Treatment with bone maturation and average lifespan of HPP model mice by AAV8-mediated neonatal gene therapy via single muscle injection

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Hypophosphatasia (HPP) is an inherited skeletal disease characterized by a deficiency in tissue-nonspecific alkaline phosphatase (TNALP). Patients with the severe infantile form of HPP may appear normal at birth, but their prognosis is very poor. To develop a practical gene therapy for HPP, we endeavored to phenotypically correct TNALP knockout (Akp2<sup>−/−</sup>) mice through adeno-associated virus type 8 (AAV8) vector-mediated, muscle-directed, TNALP expression. Following treatment of neonatal Akp2<sup>−/−</sup> mice with a single intramuscular injection of ARU-2801 (AAV8-TNALP-D10-vector) at 1.0 × 10<sup>12</sup> vector genomes/body, high plasma ALP levels (19.38 ± 5.02 U/mL) were detected for up to 18 months, and computed tomography analysis showed mature bone mineralization. Histochemical staining for ALP activity in the knee joint revealed ALP activity on the surface of the endosteal bone of mice. Throughout their lives, the surviving treated Akp2<sup>−/−</sup> mice exhibited normal physical activity and a healthy appearance, whereas untreated controls died within 3 weeks. No ectopic calcification or abnormal calcium metabolism was detected in the treated mice. These findings suggest that ARU-2801-mediated neonatal intramuscular gene therapy is both safe and effective, and that this strategy could be a practical option for treatment of the severe infantile form of HPP.

INTRODUCTION

Hypophosphatasia (HPP) is an inherited systemic skeletal disease characterized by a deficiency in tissue-nonspecific alkaline phosphatase (TNALP) due to TNALP gene mutations, which lead to abnormal mineralization of bones and teeth.1–3 Patients with the infantile form may appear normal at birth, but they gradually develop rickets as a clinical symptom within 6 months of age. Subsequent craniostenosis and nephrocalcinosis follow hypercalcemia and hypercalciuria in many cases.4 In addition, pyridoxine-dependent seizures preceding skeletal change are a predictor of lethal outcome.5 Skeletal changes leading to thoracic deformity and rib fracture cause respiratory problems, which sometimes threaten the lives of these patients.

In adult HPP patients, some benefit has been derived from several treatment approaches, including enzyme replacement therapy (ERT) to increase plasma ALP levels6–8 and administration of parathyroid hormone.9,10 However, there are some problems about ERT for HPP patients, such as the emergence of antibodies for the recombinant enzyme (which may be exacerbated by chronic exogenous dosing) and difficulty transferring the enzyme to the central nervous system. Improvement of infantile HPP patients has reportedly been achieved through bone marrow transplantation.11,12 Allogenic mesenchymal stem cell transplantation was proven for perinatal lethal HPP apathanasia and bone mineralization for 3 years, although cell therapy is always accompanied with chemotherapy, radiation therapy, and immunotherapy before the therapy and possible graft-versus-host disease after transplantation, which threaten patients’ lives.13 Even better results were observed in mice administered recombinant TNALP fused with deca-aspartate (D10) to target bone.14 Those results led to the development of an ERT with TNALP-D10 for human HPP patients.15 This important breakthrough improved survival times for patients with severe infantile HPP as well as the quality of life of both children and adults with HPP. However, ERT requires frequent subcutaneous injections (up to six times per week), which is burdensome for patients in terms of time, pain, and costs. The most frequent adverse effect of ERT is injection site reactions related to the frequency of the subcutaneous injections.16

Gene therapy has proved to be a useful tool for the treatment of several inherited diseases. In an earlier study, we succeeded in treating TNALP knockout (Akp2<sup>−/−</sup>) mice, a rodent model of HPP, with a single intravenous injection of a lentiviral or adeno-associated virus
type 8 (AAV8) vector encoding TNALP-D10, which led to sustained expression of TNALP and phenotypic correction of Akp2–/– mice.14,15 However, intravenous injection of AAV vector leads to distribution of the vector to the whole body, which could potentially cause cancer or transduction into germ cells. Intramuscular injection may limit the vector’s distribution. We therefore treated Akp2–/– mice through intramuscular injection of a vector encoding TNALP with a muscle-specific creatine kinase (MCK) promotor and achieved prolonged survival of Akp2–/– mice.18,19 However, the muscle-specific expression was not sufficient to mediate bone maturation in Akp2–/– mice comparable to that in Akp+/+ wild-type (WT) mice. In the present study, therefore, to develop a practical and safe gene therapy for HPP, we ventured to inject ARU-2801, an AAV8-TNALP-D10-vector with a non-tissue-specific constitutive promotor, into the right quadriceps femoris muscle and evaluated the benefits and adverse effects in Akp2–/– mice over a prolonged period of time.

RESULTS

Survival and sustained TNALP expression in mice intramuscularly injected with more than 3.0 × 10^1¹ vector genomes (vg)/body ARU-2801

From our previous intravenous treatment studies,17,20 we expected that the exogenous plasma ALP activity needed for survival of Akp2–/– mice would be around 10 U/mL, which is 100-fold higher than the endogenous activity in WT mice. To determine the amount of vector needed to achieve a titer able to support Akp2–/– mouse survival, neonatal mice were first intramuscularly injected with ARU-2801 (1.0 × 10^1² vg/body). The mice were then monitored to determine whether TNALP delivery via intramuscular ARU-2801 injection could phenotypically correct Akp2–/– mice. For 2 months after ARU-2801 injection, plasma ALP activity in the treated mice remained markedly higher than in WT mice (14.8 ± 4.3 versus 0.1 ± 0.004 U/mL at 2 months) (Figure 1A). Moreover, five of the seven treated mice survived for more than 18 months (Figure 1B), and sustained expression of ALP activity was confirmed, even at 18 months (19.38 ± 5.02 U/mL) (Figure 1A). Treatment failed in two mice; one died on day 25 and the second died on day 393. In similar fashion, we also tested the efficacy of administering lower doses of 3.0 × 10^1¹ or ALP activity (Figure 1A) and lived for more than 12 months until sacrifice (n = 3/7 survived) (Figure 1B). In contrast, with one exception, mice administered 1.0 × 10^1¹ vg/body died within 3 weeks (n = 1/5 survived), and in the remaining mouse, ALP activity reached 0.7 U/mL at 2 months, and the mouse died soon thereafter.

This prolonged survival showed that at vector doses of 3.0 × 10^1¹ vg/body or higher, plasma ALP activity reaches a plateau sufficient to support the treated mice throughout their life. We did not find any convulsion in these treated Akp2–/– mice. We did not observe the expected dose dependency of plasma ALP activity. Although 3.0 × 10^1¹ vg/body ARU-2801 showed significant effects, 1.0 × 10^1² vg/body ARU-2801-treated mice were further analyzed given their higher survival rate.

Mature bone mineralization in mice treated with 1.0 × 10^1² vg/body ARU-2801 Akp2–/– at 18 months

Sustained ALP activity not only prolonged the lives of Akp2–/– mice, it also improved the maturity of their bone mineralization. There was no significant difference in average body weight between WT mice (n = 3) and 1.0 × 10^1² vg/body ARU-2801-treated Akp2–/– (n = 5) mice (Figures 2A and 2B). Secondary ossification centers were detected in all of the WT mice (n = 8/8) 10 days after birth. Alternatively, most of the untreated Akp2–/– mice did not have secondary ossification centers on day 10 (n = 3/10) (Figure 2C). After treatment, secondary ossification centers were found on day 10 in 1.0 × 10^1² vg/body ARU-2801-treated Akp2–/– mice (n = 9/10) similar to those seen in WT mice. When grown to adulthood, on day 56, X-ray analysis of the knee joint showed mature bone in 1.0 × 10^1² vg/body ARU-2801-treated Akp2–/– mice, the same as found in WT mice (Figure 2D). Untreated Akp2–/– mice, however, did not survive to adulthood (no survival by day 21) and were therefore not possible to analyze.

We next used computed tomography (CT) to analyze the structure of the femurs of 18-month-old 1.0 × 10^1² vg/body ARU-2801-treated Akp2–/– mice (n = 4) and AAV8-GFP-vector-treated WT mice as control (n = 3). At 18 months, bone mineral densities (673.7 ± 41.6
versus 620.8 ± 52.9 mg/cm³; p = 0.26) in the treated mice were not significantly different from those in the WT controls (Figure 2E).

ALP activity in the bones of mice treated with $1.0 \times 10^{12}$ vg/body ARU-2801

Histological images of the knee joint are shown in Figure 3. ALP activity is revealed by fast blue staining. Blue-stained areas were seen in the surface of the endosteal bone and in the zone of resorption, where cartilage was calcifying in WT and $1.0 \times 10^{12}$ vg/body ARU-2801-treated Akp2−/− mice.

Vector distribution shows restriction to injected muscle

Although the vector was injected intramuscularly, vector volume beyond the muscle’s capacity may leak into the blood and circulate systemically. We previously reported that low AAV8-MCK-TNALP-D10 copy numbers were detected in the heart, liver, and bone following injections of 15 μL of AAV8-MCK-TNALP-D10 into the neonatal quadriceps femoris muscle. Therefore, the distribution of $1.0 \times 10^{12}$ vg/body ARU-2801 was analyzed in the liver, muscle, heart, and bone of treated mice. Using real-time PCR, we detected no AAV vector genome in any organ other than the ARU-2801-injected muscle. Since germline insertion of viral vector is important to assess for clinical use, we also analyzed the AAV vector genome in the testis and ovary of treated mice. ARU-2801 was not detected in the gonads and reproductive systems of treated animals. Real-time PCR revealed the presence of AAV vector only in muscle at the site of vector injection (data not shown).

No liver or kidney dysfunction was detected in treated Akp2−/− mice

In $1.0 \times 10^{12}$ vg/body ARU-2801-treated Akp2−/− mice, biochemical data, liver and kidney function, and calcium levels were examined. Plasma calcium metabolism in all treated mice was similar to that in WT mice (calcium, 9.5 ± 0.45 versus 9.5 ± 0.63 mg/dL; p = 0.5). Liver...
and kidney function were in the normal range. Although the mice treated with $1.0 \times 10^{12}$ vg/body ARU-2801 had plasma ALP activity (19.38 ± 5.02 U/mL) that was as much as 397-fold higher than endogenous levels in WT mice, there were no obvious problems in ARU-2801-treated mice. Furthermore, all organs looked normal macroscopically and no abnormal cell growth or tumors were observed.

**Ectopic calcification seen on the paws of WT high-dose ARU-2801-treated mice with extremely high plasma ALP activity, but not with disease model ARU-2801-treated Akp2−/− mice**

Constitutively high plasma ALP activity is not a physiological state. Therefore, to assess the safety and adverse effect of constitutively high plasma ALP activity, we injected large amounts of ARU-2801 into 6-week-old WT C57BL/6 mice. Mice injected with $5.5 \times 10^{13}$ vg/body ($n = 3$), $55 \times$ the highest dose used in Akp2−/− mice, and followed until morbidity were seen around 6 months of age with high plasma ALP activity ($8,347.3 \pm 5,738.4$ U/mL) (Figure 4A). All three mice administered this high-vector dosage developed calculi. One had a calculus in each metacarpal pad of the forepaw 2 months after injection and a small calculus in a digital pad of the left hind paw at 6 months. Another mouse had two calculi in each metacarpal pad at 2 months, while the third mouse had a small calculus in the digital pad of the left forepaw at 3 months. When mice ($n = 3$) were injected with a lower dose of vector ($2.8 \times 10^{13}$ vg/body, ~28× the highest dose used in Akp2−/− mice), the level of ALP activity was about an order of magnitude less than with the $5.5 \times 10^{13}$ vg/body dosage (373.0 ± 215.3 U/mL). In these mice, ectopic calcification started to appear about 4 months after injection. The calculi were solid and opaque to X-ray (Figure 4B). Infrared spectroscopy analysis of the stones by SRL (Tokyo, Japan) showed that they were composed mainly of calcium phosphate (55%) and calcium carbonate (45%). X-ray analysis revealed no calcification of blood vessels. Von Kossa staining showed no ectopic calcification in liver, heart, muscle, kidney, and blood vessels of these high-dose ARU-2801-treated WT mice. Additional WT C57BL/6 mice were also injected with lower doses of $1.1 \times 10^{13}$ and $2.8 \times 10^{12}$ vg/body (10× and ~3× the highest dose used in Akp2−/− mice), and they showed no stones in their paws or any other surface of the body throughout the experiment period, i.e., 6 months. Importantly, note that in ARU-2801-treated Akp2−/− mice, no ectopic or vessel calcification (data not shown) was observed at the highest efficacious dose, i.e., $1.0 \times 10^{12}$ vg/body.

**DISCUSSION**

We previously reported that intravenous AAV8 vector-associated systemic ERT achieved phenotype correction in Akp2−/− mice.20 However, local intramuscular injection may prevent off-target effects, which have occurred with systemic AAV vector transfer.21 Moreover, transduction to muscle cells with AAV vectors is likely to support sustained transgene expression because muscle cells are not dividing. AAV type 1-based intramuscular gene therapy is already certified to be safe for humans.22 In the present study, we treated Akp2−/− mice with a single intramuscular injection of $1.0 \times 10^{12}$ vg/body ARU-2801.

ARU-2801 was the same vector driven by the nonspecific constitutive promoter we used for intravenous systemic injection.20 As with systemic administration, Akp2−/− mice treated with intramuscular injection exhibited high plasma ALP activity during 12 months (until sacrifice) and phenotypic correction. This result shows the efficacy of localized vector injection for treatment of Akp2−/− mice. Ideally, the AAV8 vector would remain in the muscle, producing enough TNALP to keep plasma ALP activity at a level sufficient to support bone mineralization, suppress seizures, and enable Akp2−/− mice to survive.

We previously observed that although the MCK promoter has the advantage of driving muscle-directed TNALP expression, the treated mice did not reach satisfactory bone maturity as compared to WT mice.18,19 The promoter activity of tissue-specific promoter is weaker than that of tissue-nonspecific promoter. Moreover, tissue-nonspecific constitutive promoter has strong promoter activity in mammalian cells. We therefore aimed to achieve higher ALP expression and better bone maturation using an AAV vector encoding TNALP-D10 driven by the tissue-nonspecific constitutive promoter, which yielded high plasma ALP activity after systemic administration. To achieve sufficient plasma ALP activity with the MCK promoter, it was necessary to inject as much as 15 μL of vector into both quadriceps femoris muscles of neonatal mice ($5.0 \times 10^{12}$ vg/body). However, bone calcification still remained unsatisfactory. In the present study, in contrast, Akp2−/− mice lived healthily for 18 months, or nearly their entire lifetime, after a single intramuscular injection of only 2 μL of ARU-2801 administered during the neonatal period. The therapeutic effect of intramuscular injection was not inferior to that of systemic injection in terms of lifespan or physical phenotype. This suggests that the 2 μL of vector injected into the quadriceps femoris muscle of neonatal mice localized where it was injected. Muscular injection may be safer than intravenous injection because the vector
stays in the muscle and is not distributed systemically. We also detected no liver or kidney dysfunction. Statistical analysis of CT data revealed increases in bone width and hyperplasia in the femurs of treated mice, and epiphysial cartilage structure was irregular. Diaphysis volume was nearly the same as in WT femurs. Given these results, it is our conjecture that the quality of the trabeculae in treated mice was nearly the same as in WT mice, although accelerated widening similar to flaring in rickets was found. Thus, a single intramuscular ARU-2801 injection during the neonatal period appears to be safe and effective for treatment of HPP.

The dose dependency of the response to systemic treatment for Akp2<sup>−/−</sup> mice was described previously. It was therefore unexpected that after intramuscular injection of 3.0 × 10<sup>11</sup> or 1.0 × 10<sup>12</sup> vg/body ARU-2801, plasma ALP activity reached similar plateaus. Restricted to the muscle, the injected vector volume and thus TNALP expression likely reached an upper limit. If too large a vector volume was injected into the quadriceps, it would leak out of the muscle compartment and into circulation, effectively making the administration systemic. Consistent with that idea, we previously detected low AAV vector copy numbers in heart, liver, and bone after injection of 15 μL of AAV vector into the neonatal quadriceps femoris muscle. By injecting only 2 μL of ARU-2801 in the present study, we limited the vector distribution to the muscle where it was injected; that is, there was no meaningful leakage of AAV8-TNALP-D10 from the muscle. Thus, although the constitutive promoter is not tissue specific, physical targeting to muscle was successfully achieved by injecting only a small volume of ARU-2801. This suggests that for clinical application of vector-related gene therapy, safer protocols may entail administration of smaller volumes capable of higher expression.
High-dose ARU-2801 injection led to unphysiologically high plasma ALP activity in 6-week-old WT C57BL/6 mice (~55× the dose used in Akp2<sup>−/−</sup> mice). Plasma ALP activity more than 1,000 U/mL after injection of 5.5 × 10<sup>13</sup> vg/body vector into WT mice led to the emergence of soft tissue calcification within 2–3 months. WT mice injected with 2.8 × 10<sup>13</sup> vg/body vector had lower plasma ALP levels, which delayed the soft tissue calcification, but calcification eventually developed after about 4 months. More investigation is needed to determine the threshold at which ALP activity begins to induce calcification of soft tissues. The reason why no calcification is found in other tissues except for the paw is unclear. It was reported that calci…

patibility, and continuous irritation. Our preliminary experiments showed that extremely high ALP activity in WT mice (10,000 U/mL) causes ectopic calcification, regardless of the route of administration or the age of the mice at the time of administration (data not shown). The WT mouse with highest ALP activity suffered from calcification of the tip of its nose (Figure 4B, right panel), but an X-ray survey and Von Kossa staining showed no evidence of ectopic calcification elsewhere, such as the kidneys or blood vessels. Note that all calcification findings, however, were in high-dose-treated WT mice, and we did not observe calcification at any dose in Akp2<sup>−/−</sup> mice.

It has been reported that upregulation of TNALP plays an important role in medial vascular calcification. Hyperphosphatemia is one of the symptoms associated with a disease state such as a malignant tumor or a metabolic bone disease such as osteomalacia, rickets, Paget disease, or osteoporosis, although a high plasma ALP level is not usually a cause of severe illness. Transient hyperphosphatasemia is sometimes seen in infants and during early childhood. These children exhibit plasma phosphatase levels that are about 5-fold higher than the normal range. This is usually referred to as “benign” hyperphosphatasemia. Nonetheless, among the young patients receiving ERT employing asfotase alfa, a bone-targeted recombinant human TNALP, none has experienced ectopic calcification or vascular calcification with plasma ALP activities as high as ~24 U/mL within 4 weeks of starting ERT or 3–6 U/mL for 5 years after starting ERT therapy. In the present study, we observed that plasma ALP activities of 5–10 U/mL after intramuscular ARU-2801 injection is sufficient for phenotypic correction of Akp2<sup>−/−</sup> mice. Although plasma ALP activities were up to 10-fold or more higher than the endogenous activity in untreated WT mice, no ectopic or aortic calcification was detected, nor were significantly abnormal calcium levels. This suggests that while high plasma ALP activity may entail a risk of ectopic calcification, the clinically necessary ALP activity of around 5–10 U/mL appears safe and effective. The clinical application of ERT, asfotase alfa, was an important development for HPP patients, while interruption meant sometimes death. Frequent treatment for life could be a burden to the patients. We need to develop a new treatment with a simple method such as gene therapy.

In summary, we treated Akp2<sup>−/−</sup> mice with ARU-2801 gene therapy entailing a single intramuscular injection during the neonatal period. Treated mice exhibited high plasma ALP activity and lived for at least 18 months after injection, exhibiting normal function and behavior throughout their lives. This approach may be potentially safer than systemic administration, as there is no vector transfer to the brain or gonads and no oncogenic effects. This suggests AAV vector-related ERT may be a practical and useful approach to treating HPP patients.

### MATERIALS AND METHODS

#### Cell culture

The HEK293 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 μg/mL streptomycin under an atmosphere enriched with 5% CO₂.

#### Plasmid construction and vector production

To construct an AAV vector plasmid containing cDNA for expression of the human TNALP gene with a constitutive promoter, TNALP-D10 was prepared as described previously. ARU-2801 was generated using the HEK293 cell line by the triple transfection method and purified as described previously. Recombinant AAV8 vector encoding GFP (AAV8-GFP) was used as a control for the evaluation of bone maturity at 18 months. AAV vector titers were determined using real-time PCR (7500 Fast, Applied Biosystems, Tokyo, Japan) as described previously.

#### Enzyme activity and protein assay

ALP activity was determined as previously described. One unit (U) was defined as the amount of enzyme needed to catalyze production of 1 μmol of p-nitrophenol per min. ALP activity in plasma was calculated as U/mL. ALP activity in organs was assayed in the supernatants of homogenized organs and standardized per mg of protein.

#### Animal experiments

All animal experiments were performed using protocols approved by the Nippon Medical School Animal Ethics Committee. The generation and characterization of Akp2<sup>−/−</sup> mice were described previously. To select Akp2<sup>−/−</sup> mice, all newborn TNALP/129/Bl6 mice were genotyped using PCR as described previously. The primers used were 5′-AGTCCGTGGGCATTTGACTA-3′ and 5′-TGCTGCTCCACTCAGTCGAT-3′. The Akp2<sup>−/−</sup> mouse is a close phenotypic mimic of infantile HPP and has a healthy appearance when born. Growth retardation becomes apparent within the first week, and most Akp2<sup>−/−</sup> mice die within 2–3 weeks. About half of these mice have severe symptoms and experience epileptic seizures before death. For this study, neonatal (1, 2 or 3 days after birth) Akp2<sup>−/−</sup> mice were injected with ARU-2801 (1.0 × 10<sup>11</sup>, 3.0 × 10<sup>11</sup>, or 1.0 × 10<sup>12</sup> vg in 2 μL of PBS). We utilized TNALP/129/Bl6 Akp2<sup>−/−</sup> WT mice as controls for treatment experiment. WT mice were injected with AAV8-GFP vector (1.0 × 10<sup>12</sup> vg/body in 2 μL of PBS) for 18 months of bone analysis as controls. The injections were made into the right quadriceps femoris muscle using a 22G Hamilton syringe (Osaka Chemical, Osaka, Japan). Blood samples were collected from the orbital sinus of anesthetized animals using heparin-coated capillaries, and plasma ALP activity was assessed at 1, 2, 3, 6, and 12 months (3.0 × 10<sup>11</sup> vg/body in 2 μL.
of PBS) and 1, 2, 3, 6, 12, and 18 months (1.0 × 10^{12} \text{ vg/body in 2 μL of PBS}). The behavior of the mice was also observed. Mice were sacrificed under deep anesthesia by perfusion with PBS containing heparin (10 U/mL). Then, the organs of the mice were examined for macroscopic lesions. The organs were kept in a freezer at −80°C until analyzes.

To analyze the potential adverse effects of hyper-elevated plasma TNALP, 6-week-old male C57BL/6 mice purchased from Saitama Dobutsu Zikken were injected with a high dose of ARU-2801 (3.0 × 10^{12}, 5.5 × 10^{12}, 3.0 × 10^{13}, or 5.5 × 10^{13} \text{ vg/body in 50 μL of PBS}) into the right quadriceps muscle using a 29G insulin syringe. The mice were then monitored for the appearance of ectopic calcification in their body, and analyzed for plasma ALP activity, plasma X-ray analysis.

X-ray analysis

X-ray analysis was performed as previously described. In brief, radiographic images of adult mice were obtained on μFX-1000 film (Fujifilm, Tokyo, Japan) at an energy level of 25 kV and an exposure time of 10 s.

Bioluminescent imaging

The biodistribution of AAV vector was determined in Akp2^{−/−} mice treated with ARU-2801. Genomic DNA was extracted from heart, liver, bone, and muscle and then subjected to real-time PCR as described previously. The quadriceps from both legs were analyzed to compare the injected muscle with the contralateral untreated muscle.

Histological examination

To detect ALP activity, bone was directly stained without fixation or decalcification. Sections (10 μm thick) of the knee joints were cut using the Kawamoto film method. ALP activity was assayed in the supernatant after biochemical tissue homogenization, and was histologically examined under a light microscope (BX60; Olympus, Tokyo, Japan) in tissues stained with fast blue. Von Kossa staining on liver, bone, and muscle and then subjected to real-time PCR as described previously. The quadriceps from both legs were analyzed to compare the injected muscle with the contralateral untreated muscle.

CT imaging and morphometric evaluation of cancellous bone

CT was carried out using a Latheta experimental animal CT system (LCT-200; Hitachi Healthcare, Tokyo, Japan) as described previously. Bone morphometry was performed using Latheta software version 3.44 (Hitachi Healthcare) to determine bone mineral density (BMD). Continuous 48-μm slice images were utilized for quantitative assessments, and the cortical BMD was evaluated in 10 slices of the central part of the femur diaphysis.

Statistical analysis

Data from the experiments are expressed as means ± SD. Differences between groups were compared using Student’s t test. p values less than 0.05 were considered statistically significant. Survival rates were analyzed using the Kaplan-Meier method, and differences in survival rates were assessed using the Wilcoxon test.

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AUTHOR CONTRIBUTIONS

T.M., K.M., and T.S. conceived, designed, and administered the study, and all other authors assisted in its design. T.M., K.M., N.M., O.I., and K.A. performed experiments and analyzed data. O.I. and K.A. validated the experimental data. S.N. and J.L.M. provided the original construct and all other authors assisted in its design. T.M., K.M., N.M., O.I., and K.A. performed experiments and analyzed data. O.I. and K.A. validated the experimental data. S.N. and J.L.M. provided the original construct and all other authors assisted in its design. T.M., K.M., N.M., O.I., and K.A. performed experiments and analyzed data. O.I. and K.A. validated the experimental data. S.N. and J.L.M. provided the original construct and all other authors assisted in its design. T.M., K.M., N.M., O.I., and K.A. performed experiments and analyzed data. O.I. and K.A. validated the experimental data. S.N. and J.L.M. provided the original construct and all other authors assisted in its design.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Mornet, E. (2007). Hypophosphatasia. Orphanet J. Rare Dis. 2, 40.
2. Henthorn, P.S., and Whyte, M.P. (1992). Missense mutations of the tissue-nonspecific alkaline phosphatase gene in hypophosphatasia. Clin. Chem. 38, 2501–2505.
3. Mülân, J.L., and Whyte, M.P. (2016). Alkaline phosphatase and hypophosphatasia. Calcif. Tissue Int. 98, 398–416.
4. Alleggiani, A., and Cataldo, F. (1982). Infantile hypophosphatasia diagnosed at 4 months and surviving at 2 years. Helv. Paediatr. Acta 37, 49–58.
5. Whyte, M.P. (2016). Hypophosphatasia—Etiology, nosology, pathogenesis, diagnosis and treatment. Nat. Rev. Endocrinol. 12, 233–246.
6. Whyte, M.P., McAlister, W.H., Patton, L.S., Magill, H.L., Fallon, M.D., Lorentz, W.B., Jr., and Herrod, H.G. (1984). Enzyme replacement therapy for infantile hypophosphatasia attempted by intravenous infusions of alkaline phosphatase-rich Paget plasma: Results in three additional patients. J. Pediatr. 105, 926–933.
7. Whyte, M.P., Magill, H.L., Fallon, M.D., and Herrod, H.G. (1986). Infantile hypophosphatasia: Normalization of circulating bone alkaline phosphatase activity followed by skeletal remineralization. Evidence for an intact structural gene for tissue nonspecific alkaline phosphatase. J. Pediatr. 108, 82–88.
8. Weninger, M., Stinson, R.A., Plenk, H., Jr., Böck, P., and Pollak, A. (1989). Biochemical and morphological effects of human hepatic alkaline phosphatase in a neonate with hypophosphatasia. Acta Paediatr. Scand. Suppl. 360, 154–160.
9. Camacho, P.M., Painter, S., and Kadanoff, R. (2008). Treatment of adult hypophosphatasia with teriparatide. Endocr. Pract. 14, 204–208.
10. Whyte, M.P., Mumm, S., and Deal, C. (2007). Adult hypophosphatasia treated with teriparatide. J. Clin. Endocrinol. Metab. 92, 1203–1208.
11. Whyte, M.P., Kurtzberg, J., McAlister, W.H., Mumm, S., Podgornik, M.N., Coburn, S.P., Ryan, L.M., Miller, C.R., Gottesman, G.S., Smith, A.K., et al. (2003). Marrow cell transplantation for infantile hypophosphatasia. J. Bone Miner. Res. 18, 624–636.
