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TNALP: tissue-nonspecific alkaline phosphatase
Successful treatment with bone maturation and average life expectancy of hypophosphatasia model mice by adeno-associated virus type 8-mediated neonatal gene therapy via single muscle injection

Tae Matsumoto¹,², Koichi Miyake¹, Noriko Miyake³, Osamu Iijima³, Kumi Adachi³, Sonoko Narisawa⁴, José Luis Millán⁴, Hideo Orimo⁵, and Takashi Shimada³

¹Department of Gene Therapy, Graduate School of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-8602, Japan. ²Department of Pediatrics, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-8603, Japan. ³Department of Biochemistry and Molecular Biology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-8602, Japan. ⁴Sanford Children’s Health Research Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, 92037, USA. ⁵Department of Metabolism and Nutrition, Graduate School of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-8602, Japan.

Corresponding author: Koichi Miyake, M.D., Ph.D., Department of Gene Therapy, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, JAPAN
TEL: +81-3-3822-2131 (ext. 5350)
FAX: +81-3-5814-8156
E-mail: kmiyake@nms.ac.jp

Short title: Muscle-directed gene therapy for hypophosphatasia
ABSTRACT

Hypophosphatasia (HPP) is an inherited skeletal disease characterized by defective bone and tooth mineralization due to 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 1.0x10<sup>12</sup> vector genome/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.\textsuperscript{1-3} Patients with the infantile form may appear normal at birth, but gradually develop rickets as a clinical symptom within six months of age. Subsequent craniosynostosis and nephrocalcinosis follow hypercalcemia and hypercalciuria in many cases.\textsuperscript{4} In addition, pyridoxine-dependent seizures preceding skeletal change is a predictor of lethal outcome.\textsuperscript{5} Skeletal change leading 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 alkaline phosphatase (ALP) levels\textsuperscript{6-8} and administration of parathyroid hormone.\textsuperscript{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 central nervous system. Improvement of infantile HPP patients has reportedly been achieved through bone marrow transplantation.\textsuperscript{11, 12} Allogenic mesenchymal stem cell transplantation was
proven for perinatal lethal HPP apothanasia and bone mineralization for three years though cell therapy is always accompanied with chemotherapy, radiation therapy, immunotherapy before the therapy and possible graft-versus-host disease after transplantation, which threat patients’ lives.\textsuperscript{13} Even better results were observed in mice administered recombinant TNALP fused with deca-aspartate (D10) to target bone.\textsuperscript{14} Those results led to the development of an ERT with TNALP-D10 for human HPP patients.\textsuperscript{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 6 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.\textsuperscript{16} Gene therapy has proved to be a useful tool for treatment of several inherited diseases. In an earlier study, we succeeded in treating TNALP knockout (\textit{Akp2}\textsuperscript{-/-}) 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 \textit{Akp2}\textsuperscript{-/-} mice.\textsuperscript{14, 17} 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<sup>−/−</sup> mice through intramuscular injection of a vector encoding TNALP with a muscle-specific creatine kinase (MCK) promoter and achieved prolonged survival of Akp2<sup>−/−</sup> mice. However, the muscle-specific expression was not sufficient to mediate bone maturation in Akp2<sup>−/−</sup> mice comparable to that in Akp<sup>+/+</sup> 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, a AAV8-TNALP-D10-vector with a non-tissue-specific constitutive promotor, into the right quadriceps femoris muscle and evaluated the beneficial and adverse effects in Akp2<sup>−/−</sup> mice over a prolonged period of time.
Results

Survival and sustained TNALP expression in mice intramuscularly injected with over 3.0 x 10^{11} vg/body ARU-2801.

From our previous intravenous treatment studies,\textsuperscript{17, 20} we expected that the exogenous plasma ALP activity needed for survival of Akp2\textsuperscript{-/-} mice would be around 10 U/ml, which is 100 times higher than the endogenous activity in WT mice. To determine the amount of vector needed to achieve a titer able to support Akp2\textsuperscript{-/-} mouse survival, neonatal mice were first intramuscularly injected with ARU-2801 (1.0x10^{12} vector genome (vg)/body). The mice were then monitored to determine whether TNALP delivery via intramuscular ARU-2801 injection could phenotypically correct Akp2\textsuperscript{-/-} 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 vs. 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 3.0x10^{11} vg/body or 1.0x10^{11} vg/body ARU-2801 to determine the lowest vector strength needed for phenotypic correction of these mice.
Akp2−/− mice treated with 3.0x10^{11} vg/body also showed strong ALP activity (Figure 1A) and lived for more than 12 months until sacrifice (n=3/7 survived) (Figure 1B). By contrast, with one exception, mice administered 1.0x10^{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 3.0x10^{11} vg/body or higher, plasma ALP activity reaches a plateau sufficient to sustain 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 3.0x10^{11} vg/body ARU-2801 showed significant effects, 1.0x10^{12} vg/body ARU-2801 treated mice were further analyzed given their higher survival rate.

Mature bone mineralization in treated with 1.0x10^{12} vg/body ARU-2801 Akp2−/− mice 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 1.0x10^{12} vg/body ARU-2801 treated Akp2−/− (n=5) mice (Figure 2A, B). Secondary ossification centers were detected
in all the WT mice (n=8/8) 10 days after birth. On the other hand, most of untreated 
Akp2\(^{-/-}\) mice do not have secondary ossification centers on day 10 (n=3/10) (Figure 2C).

After treatment, secondary ossification centers were found on day 10 in 1.0x10\(^{12}\) 
vg/body ARU-2801 treated Akp2\(^{-/-}\) mice (n=9/10) similar to those seen in WT ones.

Grown to adult on day 56, X-ray analysis of knee joint showed mature bone in 1.0x10\(^{12}\) 
vg/body ARU-2801-treated Akp2\(^{-/-}\) mice as same as WT mice (Figure 2D). Untreated 
Akp\(^{-/-}\) mice alternatively 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 1.0x10\(^{12}\) 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 
density (673.7 ± 41.6 vs. 620.8 ± 52.9 mg/cm\(^{3}\); p=0.26) in the treated mice were not 
significantly different from those of the WT controls (Figure 2E).

**ALP activity in the bones of mice treated with 1.0x10\(^{12}\) 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
1.0x10^{12} \text{vg/body ARU-2801 treated} \text{Akp2}^{+/-} \text{mice.}

\textit{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 \mu l of AAV8-MCK-TNALP-D10 into the neonatal quadriceps femoris muscle.\textsuperscript{18} Therefore, the distribution of 1.0x10^{12} \text{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 AAV vector genome in 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).}

\textit{No liver or kidney dysfunction was detected in treated Akp2}^{+/-} \text{mice.}

In 1.0x10^{12} \text{vg/body ARU-2801 treated Akp2}^{+/-} \text{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 vs. 9.5±0.63 mg/dl; P=0.5). Liver and kidney function were in the normal range. Although the mice treated with 1.0x10^{12} vg/body ARU-2801 plasma ALP activity (19.38 ± 5.02 U/ml) was as much as 397 times 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 or 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 5.5x10^{13} vg/body (n=3), 55X the highest dose used in Akp2^{-/} mice, and followed until morbidity was seen around 6 months of age with high plasma ALP activity (8347.3 ± 5738.4 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 (2.8x10^{13} vg/body, ~28X the highest dose used in Akp2\(^{-/-}\) mice), the level of ALP activity was about an order of magnitude less than with the 5.5x10^{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, Inc. (Tokyo, Japan) showed they were composed of 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 1.1x10^{13} vg/body and 2.8x10^{12}/body (10X and ~3X the highest dose used in Akp2\(^{-/-}\) mice), and showed no stones in their paws or any other surface of the body throughout the experiment period, i.e. 6 months. It is important to note that in ARU-2801 treated Akp2\(^{-/-}\) mice, no ectopic or vessel calcification (data are not shown) were observed at the highest efficacious dose, i.e. 1.0x10^{12} vg/body.
Discussion

We previously reported that intravenous AAV8 vector-associated systemic enzyme replacement therapy achieved phenotype correction in Akp2−/− mice. However, local intramuscular injection may prevent off target effects which have occurred with systemic AAV vector transfer. 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 human. In the present study, we treated Akp2−/− mice with a single intramuscular injection of 1.0x10^{12} vg/body ARU-2801.

ARU-2801 was the same vector driven by the nonspecific constitutive promotor we used for intravenous systemic injection. As with systemic administration, Akp2−/− mice treated with intramuscular injection exhibited high plasma ALP activity over 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. 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 (5.0x10^{12} vg/body). However, bone calcification still remained
unsatisfactory. In the present study, by 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. Statistically
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, though 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−/− mice was described previously.20, 23 It was therefore unexpected that after intramuscular injection of 3.0x10^{11} or 1.0x10^{12} 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 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.18 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 (~55X the dose used in Akp2-/- mice). Plasma ALP activity over 1,000 U/ml after injection of 5.5x10^{13} vg/body vector into WT mice led to the emergence of soft tissue calcification within 2-3 months. WT mice injected with 2.8x10^{13} vg/body vector had lower plasma ALP levels, which delayed the soft tissue calcification, but calculi 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 paw is unclear. It was reported that calcification in the paw is not very rare in animals because circulation is poor in pads due to its fiber rich tissue, especially after trauma.\(^{24}\) We speculate that the calcification in the paw can be occurred from poor circulation, gravity, 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 though were in high-dose treated WT mice, and we did not observe calcification at any dose in Akp2-/- 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 osteomalasia, rickets, Paget disease, or osteoporosis, though 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 five times higher than the normal range. This is usually referred to as “benign” hyperphosphatasemia. Nonetheless, among the young patients receiving enzyme replacement therapy 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 times or more higher than the endogenous activity in untreated WT mice, no ectopic or aortic calcification was detected, nor were significantly abnormal calcium level. 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 all the life could be a burden of the patients. We need to develop a new treatment with simple method 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 enzyme replacement therapy 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 the a constitutive promotor, 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 AAV type 8 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.$^{20}$

Animal experiments. All animal experiments were performed using protocols approved by the Nippon Medical School Animal Ethics Committee. The generation and characterization of Akp2$^{-/-}$ mice was described previously.$^{36}$ To select Akp2$^{-/-}$ mice, all newborn TNALP/129/Bl6 mice were genotyped using PCR as described previously.$^{20}$ The primers used were 5’-AGTCCGTGGGCAATGTGACTA-3’ and 5’-TGCTGCTCCACTCACGTCGAAT-3’. The Akp2$^{-/-}$ 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$^{-/-}$ mice die within 2-3 weeks. About half of these mice have severe symptoms and experience epileptic seizures before death.$^{36}$ For this study, neonatal (1, 2 or 3 days after birth) Akp2$^{-/-}$ mice were injected with ARU-2801 (1.0x10$^{11}$, 3.0x10$^{11}$ or 1.0x10$^{12}$ vector genomes (vg) in 2 µl of PBS). We utilized TNALP/129/Bl6 Akp2$^{+/+}$ wild-type (WT) mice as control for treatment experiment. WT mice were injected with AAV8-GFP vector (1.0x10$^{12}$ vg/body in 2 µl of PBS) for 18 months bone analysis as control. The injections were made into the right quadriceps femoris muscle using a 22 gauge 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, 6,12 (3.0x10^{11} \text{vg/body in 2 } \mu\text{l of PBS}) and 1, 2, 3, 6, 12, 18 months (1.0x10^{11} and 1.0x10^{12} \text{ vg/body in 2 } \mu\text{l 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 Co. were injected with a high dose of ARU-2801 (3.0x10^{12}, 5.5x10^{12}, 3.0x10^{13} or 5.5x10^{13} \text{ vg/body in 50 } \mu\text{l of PBS}) into the right quadriceps muscle using a 29 gauge insulin syringe. The mice were then monitored for the appearance of ectopic calcification in their body, and analyzed for plasma ALP activity, plasma transaminase, and kidney function. Stones were analyzed at SRL Co. (Tokyo, Japan).

X-ray analysis. X-ray analysis was performed as previously described.\textsuperscript{20} In brief, radiographic images of adult mice were obtained on \( \mu \text{FX-1000 film} \) (Fujifilm Cooperation, Tokyo, Japan) at an energy level of 25 kV and an exposure time of 10 s.
Biodistribution of AAV vector. The biodistribution of AAV vector was determined in Akp2<sup>−/−</sup> 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 muscles 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 Ltd., Tokyo, Japan) in tissues stained with Fast Blue. Von Kossa staining on liver, heart, muscle, kidney, and blood vessels sections were performed using Von Kossa staining Kit (ab150687, Abcam, Cambridge, MA, USA) according to manufacturer's instructions.

Computed tomography imaging and morphometric evaluation of cancellous bone. Computed tomography (CT) was carried out using a Lathaeta experimental animal CT system (LCT-200; Hitachi Healthcare BU, Tokyo, Japan) as described previously.
Bone morphometry was performed using a Latheta software version 3.44 (Hitachi Healthcare BU) 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 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.
Author Contributions

T.M., K.M., and T.S. conceived, designed, and administered the study, and all other authors assigned 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 experiments data. S.N. and J.L.M. provided the original HPP model Akp−/− mice. S.N. and J.L.M. H.O., and T.S. made the original experimental methodology. T.M. wrote the manuscript with review comments from all authors. T.M. K.M. edited the paper. All authors read and approved the final draft of the manuscript.

Conflict of Interest

The other authors declare no competing interests.

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**Keywords**

Hypophosphatasia, Adeno-associated virus vector, Neonatal gene therapy, Muscle injection, Tissue-nonspecific alkaline phosphatase
References


activity followed by skeletal remineralization. Evidence for an intact structural


Bone Miner Res 26: 135-142.


Results from a Japanese clinical trial. *Clin Endocrinol (Oxf)* **87**: 10-19.


alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Dev Dyn* **208**: 432-446.

Figure Legends

Figure 1. Plasma alkaline phosphatase (ALP) concentration and the life span of ARU-2801-vector (AAV8-TNALP-D10) treated Akp2^{−/−} mice.

(A) ARU-2801 (1.0x10^{11}, 3.0x10^{11} or 1.0x10^{12} vg/body) was injected into the right quadriceps of neonatal Akp2^{−/−} mice. ALP activity was determined up to 18 months.

(B) The life span up to 18 months is shown. The numbers in each mice group were as follows: 1.0x10^{11}, n=5; 3.0x10^{11}, n=7; 1.0x10^{12}, n=7; wild-type (WT), n=5, non-treated Akp2^{−/−} mice, n=4.

Figure 2. X-ray images (A, C, D) and bone maturity (E) of ARU-2801-treated Akp2^{−/−} mice compared to WT with body weight growth (B).

(A) X-ray images at 2 months of age. Comparison of the posture and rough size of an Akp2^{−/−} mouse treated with 1.0x10^{12} vg/body ARU-2801 and a WT mouse (at 100 mA, 40 kV for 30 s with μFX-1000 film).

(B) Body weight development of WT (n=3) and 1.0x10^{12} vg/body ARU-2801 (n=5) treated Akp2^{−/−} mice at 2 months of age.

(C) X-ray images of secondary ossification centers (arrow) on day 10 after birth.

Secondary ossification centers were detected in all WT mice (left panel) but not in most
Akp2\(^{-/-}\) mice (middle panel). In contrast, secondary ossification centers were detected on day 10 after birth in \(1.0 \times 10^{12}\) vg/body ARU-2801 treated Akp2\(^{-/-}\) mice (right panel).

(D) X-ray analysis of knee joint at 2 months of age. X-ray images showed mature bone in \(1.0 \times 10^{12}\) vg/body ARU-2801 treated Akp2\(^{-/-}\) mice (right panel) as same as WT mice (left panel).

(E) Bone mineral density (BMD) at 18 months of age. CT reconstruction of a femur in a 1.0 \times 10^{12}\) vg/body ARU-2801 treated Akp2\(^{-/-}\) (n=3) and GFP treated WT (n=3) mouse.

Morphometric evaluation of cancellous bone. Data represent the mean ± SD.

Figure 3. Histochemical staining of ALP activity in tibia.

Fast Blue staining of ALP activity in tibia at 2 months of age in WT mice and Akp2\(^{-/-}\) mice treated with \(1.0 \times 10^{12}\) vg and \(1.0 \times 10^{11}\) vg of ARU-2801-vector. Original magnification, x100.

Figure 4. Calculi appeared on the ARU-2801 injected C57BL/6 mice paws with extremely high plasma ALP activity.

(A) Time-series plasma ALP activity after injection. ARU-2801 was injected into the right quadriceps femoris muscles of six-week-old male C57BL/6 mice at a dose of
2.8x10^{12}, 1.1x10^{13} or 2.8x10^{13} vg/body in 50 µl of PBS, or at a dose of 5.5x10^{13} vg/body in 100 µl of PBS injected into both quadriceps (n=3 in each group).

(B) Shown are calculi in the forepaw and hind paw of 6-week-old C57BL/6 mice treated with 2.8x10^{13} (left panel) and 5.5x10^{13} (right panel) vg/body ARU-2801 vector. Note the presence of calculi at various sites.
We succeeded in treating hypophosphatasia (HPP) model mice by single intramuscular injection of adeno associated virus type 8 (AAV8) vector expressing tissue-nonspecific alkaline phosphatase (TNALP) without any adverse effects. This AAV8 vector-related enzyme replacement therapy is a practical and useful approach to treating HPP patients.