fibroblast growth factor. Elife. 2017; 6.

63. Mizuhashi K, Ono W, Matsushita Y, Sakagami N, Takahashi A, Saunders TL, et al. Resting zone of the growth plate houses a unique class of skeletal stem cells. Nature. 2018; 563(7730):254–8. https://doi.org/10.1038/s41586-018-0662-5 PMID: 30401834

64. Bull CK, Aubdool AA, Moyes AJ, Lewis S, Drayton JP, Tang O, et al. Endothelial C-Type Natriuretic Peptide Is a Critical Regulator of Angiogenesis and Vascular Remodeling. Circulation. 2019; 139 (13):1612–28. https://doi.org/10.1161/CIRCULATIONAHA.118.036344 PMID: 30586761