Meclozine ameliorates bone mineralization and growth plate structure in a mouse model of X-linked hypophosphatemia

YASUNARI KAMIYA[1,2], MASAKI MATSUSHITA[1,2], KENICHI MISHIMA[1,2], BISEI OHKAWARA[2], TOSHIMI MICHIGAMI[3], SHIRO IMAGAMA[1], KINJI OHNO[2] and HIROSHI KITOH[4,5]

[1]Department of Orthopaedic Surgery; [2]Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8550; [3]Department of Bone and Mineral Research, Research Institute, Osaka Women's and Children's Hospital, Osaka Prefectural Hospital Organization, Izumi, Osaka, 594-1101; [4]Department of Orthopaedic Surgery, Aichi Children's Health and Medical Center, Obu, Aichi 474-8710; [5]Department of Comprehensive Pediatric Medicine, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8550, Japan

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Abstract. X-linked hypophosphatemic rickets (XLH) is characterized by hypo-mineralization of the bone due to hypophosphatemia. XLH is caused by abnormally high levels of fibroblast growth factor 23, which trigger renal phosphate wasting. Activated fibroblast growth factor receptor 3 (FGFR3) signaling is considered to be involved in XLH pathology. Our previous study revealed that meclozine attenuated FGFR3 signaling and promoted longitudinal bone growth in an achondroplasia mouse model. The present study aimed to examine whether meclozine affected the bone phenotype in a mouse model of XLH [X-linked hypophosphatemic (Hyp) mice]. Meclozine was administered orally to 7-day-old Hyp mice for 10 days, after which the mice were subjected to blood sampling and histological analyses of the first coccygeal vertebra, femur and tibia. Villanueva Goldner staining was used to assess bone mineralization, hematoxylin and eosin staining was used to determine the growth plate structure and tartrate-resistant acid phosphatase staining was used to measure osteoclast activity. The osteoid volume/bone volume of cortical bone was lower in meclozine-treated Hyp mice compared with untreated Hyp mice. Meclozine treatment improved the abnormally thick hypertrophic zone of the growth plate and ameliorated the downregulation of osteoclast surface/bone surface in Hyp mice. However, meclozine had only a marginal effect on mineralization in the trabecular bone and on calcium and phosphate plasma levels. A 10-day-tratment with meclozine partially ameliorated bone mineralization in Hyp mice; hence, meclozine could alleviate XLH symptoms.

Correspondence to: Dr Masaki Matsushita, Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi, 466-8550, Japan
E-mail: masakim@med.nagoya-u.ac.jp

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Introduction

X-linked hypophosphatemic rickets (XLH) is the most common hereditary hypophosphatemic disorder. Patients with XLH suffer from short stature, deformed lower extremities, dental abnormalities, bone and joint pain, tinnitus, and hearing loss (1). These manifestations result primarily from hypophosphatemia caused by renal phosphate wastage. At the molecular level, XLH is caused by loss-of-function mutations in the phosphate-regulating endopeptidase homolog of the X-chromosome (*Phex*) gene (2). Mutation-driven downregulation of *Phex* promotes the expression of fibroblast growth factor 23 (FGF23) (3-5). FGF23 reduces renal tubule phosphate reabsorption, leading to low serum inorganic phosphorus. FGF23 also alters the production and degradation of 1,25-dihydroxyvitamin D by activating intracellular signaling through binding of fibroblast growth factor receptor 1 (FGFR1) to α-klotho (6-8). In contrast, excessive FGF23 in a mouse XLH model (Hyp mice) was indicated to activate ERK phosphorylation in the FGFR3 signaling pathway in an α-klotho-independent manner (9,10). Moreover, Kawai *et al* (11) demonstrated that activated FGF23 attenuated endochondral ossification by enhancing FGFR3 signaling.

Activating mutations in the *FGFR3* gene cause achondroplasia, which is the most common form of short-limbed skeletal dysplasia. We previously described how meclozine, an antihistamine medicine for motion sickness, attenuated ERK phosphorylation in chondrocytes with overactive ERK signaling (12). We subsequently demonstrated that oral administration of meclozine increased longitudinal bone growth and suppressed the short-statured phenotype in a mouse model of achondroplasia in a dose-dependent manner (13). Thus, the inhibitory effect of meclozine on FGFR3 was confirmed both *in vivo* and *in vitro*. Komla-Ebri *et al* (14) developed NVP-BGJ398, an FGFR tyrosine kinase inhibitor,

which improved the skeletal phenotype in a mouse model of achondroplasia; whereas Wohrle *et al* (15) used NVP-BGJ398 to suppress the hypophosphatemic phenotype of Hyp mice. Hence, inhibition of FGFR3 signaling could present an alternative treatment for patients with XLH. Herein, we conducted an *in vivo* study to examine the effect of meclozine on bone mineralization associated with hypophosphatemia in Hyp mice.

Materials and methods

Mice. Hyp mice were obtained from the Jackson Laboratory. Hyp mice have a large deletion in the 3' untranslated region of the *Phex* gene, which results in a loss-of-function mutation. Female Hyp mice were bred with male wild-type (WT) C57BL/6J mice to obtain male Hyp mice and their non-Hyp littermates. All mice were housed in a facility with a 12/12 h light/dark cycle, stable humidity and temperature, and were fed standard mouse chow (Oriental Yeast Co.) *ad libitum*. The animals were genotyped at 4-6 days by mixing finger tissue with the Viagen DirectPCR Lysis Reagent (Viagen Biotech, Inc.) and proteinase K solution, followed by incubation at 55˚C with agitation at 1,000 rpm for 3 h, and 85˚C without agitation for 45 min (Thermo Mixer C, Eppendorf). The DNA in the supernatant was collected after centrifugation at 10,000 rpm for 15 min (Model 5424; Eppendorf). The forward (5'-CCA AAATTGTTCTTCAGTACACC-3') and reverse (5'-ATC TGGCAGCACACTGGTATG-3') primers were used for PCR amplification (16), and the resulting products were analyzed on a 1% agarose gel containing ethidium bromide. The presence or absence of a 258-bp band indicated WT or mutant *Phex* alleles, respectively. Given that all parameters were compared within a kinship of male Hyp mice, but not between kinships, meclozine-administration was performed only when multiple male Hyp mice were born within kinship.

Meclozine administration. Hyp mice were divided into meclozine-treated and untreated (vehicle only) groups; whereas WT mice were treated with vehicle only. The dose-finding study using a mouse model indicated that meclozine at 1 and 2 mg/kg/day attenuated FGFR3 signaling in a dose-dependent manner (13). Therefore, meclozine was administered at 2 mg/kg/day twice per day by dissolving it in 0.5% methylcellulose. Body weight was measured daily before oral administration, which was started at 7 days of age and continued for 10 consecutive days as described previously (13).

Serum parameters. After the 10-day-treatment period, mice were subjected to whole blood collection from the abdominal aorta using a 26-gauge needle and 1-ml syringe under general anesthesia with isoflurane. Mice were initially exposed with 3.5 to 4.0% of isoflurane to fall asleep, then the blood correction was performed with keeping exposure with 2 to 3% of isoflurane. Mice were euthanized caused by this exsanguination procedure. Collected blood samples were left at room temperature for more than 3 h to allow clotting, and were thereafter centrifuged and the obtained serum samples were preserved at -20˚C. Serum calcium and phosphate levels were measured using a DRI-CHEM 7000V biochemical analyzer (Fujifilm). Serum FGF23 levels were measured using the FGF-23 ELISA Kit (RRID: AB_2782966; Kainos Laboratories). Serum samples were excluded from the analysis when the amount of blood drawn was insufficient for measurement, hemolysis was obvious, the blood vessel was damaged, or the body fluid in the abdominal cavity was contaminated.

Histological analysis. Following treatment, mice were subjected to histological analysis of the first coccygeal vertebra, femur, and tibia. These samples were collected immediately after euthanasia and fixed with 4% paraformaldehyde at 4˚C. The samples of the first coccygeal vertebra were cut in the sagittal plane, while those of the femur and tibia were cut in the coronal plane; thin sections were stained with Villanueva Goldner (Kureha Special Laboratory). The medial mid-shaft cortical bone of the femur, the central part of the distal metaphyseal cancellous bone (primary spongiosa) of the femur, and the proximal central part of the first coccygeal vertebra were identified in low-magnification images. Osteoid volume (OV) and bone volume (BV) were measured using Image J software, and the OV/BV served as an index of bone mineralization (17-19). The osteoclast number (N.Oc), osteoclast surface (Oc.S), and osteoclast surface/bone surface (Oc.S/BS) were measured at the central part of the distal metaphyseal cancellous bone of the femur with tartrate-resistant acid phosphatase (TRAP) staining to assess osteoclast activity (19). The average growth plate thickness, proliferative zone (Pz) thickness, and hypertrophic zone (Hz) thickness (20) were calculated by measuring the center, medial quarter, and lateral quarter of the growth plate at the distal femur stained with hematoxylin and eosin. The percentage proliferative zone thickness (%Pz) and percentage hypertrophic zone thickness (%Hz) were calculated relative to the thickness of the entire growth plate.

Statistical analysis. Histological and serum parameters were normalized to those of untreated littermate Hyp mice to adjust for environmental differences such as litter skeletal size among kinship. All data are expressed as the mean ± SD. Outliers were removed from analysis using the interquartile range method (21). When equality of variance was confirmed by Levene's test, statistical differences were determined by one-way ANOVA with post-hoc Tukey's honest significance test. When equality of variance was not established by Levene's test, one-way ANOVA with Welch's correction was performed, and the Games-Howell method was applied for post-hoc testing. All analyses were conducted using IBM SPSS Statistics version 27 (IBM). Statistical significance was set at $P<0.05$.

Results

Meclozine significantly improves cortical bone mineralization in Hyp mice. The OV of the femoral and tibial cortical bones, which was higher in untreated Hyp mice than WT mice, decreased after treatment with 2 mg/kg/day of meclozine (Fig. 1A and B). Specifically, the OV/BV was 42% lower in the femur ($P<0.01$) and 26% lower in the tibia ($P<0.01$) of meclozine-treated Hyp mice compared to untreated Hyp mice (Fig. 1C).

Meclozine treatment significantly improves growth plate parameters in Hyp mice. The Hz of the growth plate in the

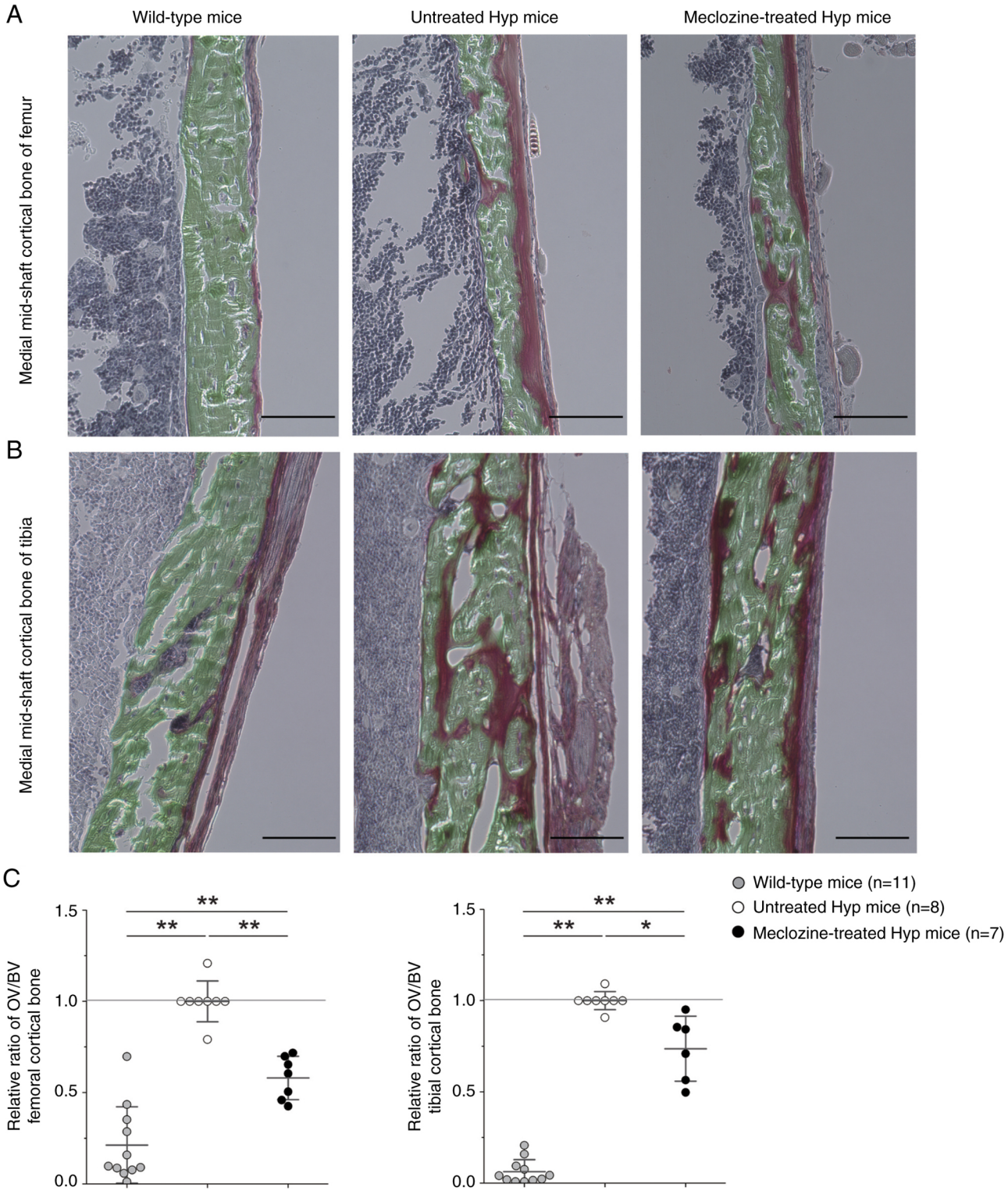

Figure 1. Meclozine rescues impaired cortical bone mineralization in Hyp mice. Representative non-decalcified histological images of the (A) femur and (B) tibia stained with Villanueva Goldner. The red and green signals indicate osteoid and bone tissues, respectively. The osteoid signal at the medial mid-shaft cortical bone was lower in meclozine-treated Hyp mice compared with untreated Hyp mice. Scale bars, 100 μm. (C) OV/BV. Dots indicate the OV/BV of each sample, and bars indicate the mean ± SD. Statistical significance was analyzed using one-way ANOVA with or without Welch's correction. *P<0.05, **P<0.01. Hyp mice, X-linked hypophosphatemic mice; OV/BV, osteoid volume/bone volume.

distal femur and proximal tibia was markedly thickened and presented with an increased cell size in untreated Hyp mice (Fig. 2A and C). Meclozine treatment decreased the %Hz in the femur by 18.5% (P<0.01) and in the tibia by 21.2% (P<0.01) compared to untreated Hyp mice (Fig. 2B and D). Instead, the %Pz of Hyp mice tended to increase following meclozine treatment.

Osteoclast activity in Hyp mice tends to be enhanced by meclozine treatment. Fig. 3 displays the histological

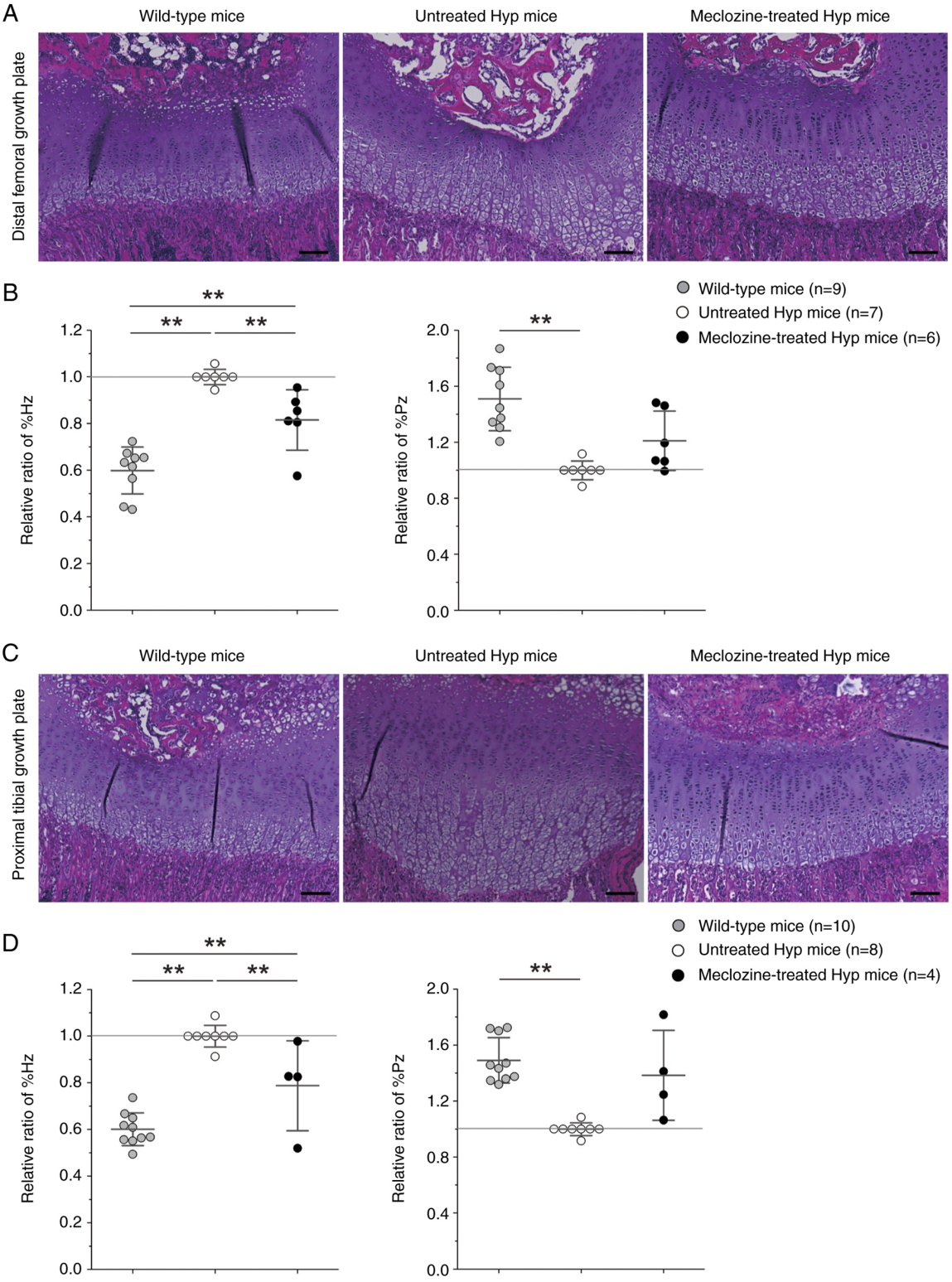

Figure 2. Meclozine improves the structure of the growth plate in Hyp mice. Representative histological images of the (A) distal femoral growth plate and (C) proximal tibial growth plate indicating the width of proliferative and hypertrophic zones in meclozine-treated Hyp mice and untreated Hyp mice. Scale bars, 100 μm. %Hz and %Pz in the (B) distal femur and (D) proximal tibia. Dots indicate the relative %Hz and %Pz of each sample, and bars indicate the mean ± SD. Statistical significance was analyzed using one-way ANOVA with or without Welch's correction. **P<0.01. Hyp mice, X-linked hypophosphatemic mice; %Hz, percentage hypertrophic zone thickness; %Pz, percentage proliferative zone thickness.

appearance of the distal femoral growth plate after TRAP staining. TRAP-positive cells were abundant among hypertrophic and calcified chondrocytes in WT mice, while they were remarkably reduced in untreated Hyp mice (Figs. 3A and S1). The Oc.S and Oc.S/BS were 2.8-fold and 3.4-fold larger in meclozine-treated Hyp mice than in untreated Hyp mice, respectively, although the difference was not statistically significant (Fig. 3B).

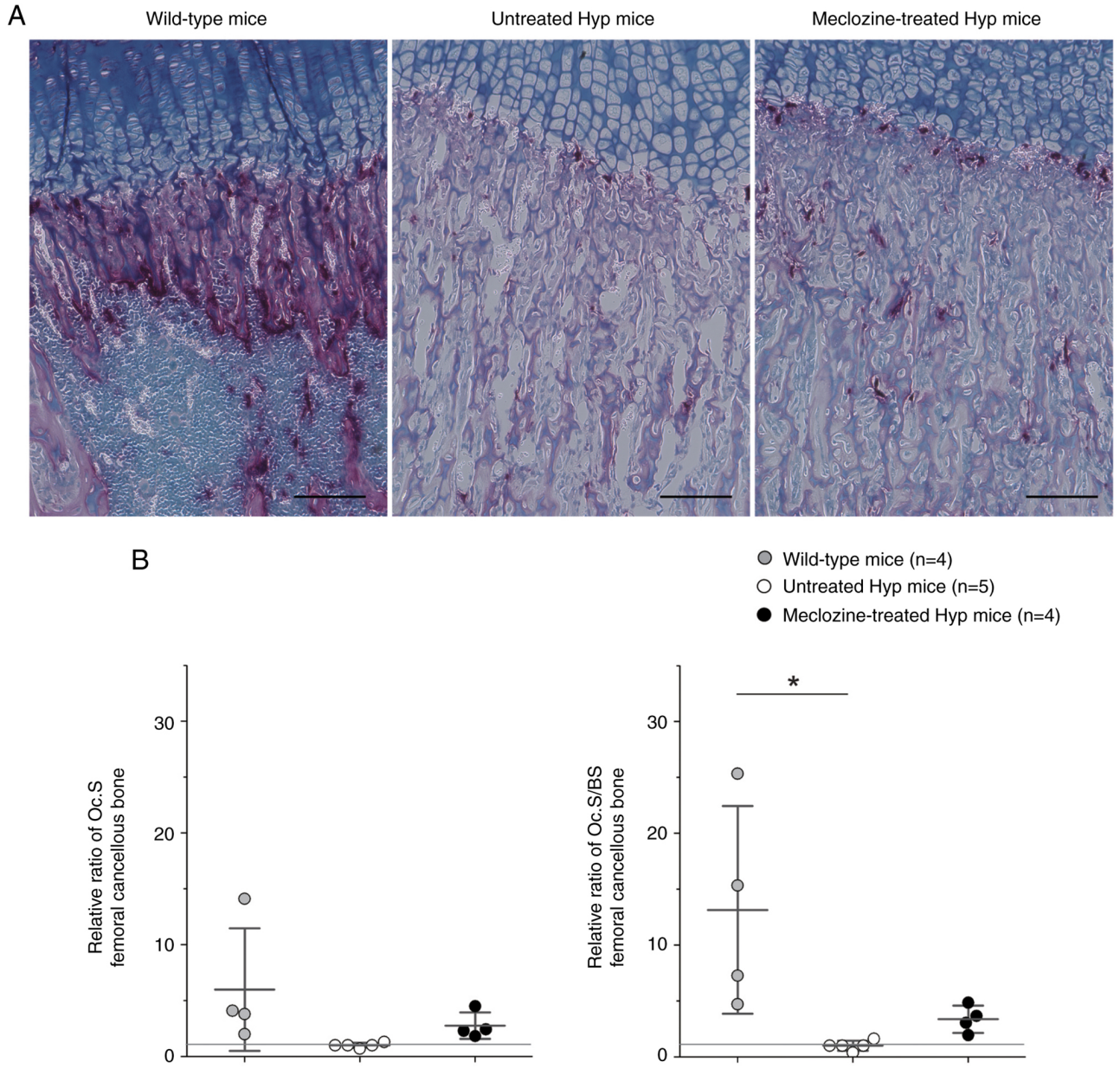

Figure 3. Meclozine slightly augments the surface of osteoclasts in Hyp mice. (A) Representative histological images of distal femoral metaphysis stained with tartrate-resistant acid phosphatase in meclozine-treated and untreated Hyp mice. The purple signal indicates osteoclasts. Scale bars, 100 μm. (B) Oc.S and Oc.S/BS. Dots indicate the Oc.S and the Oc.S/BS of each sample, and bars indicate the mean ± SD. Statistical significance was analyzed by one-way ANOVA with post-hoc Tukey's honest significance test. *P<0.05. Hyp mice, X-linked hypophosphatemic mice; Oc.S, osteoclast surface; Oc.S/BS, osteoclast surface/bone surface.

Meclozine has a marginal effect on metaphyseal bone mineralization in Hyp mice. In the trabecular bone, untreated Hyp mice presented larger OV and smaller BV than WT mice in the distal femur (Fig. 4A), proximal tibia (Fig. 4B), and first coccygeal vertebra (Fig. 4C). The OV/BV was reduced by 46% (P<0.01) in the coccygeal vertebra of meclozine-treated Hyp mice compared to untreated Hyp mice; whereas no statistical difference was detected in the distal femur and proximal tibia (Fig. 4D).

Meclozine slightly ameliorates serum calcium and phosphate levels, while boosting FGF23 secretion in Hyp mice. Serum calcium and phosphate levels were significantly higher in WT mice than in untreated Hyp mice (Fig. 5A and B). The 10-day-treatment with meclozine slightly increased serum calcium and phosphate levels, but had an even greater effect on serum FGF23 levels in Hyp mice (Fig. 5C).

Discussion

Burosumab, a recombinant human monoclonal antibody that targets FGF23, remains very expensive to administer to all patients with XLH. Additionally, some children fear the burosumab administration via injection, while oral administration of sodium phosphate does not always achieve the correct dosage due to increased renal phosphate wasting (22). We previously demonstrated that meclozine suppressed the short-statured phenotype of achondroplasia mice by attenuating FGFR3 signaling (12,13,23), and clinical trials

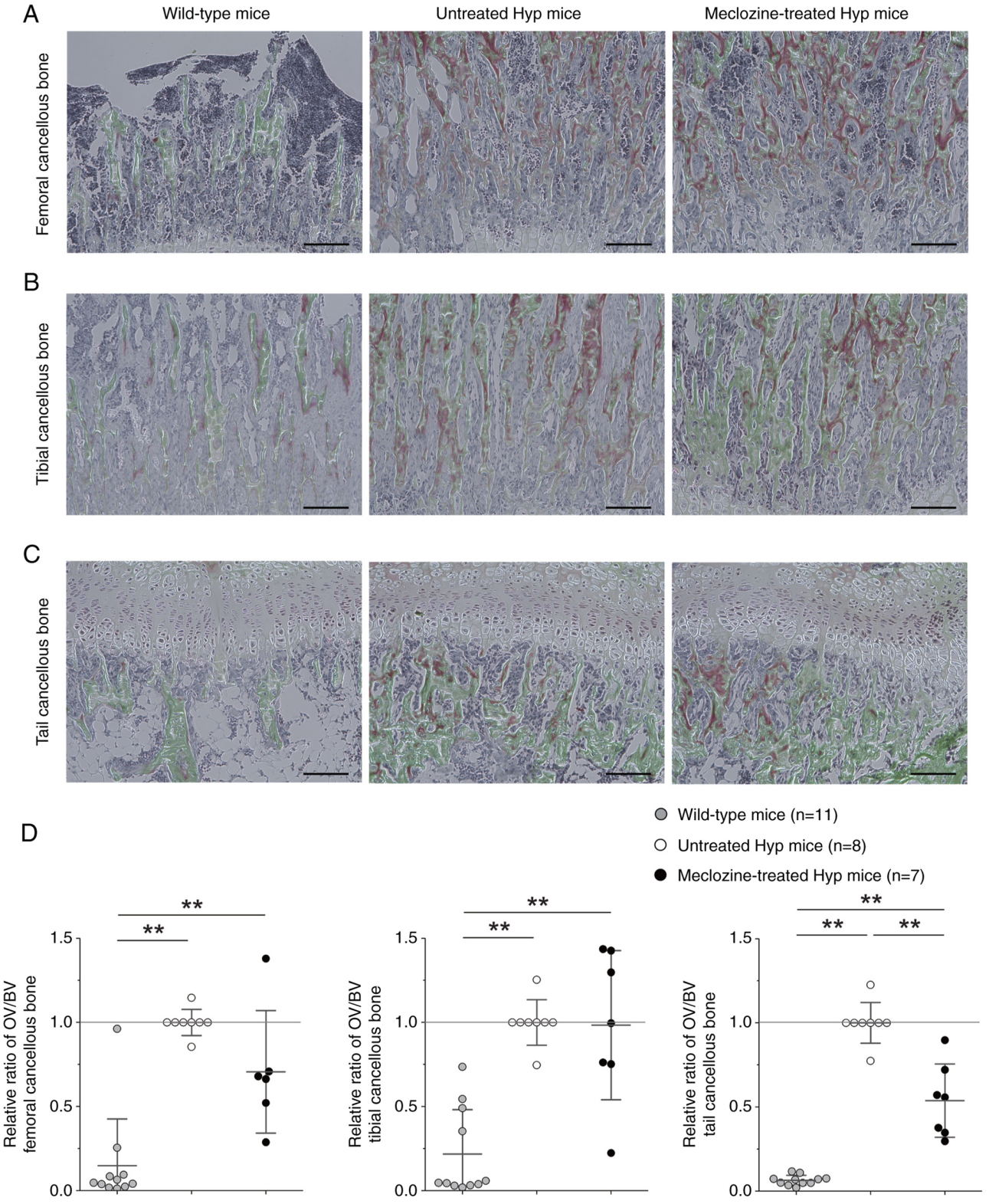

Figure 4. Meclozine partially rescues impaired mineralization of cancellous bone in Hyp mice. Representative non-decalcified histological images of the (A) distal trabecular bone of the femur, (B) proximal trabecular bone of the tibia and (C) proximal trabecular bone of the first tail vertebrae stained with Villanueva Goldner. The red and green signals indicate osteoid and bone tissue, respectively. Scale bars, 100 μm. (D) OV/BV in the tail, femoral and tibial bones. Dots indicate the OV/BV of each sample, and bars indicate the mean ± SD. Statistical significance was analyzed using one-way ANOVA with or without Welch's correction. **P<0.01. Hyp mice, X-linked hypophosphatemic mice; OV/BV, osteoid volume/bone volume.

of meclozine in children with achondroplasia are under way (24). To the best of our knowledge, current study demonstrated the first evidence of the effect of meclozine on improving bone mineralization in a mouse model of XLH. Oral administration of meclozine, which can be a cost-reduced treatment, would be reasonable considering long-term administration to patients with XLH from children to adults. The present study provides evidence that meclozine promotes bone mineralization, improves growth plate

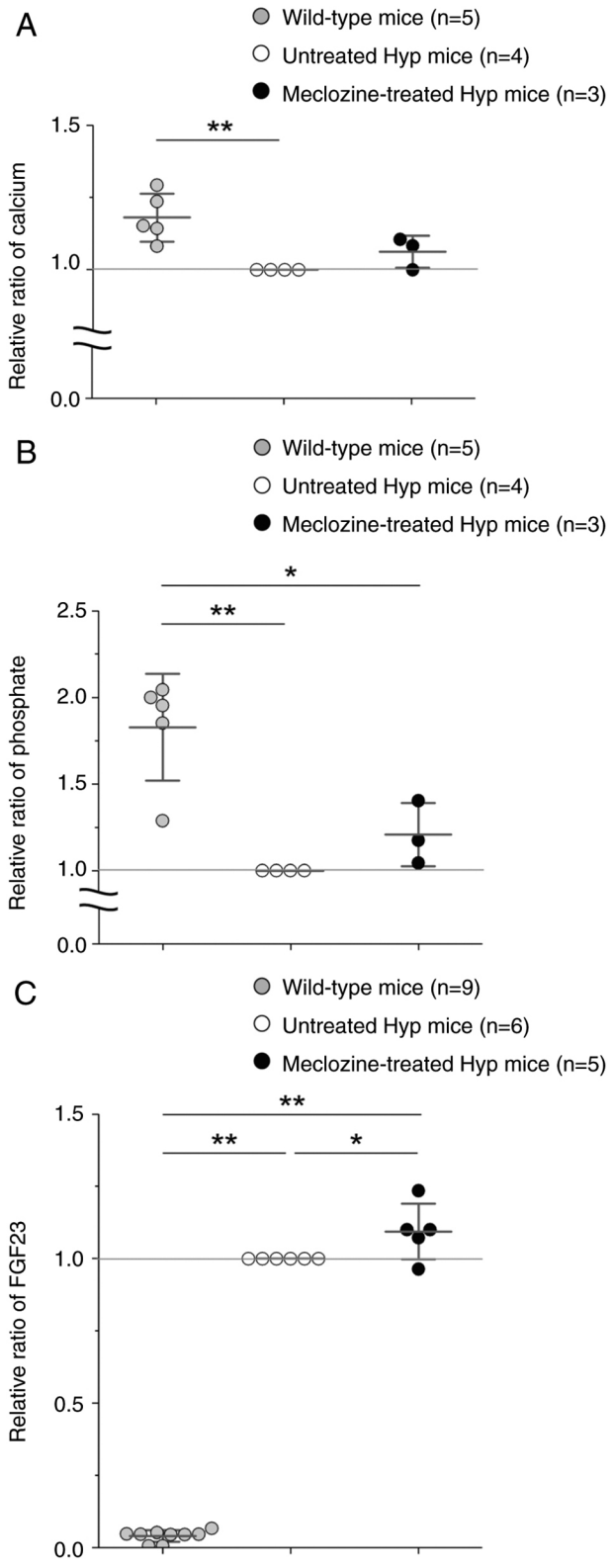

Figure 5. Meclozine tends to augment serum calcium, phosphate and FGF23 levels in Hyp mice. Relative serum (A) calcium, (B) phosphate and (C) FGF23 levels in meclozine-treated or untreated Hyp mice. Dots indicate the relative serum parameters of each sample, and bars indicate the mean ± SD. Statistical significance was analyzed using one-way ANOVA with post-hoc Tukey's honest significance test. *P<0.05, **P<0.01. Hyp mice, X-linked hypophosphatemic mice; FGF23, fibroblast growth factor 23.

structure, and marginally increases serum calcium and phosphorus levels in Hyp mice. The limited effect of meclozine on calcium and phosphorus may be explained by the partial contribution of FGFR3 hyperactivation to the etiology of hypophosphatemic rickets.

Histological analyses demonstrated that meclozine decreased OV in both cortical and cancellous bones, and realigned the growth plate structure of Hyp mice. Similar findings have previously been reported after treatment of Hyp mice with other FGFR3 inhibitors, such as NVP-BGJ398 and MAPK inhibitors (15,25). Downregulation of indian hedgehog, which is in the downstream of FGFR3, has also been shown involved in FGF23/α-klotho signaling (11). These results indicate that hyperactivation of FGFR3 signaling could be related to abnormal bone metabolism and disturbed growth plate structure including Pz and Hz through the MAPK pathway in hypophosphatemic rickets. The role of osteoclasts in the pathogenic skeletogenesis of Hyp mice is unclear. The number of osteoclasts was reported to be lower in 4-week-old Hyp mice than in WT mice (26). Administration of a MAPK inhibitor during lactation was demonstrated to inhibit osteoclastogenesis in Hyp mice (25). We confirmed that osteoclast activity was reduced in 17-day-old Hyp mice but could be slightly rescued in the distal femoral growth plate upon meclozine administration. Endochondral ossification requires precise orchestration of vascularization in hypertrophic chondrocytes, extracellular matrix remodeling, and recruitment of osteoclasts and osteoblasts. Osteoclast deficiency causes impaired growth plate ossification, resulting in the accumulation of hypertrophic chondrocytes and inhibition of vascularization. Increased osteoclast activity at the growth plate could be related to the reduced width of hypertrophic chondrocytes and accelerated calcification after treatment of Hyp mice with meclozine.

One-week treatment with a MAPK inhibitor was depicted to reduce serum FGF23 levels in Hyp mice (25). However, abnormally elevated serum FGF23 levels in Hyp mice were upregulated by 8-week administration of NVP-BGJ398 and 4-week administration of a MAPK inhibitor (15,27). Here, we indicate that repeated administration of meclozine slightly augmented serum FGF23 levels in Hyp mice. These conflicting outcomes could result from the complicated feedback loop controlling FGF23 metabolism. Meclozine, as well as other FGFR inhibitors, have been presented to elevate FGF23 and serum phosphorus levels in Hyp mice (15,27). Downstream signaling of highly activated FGF23 in Hyp mice may be disturbed by these FGFR inhibitors, leading to the suppression of renal phosphate excretion and consequent increase in serum phosphorus levels, although the exact mechanism of phosphate homeostasis has not been fully elucidated (27). In theory, elevated serum phosphorus levels stimulate the phosphorus-regulating hormone FGF23.

The current study has several limitations. First, we administered 2 mg/kg/day of meclozine to 7-day-old Hyp mice for 10 days, thus mimicking the protocol used for achondroplasia mice (13). The peak drug concentration of meclozine at 2 mg/kg/day in mice (13) was lower than that of meclozine 25 mg/body in adult humans (28). Administration of meclozine at 2 mg/kg/day to mice is likely to be clinically relevant, although different doses of meclozine, age of Hyp mice, and treatment protocols would likely lead to different results. Further studies such as a long-term toxicity tests using several kinds of animals are required to apply meclozine treatment to the human clinical setting. Second, the detailed biological

molecular mechanism of action of meclozine remains unknown, although we previously demonstrated that meclozine attenuates ERK phosphorylation downstream of FGFR3 signaling in chondrocytes (12). Meclozine was found to inhibit both ERK and p38 phosphorylation downstream of receptor activator of nuclear factor-κB ligand signaling in an OVX mouse model of postmenopausal osteoporosis (29). Moreover, we recently suggested that meclozine attenuated ERK and p38 pathways in FGF2-treated murine chondrocytes (30).

In conclusion, repeated administration of meclozine for 10 days partially ameliorated bone quality and growth plate structure in Hyp mice, probably due to inhibition of FGFR3 signaling. Thus, this mechanism successfully suppressed pathological phenotypes in mouse models of both achondroplasia and hypophosphatemic rickets.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YK, MM and HK designed the study. YK and KM acquired the data. YK, BO, TM, SI and KO analyzed and interpreted the data. YK and MM drafted the paper, while TM, SI, KO and HK revised it critically. YK and MM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal care and experiments conformed to the institutional guidelines of Nagoya University, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University (approval no. M210231; Nagoya, Aichi, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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