

# Teriparatide Improves Trabecular Osteoporosis but Simultaneously Promotes Ankylosis of the Spine in the Twy Mouse Model for Diffuse Idiopathic Skeletal Hyperostosis

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**Abstract** Diffuse idiopathic skeletal hyperostosis (DISH) is a common skeletal disorder in the elderly, which can develop into periosteal hyperostosis and paradoxically into immobilization-associated trabecular osteoporosis. The bone anabolic agent, teriparatide (TPD), seems to be a rational treatment for the immobilization-associated osteoporosis. However, it can lead to development of hyperostosis lesions in DISH patients. Here, we demonstrate TPD effectively treats trabecular osteoporosis while simultaneously promoting ankylosis of the spine in DISH model tiptoe-walking Yoshimura (twy) mice, compared with the ICR mice. Eighteen male twy mice were divided into three groups, and ICR mice were used as a normal control. Subcutaneous injections of TPD or phosphate-buffered saline (PBS) were performed according to three dosing regimens; 40 µg/kg once daily (TPD × 1 group), 40 µg/kg three times daily (TPD × 3 group), and PBS (control; Ctl group). Treatment was commenced at the age of 7 weeks and continued for 5 weeks. Micro-computed tomography (µCT) and histological analysis were performed. Longitudinal µCT study revealed that trabecular bone volume in both the vertebral body and distal femur decreased with time in the Ctl group, but increased dramatically in the TPD × 3 group. The twy mice developed ankylosis of the spine, the progression of which was

accelerated with TPD therapy. We also confirmed that TPD therapy promoted ossification of spinal ligaments. Histomorphometrical study revealed that TPD treatment increased bone formation at the vertebrae entheses region and in the trabecular bone. TPD therapy effectively treats trabecular osteoporosis, but potentially promotes ankylosis of the spine in patients with DISH.

**Keywords** Teriparatide · Diffuse idiopathic skeletal hyperostosis (DISH) · Osteoporosis · Spinal hyperostosis · Ankylosis of the spine

## Introduction

Diffuse idiopathic skeletal hyperostosis (DISH) is a skeletal disorder of unknown etiology characterized by ossification/calcification of ligaments and joint capsules [1–4]. DISH is very common in the spine of the elderly, with prevalence estimates of 27.3 % in men and 12.8 % in women aged 50 years and older, which increase with age [5]. Spinal ossification leads to immobilization of the spine, dysphagia, and respiratory tract disorder. Despite abundant bone formation in the spinal ligaments, elderly people with DISH are more likely to experience vertebral fractures compared with those without DISH [6–8]. One reason for this is that ankylosis of the spine develops paradoxically to trabecular osteoporosis owing to the stress-shielding effect. To date, however, there is no consensus on whether osteoporosis medication is recommended for fracture prevention in patients with DISH.

Intermittent administration of teriparatide (TPD), an active recombinant human peptide sequence of parathyroid hormone (PTH), is the only anabolic therapy available for osteoporosis. TPD has been proven to increase bone mass,

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improve bone microarchitecture, and reduce non-vertebral fractures as well as vertebral fractures [9, 10]. Hence, TPD is licensed for the treatment of osteoporosis in both men and postmenopausal women who are at high risk of incurring fractures. Consequently, administration of TPD seems to be a rational therapy for immobilization-associated osteoporosis. However, it potentially accelerates hyperostosis lesions of the joints and spine in patients with DISH.

In the current study, we aimed to clarify the therapeutic effect of TPD on trabecular osteoporosis in DISH and to investigate the adverse effect of TPD therapy on spinal hyperostosis using the tiptoe-walking Yoshimura (twy) mouse model for DISH.

## Materials and Methods

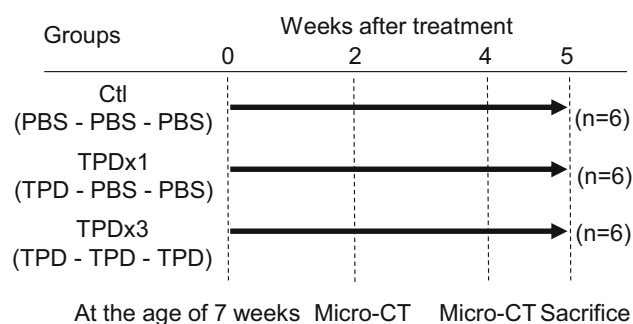
### Mice

The twy mice, introduced in Japan in 1978 by Hosoda et al. [11], are mutant mice showing multiple osteochondral lesions, and have been used as a model for DISH and ossification of the posterior longitudinal ligament [12–18]. Natural mutant twy mice are maintained by brother-sister mating of heterozygotes ICR mice at the Central Institute for Experimental Animals (Kawasaki, Japan). The twy mice harbor an autosomal recessive mutation in the nucleotide pyrophosphatase gene [19]. Systemic calcification and tip-toe walking of the twy mice occurs immediately after weaning and progresses within a short period of time. From 8 weeks of age, abnormal gait, stiffness of the vertebral column, contracture of limb joints, and trabecular bone loss [12] develop in the twy mice, and they begin to die at 12 weeks of age, with the majority dying before 30 weeks of age.

We purchased 18 twy mice from the Central Institute for Experimental Animals. The twy mice were housed in groups under a 12-h light/dark cycle, with access to food and water ad libitum.

### Study Design

Subcutaneous injections three times per day of TPD (human PTH<sub>1-34</sub>) (Forteo<sup>TM</sup>, Eli Lilly and Co., Indianapolis, IN, USA) or phosphate-buffered saline (PBS) were performed according to the dosing regimens described in Fig. 1. PBS three times per day was administered to the twy mice (control group: Ctl) and to the ICR mice as a normal healthy control (ICR). TPD was administered to twy and ICR mice according to the two dosing regimens; 40 µg/kg once daily (TPD × 1; TPD-PBS-PBS) and



**Fig. 1** Experimental protocol. Ctl: PBS control treatment; TPD × 1: treatment with TPD once daily and PBS twice per day; TPD × 3: treatment with TPD three times per day. Treatment was performed for 5 weeks. TPD: teriparatide 40 µg/kg. PBS: Phosphate-buffered saline

40 µg/kg three times daily (TPD × 3; TPD-TPD-TPD). Treatments were commenced at 7 weeks of age and were continued for 5 weeks ( $n = 6$  per group). After 5 weeks, the mice were anesthetized with a 5 µL/g cocktail of ketamine (20 mg/mL)/xylazine (2 mg/mL) and blood was collected via the inferior vena cava before euthanasia. The cervical and lumbar vertebrae, as well as the left hind limbs, were harvested, cleaned of all soft tissues, and fixed in 70 % ethanol for 3 days. Undecalcified sections were stained in Villaneuva Bone Stain. Bone section preparation was performed at the Ito Bone Histomorphometry Institute (Niigata, Japan).

### Micro-computer Tomography (µ-CT) Analysis

Cervical and lumbar spines and left femurs were scanned individually by µ-CT (R\_mCT2; Rigaku, Tokyo, Japan) at a 10-µm isotropic resolution. Because the cervical vertebrae were too small to evaluate trabecular structure, we measured the structural parameters of the 5th lumbar vertebral body for the spine. Measurements were performed using a TRI/3D-BON (Ratoc System Engineering Co., Tokyo, Japan) in accordance with the guidelines described in Bouxsein et al. [20]. For the 5th lumbar vertebral body, the centrum of each specimen, from the cephalad to caudal endplate and inside the endosteal margin, was used to assess trabecular bone morphology. For the femur, a 1000-µm area of interest from 100 slices encompassing the region of the distal metaphysis, starting from 300 µm proximal to the growth plate, was used to assess trabecular bone morphology. The following indices were calculated; trabecular bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), bone mineral density (BMD), and average cortical thickness (Ct.Th).

Ankylosis of the cervical spine was evaluated according to Mata's score [21]. Mata et al. have developed a scoring

system to grade DISH from 0 to 4 based on ossification at each disk space level, where 0 is defined as no ossification, 1 = ossification without bridging, 2 = ossification with incomplete bridging, 3 = complete bridging of the disk space, and 4 = severe ossification. We scored from the second to seventh cervical vertebra at each disk space level, with a maximum score of 20.

In addition, quantitative analysis on the volume of ectopic calcification at the posterior atlantoaxial membrane was performed based on CT-DICOM data using the TRI/3D-BON.

### Histological Analysis

After 5 weeks of injections of TPD or PBS, mice underwent double labeling with tetracycline and calcein prior to sacrifice. Double labeling was carried out by subcutaneous injections of tetracycline (20 mg/kg) 3 days before sacrifice, and calcein (20 mg/kg) the day before sacrifice. The purpose of double labeling is to analyze dynamic bone formation parameters between tetracycline and calcein labeling, as well as bone resorption, which is judged from the disappearance of the tetracycline labeling outlines.

Undecalcified sections of the cervical vertebrae were measured to determine the percentage of trabecular BV/TV, bone surface (BS), single-labeled surface (sLS), and double-labeled surface (dLS), and were used to calculate mineral apposition rate (MAR) and bone formation rate (BFR).

### Statistical Analysis

One-way analysis of variance with Turkey's multiple comparison test was performed to compare data in all groups. All data are expressed as means ± standard

deviations (SD) and a p value of less than 0.05 was considered statistically significant.

## Results

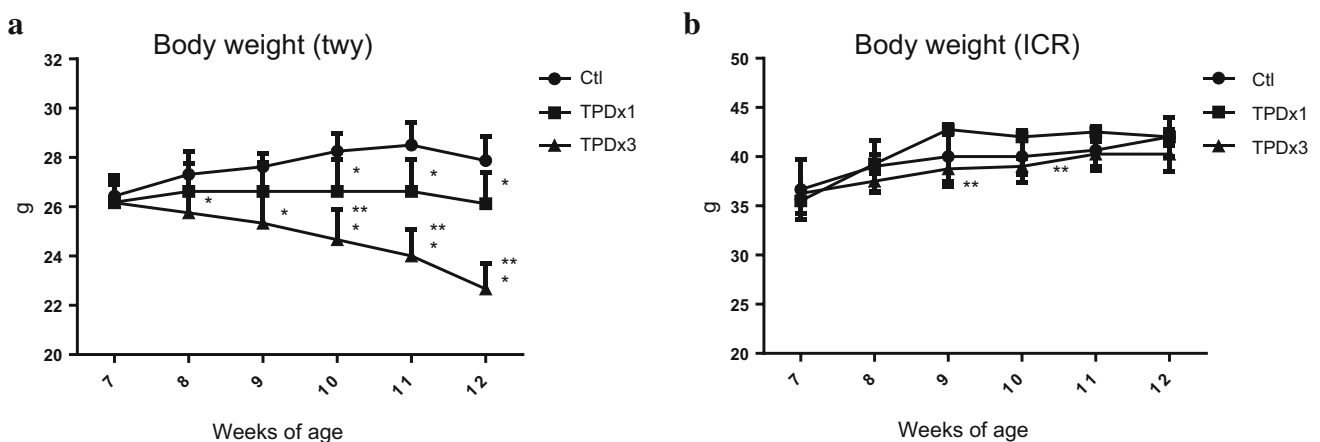
### General Observation

In the twy mice, ankylosis of the spine and contracture of lower extremity developed after 6 weeks of age, and presented with stiff neck and trunk, tip-toe walking, and less physical activity compared with the ICR mice. During the treatment period from 7 to 12 weeks of age, body size, and weight increased in the twy mice in the Ctl group, but body weight gain was much smaller than that in the ICR mice (Fig. 2). TPD treatment prevented increased body weight in the twy mice, while it did not have significant effect on body weight gain in the ICR mice. Body weight of the twy mice following TPD treatment decreased in a dose-dependent manner.

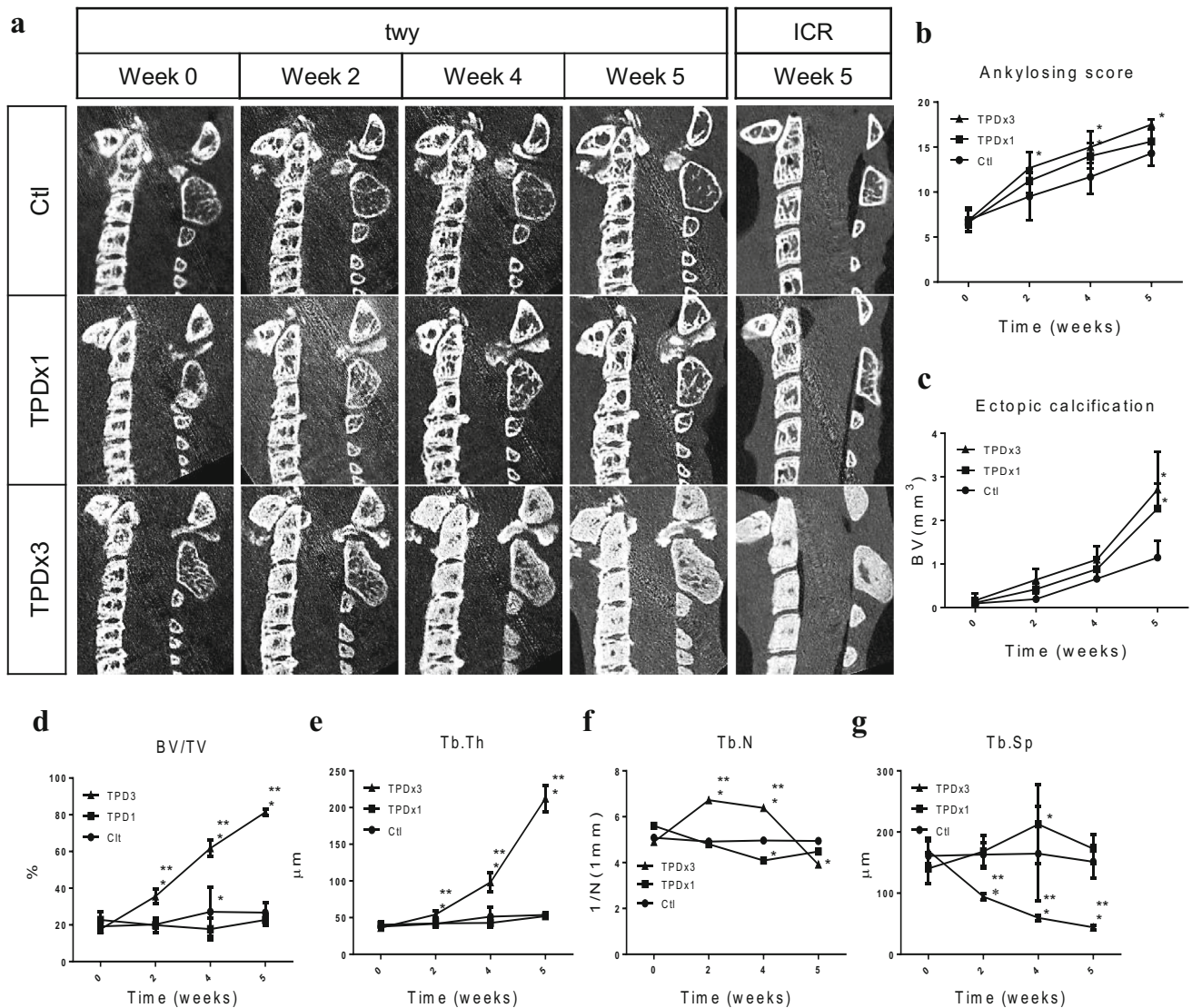
We also carefully observed behavior of the mice during the treatment period, because the twy mice develop paralysis due to spinal cord compression by ectopic ossification or calcification in the cervical spine [15], and TPD therapy could promote the incidence of paralysis. Although it was difficult to evaluate their paralysis because of joint contractures, the twy mice in the TPD × 3 group displayed more severe contracture of the knee joint and less physical activity compared with those in the Ctl group.

### Effect of TPD on Osteoporosis in Twy Mice

To elucidate the effect of TPD treatment on osteoporosis in the twy mice, we longitudinally measured the structural



**Fig. 2** Body weight changes during treatment in **a** twy and **b** ICR mice. In the twy mice, TPD therapy prevented body weight gain in accordance with the dose of TPD administration. Data represent mean ± SD (\**p* < 0.05 vs. Ctl, \*\**p* < 0.05 vs. TPD × 1)



**Fig. 3** Longitudinal dose–response radiographical assessment of osteoporosis and ankylosis of the spine of the mice using micro-CT imaging. Data represent mean  $\pm$  SD (\* $p < 0.05$  vs. Ctl, \*\* $p < 0.05$  vs. TPD  $\times$  1). **a** Representative sagittal reconstruction images of the cervical spine from each treatment and each time point analyzed (the same animal and cervical spine were used for longitudinal series);

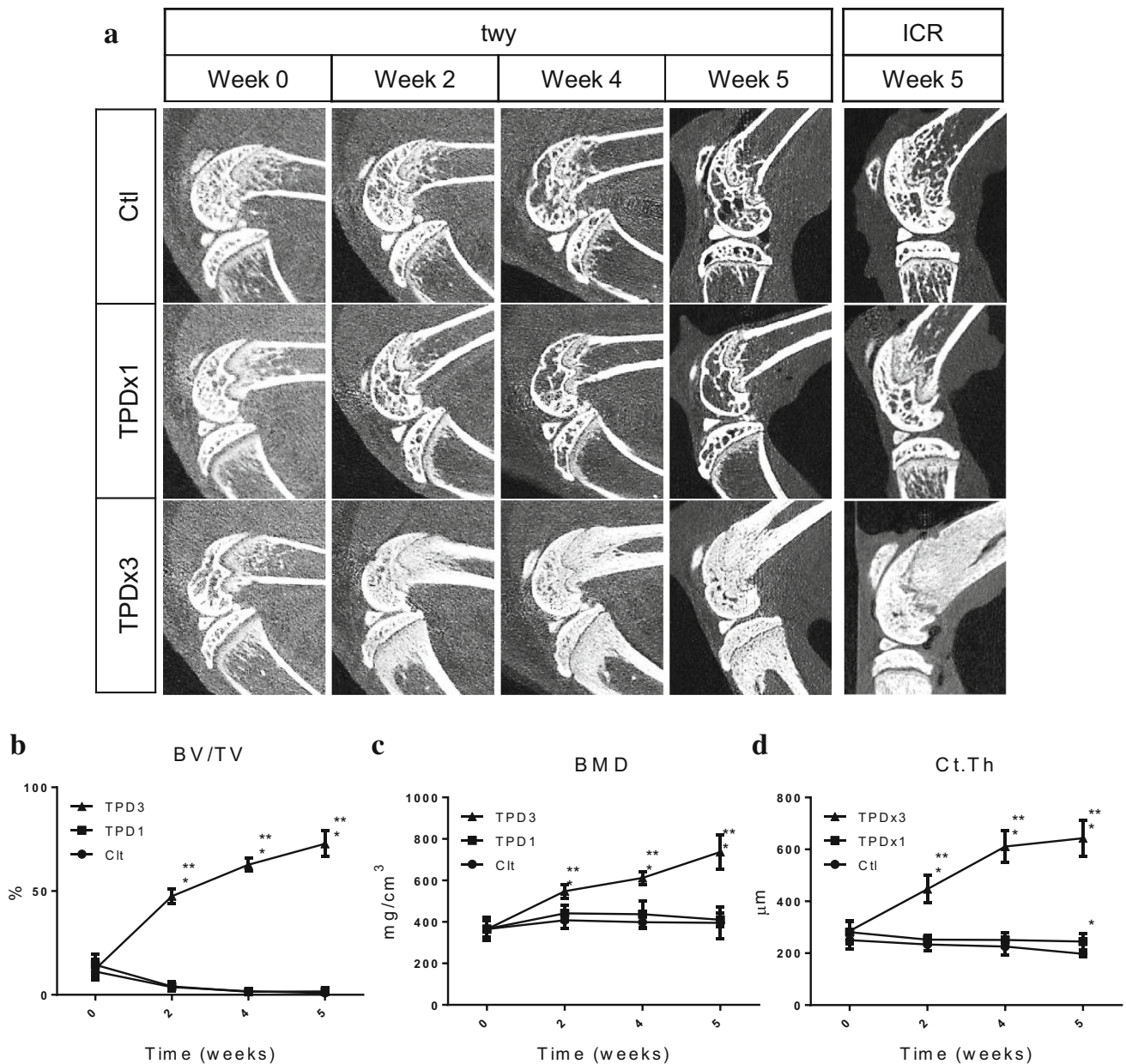
**b** Ankylosing score of the cervical spine according to Mata’s scoring system; **c** The volume of ectopic calcification of the posterior atlantoaxial membrane; **d–g** Trabecular bone volume/tissue volume (BV/TV), trabecular thickness (Tb·Th), trabecular number (Tb·N), and trabecular separation (Tb·Sp) of the 5th lumbar vertebral body

parameters of the 5th lumbar vertebral body and distal femur using  $\mu$ -CT imaging. The twy mice in the Ctl group developed osteoporosis in both the vertebral body and distal femur over time, despite their younger age. TPD  $\times$  3 treatment showed dramatic increases in both trabecular bone density and cortical bone thickness, while the effect of TPD  $\times$  1 treatment showed only a slight increase in these parameters in the twy mice (Figs. 3a, 4a).

Although BV/TV, Tb·Th, and Tb·N increased, and Tb·Sp decreased from 7 to 12 weeks of age in ICR mice (data not shown) as previously reported [22], these age-related changes in BV/TV and trabecular parameters were not seen in the twy mice. In the 5th lumbar vertebral

bodies, TPD  $\times$  3 treatment significantly increased BV/TV and Tb·Th, but decreased Tb·N and Tb·Sp in the twy mice. The decrease in Tb·N was possibly due to increased thickness and connectivity of trabecular bones. However, TPD  $\times$  1 treatment showed no significant effect in these parameters, despite  $\mu$ -CT imaging of the cervical spine showing a slight increase in bone density (Fig. 3d–g). In the distal femur, we identified a significant increase in BV/TV, BMD, and Ct.Th after TPD  $\times$  3 treatment, but not after TPD  $\times$  1 treatment in the twy mice (Fig. 4b–d).

To identify the effect of TPD on dynamic bone formation, we assessed under-calcified sections of cervical vertebrae (Fig. 5). TPD  $\times$  3 treatment significantly increased



**Fig. 4** Longitudinal dose–response radiographical assessment in the knee joint of treated mice using micro-CT imaging: **a** Representative sagittal reconstruction images of the knee joint from each treatment and each time point analyzed (the same animal and knee were used

for longitudinal series); **b–d** trabecular bone volume/tissue volume (BV/TV), bone mineral density (BMD), and average cortical thickness (Ct.Th) of distal metaphysis of femur. Data represent mean  $\pm$  SD (\* $p < 0.05$  vs. Ctl, \*\* $p < 0.05$  vs. TPD  $\times$  1)

labeled surfaces, MAR, and BFR/BS, leading to an increase in BV/TV compared with the Ctl (Fig. 5 the bottom row). TPD  $\times$  1 treatment increased the labeled surface but induced no statistically significant effect on BFR/BS, despite a trend toward an increase (Fig. 5x–z). Micro-CT analysis showed that BV/TV in the 5th lumbar vertebral body in the TPD  $\times$  1 group did not increase significantly compared with the Ctl, while BV/TV in the 6th cervical vertebral body was significantly increased histologically (Fig. 5u). Taken together, TPD  $\times$  3

treatment significantly enhanced bone formation, and TPD  $\times$  1 treatment mildly enhanced bone formation in the twy mice.

#### Effect of TPD on Ankylosis of the Spine and Ectopic Calcification in Twy Mice

Concerning the potential adverse effect of TPD on hyperostosis lesions, we assessed the effect of TPD on ankylosis of the spine and ectopic calcification in the twy mice.

Although we were unable to quantitatively measure the range of motion of the cervical spine, cervical motion of the twy mice was obviously restricted compared with the ICR mice. Moreover, cervical spine immobilization of the twy mice appeared accelerated following TPD  $\times$  3 treatment, as determined by manual palpation.

Reconstructed  $\mu$ -CT images and Mata's scoring system showed that TPD treatment accelerated ankylosis of the cervical spine in the twy mice (Fig. 3b). Histologically unorganized cartilage and irregular calcification were seen around the protruded disks in the twy mice, although no pronounced inflammatory cell infiltration was observed (Fig. 5f–h). Moreover, osseous bridges were observed across the outer layer of the annulus fibrosus of the intervertebral disks and hypertrophied spinal ligaments. These histological features were not observed in the ICR mice. After TPD treatment, larger osseous bridges and more cartilage formation was observed in the entheses lesions in the TPD groups compared with the Ctl group in the twy mice (Fig. 5f–h, j–l). These changes were more evident in the TPD  $\times$  3 group than in the TPD  $\times$  1 group. Fluorescent microscopy showed that the labeled bone surfaces in the entheses lesions increased in accordance with TPD treatment (Fig. 5b–d).

The volume of ectopic calcification at the posterior atlantoaxial membrane also increased in the twy mice with time, and both TPD  $\times$  1 and TPD  $\times$  3 treatments promoted ectopic calcification (Fig. 3c). Histologically, there were no cells inside the ectopic calcification but there were fibroblast- or osteoblast-like cells around the ectopic calcification. Calcification areas at the posterior atlantoaxial membrane were so fragile that they peeled off during tissue processing. The ectopic calcification lesions were not labeled with calcein and tetracycline, suggesting that calcification at the posterior atlantoaxial membrane developed in a different fashion from ossification (Supplementary Fig. 1).

## Discussion

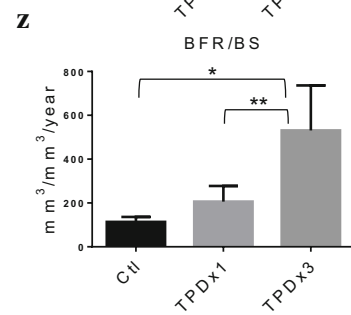
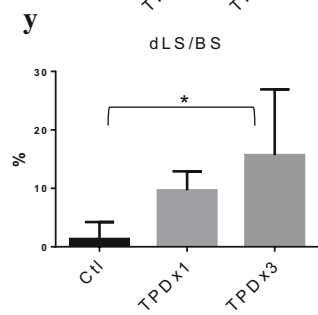
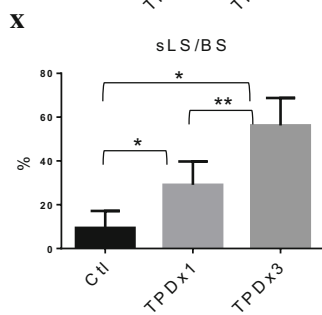
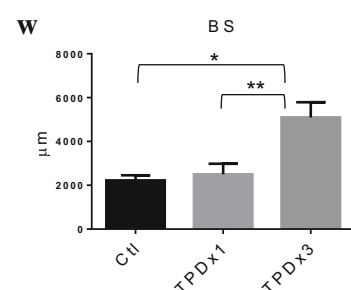
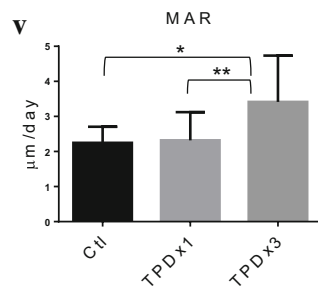
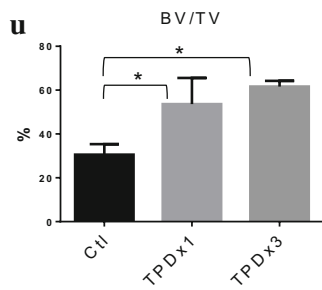
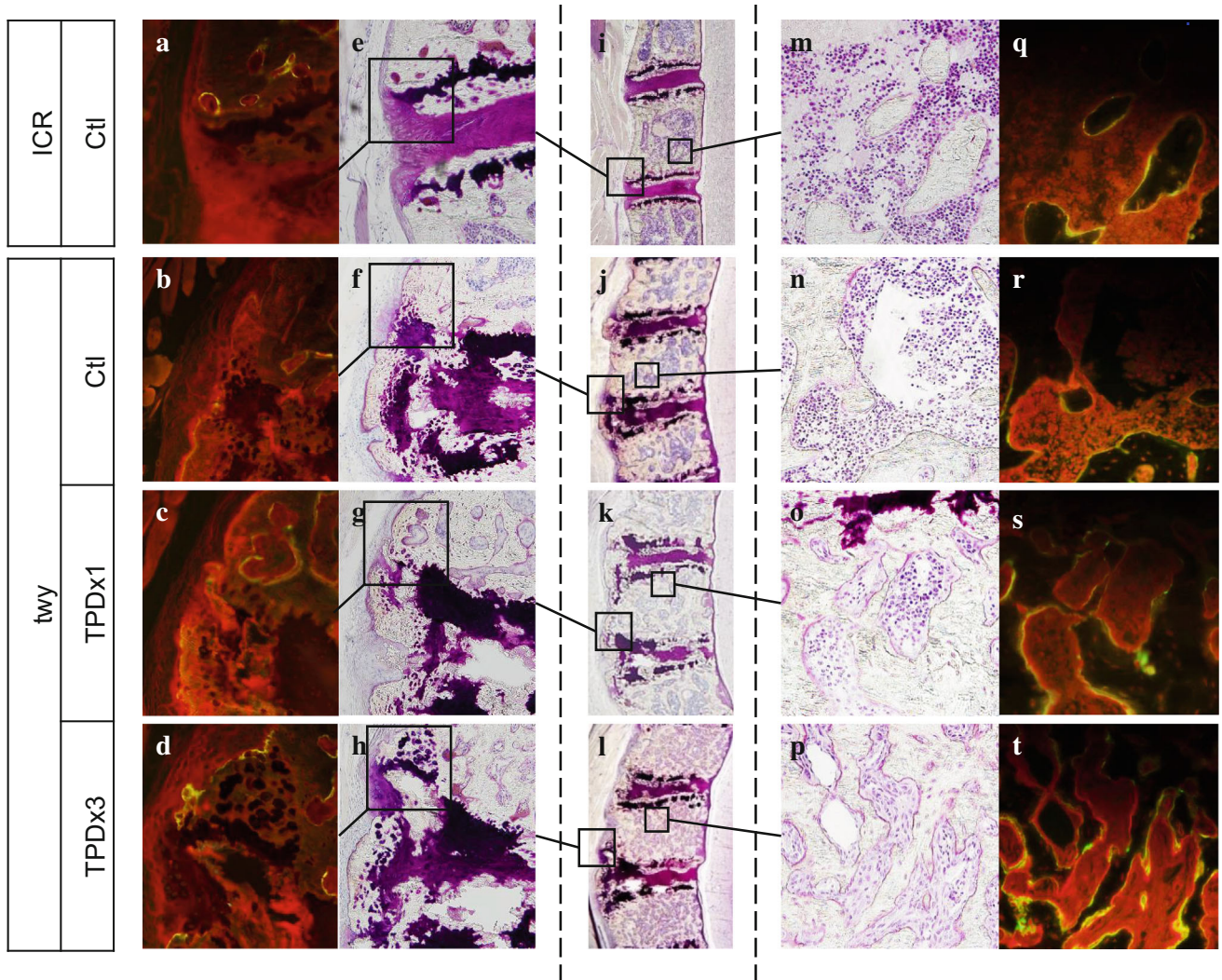
The results of this study suggest that TPD therapy exerts therapeutic effect on trabecular osteoporosis in DISH, which is mainly caused by decreased mechanical loading resulting from ankylosis of the spine. Although there is less evidence regarding the efficacy of TPD therapy in male osteoporosis compared with postmenopausal osteoporosis, there is a rationale for PTH therapy for the treatment of osteoporosis in DISH. Immobilization-associated osteoporosis is attributable to a decrease in bone formation via down-regulation of wnt/ $\beta$ -catenin signaling and TPD exerts its anabolic effects on bone formation by the induction of wnt/ $\beta$ -catenin pathways [23–26]. Therefore, TPD should

**Fig. 5** Histology and histomorphometric study of the 6th cervical vertebra. **a–h** Microphotographs at the entheoperidiscal region of the 6th cervical vertebra under high magnification, **a–d** fluorescent field imaging, **e–h** bright field imaging with Villanueva staining; **i–l** Bright field sagittal images of 5–7th cervical vertebral bodies under low magnification with Villanueva staining; **m–t** Microphotographs of the 6th cervical vertebral body under high magnification with Villanueva staining, **m–p** bright field imaging with Villanueva staining, **q–t** fluorescent field imaging; **u–z** Bone histomorphometry data, including trabecular bone volume/tissue volume (BV/TV), mineral apposition rate (MAR), bone surface (BS), single-labeled surface/bone surface (sLS/BS), double-labeled surface/bone surface (dLS/BS), bone formation rate/bone surface (BFR/BS). Data represent mean  $\pm$  SD (\* $p$  < 0.05 vs. Ctl, \*\* $p$  < 0.05 vs. TPD  $\times$  1)

counteract the stress-shielding effect on bone. This is also supported by a previous study demonstrating that TPD therapy is effective for immobilization-associated osteoporosis in the neurectomized hindlimb murine model [27]. It should be noted, however, that bone environment of ankylosed spine may blunt response to TPD therapy because 40  $\mu$ g/kg once daily injection of TPD, which is a promising dose for anabolic effects on rodents osteoporosis, showed only a slight increase in bone volume in the twy mice.

Despite the therapeutic effect of TPD on trabecular osteoporosis in DISH, our data suggest a potential adverse effect of TPD therapy on ankylosis of the spine and ectopic calcification. The Mata's score data based on  $\mu$ -CT and histological observation support this idea. Although we did not obtain quantitative data regarding stiffness of the spine because of a technical difficulty, cervical spines of TPD-treated twy mice were stiffer than those of PBS-treated twy mice, as determined by manual palpation at sacrifice. As a result of enhanced development of ankylosis of the spine, TPD-treated twy mice lost body weight with time, while PBS-treated twy mice did not lose body weight. Given that TPD-treated ICR mice did not lose body weight, we can say that TPD therapy does not directly reduce body weight but reduces it as a result of promoting ankylosis of the spine, which in turn leads to muscle disuse atrophy, dysphagia, and difficulty of mastication. In clinical practice, TPD therapy might increase the risk for dysphagia, and respiratory tract disorder by promoting development of ossification of spinal ligaments in patients with DISH. The adverse effect of TPD therapy may be applicable to the patients with ankylosing spondylitis, who develop ankylosis of the spine as a result of chronic inflammation in the entheses of the spine.

Although the mechanism of ectopic ossification in spinal ligaments remains unknown, several studies using the twy mouse model have demonstrated that osteoporosis medication can have an effect on spinal hyperostosis lesions. Hirakawa et al. reported that administration of etidronate, which is a non-nitrogen-containing bisphosphonate, accelerated prolapse of intervertebral disks, and increased the thickness



of posterior longitudinal ligaments in the twy mice [13]. Okawa et al. showed that calcitonin suppressed periosteal bone formation in the twy mice regardless of age [16]. Although we observed a promotion effect of TPD on ossification of spinal ligaments in the twy mice, Sampson et al. demonstrated that TPD did not enhance osteophyte formation in the mouse model of knee osteoarthritis using C57B16 mice [28]. This indicates that spinal hyperostosis lesions or the twy mice are susceptible to these osteoporosis medications.

This study has several limitations. First, it is inconclusive whether the dosing regimens used correspond to clinical dosing for osteoporosis. Rodent models typically report the use of doses as high as 40–200 µg/kg per day, which are 100- to 500-fold greater than the recommended clinical dose in humans (20 µg per day total, which in a 40–80 kg person translates to 0.2–0.4 µg/kg per day). These differences are thought to arise from differences in metabolism and clearance of TPD between humans and rodents. Bellido et al. indicated that daily injection of as little as 30 µg/kg of TPD for 28 days increases spine and hindlimb BMD, using 3–300 µg/kg of TPD to determine the minimum effective dose requirement for an anabolic effect of TPD in mice. Additionally, they reported that four-times-daily injections of TPD reduce the number of apoptotic osteoblasts by 50 % [29]. Dobning et al. found that the optimal response of the rodent skeleton to TPD require a three-times-daily subcutaneous dose regimen, given the rapid clearance of TPD [30]. However, to the best of our knowledge, there are few reports that demonstrated anabolic responses to TPD using ICR mice [31], we tested a three-times-daily injection of TPD in addition to a once-daily injection of TPD (40 µg/kg) in this study based on these previous studies. However, given that mice and human react differently to TPD, conclusive statements about the effect of TPD on osteoporosis and ankylosis of the spine in patients with DISH requires investigation in larger animal species, which might better approximate the metabolism of the drug in humans. The problem is that there are currently no animal models for DISH other than mouse models.

Another limitation of this study is the young age of the mice, while the human age at onset of DISH is predominantly over 50 years [1] [3]. The reason we commenced TPD treatment from the age of 7 weeks is that the twy mice sometimes die after the age of 12 weeks because of paraplegia, which is caused by spinal cord compression with the development of ossification or calcification of spinal ligaments. Although it is of great interest whether TPD exacerbates paralytic symptoms or not, we did not perform MRI or histological observation on spinal cord in this study because the twy mice usually develop profound motor paresis at the age of 18–24 weeks [32]. We carefully observed behavior of the mice during the treatment period from the age of

7–12 weeks; however, we could not find apparent motor paralysis in the twy mice regardless of treatment.

In conclusion, we have investigated the effect of TPD on skeletal hyperostosis lesions using the twy mice, which is the murine model for DISH. The data obtained in this study suggest that TPD therapy effectively treats trabecular osteoporosis, while it potentially promotes ossification/calcification of spinal ligaments and ankylosis of the spine in patients with spinal hyperostosis.

#### Compliance with Ethical Standards

**Conflict of Interest** Hiroki Hamano, Masahiko Takahata, Masahiro Ota, Shigeto Hiratsuka, Tomohiro Shimizu, Yusuke Kameda, and Norimasa Iwasaki declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** The Ethics Review Committee for Animal Experimentation of Hokkaido University approved the experimental protocol.

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