

# **C-type natriuretic peptide restores growth impairment under enzyme replacement in mice with mucopolysaccharidosis VII**

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Keywords: C-type natriuretic peptide, mucopolysaccharidosis VII, enzyme replacement therapy, gene delivery, growth impairment, short stature

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Funding:

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (JP25860765 and JP17K09881).

Disclosure summary:

The authors have nothing to disclose.

## Abstract

Growth impairment in mucopolysaccharidoses (MPSs) is an unresolved issue as it is resistant to enzyme replacement therapy (ERT) and growth hormone therapy. C-type natriuretic peptide (CNP) is a promising agent that has growth promoting effects. Here, we investigate the effects of CNP on growth impairment of MPSs using *Gusb<sup>mps-2J</sup>* mice, a model for MPS type VII, with combination therapy of CNP and ERT by hydrodynamic gene delivery. Although monotherapies were not sufficient to restore short statures of treated mice, combination therapy resulted in successful restoration. The synergistic effects of CNP and ERT were not only observed in skeletal growth but also in growth plates. ERT reduced cell swelling in the resting zone and increased cell number by accelerating proliferation or inhibiting apoptosis. CNP thickened the proliferative and hypertrophic zones. Regarding changes in the bone, ERT restored bone sclerosis through decreased bone formation and increased bone resorption, and CNP did not adversely affect this process. In addition, improvement of joint deformation by ERT was suggested by analyses of joint spaces and articular cartilage. CNP additively provided restoration of the short stature of MPS VII mice in combination with ERT which improved abnormalities of growth plates and bone metabolism.

## Introduction

Mucopolysaccharidoses (MPSs), a group of lysosomal storage disorders, are caused by deficiencies of enzymes that degrade glycosaminoglycans (GAGs). Accumulation of undegraded or partially degraded GAGs in the lysosome injures multiple organs resulting in sequelae such as mental retardation, corneal clouding, deafness, joint stiffness, obstructive airway disease, valvular disease, organomegaly, and growth impairment (1). MPSs are classified into 7 types (I, II, III, IV, VI, VII, and IX) according to the deficient enzymes. MPS I (Hurler syndrome) and MPS II (Hunter syndrome) involve accumulation of dermatan sulfate (DS) and heparan sulfate (HS), MPS III involves HS, MPS IV involves keratan sulfate, MPS VI involves DS and chondroitin sulfate (CS), and MPS IX involves hyaluronan (1). In MPS VII (Sly syndrome), caused by genetic mutations in the *GUSB* gene leading to  $\beta$ -glucuronidase (*GUSB*) deficiency, accumulated GAGs consist of CS as well as DS and HS observed in MPS I and II (2-4).

Enzyme replacement therapy (ERT) was developed to specifically treat several types of MPSs; more recently, hematopoietic stem cell transplantation can be considered. In MPS I, II, IVA and VI, ERT reduced urinary GAG excretion and improved some clinical manifestations of the deficiency (5-9). However, growth impairment seemed to remain: for example, a double-blind, randomized placebo-controlled study of ERT in patients with MPS IVA showed no significant improvement in their standing heights and growth rates (9).

Short stature and skeletal dysplasia resulting from growth impairment need to be treated in patients with MPSs (10). MPSs are characterized by disproportionate dwarfism with short neck and trunk accompanied by a prominent forehead and pectus carinatum. The short stature is severe as: the mean final height was  $125.63 \pm 9.09$  cm in MPS II (11), and  $119.3 \pm 22.6$  and  $113.5 \pm 23.1$  cm in male and female patients with MPS IVA, respectively

(12). Furthermore, kyphoscoliosis and platyspondyly cause pain and neurodegeneration, and tracheal obstruction is a fatal risk. Motor function is disturbed by the rigidity of joints. Thus, quality of life and activity of daily living decrease proportionally with growth impairment in patients with MPSs (10).

Growth hormone (GH) is a current therapeutic agent in clinical settings to treat short stature (13). GH therapy has been applied for both patients with and without disorders of the GH axis, such as Turner syndrome, Prader-Willi syndrome, and Noonan syndrome (14). Polgreen *et al.* reported that there was no difference in growth velocity between children with MPS I or MPS II who were treated with GH and untreated children (15). Insulin-like growth factor 1 (IGF1) is applied for some GH resistant disorders (16-18). However, a thesis demonstrated that their IGF1 treatment did not promote proliferation of chondrocytes in MPS VII model mice (19). Growth impairment of MPSs might be resistant to GH and IGF1 therapy, so it is worth developing alternative agents.

C-type natriuretic peptide (CNP), a member of the natriuretic peptide family, has growth promoting effects, verified by skeletal phenotypes of transgenic mice with overexpressed CNP (20), CNP knockout mice (21), and through exogenous administration (22). Skeletal growth mediated by CNP is also successfully observed in achondroplasia, one type of skeletal dysplasia (20). In addition, glucocorticoid-induced growth retardation, resistant to GH therapy (23), was restored by CNP overexpression (24). Therefore, CNP could potentially be used to treat the short stature of MPSs involving skeletal dysplasia and GH resistance.

In the present study, we investigated effects of CNP on growth impairment of MPSs. Combination therapy of CNP and GUSB, as CNP add-on therapy to ERT, was performed for MPS VII model mice which have an accumulation of more kinds of GAGs than MPS I or II.

Mechanistic insights were provided by histological analyses of growth plates and articular cartilage and by analyses of bone formation and metabolism. As a result, CNP added on to ERT caused independent changes in growth plates and bones, leading to their synergistic effects on skeletal growth.

## Materials and Methods

### *Animals*

*Gusb*<sup>mps-2J</sup> mice, the MPS VII model mice, were purchased from the Jackson Laboratory (Bar Harbor, ME) (25). Spontaneous insertion of a 5.4kb intracisternal A particle transposon into the 3' end of intron 8 of *Gusb* gene depletes its expression. In the present experiments, we only used homozygous mice that had less than 1% of the normal levels of GUSB activity (26). The genetic background of this strain is C57BL/6 (25). The C57BL/6 mice used in the present study were generated by mating *Gusb*<sup>mps-2J</sup> heterozygous mice. The mice were genotyped by PCR at 4 weeks old using following primers; 5'-CAAGGGTATTTACGACCACTTGTACTCTGT-3' (Forward for *Gusb*<sup>mps-2J</sup>); 5'-CCAGAACAAGATCTCAATCGGGTAGCACAG-3' (Forward for C57BL/6); 5'-TAGCATGCACAGGCAAGGCCCTGAACTGGA-3' (Reverse for both *Gusb*<sup>mps-2J</sup> and C57BL/6). All animals were housed at 23 °C in a 14:10-hour light/dark cycle with free access to water and standard chow in the same manner as our previous study (27).

### *Plasmids construction and preparation*

The coding sequence of the human *NPPC* gene (28) encoding preproCNP was obtained by PCR technique from cDNA of TSA201 cells as a template and was cloned into the pMD20-T vector (Takara Bio, Shiga, Japan): the forward primer 5'-

AATAGGATCCGCCACCATGCATCTCTCCCAGCTG-3' containing a *BamH I* linker and Kozak sequence, and the reverse primer 5'-AATAGAGCTCCTAACATCCCAGGCCGCTC-3' containing a *SacI* linker were used. Coding sequence of murine *Gusb* gene (29) was obtained from hypothalamic cDNA of C57BL/6 mice and cloned into the pMD20-T vector: the forward primer 5'-AATAGCTAGCGCCACCATGTCCCTAAAATGGAGTG-3' containing a *Nhe I* linker and Kozak sequence, and the reverse primer 5'-AATACTCGAGTTAGAACGTGAACGGTCTG-3' containing a *XhoI* linker were used. These cloned pMD20-T vectors were treated with *BamHI/SacI* for human *NPPC* and *NheI/XhoI* for murine *Gusb*, and the obtained DNA inserts were subcloned into the *BamHI/SacI*-digested and *NheI/XhoI*-digested pLIVE-Empty vector (Mirus Bio, Madison, WI), respectively. Thus, we constructed pLIVE-*NPPC* vector and pLIVE-*Gusb* vector, and verified these by sequencing.

We prepared a large amount of these three pLIVE plasmid vectors using NucleoBond Xtra Midi Plus kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Concentrations of plasmid DNA were measured with a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

#### *Hydrodynamic tail-vein injection*

Six-week-old C57BL/6 and *Gusb*<sup>mps-2J</sup>/*Gusb*<sup>mps-2J</sup> homozygous mice were injected with plasmid DNA using a hydrodynamic-based procedure as previously described (30,31). At first, a solution of plasmid DNA and sterilized saline (Otsuka Pharmaceutical Factory, Tokyo, Japan) was prepared as a total volume equivalent to 10% of mouse weight. The solution was rapidly injected within 10 seconds into the tail-vein of mice using a 27-gauge needle (Terumo Corporation, Tokyo, Japan). Amounts of vectors were fixed; pLIVE-*NPPC*

vector was 25 µg and pLIVE-*Gusb* vector was 100 µg. pLIVE-Empty vector was mixed to align with the total amount of injected vectors. Four weeks after injection (at 10 weeks old), we sacrificed and collected blood and tissues as described below.

#### *Growth curve and skeletal analysis*

Body weight, naso-anal length, and naso-tail length were measured under anesthesia with isoflurane every week from 6 to 10 weeks old. The anteroposterior and transverse diameters of the cranial bone, the lengths of the upper limb (humerus and radius) and lower limb (femur and tibia), and the lengths of lumbar vertebrae were measured on soft x-ray film at 10 weeks old. The lengths of the upper and lower limbs were presented as averages of their right and left values. Lumbar vertebrae were defined as the span from the first to the fifth lumbar vertebra. Shoulder joint spaces were also measured on soft x-ray film at 10 weeks old.

#### *Measurement of serum levels of NT-proCNP, osteocalcin, and TRAP5b*

To obtain serum, blood samples of the treated mice were collected in microtubes and left undisturbed for 15 minutes at room temperature. Subsequently, these samples were left on ice for 30 minutes, and centrifuged at 1,500 *g* for 20 minutes at 4 °C. The supernatants were collected and stored at –80 °C until use. Serum levels of N-terminal proCNP (NT-proCNP) (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria) (32,33), osteocalcin (Alfa Aesar, Ward Hill, MA) (34), and tartrate-resistant acid phosphatase form 5b (TRAP5b) (Immunodiagnostic Systems Ltd., Boldon, UK) (35) were measured using the each commercial assay kit based on the enzyme immunoassay, according to the manufacturer's instructions. Regarding NT-proCNP measurements were performed using the

first generation kit (32) in an examination of C57BL/6 mice and the second generation kit (33) in a dilution series of C57BL/6 mice and an examination of MPS mice.

#### *Glycosaminoglycan assay*

We mechanically homogenized 100 mg of frozen samples of liver in 400  $\mu$ L of RIPA buffer (Nacalai Tesque, Kyoto, Japan) using TissueLyser LT (QIAGEN, Venlo, Netherlands), and left these on ice for 30 minutes. The supernatants were centrifuged at 10,000  $g$  for 10 minutes at 4 °C. We collected supernatants of these samples in microtubes, added 400  $\mu$ L of methanol (Nacalai Tesque), and then vortexed the resultant mixture. Mixtures of these and 800  $\mu$ L of chloroform (Nacalai Tesque) were vortexed and centrifuged at 15,000  $g$  for 2 minutes at 4 °C. The upper water/methanol phase was collected in new microtubes and dried using a Speed Vac™ DNA 110 Concentrator (Savant, Farmingdale, NY). The pellets were suspended in 1 ml of papain extraction reagent; 50 ml of a 0.2 M sodium phosphate buffer, pH 6.4, containing 250  $\mu$ g papain crystallized suspension (Sigma Aldrich, St. Louis, MO), 400 mg of sodium acetate, 200 mg of EDTA disodium salt, and 40 mg of cysteine hydrochloride. After digestion at 65 °C overnight and being centrifuged at 10,000  $g$  for 10 minutes, we discarded the supernatants and added dimethylmethylene blue dye solution supplied in Blyscan Sulfated Glycosaminoglycan Assay (Biocolor Ltd., County Antrim, UK). Concentrations of GAG were determined by measuring the absorbance at 650 nm using an iMark microplate reader (Bio-Rad, Hercules, CA).

### *Histological analysis*

The tibias and femurs of the treated mice were fixed in 10% formaldehyde neutral buffer solution (Nacalai Tesque) and decalcified with 10% EDTA for 2 weeks before they were embedded in paraffin and sagittally sliced to evaluate their growth plates. Alcian blue staining were performed for morphologic analyses. Ki-67 immunohistochemistry was performed using a monoclonal antibody against Ki-67 SP6 (Nichirei, Tokyo, Japan) (36). Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed using Apop Tag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA) (37). We counted Ki-67 positive cells and TUNEL positive cells over the whole area of the growth plate of tibias.

### *RNA extraction and quantitative RT-PCR analysis*

Treated 10-week-old male mice were sacrificed and resected their lumbar vertebrae as our previous study (38). Total RNA of lumbar vertebrae was extracted using RNeasy Lipid Tissue Mini Kit (QIAGEN) and then was reverse-transcribed using ReverTra Ace (TOYOBO Life Science, Osaka, Japan). Quantitative PCR analyses using 20 ng of total RNA were performed using THUNDERBIRD SYBR qPCR MIX (TOYOBO Life Science) with the StepOnePlus™ Real-time PCR System (Thermo Fisher Scientific). Results were normalized using *Ppia* and *Hprt* as reference genes and relative mRNA expression of target genes was evaluated using a comparative threshold cycle method. The primers used in this analysis were as follows. *Nppc* forward: 5'-AGCGGTCTGG GATGTTAGTG-3', *Nppc* reverse: 5'-CCTCCCCTCCCCAAATAATA-3'; *Npr2* forward: 5'-GAGGCCCTGCTGTACCAAATTCTA-3', *Npr2* reverse: 5'-GAAGCCCACGATGTCAGTGAAG-3'; *Ppia* forward: 5'-

CGCGTCTCCTTCGAGCTGTTTG-3', *Ppia* reverse: 5'-  
TGTAAGTCACCACCCTGGCACAT-3'; *Hprt* forward: 5'-  
GGACCTCTCGAAGTGTTGGATAC-3', and *Hprt* reverse: 5'-  
GCTCATCTTAGGCTTTGTATTTGGCT-3'.

### *Bone microstructural analysis*

Micro-CT (SMX-100CTSV3; Shimadzu Co., Kyoto, Japan) scans of femurs of treated mice fixed in 10% formaldehyde neutral buffer solution were performed. The scan area was set as the 1.0 mm region from 0.2 mm proximal of the distal growth plates of the femurs. Bone mineral densities (BMDs) were calculated using a three-dimensional trabecular bone analysis software (TRI/3D-BON, Ratoc System Engineering Co., Tokyo, Japan).

### *Statistics*

All results are expressed as the mean  $\pm$  SEM. Statistical analysis of the data was performed using Student's t-test, one-way ANOVA followed by the Tukey-Kramer test or Dunnett test, and two-way factorial ANOVA. JMP pro version 11.3.0 (SAS Institute Inc., Cary, NC) was used for all statistical analyses. Statistical significance was defined as  $p < 0.05$ .

### *Study approval*

All experimental procedures involving animals were approved by the Animal Research Committee, Kyoto University Graduate School of Medicine (Permit number:

MedKyo07598). Care of animals and all animal experiments were conducted in accordance with the institutional guidelines of the Kyoto University Graduate School of Medicine and the United States NIH *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011).

## Results

### *Establishment of a CNP administration model using hydrodynamic gene delivery.*

In MPS VII, developing treatments include gene therapy that provides sustained GUSB expression. Richard *et al.* reported on the hydrodynamic gene delivery of murine *Gusb*, which successfully reduced GAG accumulation in MPS VII model mice (39). Based on their research, we planned to perform the combination therapy of CNP and GUSB by hydrodynamic gene delivery (30).

To establish a CNP administration model that promotes skeletal growth using hydrodynamic gene delivery, we introduced 25  $\mu$ g of pLIVE-NPPC vector by hydrodynamic tail-vein injection to 6-week-old C57BL/6 male mice. Mean serum NT-proCNP levels were  $3.5 \pm 1.4$  pmol/L ( $n = 3$ ),  $913.8 \pm 45.4$  pmol/L ( $n = 3$ ),  $582.3 \pm 44.6$  pmol/L ( $n = 3$ ) in 6- (without injection), 7- (1 week after injection), and 10-week-old (4 weeks after injection), respectively (Fig. 1A). NT-proCNP levels were significantly higher in 7- ( $p < 0.001$ ) and 10-week-old mice ( $p < 0.001$ ), compared to 6-week-old mice. We examined a dilution series of a sample of 10-week-old mice with injection and validated that NT-proCNP levels were specifically measured: NT-proCNP levels were  $103.5 \pm 3.0$  pmol/L,  $50.0 \pm 4.8$  pmol/L,  $21.8 \pm 1.2$  pmol/L, and  $11.6 \pm 3.5$  pmol/L in a serial 2-fold dilution series. These observations

suggest that hydrodynamic gene delivery can guarantee prolonged CNP supply for at least 4 weeks.

In addition, we verified that the increased CNP levels actually resulted in promotion of skeletal growth. Likewise, hydrodynamic tail-vein injection to 6-week-old C57BL/6 male mice was performed using 25  $\mu$ g of pLIVE-*NPPC* vector (WT-CNP mice,  $n = 5$ ) and 25  $\mu$ g of pLIVE-Empty vector (WT-Empty mice,  $n = 6$ ), and compared with non-injected mice (WT mice,  $n = 5$ ). In gross appearances (Fig. 1B) and soft x-ray images (Fig. 1C), WT-CNP mice showed markedly longer body length than WT-Empty and WT mice. The growth curves confirmed that naso-anal lengths of WT-CNP mice ( $10.6 \pm 0.1$  cm at 10 weeks old) were significantly longer than those of WT-Empty ( $9.5 \pm 0.0$  cm) and WT ( $9.5 \pm 0.0$  cm) mice (Fig. 1D). Naso-tail lengths showed similar elongation in WT-CNP mice, while there were no differences among body weights of the three groups (Fig. 1E and 1F). The injection protocol had no significant effect on any of the parameters we measured as there were no differences in naso-anal lengths, naso-tail lengths, or body weights (Fig. 1D-1F) between WT-Empty and WT mice. Analyses of soft x-ray images revealed that bone lengths of the front limb, hind limb, and lumbar vertebrae in WT-CNP mice were significantly longer than those in WT-Empty and WT mice ( $p < 0.001$  in all significant changes) (Fig. 1G).

Furthermore, we injected various amounts of pLIVE-*NPPC* vectors (1, 5, and 25  $\mu$ g). Mice injected with pLIVE-*NPPC* vectors showed elongation of naso-anal and naso-tail lengths in a dose dependent manner (Fig. 1H-1K). We adopted 25  $\mu$ g as the optimal amount of pLIVE-*NPPC* vector for the following study because of its ability to sufficiently and stably promote skeletal growth.

### *Combination therapy of CNP and GUSB and its skeletal effects in MPS VII mice.*

The  $Gusb^{mps-2J}$  mouse is a spontaneously derived MPS VII model:  $Gusb^{mps-2J}/Gusb^{mps-2J}$  homozygous mice have less than 1% of the normal levels of GUSB activity of wild type mice (26). As previously reported (40), these homozygous mice showed the dwarf phenotype (Fig. 2A). We performed combination therapy of CNP and GUSB for 6-week-old  $Gusb^{mps-2J}/Gusb^{mps-2J}$  homozygous mice using hydrodynamic gene delivery (male:  $n = 8$ , female:  $n = 7$  in each group) and observed for 4 weeks. According to injected vectors, we divided the mice into 4 groups; 125  $\mu$ g of pLIVE-Empty vector (MPS-Empty mice); 100  $\mu$ g of pLIVE-*Gusb* vector and 25  $\mu$ g of pLIVE-Empty vector (MPS-GUSB mice); 25  $\mu$ g of pLIVE-*NPPC* vector and 100  $\mu$ g of pLIVE-Empty vector (MPS-CNP mice); and 100  $\mu$ g of pLIVE-*Gusb* vector and 25  $\mu$ g of pLIVE-*NPPC* vector (MPS-GUSB/CNP mice). Numbers of mice (eight male mice in each group were tested) with more than 40 pmol/L of serum NT-proCNP levels at 4 weeks after injection were significantly larger in a MPS-CNP group ( $n = 4$ ,  $182.5 \pm 116.8$  pmol/L) and a MPS-GUSB/CNP group ( $n = 4$ ,  $189.0 \pm 152.0$  pmol/L) than in a MPS-Empty group ( $n = 0$ ,  $23.9 \pm 3.1$  pmol/L) and a MPS-GUSB group ( $n = 0$ ,  $21.0 \pm 2.1$  pmol/L) ( $p < 0.01$  in a chi-square test).

To validate whether introduction of pLIVE-*Gusb* vector supplied sufficient GUSB activity, we evaluated liver GAG contents in male mice at 4 weeks after injection (Fig. 2B). As a result, liver GAG content of MPS-GUSB mice ( $28.4 \pm 8.5$  ng/mg liver) was significantly less than that of MPS-Empty mice ( $238.3 \pm 11.2$  ng/mg liver) ( $p < 0.001$ ). MPS-GUSB/CNP mice ( $56.5 \pm 9.6$  ng/mg liver) also showed a significant decrease compared to MPS-Empty mice ( $p < 0.001$ ), but did not show a difference compared to MPS-GUSB mice. Likewise, liver GAG content of MPS-CNP mice ( $254.8 \pm 22.0$  ng/mg liver) was not different from MPS-Empty mice. These results were reproduced in female mice (Fig. 2C). Thus, liver GAG contents were reduced by GUSB overexpression and but not by CNP.

Representative skeletal appearances at 10 weeks old are shown as soft x-ray images (Fig. 2D). The MPS-GUSB/CNP mouse was markedly longer than the other mice. Analyses of growth curves revealed that only MPS-GUSB/CNP mice had significantly longer naso-anal lengths than MPS-Empty mice in both sexes (male:  $p < 0.05$ , female:  $p < 0.001$  at 10 weeks old) (Fig. 2E and 2F). Naso-tail lengths showed a similar tendency in male mice ( $p = 0.062$ , MPS-GUSB/CNP versus MPS-Empty at 10 weeks old) and significant elongation in female mice ( $p < 0.001$ , MPS-GUSB/CNP versus MPS-Empty at 10 weeks old) (Fig. 2G and 2H). Meanwhile, there were no significant differences in body weight (Fig. 2I and 2J).

Analyses of soft x-ray images at 10 weeks old revealed that the anteroposterior diameter of the cranium ( $p < 0.05$ ), and bone lengths of the front limb ( $p < 0.05$ ), hind limb ( $p < 0.05$ ), and lumbar vertebrae ( $p < 0.01$ ) in MPS-GUSB/CNP male mice were significantly longer than those in MPS-Empty male mice (Fig. 2K). Compared to MPS-GUSB mice, only bone lengths of the lumbar vertebrae were significantly longer than MPS-GUSB/CNP male mice ( $p < 0.01$ ). Regarding female mice, the hind limb and lumbar vertebrae of MPS-GUSB/CNP mice were significantly longer than those of MPS-Empty mice ( $p < 0.05$  and  $p < 0.01$ , respectively) (Fig. 2L). In the lumbar vertebrae, significant elongation of MPS-CNP mice compared to MPS-Empty mice and that of MPS-GUSB/CNP mice compared to MPS-GUSB mice were also observed ( $p < 0.05$  and  $p < 0.01$ , respectively).

We generated WT-Empty mice injected with 125  $\mu$ g of pLIVE-Empty vector as controls for treated MPS mice and used them for the following analyses. Naso-anal length in MPS-GUSB/CNP mice was comparable to WT-Empty mice at 10 weeks old in both sexes (Fig. 2M and 2N). Therefore, skeletal growth observed in MPS-GUSB/CNP mice was statistically and biologically significant.

### *Histological characteristics of growth plates of MPS VII mice.*

The combination of CNP and GUSB therapy was crucial to promote skeletal growth in  $\text{Gusb}^{\text{mps-2J}}/\text{Gusb}^{\text{mps-2J}}$  homozygous mice. To clarify mechanisms, we elucidated the histological structures of tibial growth plates of 10-week-old male mice. Representative images stained by alcian blue are shown in Fig. 3A. In particular, the resting zone in growth plates of MPS-Empty mice included swelling cells that were not observed in WT-Empty mice. Consequently, growth plates were thicker in MPS-Empty mice than in WT-Empty mice ( $p < 0.001$ ) (Fig. 3B). In addition, we evaluated the width of each of the resting, proliferative, and hypertrophic zones (Fig. 3C). The resting ( $p < 0.001$ ) and proliferative zones ( $p < 0.01$ ) were thicker in MPS-Empty mice, while the hypertrophic zone showed no difference.

Importantly, we found that the number of cells in the proliferative and hypertrophic zones was smaller in MPS-Empty mice compared to WT-Empty mice ( $p < 0.001$ ) (Fig. 3D). Therefore, we examined alterations in the proliferation and apoptosis of chondrocytes using Ki-67 staining and a TUNEL assay, respectively. Ki-67 positive cells were mostly located in the proliferative zone (Fig. 3E). The number of Ki-67-positive cells per growth plate of MPS-Empty mice ( $40.8 \pm 5.7$  per growth plate) was significantly smaller than that of WT-Empty mice ( $94.2 \pm 7.9$  per growth plate) ( $p < 0.001$ ) (Fig. 3F). Meanwhile, TUNEL-positive cells were predominantly located in the hypertrophic zone (Fig. 3G). The number of TUNEL-positive cells per growth plate of MPS-Empty mice ( $9.0 \pm 0.5$  per growth plate) was significantly larger than that of WT-Empty mice ( $3.3 \pm 0.3$  per growth plate) ( $p < 0.001$ ) (Fig. 3H).

### *Effects of combination therapy on histological structure of growth plates.*

Characteristic structural changes in the growth plates of  $Gusb^{mps-2J}/Gusb^{mps-2J}$  homozygous mice include thicker resting zones with swelling cells, thicker proliferative zones, impaired cell proliferation, and accelerated cell apoptosis. To verify whether these changes were restored by combination therapy of CNP and GUSB, we similarly analyzed tibial growth plates of treated 10-week-old male mice. These data were followed by statistical analyses using two-way ANOVA to assess the effects of CNP and GUSB independently. In alcian blue staining, the widths of the growth plates were not changed by either CNP or GUSB therapy (Fig. 4A and 4B). However, focusing on each zone of the growth plate (Fig. 4C-4E), GUSB therapy did not change the widths of the proliferative and hypertrophic zones, but significantly thinned the resting zone. Swelling cells in the resting zone were made smaller by GUSB therapy (Fig. 5). In contrast, CNP therapy significantly thickened the proliferative and hypertrophic zones, but did not affect the resting zone. There were no interactions between CNP and GUSB therapy regarding changes in the thickness of the growth plates and each zone.

The number of cells in the proliferative and hypertrophic zones were increased by both CNP and GUSB therapy, and this CNP effect was augmented by GUSB (Fig. 4F). The number of Ki-67-positive cells (MPS-Empty,  $40.8 \pm 5.7$  per growth plate; MPS-GUSB,  $80.4 \pm 3.4$  per growth plate; MPS-CNP,  $28.2 \pm 1.3$  per growth plate; MPS-GUSB/CNP,  $86.5 \pm 10.9$ ) was significantly increased by GUSB therapy ( $p < 0.001$ ) (Fig. 4G and 4H). Meanwhile, the number of TUNEL-positive cells (MPS-Empty,  $9.0 \pm 0.6$  per growth plate; MPS-GUSB,  $5.7 \pm 0.9$  per growth plate; MPS-CNP,  $10.3 \pm 1.2$  per growth plate; MPS-GUSB/CNP,  $5.0 \pm 1.2$  per growth plate) was significantly decreased by GUSB therapy ( $p < 0.01$ ) (Fig. 4I and 4J). Regarding the results of Ki-67 staining and the TUNEL assay, there was no change with CNP therapy and no interaction between CNP and GUSB therapy. In

addition, mRNA levels of murine *Nppc*, as a surrogate for endogenous CNP production, and those of *Npr2*, a gene coding natriuretic peptide receptor B (NPR-B), were evaluated using lumbar vertebrae of MPS-Empty and MPS-GUSB male mice. *Nppc* mRNA showed no changes, while *Npr2* mRNA significantly increased by GUSB therapy ( $p < 0.05$ ) (Fig. 4K). Data shown in Fig. 4K were normalized by *Ppia* and the similar result was obtained when normalized by *Hprt* (*Nppc*, not significant; *Npr2*,  $p < 0.01$ ).

Following these effects, cells in the hypertrophic zone presented drastic changes. As shown in Fig. 6, GUSB therapy restored the disruption of column formation in addition to increasing the number of cells, while CNP therapy enlarged cells. Consequently, combination therapy of CNP and GUSB enlarged hypertrophic chondrocytes organized into columns.

#### *Analysis of changes in bone structure by combination therapy.*

Several of the beneficial changes caused by combination therapy of CNP and GUSB were observed in growth plates. We examined how consequent bone structure was altered. Analyses of the femur of 10-week-old male mice using micro CT revealed that bone trabeculae in MPS-Empty mice were markedly increased compared to WT-Empty mice (Fig. 7A). BMD of MPS-Empty mice was significantly higher than that of WT-Empty mice (Fig. 7B). As for bone turnover markers, serum osteocalcin levels of MPS-Empty mice ( $48.8 \pm 5.7$  ng/ml,  $n = 8$ ) were significantly higher than those of WT-Empty mice ( $24.8 \pm 4.4$  ng/ml,  $n = 5$ ;  $p < 0.01$ ) (Fig. 7C). Conversely, serum TRAP5b levels were significantly lower in MPS-Empty mice ( $2.5 \pm 0.1$  U/L,  $n = 8$ ) than in WT-Empty mice ( $3.2 \pm 0.3$  U/L,  $n = 6$ ;  $p < 0.05$ ) (Fig. 7D). Thus, MPS-empty mice presented excessive bone sclerosis associated with increased bone formation and decreased bone absorption.

We evaluated the effects of combination therapy in a similar manner. Bone trabeculae were markedly reduced in MPS-GUSB and MPS-GUSB/CNP mice compared to MPS Empty mice, and were not changed in MPS-CNP mice (Fig. 7E). BMD was significantly decreased by GUSB therapy and not changed by CNP therapy (Fig. 7F). Serum osteocalcin levels were decreased by GUSB therapy and increased by CNP therapy (MPS-Empty,  $48.8 \pm 5.7$  ng/ml; MPS-GUSB,  $31.6 \pm 4.6$  ng/ml; MPS-CNP,  $77.4 \pm 14.1$  ng/ml; MPS-GUSB/CNP,  $46.1 \pm 5.1$  ng/ml; Fig. 7G). Serum TRAP5b levels were increased by GUSB therapy and were not changed by CNP therapy (MPS-Empty,  $2.5 \pm 0.1$  U/L; MPS-GUSB,  $3.8 \pm 0.2$  U/L; MPS-CNP,  $2.9 \pm 0.3$  U/L; MPS-GUSB/CNP,  $4.0 \pm 0.2$  U/L; Fig. 7H). There were also no interactions between CNP and GUSB therapy in these analyses.

#### *Restoration in articular cartilage by combination therapy.*

Considering the articular abnormality of MPSs, we evaluated shoulder joint space and histology of the distal femur cartilage. In MPS-Empty mice, shoulder joint space was wider than in WT-Empty mice (Fig. 8A and 8B). In the distal femur cartilage of MPS-Empty mice, swelling cells, strong staining by alcian blue, and thicker cartilage layer were observed (Fig. 8C and 8D).

By comparing treated MPS mice, shoulder joint space was shortened by GUSB therapy and not changed by CNP therapy (Fig. 8E and 8F). Histology of the distal femur cartilage showed that GUSB therapy decreased the number of swelling cells and weakened alcian blue staining (Fig. 8G). The width of the articular cartilage was reduced by GUSB therapy (Fig. 8H). There were no interactions between CNP and GUSB therapy in these analyses. Articular abnormality was improved by GUSB therapy and was not interfered with by CNP therapy.

Above results are summarized in Table 1 as focusing individual GUSB and CNP effects. Combination therapy of CNP and GUSB mostly presented combined changes derived from GUSB and CNP monotherapies.

## Discussion

In the present study, we demonstrated the effects of combination therapy of CNP and GUSB on growth impairment of *Gusb*<sup>mps-2J</sup> mice, a model for MPS VII. The combination therapy administered by hydrodynamic gene delivery resulted in further skeletal growth than the monotherapies. Histological analyses of tibial growth plates revealed that GAG accumulation caused swelling of resting zone cells and was accompanied by less proliferative and more apoptotic chondrocytes. Despite the restorative changes of growth plates, GUSB therapy did not promote skeletal growth. On the other hand, CNP therapy thickened the proliferative and hypertrophic zones and its growth-promoting effects were augmented by concomitant GUSB therapy. In addition, GUSB therapy improved abnormal bone structure and metabolism characterized by increased BMD with more osteogenesis and less resorption, and also improved articular abnormalities involving overgrowth of cartilage. CNP therapy did not interfere with these beneficial effects of GUSB therapy. Thus, combination therapy of CNP and GUSB was used to successfully treat growth impairment issues of *Gusb*<sup>mps-2J</sup> mice including short stature, bone sclerosis, and arthropathy.

One of reasons we used gene therapy to treat these mice was derived from some reports of MPS VII model mice: GUSB activity supplied by gene therapy including hydrodynamic methods and viral infection significantly reduced GAGs (39,41-44). Our assays provided enough enzymatic activities for treated *Gusb*<sup>mps-2J</sup> mice to reduce GAGs throughout the study period. In the present study, we established a novel CNP administration model by utilizing the versatility of hydrodynamic methods (45,46). Gene delivery by the

hydrodynamic method occurred predominantly in the liver (30). As we previously reported, serum amyloid P-CNP transgenic mice with secretion of CNP overexpressed in the liver also presented skeletal overgrowth (47,48). Although these two CNP administration models have the same source of CNP, hydrodynamic gene delivery was more suitable for the present study as we could achieve acquired and sustained CNP secretion for at least 4 weeks.

Regarding skeletal growth, our GUSB monotherapy failed to restore the short stature of *Gusb<sup>mps-2J</sup>* mice. There were several previous reports that evaluated the skeletal effects of ERT in MPS type VII model mice: e.g. ERT by gene therapy using lentiviral vectors initiated at 2 days as well as 7 weeks after birth did not change bone lengths including lumbar vertebra and femur (42); ERT by gene therapy using adeno-associated viral vectors initiated at 2 days after birth improved bone lengths of the ulnar, tibia, and humerus (49). The discrepancy among previous reports and our results may be due to the age of the mice when treatment was initiated or efficiency of gene delivery. No attempts to apply CNP for MPSs had been reported.

Through combination therapy of CNP and GUSB, for the first time, short stature of *Gusb<sup>mps-2J</sup>* mice was restored to that in wild type mice. Even though CNP monotherapy elongated some bones, simultaneous overexpression of CNP and GUSB showed synergistic skeletal effects that were suggested by the negative results of GUSB monotherapy. Furthermore, despite delayed initiation at 6 weeks of age, this combination therapy was effective. The growth rate of body lengths in C57BL/6 mice generally becomes much slower after 6 weeks of age, which was even seen in WT mice of the present study. CNP has a distinctive effect that increases the body length of mice after 6 weeks of age, as observed in serum amyloid P-CNP transgenic mice and in mice that received an exogenous administration of CNP (24,27,38).

Histological analyses of growth plates provided interesting and clear mechanistic insights of growth impairment of MPSs. Swelling of chondrocytes in the resting zone had been clarified, but its pathogenic roles remained unknown (50). To discuss this issue, the important finding of the present study was changes in the number of chondrocytes in the proliferative and hypertrophic zones. In *Gusb<sup>mps-2J</sup>* mice, Ki-67 positive cells, through the cell proliferation cycle, were decreased, and the number of chondrocytes per growth plate was decreased, as previously reported (51,52). GAG accumulation was implicated as GUSB overexpression restored these changes. To note, there was a report that the resting zone contains stem cells supplying clones of proliferative chondrocytes (53,54). Assuming that the function of swelling chondrocytes of the resting zone is impaired, proliferative chondrocytes recruited from the resting zone could decrease. ERT seemed to improve these harmful changes in the resting zone and consequently increase proliferation of growth plate chondrocytes.

Changes in the hypertrophic zone were also drastic. Firstly, CNP therapy thickened the proliferative and hypertrophic zones as observed in our previous studies (22,24,47,55). This change was important because thickness of the growth plate positively relates to skeletal growth (22,24). Secondly, GUSB therapy did not change the thickness of the proliferative and hypertrophic zones, but decreased the number of TUNEL-positive cells, through the apoptotic process. Simonaro *et al.* reported that apoptosis in articular cartilage increased in MPS VI rats (56). Essentially, apoptosis in the hypertrophic zone is a process of endochondral ossification (57). However, increases of chondrocyte apoptosis was observed in growth impairment by glucocorticoids (24) and TNF $\alpha$  (58) administration. Considering these relationships, the regulation of chondrocyte apoptosis by GUSB therapy might contribute to improve growth impairment in the present study. Thirdly, combination therapy of CNP and GUSB produced more hypertrophic chondrocytes that enlarged and organized into columns,

compared to the monotherapies. CNP monotherapy enlarged hypertrophic chondrocytes, but their number did not multiply and their columns were not in order. GUSB monotherapy increased hypertrophic chondrocytes that were in ordered columns, but their sizes were not changed. In endochondral bone formation, it is generally recognized that death is the fate of terminally differentiated hypertrophic chondrocytes. Yang *et al.* presented crucial evidence that hypertrophic chondrocytes can survive the cartilage-to-bone transition and become osteogenic cells (59). We believe that these beneficial changes in the hypertrophic zone by combination therapy of CNP and GUSB had a large impact on synergistic skeletal effects.

As for molecular mechanisms of these synergistic effects, we found alteration of NPR-B expression. CNP is a specific ligand of NPR-B. Because GUSB therapy increased *Npr2* mRNA in lumbar vertebrae that actually showed elongation by combination therapy, we considered GUSB therapy augmented responsiveness to CNP. In other words, MPS VII mice might have less sensitivity for CNP. Our examination regarding *Nppc* mRNA in lumbar vertebrae did not suggest a decrease in local CNP production. Recently, Bartolomeo *et al.* reported that autophagy induction restored decreases in thickness of hypertrophic zones of femoral growth plates in MPS VII model mice (60). We believe the restoration was not due to a direct effect of CNP that thickened hypertrophic zone as in the present study, because autophagy induction did not thicken hypertrophic zones in MPS VII model mice beyond wild type mice (60). Effects of GUSB therapy on growth plates might be mediated by changes in autophagy, but whether synergistic skeletal effects of GUSB and CNP associate with autophagy remains to be elucidated.

The bone structure of *Gusb*<sup>mps-2J</sup> mice was characterized as increased bone trabeculae and BMD. Previous reports of MPS VII model mice showed similar phenotypes (42,43,61). Decreased TRAP5b reflects deterioration of bone resorption as abnormal function of the osteoclasts in MPS VII model mice was reported (62). This increases bone trabeculae in MPS

VII model mice. In the present study, we verified GUSB therapy restored abnormalities in BMD and serum TRAP5b levels. Meanwhile, changes in serum osteocalcin levels might reflect several factors. Osteocalcin, a well-known marker of bone formation, is secreted from osteoblasts. Osteoblast proliferation certainly could elevate serum osteocalcin levels. In addition, increased bone trabeculae observed in MPS VII model mice might associate with elevated serum osteocalcin levels because osteocytes are derived from osteoblasts.

Although CNP therapy showed no relevant changes except for increasing serum osteocalcin levels, it was important that there seemed to be no interference with GUSB effects. Increases in serum osteocalcin levels by CNP therapy are consistent with previous reports (55,63). No changes in serum TRAP5b levels by CNP therapy might be due to some mechanisms related to bone abnormality in MPS VII, because the previous reports demonstrated serum TRAP5b level was elevated by CNP (55,63).

Rigidity of joints is also a severe problem of MPSs. As previously reported (64,65), joint space was wider and articular cartilage was thickened in MPS VII model mice. These changes indicated that rigidity of joints in MPSs was associated with inappropriate growth of articular cartilage. GUSB therapy succeeded in attenuating cartilaginous overgrowth. A concern that CNP has the effects of thickening cartilage layer in growth plates, shown even in the present study, was addressed by the result that CNP therapy did not thicken cartilage layer in the joint. Combination therapy of CNP and GUSB is expected to improve both short stature and motor function.

The present study proposes a novel therapy using CNP for short stature of MPSs. ERT for MPSs was not always initiated shortly after birth owing to various conditions. For example, diagnosis of MPSs is often delayed: It was reported that 43% of the patients with MPS VII were diagnosed at 3 years old or older (66). Our examination was designed as an

acquired-treatment model, that is to say, treatment duration was from 6 to 10 weeks old. Despite this delayed initiation, combination therapy of CNP and GUSB restored the short stature of *Gusb<sup>mps-2J</sup>* mice comparable to wild type mice. Moreover, in real-world settings, the introduction of ERT is generally prior to management of short stature, which is often diagnosed in the post-infant period. Considering these clinical conditions, it was quite valuable that effects of combination therapy of CNP and GUSB were observed in mice over 6 weeks of age after growth spurt.

Development of CNP or its analogue as agents for short stature is ongoing. A phase 2 clinical trial of a CNP analogue, vosoritide, has been performed for achondroplasia (67). Administration of CNP-53, a precursor of CNP, succeeded in promoting skeletal growth in rodents (38,55). Other clinical applications of CNP therapy are currently under investigation.

Whether our results of *Gusb<sup>mps-2J</sup>* mice could be applied for other MPSs is a very important matter. More kinds of GAGs accumulate in MPS VII than in MPS I and II (1) and the growth impairment of MPS II is milder than MPS VII (10). We believe there is the possibility that MPS I (mild phenotype) and MPS II as well as MPS VII are sensitive to CNP add-on therapy to ERT.

In conclusion, we demonstrated that combination therapy of CNP and GUSB using hydrodynamic gene delivery was effective for growth impairment in *Gusb<sup>mps-2J</sup>* mice, MPS VII model. Although their monotherapies were not sufficient to restore short statures, acquired combination therapy of CNP and GUSB showed synergistic effects on skeletal growth equivalent to levels seen in wild type mice. As for mechanisms, reducing cell swelling in the resting zone and accelerating cell proliferation in the proliferative zone through GUSB therapy, and thickening the proliferative and hypertrophic zones through CNP therapy were suggested from analyses of growth plates. Moreover, GUSB therapy restored

bone sclerosis accompanied with increased bone formation and decreased bone resorption, and improved inappropriate growth of articular cartilage, while CNP therapy did not adversely affect these processes. CNP added on to conventional ERT was suggested as a promising strategy for refractory short stature of MPSs.

Accepted Manuscript

## Acknowledgements

### *Author contributions*

TY conducted experiments and analyzed the data. TF designed the experiments and provided funding. IY designed the experiments and supported conducting experiments. YU, KH, and YK contributed to the discussion. AY and NI provided funding and supervised the entire study. TY, TF, and IY drafted the manuscript, and all authors reviewed, edited, and approved the manuscript.

### *Data and materials availability*

All data supporting the findings of this study are available within the article. The plasmid vectors constructed in this study are available from the corresponding author under a material transfer agreement with Kyoto University.

## References and notes

1. Stapleton M, Arunkumar N, Kubaski F, Mason RW, Tadao O, Tomatsu S. Clinical presentation and diagnosis of mucopolysaccharidoses. *Mol Genet Metab.* 2018;**125**(1-2):4-17.
2. Oshima A, Kyle JW, Miller RD, Hoffmann JW, Powell PP, Grubb JH, Sly WS, Tropak M, Guise KS, Gravel RA. Cloning, sequencing, and expression of cDNA for human beta-glucuronidase. *Proc Natl Acad Sci U S A.* 1987;**84**(3):685-689.
3. Tomatsu S, Montano AM, Dung VC, Grubb JH, Sly WS. Mutations and polymorphisms in GUSB gene in mucopolysaccharidosis VII (Sly Syndrome). *Hum Mutat.* 2009;**30**(4):511-519.
4. Sly WS, Quinton BA, McAlister WH, Rimoin DL. Beta glucuronidase deficiency: report of clinical, radiologic, and biochemical features of a new mucopolysaccharidosis. *The J Pediatr.* 1973;**82**(2):249-257.
5. Kakkis ED, Muenzer J, Tiller GE, Waber L, Belmont J, Passage M, Izykowski B, Phillips J, Doroshov R, Walot I, Hoft R, Neufeld EF. Enzyme-replacement therapy in mucopolysaccharidosis I. *N Engl J Med.* 2001;**344**(3):182-188.
6. Wraith JE, Clarke LA, Beck M, Kolodny EH, Pastores GM, Muenzer J, Rapoport DM, Berger KI, Swiedler SJ, Kakkis ED, Braakman T, Chadbourne E, Walton-Bowen K, Cox GF. Enzyme replacement therapy for mucopolysaccharidosis I: a randomized, double-blinded, placebo-controlled, multinational study of recombinant human alpha-L-iduronidase (laronidase). *J Pediatr.* 2004;**144**(5):581-588.
7. Muenzer J, Wraith JE, Beck M, Giugliani R, Harmatz P, Eng CM, Vellodi A, Martin R, Ramaswami U, Gucsavas-Calikoglu M, Vijayaraghavan S, Wendt S, Puga AC, Ulbrich B, Shinawi M, Cleary M, Piper D, Conway AM, Kimura A. A phase II/III clinical study of enzyme replacement therapy with idursulfase in

- mucopolysaccharidosis II (Hunter syndrome). *Genet Med.* 2006;**8**(8):465-473.
8. Harmatz P, Giugliani R, Schwartz I, Guffon N, Teles EL, Miranda MC, Wraith JE, Beck M, Arash L, Scarpa M, Yu ZF, Wittes J, Berger KI, Newman MS, Lowe AM, Kakkis E, Swiedler SJ. Enzyme replacement therapy for mucopolysaccharidosis VI: a phase 3, randomized, double-blind, placebo-controlled, multinational study of recombinant human N-acetylgalactosamine 4-sulfatase (recombinant human arylsulfatase B or rhASB) and follow-on, open-label extension study. *J Pediatr.* 2006;**148**(4):533-539.
  9. Hendriksz CJ, Burton B, Fleming TR, Harmatz P, Hughes D, Jones SA, Lin SP, Mengel E, Scarpa M, Valayannopoulos V, Giugliani R, Slasor P, Lounsbury D, Dummer W. Efficacy and safety of enzyme replacement therapy with BMN 110 (elosulfase alfa) for Morquio A syndrome (mucopolysaccharidosis IVA): a phase 3 randomised placebo-controlled study. *J Inherit Metab Dis.* 2014;**37**(6):979-990.
  10. Melbouci M, Mason RW, Suzuki Y, Fukao T, Oorii T, Tomatsu S. Growth impairment in mucopolysaccharidoses. *Mol Genet Metab.* 2018;**124**(1):1-10.
  11. Patel P, Suzuki Y, Maeda M, Yasuda E, Shimada T, Oorii KE, Oorii T, Tomatsu S. Growth charts for patients with Hunter syndrome. *Mol Genet Metab Rep.* 2014;**1**:5-18.
  12. S. Tomatsu AMM, H. Oikawa, R. Giugliani, P. Harmatz, M. Smith, Y. Suzuki, T. Oorii. Impairment of body growth in mucopolysaccharidoses. *Handbook of Growth and Growth Monitoring in Health and Disease.* New York, NY: Springer; 2012:2091-2117.
  13. Collett-Solberg PF, Jorge AAL, Boguszewski MCS, Miller BS, Choong CSY, Cohen P, Hoffman AR, Luo X, Radovick S, Saenger P. Growth hormone therapy in children; research and practice - A review. *Growth Horm IGF Res.* 2019;**44**(2):20-32.
  14. Pfaffle R. Hormone replacement therapy in children: The use of growth hormone and

- IGF-I. *Best Pract Res Clin Endocrinol Metab.* 2015;**29**(3):339-352.
15. Polgreen LE, Thomas W, Orchard PJ, Whitley CB, Miller BS. Effect of recombinant human growth hormone on changes in height, bone mineral density, and body composition over 1-2 years in children with Hurler or Hunter syndrome. *Mol Genet Metab.* 2014;**111**(2):101-106.
  16. Munoz-Calvo MT, Barrios V, Pozo J, Chowen JA, Martos-Moreno GA, Hawkins F, Dauber A, Domene HM, Yakar S, Rosenfeld RG, Perez-Jurado LA, Oxvig C, Frystyk J, Argente J. Treatment With Recombinant Human Insulin-Like Growth Factor-1 Improves Growth in Patients With PAPP-A2 Deficiency. *J Clin Endocrinol Metab.* 2016;**101**(11):3879-3883.
  17. Chatterjee S, Shapiro L, Rose SJ, Mushtaq T, Clayton PE, Ten SB, Bhangoo A, Kumbattae U, Dias R, Savage MO, Metherell LA, Storr HL. Phenotypic spectrum and responses to recombinant human IGF1 (rhIGF1) therapy in patients with homozygous intronic pseudoexon growth hormone receptor mutation. *Eur J Endocrinol.* 2018;**178**(5):481-489.
  18. Breil T, Kneppo C, Bettendorf M, Muller HL, Kapelari K, Schnabel D, Woelfle J. Sequential measurements of IGF-I serum concentrations in adolescents with Laron syndrome treated with recombinant human IGF-I (rhIGF-I). *J Pediatr Endocrinol Metab.* 2018;**31**(8):895-902.
  19. Jiang Z. Mechanism of growth failure in mucopolysaccharidosis VII mice (PhD Thesis). Adelaide, Australia: University of Adelaide; 2018.
  20. Yasoda A, Komatsu Y, Chusho H, Miyazawa T, Ozasa A, Miura M, Kurihara T, Rogi T, Tanaka S, Suda M, Tamura N, Ogawa Y, Nakao K. Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat Med.* 2004;**10**(1):80-86.

21. Chusho H, Tamura N, Ogawa Y, Yasoda A, Suda M, Miyazawa T, Nakamura K, Nakao K, Kurihara T, Komatsu Y, Itoh H, Tanaka K, Saito Y, Katsuki M, Nakao K. Dwarfism and early death in mice lacking C-type natriuretic peptide. *Proc Natl Acad Sci U S A*. 2001;**98**(7):4016-4021.
22. Yasoda A, Kitamura H, Fujii T, Kondo E, Murao N, Miura M, Kanamoto N, Komatsu Y, Arai H, Nakao K. Systemic administration of C-type natriuretic peptide as a novel therapeutic strategy for skeletal dysplasias. *Endocrinology*. 2009;**150**(7):3138-3144.
23. Rivkees SA, Danon M, Herrin J. Prednisone dose limitation of growth hormone treatment of steroid-induced growth failure. *J Pediatr*. 1994;**125**(2):322-325.
24. Ueda Y, Yasoda A, Yamashita Y, Kanai Y, Hirota K, Yamauchi I, Kondo E, Sakane Y, Yamanaka S, Nakao K, Fujii T, Inagaki N. C-type natriuretic peptide restores impaired skeletal growth in a murine model of glucocorticoid-induced growth retardation. *Bone*. 2016;**92**(11):157-167.
25. RRID:IMSR\_JAX:006557, <https://www.jax.org/strain/006557>.
26. Gwynn B, Lueders K, Sands MS, Birkenmeier EH. Intracisternal A-particle element transposition into the murine beta-glucuronidase gene correlates with loss of enzyme activity: a new model for beta-glucuronidase deficiency in the C3H mouse. *Mol Cell Biol*. 1998;**18**(11):6474-6481.
27. Kanai Y, Yasoda A, Mori KP, Watanabe-Takano H, Nagai-Okatani C, Yamashita Y, Hirota K, Ueda Y, Yamauchi I, Kondo E, Yamanaka S, Sakane Y, Nakao K, Fujii T, Yokoi H, Minamino N, Mukoyama M, Mochizuki N, Inagaki N. Circulating osteocrin stimulates bone growth by limiting C-type natriuretic peptide clearance. *J Clin Invest*. 2017;**127**(11):4136-4147.
28. GenBank:NM\_024409, [https://www.ncbi.nlm.nih.gov/nuccore/NM\\_024409](https://www.ncbi.nlm.nih.gov/nuccore/NM_024409).
29. GenBank:NM\_001357025, [https://www.ncbi.nlm.nih.gov/nuccore/NM\\_001357025](https://www.ncbi.nlm.nih.gov/nuccore/NM_001357025).

30. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 1999;**6**(7):1258-1266.
31. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther.* 1999;**10**(10):1735-1737.
32. RRID:AB\_2814837, [https://antibodyregistry.org/search.php?q=AB\\_2814837](https://antibodyregistry.org/search.php?q=AB_2814837).
33. RRID:AB\_2811290, [https://antibodyregistry.org/search.php?q=AB\\_2811290](https://antibodyregistry.org/search.php?q=AB_2811290).
34. RRID:AB\_2811294, [https://antibodyregistry.org/search.php?q=AB\\_2811294](https://antibodyregistry.org/search.php?q=AB_2811294).
35. RRID:AB\_2811293, [https://antibodyregistry.org/search.php?q=AB\\_2811293](https://antibodyregistry.org/search.php?q=AB_2811293).
36. RRID:AB\_2811292, [https://antibodyregistry.org/search.php?q=AB\\_2811292](https://antibodyregistry.org/search.php?q=AB_2811292).
37. RRID:AB\_2661855, [https://antibodyregistry.org/search.php?q=AB\\_2661855](https://antibodyregistry.org/search.php?q=AB_2661855).
38. Ueda Y, Yasoda A, Hirota K, Yamauchi I, Yamashita T, Kanai Y, Sakane Y, Fujii T, Inagaki N. Exogenous C-type natriuretic peptide therapy for impaired skeletal growth in a murine model of glucocorticoid treatment. *Sci Rep.* 2019;**9**(1):8547.
39. Richard M, Arfi A, Seguin J, Gandolphe C, Scherman D. Widespread biochemical correction of murine mucopolysaccharidosis type VII pathology by liver hydrodynamic plasmid delivery. *Gene Ther.* 2009;**16**(6):746-756.
40. Vogler C, Levy B, Galvin N, Sands MS, Birkenmeier EH, Sly WS, Barker J. A novel model of murine mucopolysaccharidosis type VII due to an intracisternal a particle element transposition into the beta-glucuronidase gene: clinical and pathologic findings. *Pediatr Res.* 2001;**49**(3):342-348.
41. Xu L, Mango RL, Sands MS, Haskins ME, Ellinwood NM, Ponder KP. Evaluation of pathological manifestations of disease in mucopolysaccharidosis VII mice after neonatal hepatic gene therapy. *Mol Ther.* 2002;**6**(6):745-758.
42. Macsai CE, Derrick-Roberts AL, Ding X, Zarrinkalam KH, McIntyre C, Anderson

- PH, Anson DS, Byers S. Skeletal response to lentiviral mediated gene therapy in a mouse model of MPS VII. *Mol Genet Metab.* 2012;**106**(2):202-213.
43. Derrick-Roberts AL, Panir K, Pyragius CE, Zarrinkalam KH, Atkins GJ, Byers S. Reversal of established bone pathology in MPS VII mice following lentiviral-mediated gene therapy. *Mol Genet Metab.* 2016;**119**(3):249-257.
  44. Ohashi T, Watabe K, Uehara K, Sly WS, Vogler C, Eto Y. Adenovirus-mediated gene transfer and expression of human beta-glucuronidase gene in the liver, spleen, and central nervous system in mucopolysaccharidosis type VII mice. *Proc Natl Acad Sci U S A.* 1997;**94**(4):1287-1292.
  45. Herweijer H, Wolff JA. Gene therapy progress and prospects: hydrodynamic gene delivery. *Gene Ther.* 2007;**14**(2):99-107.
  46. Suda T, Liu D. Hydrodynamic gene delivery: its principles and applications. *Mol Ther.* 2007;**15**(12):2063-2069.
  47. Kake T, Kitamura H, Adachi Y, Yoshioka T, Watanabe T, Matsushita H, Fujii T, Kondo E, Tachibe T, Kawase Y, Jishage K, Yasoda A, Mukoyama M, Nakao K. Chronically elevated plasma C-type natriuretic peptide level stimulates skeletal growth in transgenic mice. *Am J Physiol Endocrinol Metab.* 2009;**297**(6):E1339-1348.
  48. Fujii T, Komatsu Y, Yasoda A, Kondo E, Yoshioka T, Nambu T, Kanamoto N, Miura M, Tamura N, Arai H, Mukoyama M, Nakao K. Circulating C-type natriuretic peptide (CNP) rescues chondrodysplastic CNP knockout mice from their impaired skeletal growth and early death. *Endocrinology.* 2010;**151**(9):4381-4388.
  49. Daly TM, Ohlemiller KK, Roberts MS, Vogler CA, Sands MS. Prevention of systemic clinical disease in MPS VII mice following AAV-mediated neonatal gene transfer. *Gene Ther.* 2001;**8**(17):1291-1298.
  50. Kanaji A, Kosuga M, Li XK, Fukuhara Y, Tanabe A, Kamata Y, Azuma N, Yamada M,

- Sakamaki T, Toyama Y, Okuyama T. Improvement of skeletal lesions in mice with mucopolysaccharidosis type VII by neonatal adenoviral gene transfer. *Mol Ther*. 2003;**8**(5):718-725.
51. Rowan DJ, Tomatsu S, Grubb JH, Haupt B, Montano AM, Oikawa H, Sosa AC, Chen A, Sly WS. Long circulating enzyme replacement therapy rescues bone pathology in mucopolysaccharidosis VII murine model. *Mol Genet Metab*. 2012;**107**(1-2):161-172.
  52. Metcalf JA, Zhang Y, Hilton MJ, Long F, Ponder KP. Mechanism of shortened bones in mucopolysaccharidosis VII. *Mol Genet Metab*. 2009;**97**(3):202-211.
  53. Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, Bacher JD, Baron J. The role of the resting zone in growth plate chondrogenesis. *Endocrinology*. 2002;**143**(5):1851-1857.
  54. Mizuhashi K, Ono W, Matsushita Y, Sakagami N, Takahashi A, Saunders TL, Nagasawa T, Kronenberg HM, Ono N. Resting zone of the growth plate houses a unique class of skeletal stem cells. *Nature*. 2018;**563**(7730):254-258.
  55. Hirota K, Furuya M, Morozumi N, Yoshikiyo K, Yotsumoto T, Jindo T, Nakamura R, Murakami K, Ueda Y, Hanada T, Sade H, Yoshida S, Enomoto K, Kanai Y, Yamauchi I, Yamashita T, Ueda-Sakane Y, Fujii T, Yasoda A. Exogenous C-type natriuretic peptide restores normal growth and prevents early growth plate closure in its deficient rats. *PLoS One*. 2018;**13**(9):e0204172.
  56. Simonaro CM, Haskins ME, Schuchman EH. Articular chondrocytes from animals with a dermatan sulfate storage disease undergo a high rate of apoptosis and release nitric oxide and inflammatory cytokines: a possible mechanism underlying degenerative joint disease in the mucopolysaccharidoses. *Lab Invest*. 2001;**81**(9):1319-1328.
  57. Hughes A, Oxford AE, Tawara K, Jorcyk CL, Oxford JT. Endoplasmic Reticulum

- Stress and Unfolded Protein Response in Cartilage Pathophysiology; Contributing Factors to Apoptosis and Osteoarthritis. *Int J Mol Sci.* 2017;**18**(3):E665.
58. Sederquist B, Fernandez-Vojvodich P, Zaman F, Savendahl L. Recent research on the growth plate: Impact of inflammatory cytokines on longitudinal bone growth. *J Mol Endocrinol.* 2014;**53**(1):T35-44.
  59. Yang L, Tsang KY, Tang HC, Chan D, Cheah KS. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proc Natl Acad Sci U S A.* 2014;**111**(33):12097-12102.
  60. Bartolomeo R, Cinque L, De Leonibus C, Forrester A, Salzano AC, Monfregola J, De Gennaro E, Nusco E, Azario I, Lanzara C, Serafini M, Levine B, Ballabio A, Settembre C. mTORC1 hyperactivation arrests bone growth in lysosomal storage disorders by suppressing autophagy. *J Clin Invest.* 2017;**127**(10):3717-3729.
  61. Rowan DJ, Tomatsu S, Grubb JH, Montano AM, Sly WS. Assessment of bone dysplasia by micro-CT and glycosaminoglycan levels in mouse models for mucopolysaccharidosis type I, IIIA, IVA, and VII. *J Inherit Metab Dis.* 2013;**36**(2):235-246.
  62. Monroy MA, Ross FP, Teitelbaum SL, Sands MS. Abnormal osteoclast morphology and bone remodeling in a murine model of a lysosomal storage disease. *Bone.* 2002;**30**(2):352-359.
  63. Kondo E, Yasoda A, Fujii T, Nakao K, Yamashita Y, Ueda-Sakane Y, Kanamoto N, Miura M, Arai H, Mukoyama M, Inagaki N, Nakao K. Increased Bone Turnover and Possible Accelerated Fracture Healing in a Murine Model With an Increased Circulating C-Type Natriuretic Peptide. *Endocrinology.* 2015;**156**(7):2518-2529.
  64. Xing EM, Wu S, Ponder KP. The effect of Tlr4 and/or C3 deficiency and of neonatal gene therapy on skeletal disease in mucopolysaccharidosis VII mice. *Mol Genet*

- Metab.* 2015;**114**(2):209-216.
65. Mango RL, Xu L, Sands MS, Vogler C, Seiler G, Schwarz T, Haskins ME, Ponder KP. Neonatal retroviral vector-mediated hepatic gene therapy reduces bone, joint, and cartilage disease in mucopolysaccharidosis VII mice and dogs. *Mol Genet Metab.* 2004;**82**(1):4-19.
66. Montano AM, Lock-Hock N, Steiner RD, Graham BH, Szlago M, Greenstein R, Pineda M, Gonzalez-Meneses A, Coker M, Bartholomew D, Sands MS, Wang R, Giugliani R, Macaya A, Pastores G, Ketko AK, Ezgu F, Tanaka A, Arash L, Beck M, Falk RE, Bhattacharya K, Franco J, White KK, Mitchell GA, Cimbalistiene L, Holtz M, Sly WS. Clinical course of sly syndrome (mucopolysaccharidosis type VII). *J Med Genet.* 2016;**53**(6):403-418.
67. Savarirayan R, Irving M, Bacino CA, Bostwick B, Charrow J, Cormier-Daire V, Le Quan Sang K-H, Dickson P, Harmatz P, Phillips J, Owen N, Cherukuri A, Jayaram K, Jeha GS, Larimore K, Chan M-L, Huntsman Labeled A, Day J, Hoover-Fong J. C-Type Natriuretic Peptide Analogue Therapy in Children with Achondroplasia. *N Engl J Med.* 2019;**381**(1):25-35.

## Figure legends

### Figure 1.

Effects of overexpressed C-type natriuretic peptide (CNP) by hydrodynamic gene delivery in C57BL/6 mice (WT). **(A)** Serum N-terminal pro C-type natriuretic peptide (NT-proCNP) levels after injection of pLIVE-NPPC vector to 6-week-old C57BL/6 mice (WT). n = 3 each. **(B)** Gross appearance of 10-week-old WT-CNP, WT-Empty, and WT mice. **(C)** Soft x-ray images of 10-week-old WT-CNP, WT-Empty and WT mice. Scale bar: 20 mm. **(D-F)** Growth curves based on naso-anal lengths (D), naso-tail lengths (E) and body weights (F) of WT-CNP (black filled squares), WT-Empty (gray filled squares), and WT mice (black open squares) measured every week in 6 to 10 weeks old. WT-CNP, n = 5; WT-Empty, n = 6; WT, n = 6. **(G)** Bone lengths of WT-CNP (black filled bars), WT-Empty (gray filled bars), and WT mice (black open bars) measured on soft x-ray images. WT-CNP, n = 5; WT-Empty, n = 6; WT, n = 6. **(H-K)** Dose-dependent skeletal effects of overexpressed CNP by hydrodynamic gene delivery in WT mice. **(H)** Growth curves based on naso-anal lengths of mice injected with 1  $\mu$ g (WT-CNP1 $\mu$ g, n = 3, gray dotted line), 5  $\mu$ g (WT-CNP5 $\mu$ g, n = 3, black dotted line), 25  $\mu$ g (WT-CNP25 $\mu$ g, n = 5, black line) of pLIVE-NPPC vector, and 25  $\mu$ g of pLIVE-Empty vector (WT-Empty, n = 6, gray line). **(I)** Comparison of amounts of elongation in naso-anal lengths during 4 weeks ( $\Delta$ naso-anal length) among WT-Empty (gray filled bars), WT-CNP1 $\mu$ g (gray open bars), WT-CNP5 $\mu$ g (black open bars), and WT-CNP25 $\mu$ g (black filled bars) mice. **(J)** Growth curves based on naso-tail lengths. **(K)** Comparison of amounts of elongation in naso-tail lengths during 4 weeks ( $\Delta$ naso-tail length) among these four groups. Statistical analyses were performed by 1-way ANOVA followed by Tukey-Kramer test. In panels A and G, \*\*p < 0.01, \*\*\*p < 0.001. In panel D-F, \*\*\*p < 0.001 vs WT, ††p < 0.01 vs WT-Empty, and †††p < 0.001 vs WT-Empty. In panels H and J, statistical analyses were not performed. In panels I and K, \*\*p < 0.01, \*\*\*p < 0.001.

Figure 2.

Combination therapy of CNP and GUSB and its skeletal effects in MPS VII mice. **(A)** Gross appearance of 3-week-old WT (C57BL/6) and MPS ( $Gusb^{mps-2J}/Gusb^{mps-2J}$ ) mice. MPS mice were treated with CNP and  $\beta$ -glucuronidase (GUSB) which was overexpressed by hydrodynamic gene delivery in 6 to 10 weeks old. **(B, C)** Liver GAG levels after treatments in male (B) and female mice (C). MPS-Empty (black open bars): male, n = 6, female, n = 5; MPS-GUSB (black filled bars): male, n = 5, female, n = 4; MPS-CNP (red open bars): male, n = 5, female, n = 5; MPS-GUSB/CNP (red filled bars): male, n = 6, female, n = 4. **(D)** Soft x-ray images of 10-week-old treated MPS mice. Scale bar: 20 mm. **(E, F)** Growth curves based on naso-anal length of MPS-Empty (black open squares), MPS-GUSB (black filled squares), MPS-CNP (red open squares), and MPS-GUSB/CNP (red filled squares) mice. Male, n = 8 each (E); female, n = 7 each (F). **(G-J)** Growth curves based on naso-tail lengths of male (G) and female (H), and body weights of male (I) and female (J) in MPS mice ( $Gusb^{mps-2J}/Gusb^{mps-2J}$ ). MPS-Empty (black open squares), MPS-GUSB (black filled squares), MPS-CNP (red open squares), and MPS-GUSB/CNP (red filled squares) mice were generated by hydrodynamic gene delivery of each expression vector. Male, n = 8 each; female, n = 7 each. **(K, L)** Bone lengths in each group measured on the soft x-ray images. Male, n = 8 each (K); female, n = 7 each (L). **(M, N)** Comparisons of final naso-anal lengths between treated MPS mice and WT-Empty mice in male (M) and female (N). MPS mice: male, n = 8 each, female n = 7 each; WT-Empty mice: n = 6 in both sexes. Statistical analyses were performed by 1-way ANOVA followed by Tukey-Kramer test in panels B, C and E-L and Dunnett test in panels M and N. In panels B, C, K and L, \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001. In panels E-J, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for MPS-GUSB/CNP vs MPS-Empty, †p < 0.05, ††p < 0.01 for MPS-GUSB/CNP vs MPS-GUSB, and ‡p < 0.05 for MPS-CNP vs MPS-Empty.

Figure 3.

Histological characteristics of growth plates of MPS VII mice. **(A)** Histological images of the tibial growth plates of 10-week-old WT-Empty and MPS-Empty mice stained by alcian blue. Red two-way arrows, hypertrophic zones; blue two-way arrows, proliferative zones; and black two-way arrows, resting zones. Scale bars: 50  $\mu$ m. **(B, C)** Quantitative analyses for the widths of whole growth plates (B) and those of each zone (C) and comparisons between WT-Empty (black open bars) and MPS-Empty mice (black filled bars). n = 5 each. **(D)** Changes in the number of cells in proliferative and hypertrophic zone per growth plate. n = 5 each. **(E)** Representative images of Ki-67 staining. Blue two-way arrows, proliferative zones. Scale bars: 50  $\mu$ m. **(F)** Changes in the number of Ki-67 positive cells per growth plate. n = 5 each. **(G)** Representative images of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Red two-way arrows, hypertrophic zones. Scale bars: 50  $\mu$ m. **(H)** Changes in the number of TUNEL positive cells per growth plate. n = 3 each. Statistical analyses were performed by Student's t test. \*\*p < 0.01, \*\*\*p < 0.001.

Figure 4.

Beneficial changes in growth plates of MPS VII mice treated by combination therapy of CNP and GUSB. **(A)** Histological images of the tibial growth plates of 10-week-old treated MPS mice stained by alcian blue. Red two-way arrows, hypertrophic zones; blue two-way arrows, proliferative zones; and black two-way arrows, resting zones. Scale bars: 50  $\mu$ m. **(B-E)** Quantitative analyses for the widths of whole growth plates (B) and those of each zone (C-E) in MPS-Empty (black open bars), MPS-GUSB (black filled bars), MPS-CNP (red open bars), and MPS-GUSB/CNP (red filled bars) mice. n = 5 each. **(F)** Changes in the number of cells in proliferative and hypertrophic zone per growth plate. MPS-Empty, n = 5; MPS-GUSB, n = 5; MPS-CNP, n = 5; and MPS-GUSB/CNP, n = 4. **(G)** Representative images of Ki-67 staining. Blue two-way arrows, proliferative zones. Scale bars: 50  $\mu$ m. **(H)** Changes in the number of Ki-67 positive cells per growth plate. MPS-Empty, n = 5; MPS-GUSB, n = 5; MPS-CNP, n = 5; MPS-GUSB/CNP, n = 4. **(I)** Representative images of TUNEL staining. Red two-way arrows, hypertrophic zones. Scale bars: 50  $\mu$ m. **(J)** Changes in the number of terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells per growth plate. n = 3 each. **(K)** Quantitative RT-PCR analyses of lumbar vertebrae. n = 6 each. mRNA levels of target genes were normalized to those of *Ppia*, and represented as fold-change versus WT-Empty mice. Statistical analyses were performed by two-way factorial ANOVA in panel B-F, H, and J, and by Student's t test in panel K. NS: not significant. \*p < 0.05 for GUSB therapy, \*\*p < 0.01 for GUSB therapy, \*\*\*p < 0.001 for GUSB therapy, †††p < 0.001 for CNP therapy.

Figure 5.

Histological images (alcian blue, 400x) of the resting zone in the tibial growth plate were presented regarding all analyzed biological replicate samples. #1-#5 represent different samples obtained from MPS-Empty, MPS-GUSB, MPS-CNP, and MPS-GUSB/CNP mice.

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Figure 6.

Histological images (alcian blue, 400x) of the hypertrophic zone in the tibial growth plate were presented regarding all analyzed biological replicate samples. #1-#5 represent different samples obtained from MPS-Empty, MPS-GUSB, MPS-CNP, and MPS-GUSB/CNP mice.

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Figure 7.

Evaluations of bone structures and bone turnover markers of treated MPS VII mice. **(A)** Micro-CT images of proximal ends of tibial bones of 10-week-old WT-Empty and MPS-Empty mice. **(B-D)** Comparisons of bone mineral density (B), serum osteocalcin levels (C), and serum tartrate-resistant acid phosphatase form 5b (TRAP5b) levels (D). WT-Empty (black open bars), n = 6; MPS-Empty (black filled bars), n = 8. **(E)** Micro-CT images of proximal ends of tibial bones of treated MPS mice. **(F-H)** Comparisons of bone mineral density (F), serum osteocalcin levels (G), and serum TRAP5b levels (H) in MPS-Empty (black open bars), MPS-GUSB (black filled bars), MPS-CNP (red open bars), and MPS-GUSB/CNP (red filled bars) mice. n = 8 each. Statistical analyses were performed by Student's t test (in panels B, C, and D) and by using two-way factorial ANOVA (in panels F, G, and H). \*p < 0.05 for WT vs MPS, \*\*p < 0.01 for WT vs MPS and for GUSB therapy, \*\*\*p < 0.001 for WT vs MPS and for GUSB therapy, †p < 0.05 for CNP therapy.

Figure 8.

Abnormalities in articular cartilage were restored in treated MPS VII mice. **(A)** Soft x-ray images of shoulder joints of 10-week-old WT-Empty and MPS-Empty mice. Scale bars: 2 mm. **(B)** Quantitative analyses for their widths of shoulder joint spaces measured on the images. WT-Empty (black open bars), n = 6; MPS-Empty (black filled bars), n = 8. **(C)** Histological images of the distal femur cartilages of 10-week-old WT-Empty and MPS-Empty mice stained by alcian blue. Black two-way arrows indicate the widths of articular cartilages. Scale bars: 50  $\mu$ m. **(D)** Changes in the widths of articular cartilages measured on the pictures between WT-Empty (black open bars) and MPS-Empty (black filled bars) mice. n = 5 each. **(E)** Soft x-ray images of shoulder joints of treated MPS mice. Scale bars: 2 mm. **(F)** Quantitative analyses for their width of joint spaces. n = 8 each. **(G)** Histological images of the distal femur cartilages of treated MPS mice. Black two-way arrows indicate the widths of articular cartilages. Scale bars: 50  $\mu$ m. **(H)** Changes in the widths of articular cartilages of MPS-Empty (black open bars), MPS-GUSB (black filled bars), MPS-CNP (red open bars), and MPS-GUSB/CNP (red filled bars) mice. n = 5 each. Statistical analyses were performed by Student's t test (in panels B and D) and by two-way factorial ANOVA (in panels F and H). \*p < 0.05 for GUSB therapy, \*\*p < 0.01 for WT vs MPS, \*\*\*p < 0.001 for WT vs MPS and for GUSB therapy.

**Table 1. Summary of changes by GUSB and CNP therapy**

|                                                    | GUSB         | CNP          | GUSB/CNP      |
|----------------------------------------------------|--------------|--------------|---------------|
| <u>Body and bone lengths</u>                       |              |              |               |
| <u>(mm, changes from MPS-Empty)</u>                |              |              |               |
| Naso-anal length                                   | +1.5         | +3.9         | <b>+6.0</b>   |
| Cranium (APD)                                      | +0.4         | +0.7         | <b>+1.1</b>   |
| Cranium (TD)                                       | +0.1         | +0.2         | +0.2          |
| Front limb                                         | +0.4         | +0.8         | <b>+0.9</b>   |
| Hind limb                                          | +0.4         | +1.1         | <b>+1.7</b>   |
| Lumbar spine (L1-5)                                | +0.2         | +0.8         | <b>+1.3</b>   |
| <u>Growth plate (% changes from MPS-Empty)</u>     |              |              |               |
| Width of resting zone                              | <b>-28.9</b> | +10.0        | <b>-33.2</b>  |
| Width of proliferative zone                        | +2.5         | <b>+40.9</b> | <b>+36.3</b>  |
| Width of hypertrophic zone                         | +3.5         | <b>+31.8</b> | <b>+33.8</b>  |
| Cell number in proliferative and hypertrophic zone | <b>+25.8</b> | <b>+12.9</b> | <b>+58.5*</b> |
| Ki-67 positive cell number                         | <b>+97.1</b> | -30.9        | <b>+112.0</b> |
| TUNEL positive cell number                         | <b>-37.0</b> | +14.8        | <b>-44.4</b>  |
| <u>Bone and joint (% changes from MPS-Empty)</u>   |              |              |               |
| Bone mineral density                               | <b>-29.0</b> | -2.6         | <b>-30.4</b>  |
| Serum osteocalcin level                            | <b>-35.3</b> | <b>+58.5</b> | -5.5          |
| Serum TRAP5b level                                 | <b>+54.0</b> | +16.5        | <b>+63.6</b>  |
| Joint space                                        | <b>-12.1</b> | +4.0         | <b>-59.4</b>  |
| Width of articular cartilage                       | <b>-20.5</b> | -12.5        | <b>-63.1</b>  |

Data of male mice at 10 weeks old. Bold-type indicates statistical significance vs

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MPS-Empty mice.

\*interaction between GUSB and CNP therapy

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Figure 1

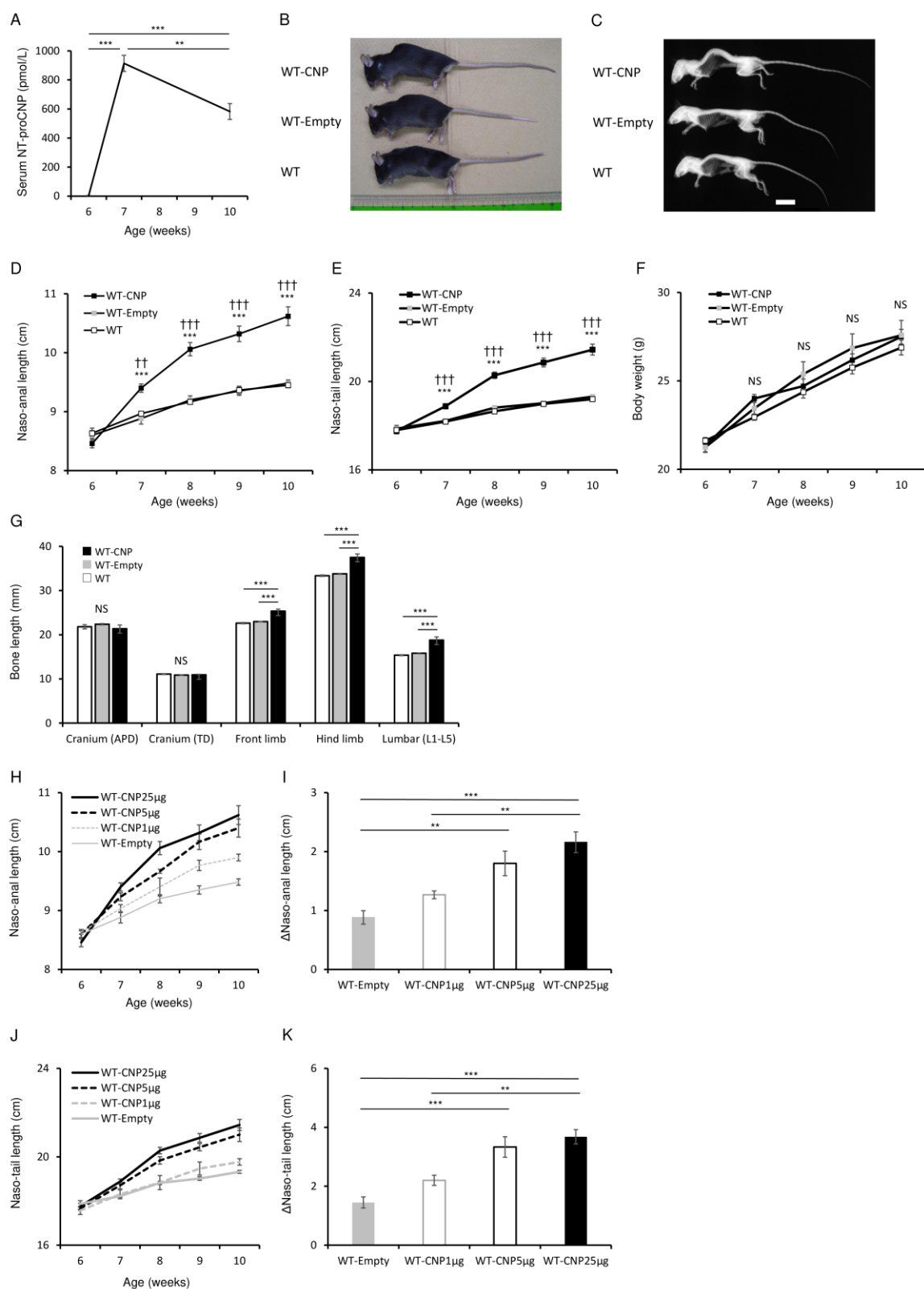


Figure 2

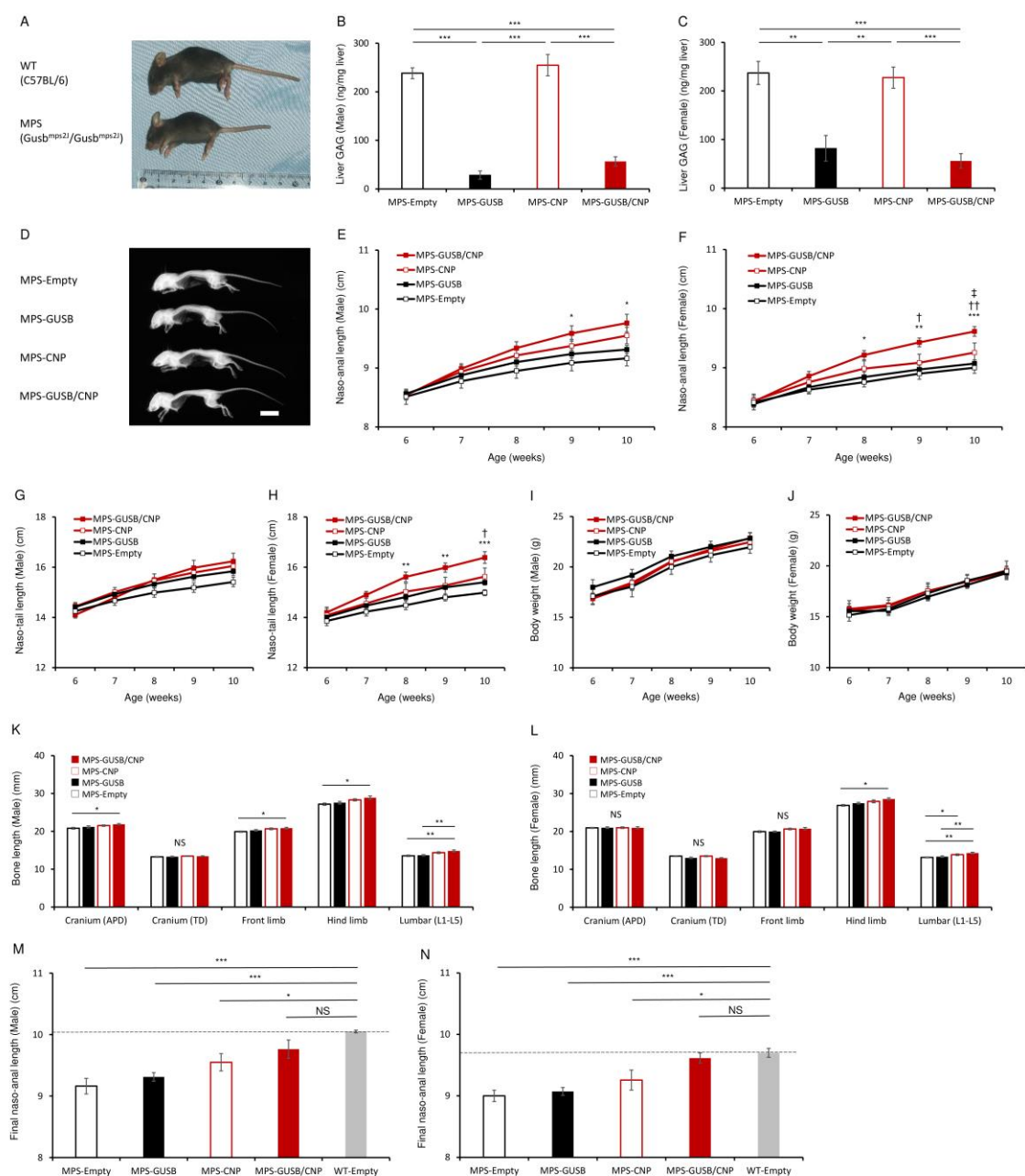


Figure 3

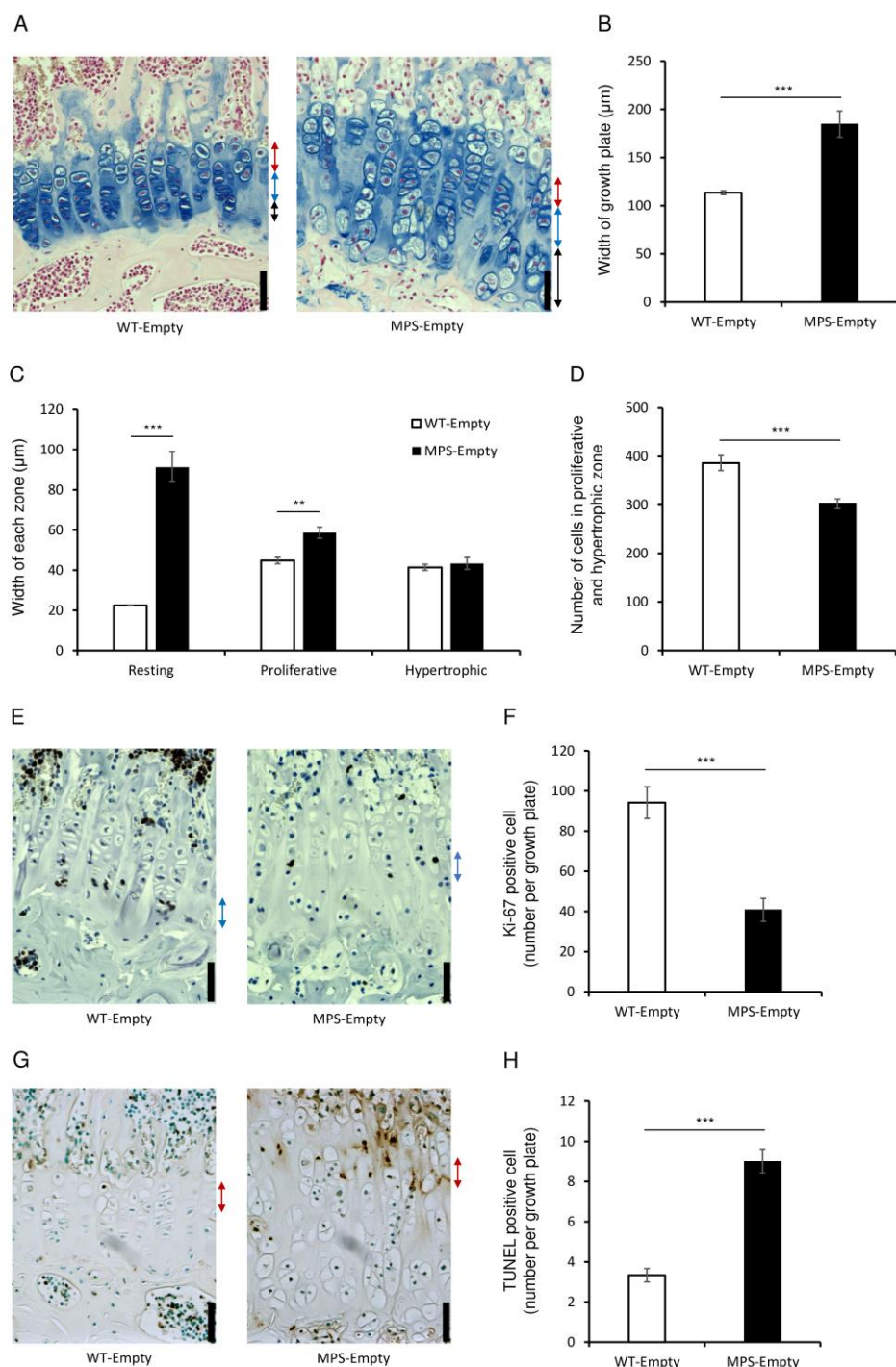
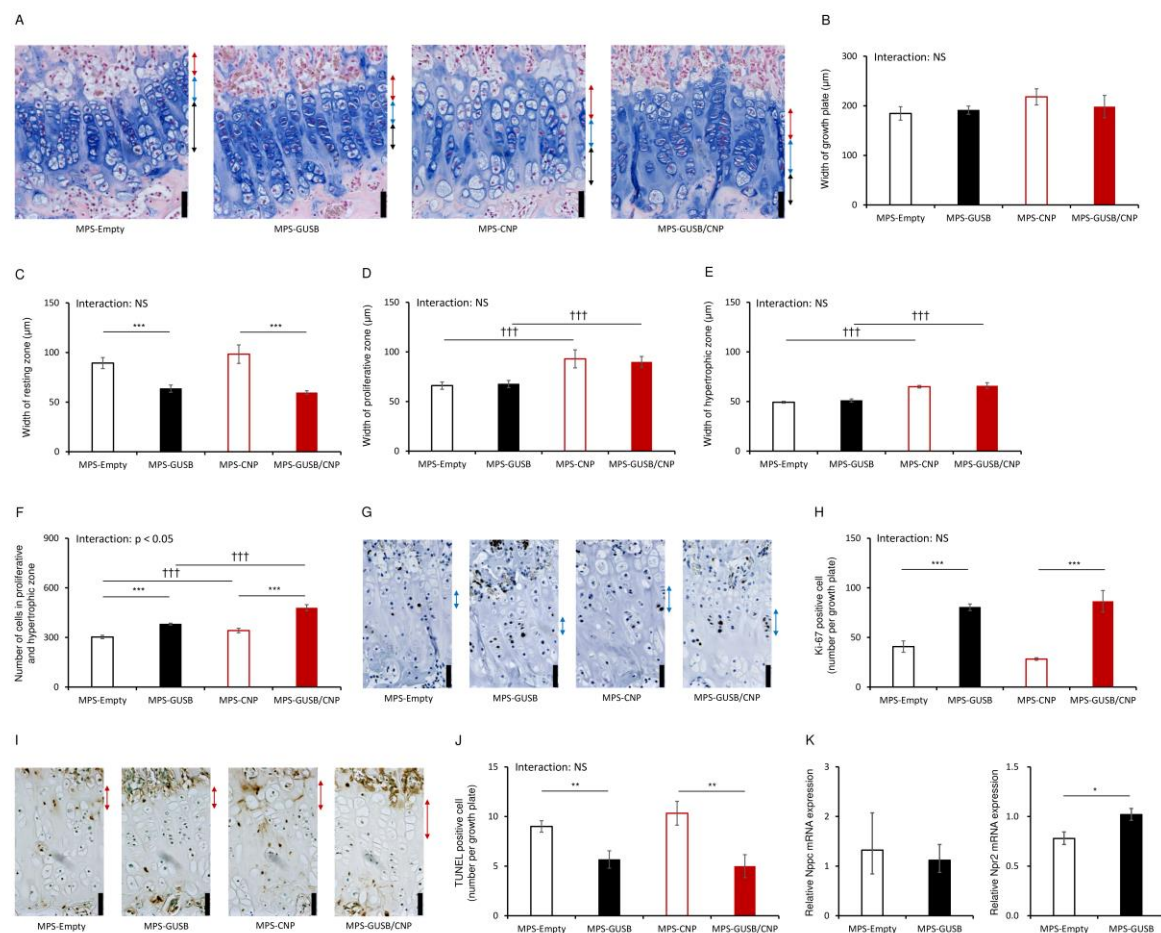
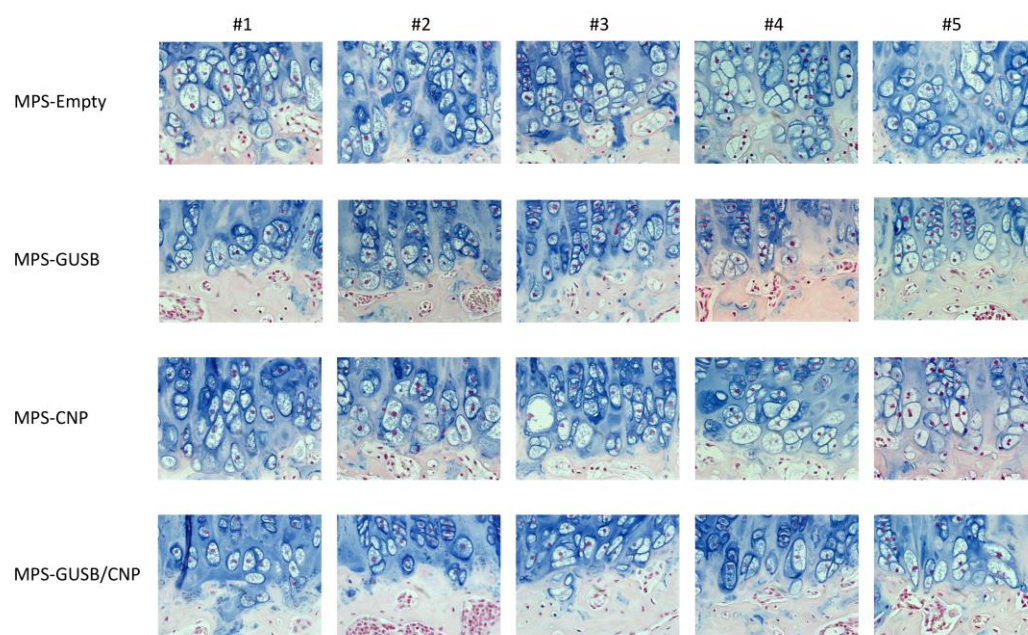


Figure 4



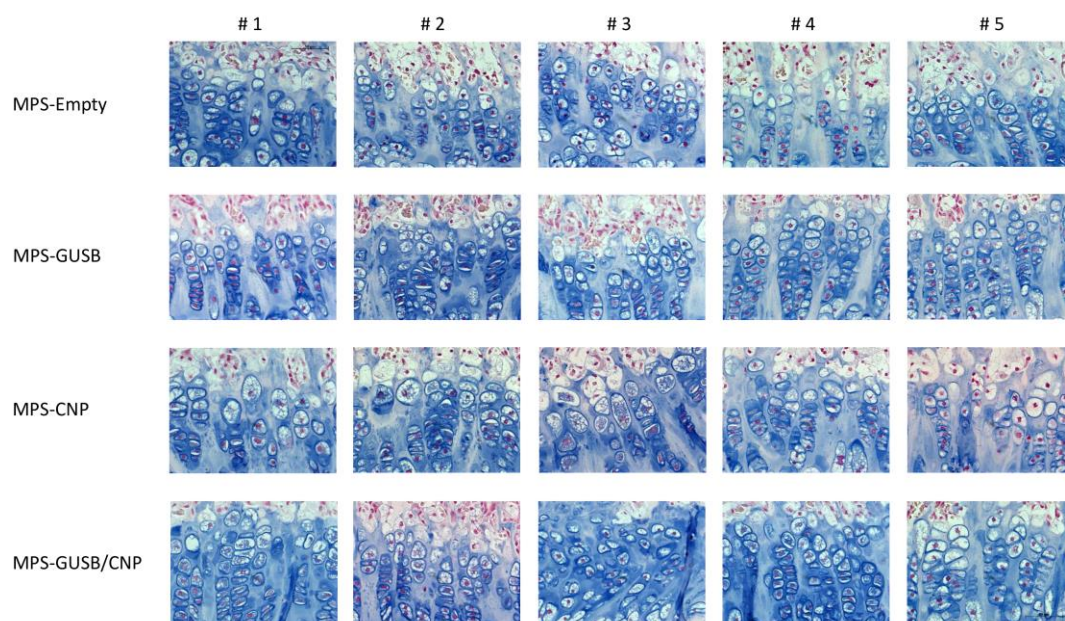
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Figure 5



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Figure 6



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Figure 7

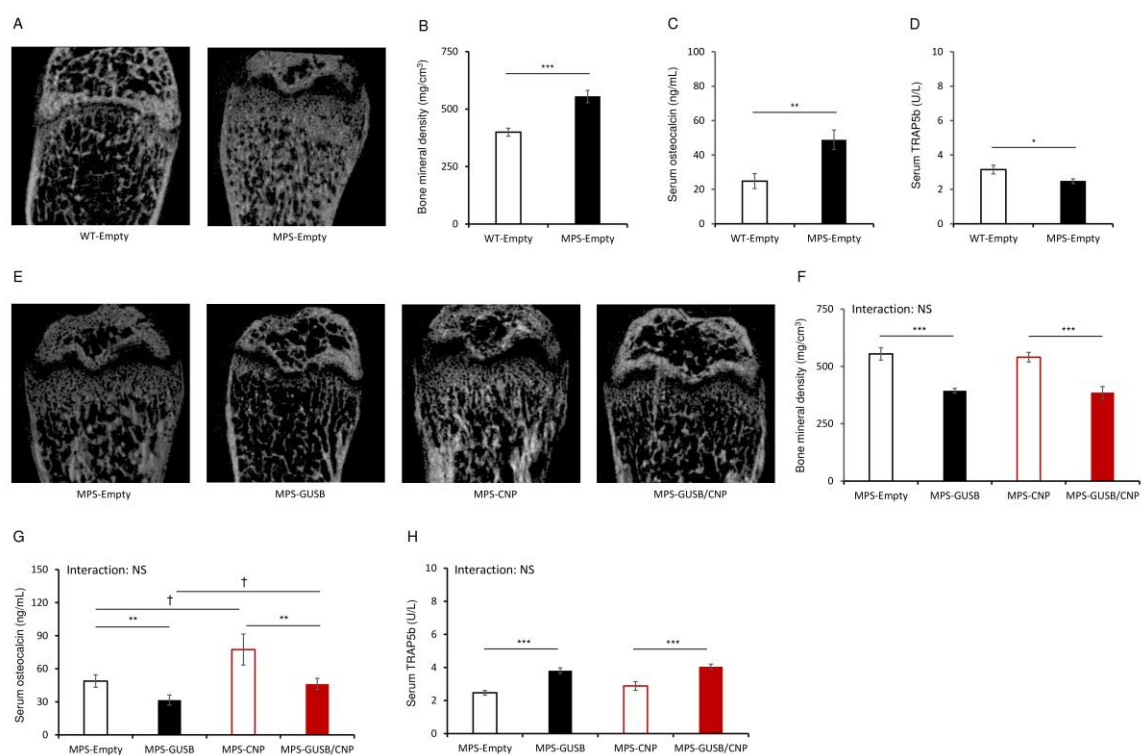


Figure 8

