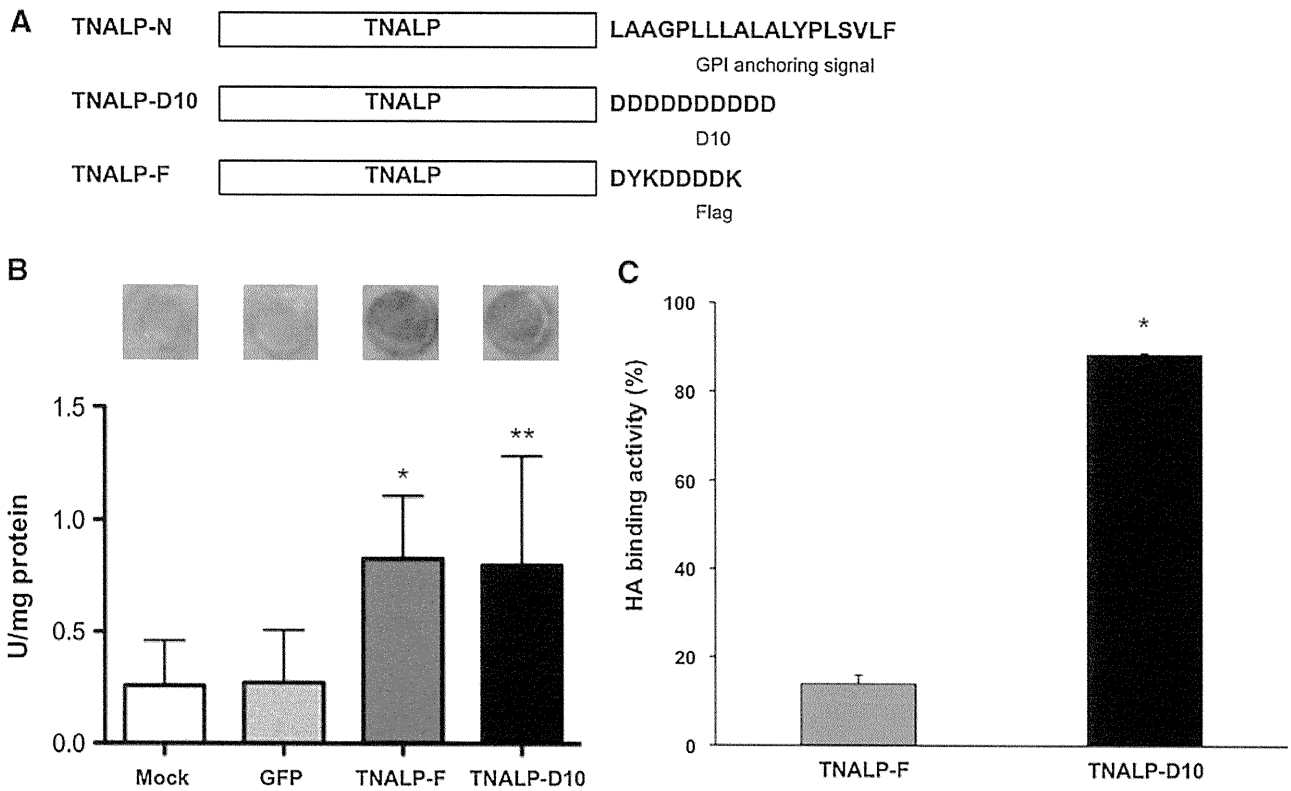

FIG. 1. Construction and characterization of TNALP. **(A)** Schematic representation of structure of native TNALP (TNALP-N), bone-targeted TNALP with D10 (TNALP-D10), and soluble TNALP with the flag epitope (TNALP-F). **(B)** *In vitro* mineralization assay. U2OS cells were incubated with the conditioned medium from C2C12 cells transduced with AAV1-TNALP-D10, AAV1-TNALP-F, or AAV1-GFP. Mock was analyzed without any vector. *Upper panel*: cell calcification stained with alizarin red S. *Lower panel*: quantitative analysis of cell calcification evaluated in cetylpyridinium chloride extraction by reading at 570 nm. $^{*}p < 0.005$, $^{**}p < 0.05$ as compared with the GFP-treated group. **(C)** *In vitro* binding assay. Hydroxyapatite was incubated with the conditioned medium from C2C12 cells transduced with AAV1-TNALP-F or AAV1-TNALP-D10. The percentage binding of TNALP was calculated by the difference in ALP activities before and after binding to hydroxyapatite. $^{*}p < 0.0001$ as compared with the TNALP-F group. HA, hydroxyapatite.

AAV8 vectors because the *in vivo* transduction efficiency of AAV8 is much higher than that of AAV1 (data not shown). AAV8-TNALP-D10 (5×10^{11} vg in 100 μl of PBS) was injected intravenously into the external jugular vein of 1-day-old *Akp2*$^{-/-}$ mice. The plasma ALP activity in these mice was markedly increased, and the superphysiologically high levels of ALP activity were sustained for at least 56 days (Fig. 2A). During this period, the mice appeared healthy. They had a normal physical activity level and no seizures, indicating that treatment by AAV-mediated gene therapy prevents *Akp2*$^{-/-}$ mice from having severe epileptic seizures. Two long-term follow-up survivors retained a high level of plasma ALP activity (9.0 ± 0.26 U/ml) and were healthy at more than 9 months after injection. Thus, a single injection of 5×10^{11} vg of AAV8-TNALP-D10 into *Akp2*$^{-/-}$ mice was sufficient for phenotypic correction and improved survival time.

TNALP dephosphorolates PPi, so that HPP patients show elevated plasma PPi concentrations, which inhibits hydroxyapatite crystal growth, leading to defective bone mineralization. Therefore, we next assayed plasma PPi levels in *Akp2*$^{-/-}$ mice. We found that, in untreated mice, plasma PPi levels were 30.0 ± 11.7 nmol/ml on day 10 after birth, but were only 24.5 ± 3.9 nmol/ml in mice administered AAV8-TNALP-D10, which is almost the same level as that in wild-type (WT) mice (25.0 ± 6.0 nmol/ml).

To determine the optimal dosage of AAV vector, we injected lower doses of AAV8-TNALP-D10 (5×10^{10} and 5×10^{9} vg/mouse) into 1-day-old *Akp2*$^{-/-}$ mice. Mice that received 5×10^{10} vg of AAV8-TNALP-D10 survived and appeared healthy, although their plasma ALP activity on day 28 was one order lower than that of mice that received 5×10^{11} vg (1.2 ± 0.7 U/ml vs. 13.7 ± 1.08 U/ml) (Fig. 2A). The mice that received the lowest dose (5×10^{9} vg) showed only a very slight increase in plasma ALP activity, and they died before weaning (similar to untreated *Akp2*$^{-/-}$ mice) (Fig. 2B). Thus, the optimal dosage of AAV8-TNALP-D10 to rescue the *Akp2*$^{-/-}$ mice was between 5×10^{10} and 5×10^{11} vg/mouse.

Therapeutic effects of TNALP-F and TNALP-N on survival

In the second series of animal experiments, we evaluated various forms of TNALP. *Akp2*$^{-/-}$ mice that received AAV8-TNALP-F (5×10^{10} vg/mouse) had a higher level of plasma ALP activity on day 28 than mice that received an equivalent dose of AAV8-TNALP-D10 (5.70 ± 2.57 U/ml vs. 1.20 ± 0.70 U/ml) (Fig. 3A). Seven of the nine mice that received

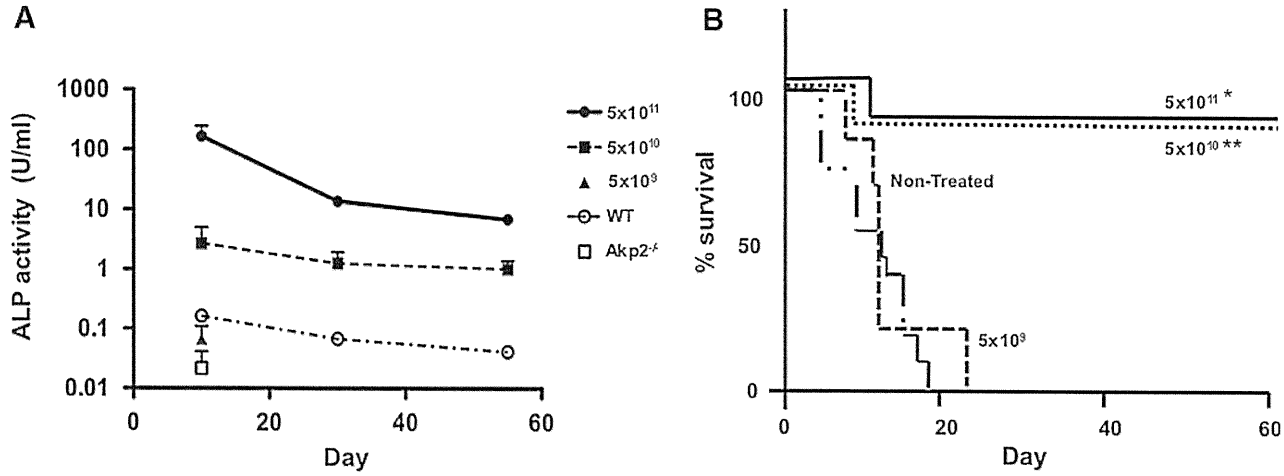

FIG. 2. Gene therapy of *Akp2*$^{-/-}$ mice with AAV8-TNALP-D10. Plasma ALP activity **(A)** and the survival curves **(B)** of *Akp2*$^{-/-}$ mice treated with AAV8-TNALP-D10 are shown. *Akp2*$^{-/-}$ neonatal mice were injected with 5×10^{11} ($n=7$), 5×10^{10} ($n=6$), and 5×10^{9} ($n=6$) vg/mouse doses of AAV8-TNALP-D10. Plasma ALP activities of treated mice, untreated *Akp2*$^{-/-}$ mice ($n=4$), and WT mice ($n=6$) were measured on days 10, 28, and 56 and are presented as the means×SD. The survival of mice was significantly prolonged by treatment with either 5×10^{11} or 5×10^{10} vg/mouse dose of AAV8-TNALP-D10. $^{*}p<0.001$, $^{**}p<0.003$ as compared with the nontreated group.

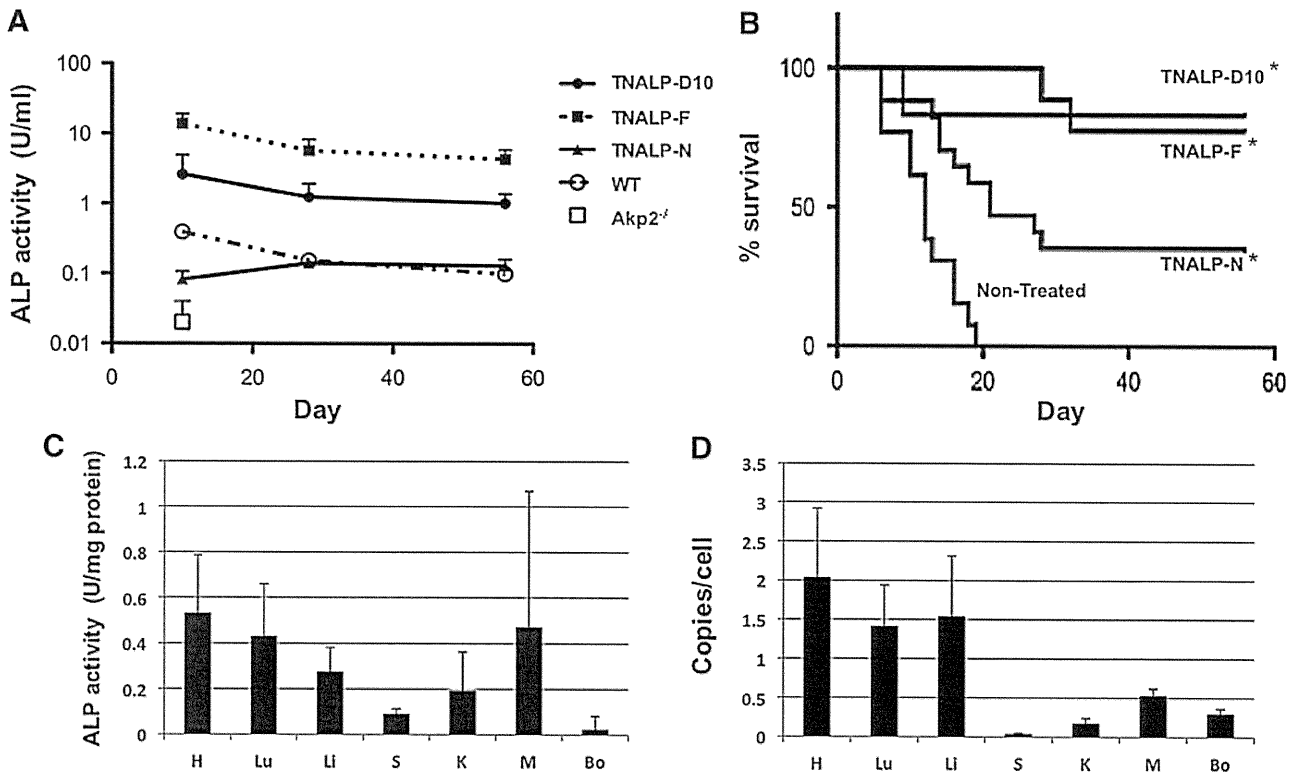

FIG. 3. Comparison of the therapeutic effects of AAV8-TNALP-D10, AAV8-TNALP-F, and AAV8-TNALP-N. Plasma ALP activity **(A)** and the survival curves **(B)** of *Akp2*$^{-/-}$ mice treated with 5×10^{10} vg/mouse doses of AAV8 vector expressing various forms of TNALP are shown. *Akp2*$^{-/-}$ neonatal mice were injected with AAV8-TNALP-D10 ($n=6$), AAV8-TNALP-F ($n=9$), or AAV8-TNALP-N ($n=17$). Plasma ALP activities of these treated mice, untreated *Akp2*$^{-/-}$ mice ($n=4$), and WT mice ($n=6$) were measured on days 10, 28, and 56 and are presented as the means×SD. The survival effects were observed in mice treated with AAV8-TNALP-D10, AAV8-TNALP-F, and AAV8-TNALP-N. $^{*}p<0.003$ as compared with the nontreated group. ALP activity **(C)** and vector distribution **(D)** in seven organs from AAV8-TNALP-N–injected **(C)** and AAV8-TNALP-D10–injected **(D)** *Akp2*$^{-/-}$ mice ($n=4$) are shown. H, heart; Lu, lung; Li, liver; S, spleen; K, kidney; M, muscle; Bo, bone.

AAV8-TNALP-F survived for at least 56 days (Fig. 3B). *Akp2*$^{-/-}$ mice that received AAV8-TNALP-N experienced a moderate increase in plasma ALP activity (0.14 ± 0.02 U/ml), indicating that some of the TNALP-N may have been solubilized by enzymatic cleavage, and five of the 17 mice in this group survived for at least 56 days (Fig. 3A and B). These results suggest that soluble TNALP, even without bone-targeted peptides, in the circulation shows favorable effects on prolongation of the lifespan of *Akp2*$^{-/-}$ mice.

To determine which organ(s) is the primary source of secreted TNALP in treated *Akp2*$^{-/-}$ mice, we investigated the ALP activity and vector distribution in seven organs from AAV8-TNALP-N– or D10–injected *Akp2*$^{-/-}$ mice. High ALP activities were detected in heart, skeletal muscle, lung, and liver (Fig. 3C). Real-time PCR analysis confirmed that these organs were efficiently transduced with AAV vector. Transduction of other organs, including bones, was very low (Fig. 3D).

Mature bone mineralization was detected in treated Akp2$^{-/-}$ *mice*

Bones in *Akp2*$^{-/-}$ mice treated with AAV vectors were evaluated by X-ray examination. *Akp2*$^{-/-}$ mice have a normal appearance at birth, but growth retardation and radiographic changes become apparent during the first 7 to 10 days of life. The severity of the mineralization defects is highly variable. One parameter for bone development is the timing of the appearance of secondary ossification centers (epiphyses) (Fedde *et al.*, 1999). Epiphyses in the knees of WT mice were well mineralized by day 10 (Fig. 4A). X-ray images of 10-day-old mice revealed that untreated and control AAV8-GFP–treated *Akp2*$^{-/-}$ mice did not have mineralized epiphyses, whereas apparent secondary ossification centers were detected in nine of 10 AAV8-TNALP-D10 (5×10^{11} vg)–treated mice. Mineralization of the epiphyses was also accelerated following AAV8-mediated expression of TNALP-D10. Untreated *Akp2*$^{-/-}$ mice died by day 20. After 56 days, epiphyses on the femurs and the tibias in treated *Akp2*$^{-/-}$ mice were well mineralized and became indistinguishable from those in WT mice (Fig. 4B). BMD showed no significant difference between treated *Akp2*$^{-/-}$ and WT mice (data not shown). These results indicate that a single injection of 5×10^{11} vg of AAV8-TNALP-D10 is effective in preventing the bone mineralization abnormalities and prolonging survival of *Akp2*$^{-/-}$ mice. We also examined the X-ray images of untreated ($n = 11$) and AAV8-TNALP-D10 (5×10^{11} vg)–treated *Akp2*$^{-/-}$ mice ($n = 9$) at day 10, and 56-day-old *Akp2*$^{-/-}$ mice successfully treated with various types of AAV8-TNALP (5×10^{10} vg, $n = 32$). No apparent bone fractures were detected in both untreated and any of the surviving mice. In addition, mineralization of epiphyses at the forepaws and the knees was present in all surviving animals (Fig. 4B).

TNALP-D10 has high in vivo *affinity for bone*

A histological study of knee joints is illustrated in Fig. 5, in which fast blue staining indicates ALP activity. Neonatal injection of 5×10^{11} vg of AAV8-TNALP-D10 resulted in positive staining on the surface of the endosteal bone. No signals were detected in untreated *Akp2*$^{-/-}$ mice. Faint ALP staining occurred in mice that received 5×10^{10} vg of AAV8-TNALP-D10. *Akp2*$^{-/-}$ mice treated with 5×10^{10} vg of AAV8-TNALP-F and AAV8-TNALP-N showed no ALP-stained areas. These results confirm that TNALP-D10 has higher *in vivo* affinity for bone than TNALP-F.

Discussion

We previously demonstrated that the phenotypes of *Akp2*$^{-/-}$ mice can be corrected by continuous subcutaneous

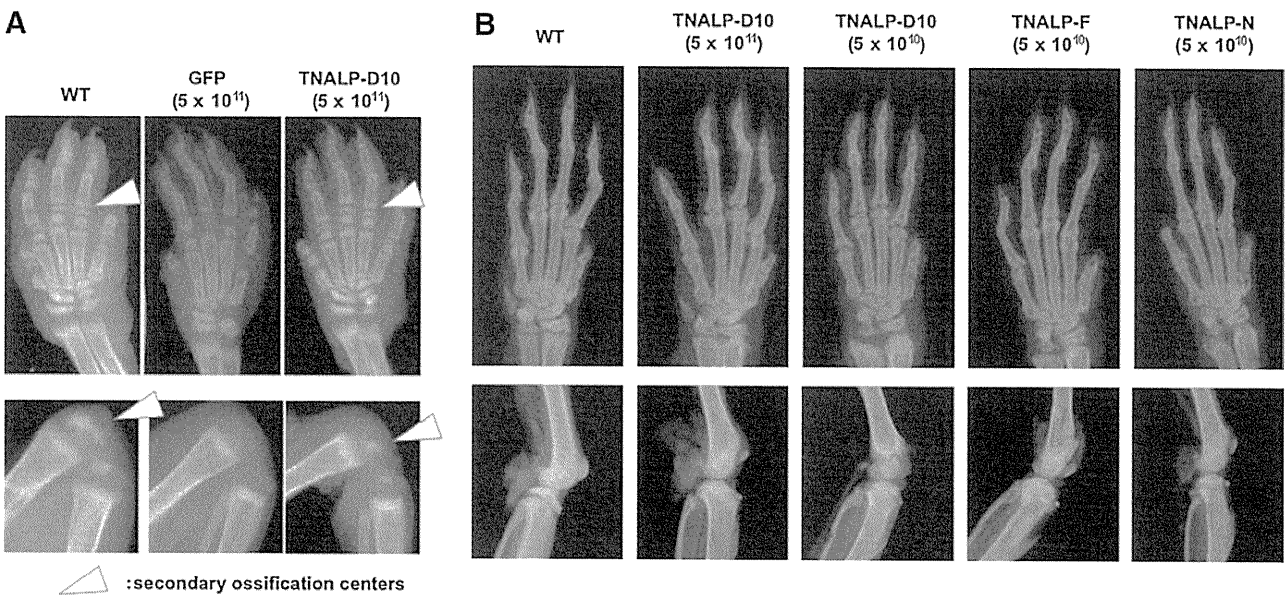

FIG. 4. X-ray images of knee joints and forepaws. **(A)** Secondary ossification centers in the femur, tibia, and digital bones were absent in the GFP-treated *Akp2*$^{-/-}$ mice (5×10^{11} vg/mouse), but were detectable in mice treated with AAV8-TNALP-D10 (5×10^{11} vg/mouse) on day 10. **(B)** Mineralization of epiphyses at the forepaws and the knee was shown in all surviving *Akp2*$^{-/-}$ mice treated with 5×10^{11} or 5×10^{10} vg/mouse doses of AAV8 vector expressing various forms of TNALP at 56 days of age.

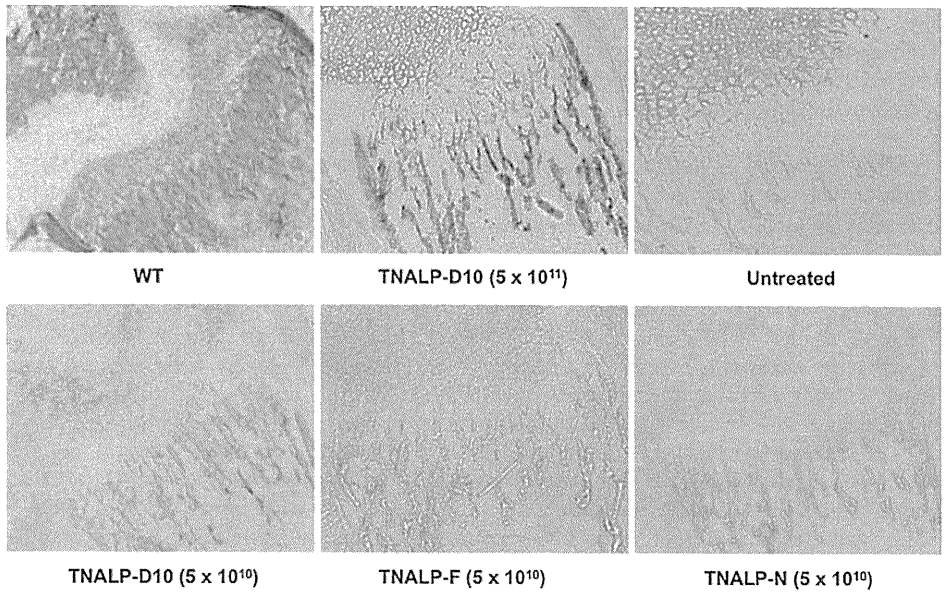

FIG. 5. Histochemical staining of ALP activity in the knee joints. ALP activity in the knee joints of *Akp2*$^{-/-}$ mice treated with various AAV vectors was directly stained with fast blue without fixation and decalcification at day 10. Blue staining was detected on the surface of the endosteal bone of WT mice and *Akp2*$^{-/-}$ mice treated with 5×10^{11} or 5×10^{10} vg/mouse doses of AAV8-TNALP-D10, but not of untreated *Akp2*$^{-/-}$ mice and *Akp2*$^{-/-}$ mice treated with 5×10^{10} vg/mouse doses of AAV8-TNALP-F and AAV8-TNALP-N. Original magnification, ×100.

injection or lentiviral-mediated expression of TNALP-D10 (Millán *et al.*, 2008; Yamamoto *et al.*, 2011). In the present study, we demonstrated that an intravenous injection of AAV8 vector expressing TNALP-D10 into neonatal *Akp2*$^{-/-}$ mice is also a highly effective treatment. These gene therapy approaches are minimally invasive, unlike classical ERT, which requires repeated injections of a large amount of enzyme (Millán *et al.*, 2008). Both lentiviral and AAV vectors worked efficiently to treat lethal HPP model mice. The high levels of ALP activity persisted for at least 9 months after a single injection of either vector in the neonatal period, but the time course of the plasma ALP activity was different between two vectors. When 5×10^{11} vg/mouse of AAV8-TNALP-D10 was injected, the plasma ALP level was initially extremely high (165.1 ± 79.8 U/ml on day 10), but decreased with time to a stable level (13.7 × 1.1 U/ml on day 28, 6.8 × 1.3 U/ml on day 56) (Fig. 2A). In contrast, ALP activities in plasma were relatively stable, ranging from 5.1 to 9.1 U/ml during 60 days after injection of 5×10^{7} TU/mouse HIV-TNALP-D10 (Yamamoto *et al.*, 2011). The rapid decline of ALP activity after AAV injection is thought to be due to a reduction of ALP expression in the liver. It was reported that AAV vector transduces the neonatal liver with high efficiency, but the expression is rapidly decreased mainly because episomal vector genomes are degraded in the liver at this developmental stage (Cunningham *et al.*, 2008; Inagaki *et al.*, 2008). There was no significant difference in bone mineralization between lentiviral- and AAV-treated mice. Integrating lentiviral vectors have been used for stable gene transfer into hematopoietic cells in *ex vivo* protocols (Cartier *et al.*, 2009; Kaiser, 2009), but their application for *in vivo* gene transfer is limited (Jarraya *et al.*, 2009). There is no clinical protocol for systemic lentiviral vector injection. In contrast, AAV vector is suitable for *in vivo* gene delivery and has been widely used for vector-mediated ERTs for genetic diseases, such as lysosomal storage disorders and hemophilia (Manno *et al.*, 2006; Kurai *et al.*, 2007; Passini *et al.*, 2007; Ogawa *et al.*, 2009). Taken together, both lentiviral and AAV vector are useful for treatment of HPP mice, but for clinical application, systemic delivery of AAV vector appears to be a more realistic approach to treat human patients.

An important finding is that soluble TNALP both with and without deca-aspartates is capable of rescuing lethal *Akp2*$^{-/-}$ mice when expressed by AAV vectors. A repeating sequence of acidic amino acids is found in several non-collagenous bone proteins and plays a key role in binding to bone (Kasugai *et al.*, 2000) and to hydroxyapatite (Nishioka *et al.*, 2006; Millán *et al.*, 2008). In an enzyme replacement approach, the use of TNALP with deca-aspartates, TNALP-D10, appears to be essential to treat HPP mice (Millán *et al.*, 2008). Previous human trials of ERT using various soluble ALP have shown very limited clinical and radiographic benefits (Whyte *et al.*, 1982, 1984, 1986; Weninger *et al.*, 1989). The main reason for these failures is thought to be the insufficient concentration of local ALP activity in skeletal tissues after systemic enzyme replacement. In contrast, TNALP-D10 has a high affinity for hydroxyapatite crystals and, therefore, ALP activity could be easily increased at the sites of skeletal mineralization. The efficacy of bone-targeted TNALP-D10 is now being evaluated in clinical trials of ERT.

In our gene therapy approach, the utility of deca-aspartates in the treatment of HPP model mice was not apparent. We confirmed that TNALP-D10 has a higher affinity for bone than TNALP-F both *in vitro* and *in vivo*. However, there was no significant difference in survival rate, convulsion frequency, and bone mineralization between mice treated with AAV8-TNALP-D10 and AAV8-TNALP-F. A potential advantage of AAV-mediated gene therapy compared with classical ERT is that a high concentration of soluble TNALP can be continuously supplied to systemic organs. Actually, very high levels of serum ALP activities were sustained in the circulation of both AAV8-TNALP-D10– and AAV8-TNALP-F–treated mice. The concentration of TNALP-F may be sufficient to keep the local ALP activity required for skeletal mineralization; even TNALP-F does not have a high affinity for bones. These data suggest that the use of soluble TNALP without additional peptides is an important option for treatment of HPP, at least in gene therapy approaches.

However, bone-targeted TNALP-D10 could be useful to reduce the vector dose and the safety concern in clinical gene therapy protocols. Further studies are required to optimize the vector construct for a safe and efficient gene therapy of HPP.

It was somewhat surprising that partial survival effects were also observed in animals treated with AAV8-TNALP-N. Synthesized TNALP-N is GPI-anchored to the cell membrane. A moderate increase in plasma ALP activity (around 0.1 U/ml) suggests that a portion of TNALP is released into the circulation by enzymatic cleavage. Only five of 17 *Akp2*$^{-/-}$ mice were rescued by neonatal injection of AAV8-TNALP-N, but surviving animals showed a healthy appearance and a normal activity with mature bone mineralization. These findings suggest that a circulating ALP activity level near 0.1 U/ml may be the threshold needed to inhibit seizures and prolong the survival of *Akp2*$^{-/-}$ mice, but sufficient to improve bone mineralization of surviving animals.

The major mechanism of gene therapy for HPP mice seems to be due to continuous supply of ALP activity from the circulation, but not genetic correction of bone cells. This speculation is strongly supported by the recent success of ERT of HPP (Millán *et al.*, 2008). Biodistribution of AAV vector demonstrated that major organs to secrete TNALP were heart and muscle. Transduction of bone tissues was very low. ALP staining in treated mice is due mainly to the circulating TNALP-D10 in the bone matrix. The contribution of *in situ* expression of TNALP in bone cells to bone mineralization is not likely to be significant. These data support the conclusion that normalizing systemic extracellular PPi concentrations is sufficient to prevent all the manifestations of HPP, in agreement with an earlier report describing correction of the HPP phenotype by the transgenic overexpression of GPI-anchored TNALP in the liver of *Akp2*$^{-/-}$ mice under the control of the apoprotein E promoter (Murshed *et al.*, 2005).

Pyridoxine-responsive seizures occur in some severe cases of human HPP, but they are not a common symptom (Mornet, 2007). The cause of these seizures is not completely understood, but they are thought to be due to reduced levels of pyridoxal 5′-phospate–dependent synthesis of the inhibitory neurotransmitter γ-aminobutyric acid in the brain (Waymire *et al.*, 1995; Narisawa *et al.*, 2001). The major clinical complications in human patients with HPP are directly related to defective skeletal mineralization (Whyte, 2002). Patients with severe infantile HPP usually die from respiratory failure caused by skeletal diseases in the chest, such as flail chest, rachitic deformity, and rib fractures (Whyte, 2002). Apnea is the major cause of death of *Akp2*$^{-/-}$ mice, but it appears to be caused by epileptic convulsions (not skeletal diseases) (Narisawa *et al.*, 1997).

It is unclear why skeletal disease is relatively mild in *Akp2*$^{-/-}$ mice, which have completely null TNALP activity. Other ALP isoenzymes or maternal TNALP may compensate for the virtual absence of endogenous TNALP. Indeed, recent findings have pointed to the nonredundant roles of PHOSPHO1 and TNALP in the initiation of endochondral ossification (Yadav *et al.*, 2011) and have also uncovered a potential compensating role of nucleoside triphosphate pyrophosphohydrolase-1 (NPP1) that might limit the severity of the TNALP null phenotype in *Akp2*$^{-/-}$ mice in the first days of life (Ciancaglini *et al.*, 2010; Yadav *et al.*, 2011). In the absence of TNALP, NPP1 has been found to act as the second best pyrophosphatase and ATPase in skeletal tissues (Ciancaglini *et al.*, 2010). Recent data indicate that skeletal mineralization is not affected in *Akp2*$^{-/-}$ mice due to the combined action of PHOSPHO1 and Pi-transporter-mediated influx of Pi into matrix vesicles (Ciancaglini *et al.*, 2010). Extravesicular propagation of hydroxyapatite deposition is impaired in *Akp2*$^{-/-}$ mice after postnatal day 6 due to the absence of TNALP's pyrophosphatase activity, but in the immediate postnatal state it has been argued that NPP1's pyrophosphatase activity can compensate for the lack of TNALP. Nevertheless, various degrees of mineralization defects are observed in untreated *Akp2*$^{-/-}$ mice after postnatal day 6. The delayed appearance of secondary ossification centers and carpal bones and the shortening of long bones are features of the neonatal period. A single injection of AAV8-TNALP-D10 prolonged survival and corrected the phenotype. Radiographic examination of these treated mice revealed accelerated mineralization of the epiphyses on day 10. No fractures were detected. Importantly, there were no significant differences in gross skeletal structure between mice treated with AAV8-TNALP-D10 and with AAV8-TNALP-F at 56 days of age. All surviving mice appeared healthy and had normal physical activity.

In conclusion, we successfully treated *Akp2*$^{-/-}$ mice with a single intravenous injection of AAV8 vector on day 1 after birth. Both bone-targeted TNALP-D10 and soluble TNALP were effective in inhibiting lethal seizures, prolonging survival, and improving bone mineralization. Thus, AAV8-mediated systemic gene therapy is a safe and effective treatment for the infantile form of HPP.

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Author Disclosure Statement

J.L. Millán is a consultant for Enobia Pharma, Inc. The other authors have no competing financial interests.

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Address correspondence to:
Dr. Takashi Shimada
Department of Biochemistry and Molecular Biology
Nippon Medical School
1-1-5 Sendagi, Bunkyo-ku
Tokyo 113-8602
Japan

E-mail: tshimada@nms.ac.jp

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ORIGINAL ARTICLE

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Prolonged Survival and Phenotypic Correction of *Akp2*$^{-/-}$ Hypophosphatasia Mice by Lentiviral Gene Therapy

Seiko Yamamoto,[1,2] Hideo Orimo,[1] Tae Matsumoto,[1] Osamu Iijima,[1] Sonoko Narisawa,[3] Takahide Maeda,[2] José Luis Millán,[3] and Takashi Shimada[1]

[1]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan
[2]Department of Pediatric Dentistry, Nihon University Graduate School of Dentistry at Matsudo, Matsudo, Japan
[3]Sanford-Burnham Medical Research Institute, La Jolla, CA, USA

ABSTRACT

Hypophosphatasia (HPP) is an inherited systemic skeletal disease caused by mutations in the gene encoding the tissue-nonspecific alkaline phosphatase (*TNALP*) isozyme. The clinical severity of HPP varies widely, with symptoms including rickets and osteomalacia. *TNALP* knockout (*Akp2*$^{-/-}$) mice phenotypically mimic the severe infantile form of HPP; that is, *TNALP*-deficient mice are born with a normal appearance but die by 20 days of age owing to growth failure, hypomineralization, and epileptic seizures. In this study, a lentiviral vector expressing a bone-targeted form of *TNALP* was injected into the jugular vein of newborn *Akp2*$^{-/-}$ mice. We found that alkaline phosphatase activity in the plasma of treated *Akp2*$^{-/-}$ mice increased and remained at high levels throughout the life of the animals. The treated *Akp2*$^{-/-}$ mice survived for more than 10 months and demonstrated normal physical activity and a healthy appearance. Epileptic seizures were completely inhibited in the treated *Akp2*$^{-/-}$ mice, and X-ray examination of the skeleton showed that mineralization was significantly improved by the gene therapy. These results show that severe infantile HPP in *TNALP* knockout mice can be treated with a single injection of lentiviral vector during the neonatal period. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: ALKALINE PHOSPHATASE; LENTIVIRAL VECTOR; ENZYME REPLACEMENT; EPILEPSY; CALCIFICATION

Introduction

Hypophosphatasia (HPP) is an inherited skeletal disease caused by mutations in the gene encoding the tissue-nonspecific alkaline phosphatase (*TNALP*) isozyme.[1] The symptoms of HPP include hypomineralization that causes rickets in infants and children and osteomalacia in adults.[2,3] The clinical severity of HPP varies widely from a lethal perinatal form to mild odontohypophosphatasia that manifests only dental abnormalities.[4] In the infantile form, postnatal development appears to proceed normally before the onset of failure to thrive and the associated development of rickets before 6 months of age. Severe infantile HPP is often fatal.[3]

TNALP is an ectoenzyme that is attached to the outer plasma membrane via a glycosylphosphatidylinositol (GPI) anchor.[5,6] Absence of TNALP activity results in extracellular accumulation of natural substrates such as inorganic pyrophosphate (PP_i),[7,8] pyridoxal 5′-phosphate (PLP),[9,10] and phosphoethanolamine (PEA).[11] Since high concentrations of PP_i result in a strong inhibition of hydroxylapatite crystal growth, normal mineralization of the systemic bones and teeth is impaired in HPP patients.[8,12] Pyridoxine-responsive seizures are also observed in some severe cases. Enzyme-replacement therapy using various types of alkaline phosphatase[13–17] and cell therapy using bone marrow cells[18,19] and mesenchymal cells[20] have been reported with no or very limited clinical benefit.

TNALP knockout mice have been established in two independent laboratories.[21,22] These mice are born with a normal appearance but, owing to deficient degradation of PP_i and abnormal metabolism of PLP, develop rickets and die by 20 days of age as a result of severe skeletal hypomineralization and epileptic seizures and represent an appropriate model of the infantile form of HPP.[23,24] Recently, Millán and colleagues[25] treated *TNALP* knockout mice with a daily subcutaneous injection of a bone-targeted form of TNALP in which a bone-targeting deca-aspartate sequence was linked to the C-terminal end of soluble TNALP.[26,27] Based on those data, clinical trials of enzyme-replacement therapy with bone-targeted TNALP in

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Address correspondence to: Takashi Shimada, MD, PhD, Department of Biochemistry and Molecular Biology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan. E-mail: tshimada@nms.ac.jp

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patients with adult and infantile HPP have been initiated.[28] A limitation of enzyme-replacement therapy for HPP is the restricted half-life of the TNALP protein in patients' fluids and tissues, which necessitates repeated administration of large amounts of the enzyme for long-term correction.

In this study, we examined viral vector–mediated gene therapy of HPP. We found that a single injection of lentiviral vector expressing bone-targeted TNALP into neonatal HPP mice resulted in long-term high levels of ALP in the serum and long-term phenotypic correction in HPP mice. We conclude that gene therapy may prove to be an important option for the treatment of human HPP.

Materials and Methods

Plasmid construction

To create *TNALP-D10* cDNA coding for TNALP lacking the GPI anchor sequence and containing 10 repeated aspartic acid (Asp) residues at its C-terminus, polymerase chain reaction (PCR) was performed using primers *TNALP-D10*-f (5′-GAA TTC ACC CAC GTC GAT TGC ATC TCT CTG GGC TCC AG) and *TNALP-D10*-r (5′-GAA TTC TCA GTC GTC ATC ATC ATC ATC GTC GTC ATC GTC GTC GCC TGC GGA GCT GGC AGG AGC ACA GTG-3′) with pcDNA3 *TNALP* cDNA plasmid as the template.[29] The PCR product then was digested with *Eco*RI and inserted into the pGEM T-easy vector (Promega Corporation, Madison, WI, USA). A second PCR was performed using primers *Eco*RI–*TNALP*-f (5′-TTT GAA TTC GCC ACC ATG ATT TCA CCA TTC TTA GTA C-3′) and *TNALP-D10-Not*I-r (5′-TTT GCG GCC GCT CAG TCG TCA TCA TCA TCA TCG). The orientation of each sequence then was confirmed.

The pHIV-TNALP-D10 plasmid was constructed by insertion of the *Eco*RI and *Not*I fragments containing the cDNA for *TNALP-D10* into pC1(–)3UTR-del, which is a newly constructed SJ1-based HIV-1 vector containing 0.25-kb insulators in the U3 and the murine stem cell virus (MSCV) long terminal repeat (LTR) as an internal promoter (Fig. 1*A*).[30]

Lentiviral vector preparation

Lentiviral vector was prepared by transient transfection in 293T cells, as described previously.[30] Vector preparation treated with Benzonase (50 μL/mL) for 1 hour at room temperature was filtrated by 0.45-μm membrane after adjustment of the pH to 8.0 with 1 N NaOH. Vector was concentrated using Acrodisc Units with Mustang Q Membranes (PALL Corporation, Ann Arbor, MI, USA).[31,32] The eluted solution containing lentiviral vector was ultracentrifuged with a 20% (w/v) sucrose underlay for purification, and the infectious vector particle (titer) was determined in HeLa cells. The titer was expressed as transducing units per milliliter (TU/mL).

Animal procedures and experiments

All animal experiments were preapproved by the Nippon Medical School Animal Ethics Committee. Wild-type (WT) *Akp2*$^{+/-}$ heterozygous (HET) and *Akp2*$^{-/-}$ knockout (HPP) mice were obtained by mating *Akp2*$^{+/-}$ heterozygous mice with mice of a mixed 129J × C57Bl/6J genetic background.[22] Lentiviral vector (5.0×10^7 TU/100 μL in PBS) was injected into the jugular vein of neonatal mice on days 1 through 3. Breeding HET pairs were fed modified Laboratory Rodent Diet 5001 (Purina Mills, St Louis, MO, USA)[35] composed of CMF laboratory feed (Oriental Yeast Co., Ltd., Tokyo, Japan) supplemented with 325 ppm pyridoxine/10 kg of feed.

ALP activity

Blood samples were collected from the tail vein or the orbital sinus. The level of ALP in the plasma was quantified using a colorimetric assay for ALP activity, as described previously.[36] ALP activity was determined using 10 mM *p*-nitrophenyl phosphate (Sigma-Aldrich, Steinheim, UK) as the substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer containing 5 mM $MgCl_2$ (pH 10.0) at 37°C. ALP enzyme activity was described in units (U) defined as the amount of enzyme needed to catalyze production of 1 μmol of *p*-nitrophenol formed per minute. ALP activity in plasma was calculated as units per milliliter (U/mL).

Biodistribution of lentiviral vector

Mice were deepley anesthesized and perfused with 15 mL of PBS containing 150 U of heparin and 15 mL of PBS. The liver, spleen, kidney, lung, heart, and bone (femur) were harvested, and homogenates were made using the Percellys-24 bead-beating homogenizer according to the company's protocol (Bertin Technologies, Paris, France). Genomic DNA was extracted from tissue homogenates using the Gentra Puregene Kit (Qiagen Siences, Germantown, MD, USA) and was subjected to real-time PCR to estimate the distribution. The primer/probe sets FPLV2 (modified at one base to 5′-ACT TGA AAG CGA AAG GGA AAC-3′ owing to a difference in the HIV-1 strain), RPLV2 (5′-CAC CCA TCT CTC TCC TTC TAG CC-3′), and LV2 (5′-AGC TCT CTC GAC GCA GGA CTC GGC -3′) were used to detect the lentiviral vector provirus, as described previously.[33] TaqMan ribosomal RNA control reagents (Applied Biosystems, Branchburg, NJ, USA) were used to quantify the amount of genomic DNA. To estimate vector distribution, genomic DNA extracted from the bone marrow cells of BL/6 wild-type mice or genomic DNA spiked with plasmid DNA was used as a standard, and average copy number per diploid were determined.[34]

X-ray analysis

Digital microradiography images were obtained using a μFX-1000 (Fujifilm, Tokyo, Japan) and imaged with FLA-7000 (Fujifilm). The X-ray energy levels were 25 kV and 100 μA, and an exposure time of 90 seconds was used for 15-day-old mice, and 15 seconds was used for adult mice. We examined a minimum of three X-ray pictures from each group at given ages.

ALP activity staining

Bone samples were fixed in neutral buffered formalin for 24 hours at 4°C. Knuckle samples then were decalcified in 10% EDTA solution with rotation for 2 to 3 days at 4°C. For the azo-dye method, knuckles were embedded in optimal-cutting-temperature (OCT) compound (Tissue-Tek, SAKUSA Finetechnical, Tokyo, Japan) and sectioned using a Leica CM1950 cryostat. Thin sections (4 μm thick) were air-dried for 10 minutes, washed in

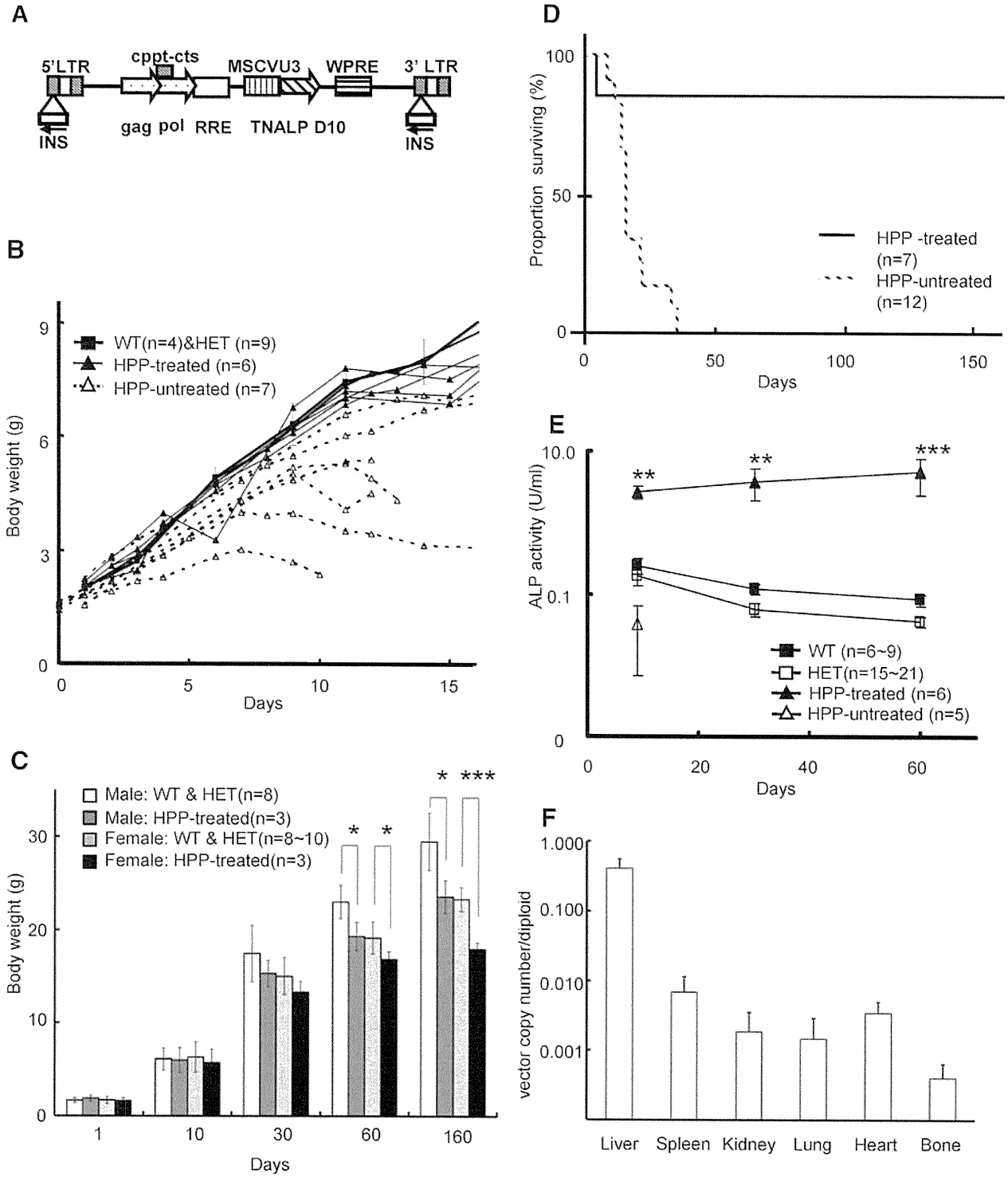

Fig. 1. Lentiviral-mediated gene therapy of *Akp2*$^{-/-}$ hypophosphatasic (HPP) mice. (*A*) Schematic diagram of HIV-TNALP-D10 lentiviral vector. LTR = long terminal repeat; MSCVU3 = U3 region of the LTR promoter of murine stem cell virus; WPRE = woodchuck hepatitis virus posttranscriptional regulatory element; INS = chicken β-globin hypersensitivity site 4 insulator; cppt-cts = central polypurine tract–central termination sequence; RRE = reverse responsive element. (*B*) Growth curves of untreated HPP mice ($n = 7$), treated HPP mice ($n = 6$), and WT ($n = 4$) and HET ($n = 9$) mice. The body weights of untreated HPP mice were recorded until spontaneous death. The weights of WT and HET (total $n = 13$) mice are presented as the average ± SD. (*C*) The comparison of average body weights of treated HPP mice (male, $n = 3$; female, $n = 3$) and WT/HET littermates (male, $n = 8$; female, $n = 8$ to 10). $^{*}p < .05$; $^{***}p < .001$. (*D*) The survival curves of treated ($n = 7$) and untreated ($n = 12$) HPP mice. (*E*) Concentration of plasma ALP in the treated ($n = 6$) and untreated ($n = 5$) HPP mice and HET ($n = 15$ to 21) and WT ($n = 6$-9) controls. $^{**}p < .01$ versus the WT group; $^{***}p < .001$ versus the WT group. (*F*) Distribution of lentiviral vector. The copy numbers of the vector genome in the organs was determined by qPCR with HIV-TNALP-D10 injected WT mice. Data are presented as mean ± SEM ($n = 4$).

PBS, and transferred to a solution of 50 mM $MgCl_2$ in 0.05 M Tris–maleic acid buffer (pH 7.4) for 30 minutes for the reactivation of ALP.[37] The sections then were incubated in a freshly prepared mixture of Naphthol AS-MX phosphate disodium salt (Sigma-Aldrich) and Fast Blue BB Salt (Sigma-Aldrich) as described previously.[38] Methyl green served as the counterstain.

Statistical analysis

Data are expressed as mean ± SD. Differences between two groups were tested for statistical significance using Student's *t* test. *p* values $< .05$ were considered statistically significant. Kaplan-Meier curves were produced and analyzed using SPSS for Windows, Version 14.0J (SPSS Japan, Tokyo, Japan).

Results

Growth and survival of *Akp2*$^{-/-}$ mice

The growth of the *Akp2*$^{+/-}$ HET mice appeared indistinguishable from that of the WT mice. The *Akp2*$^{-/-}$ HPP mice were born with a normal appearance and weight. However, HPP mice showed apparent growth failure and became progressively exhausted (Fig. 1*B*). Most of the HPP mice also developed spontaneous seizures with various clinical presentations, including tonic-clonic convulsions and abnormal running and vocalization. The mice usually died 1 to 2 days after the epileptic seizures began. The average life span of the HPP mice was 12.0 ± 4.4 days ($n = 13$). Pyridoxine supplementation of the food for the nursing mother delayed the onset of the epileptic attacks in the neonates and extended their survival to postnatal day 18.1 ± 7.6 ($n = 15$).

Lentiviral vector containing bone-targeted human *TNALP* cDNA (HIV-TNALP-D10) was injected into the jugular vein of the neonatal HPP mice on days 1 through 3 ($n = 6$). The weight and growth rates of the treated HPP mice were improved compared with the untreated HPP mice and were indistinguishable from those of their WT and HET littermates ($n = 13$; Fig. 1*B*). The long-term follow-up was done for 7 treated mice. Compared with untreated mice ($n = 12$), the life spans of treated mice ($n = 7$) were significantly extended up to at least 160 days of age, except that one treated animal died on day 6 from unknown causes (Fig. 1*D*). In the long survivors ($n = 6$), 3 were euthanized on day 160 for X-ray analysis, whereas the remaining 3 animals survived for more than 400 days with normal appearance and physical activity. Seizures were not observed in the treated mice throughout the experimental period. The average body weights of treated HPP ($n = 6$) and WT/HET ($n = 13$ to 18) were compared on days 1, 10, 30, 60, and 160 (Fig. 1*C*). The body weights differed between male and female mice after 60 days. In either gender, the slight but significant growth retardation was detected in treated HPP mice on days 60 and 160.

Lentivirus-mediated expression of ALP

At 10 to 12 days after birth, ALP activity in the plasma of WT and HET mice was 0.25 ± 0.07 U/mL ($n = 9$) and 0.16 ± 0.05 U/mL ($n = 21$), respectively, whereas that of the HPP mice was less than 0.1 U/mL ($n = 5$; Fig. 1*E*). A single injection of HIV-TNALP-D10 into the neonatal HPP mice ($n = 6$) on days 1 through 3 resulted in extremely high levels of plasma ALP (2.67 ± 0.56 U/mL). The plasma ALP activity in the WT and HET mice decreased slowly with aging, whereas the lentivirus-mediated expression of ALP remained stable, and the high levels of ALP activity persisted for at least 6 months. At 60 days of age, the average ALP activity in the treated HPP mice was 73-fold higher than that of the WT mice (5.14 ± 2.66 versus 0.07 ± 0.02).

Biodistribution of lentiviral vector was determined using quantitative PCR (qPCR) on genomic DNA isolated from the injected WT littermate mice 14 days after injection (Fig. 1*F*). The highest copy number of integrated vector was detected in liver samples (0.40 copy/diploid). Low levels of lentiviral integration also were observed in the spleen and the heart. Transduction of the bone tissue, including bone marrow cells, was very low (<0.001 copy/diploid).

Radiographic analysis

Since radiographic changes in the HPP mice were not apparent during the first 8 days of life, we examined X-ray images of the feet and legs of mice at approximately 20 days after birth. The severity of the mineralization defects in the untreated HPP mice was found to be highly variable. In the most severely affected cases, the metacarpal and digital bones were significantly shorter than those of the WT mice, and their epiphyses were not detected. In addition, some of the carpal bones were absent. We also observed the absence of secondary ossification centers in the feet (Fig. 2). The most severe phenotype was observed in approximately 10% of the *Akp2*$^{-/-}$ homozygous neonates, and these mice usually died by 10 days of age. In the milder cases, the epiphyses and all the digital bones were significantly mineralized, even though the HPP mice were smaller than the HET and WT mice. Heterogeneous radiographic changes between these two extreme phenotypes were observed in the untreated HPP mice.

X-ray images of treated HPP mice showed that mineralization was accelerated following lentivirus-mediated expression of TNALP-D10. Secondary ossification centers were detected in the feet of all treated animals at 15 days of age, although the intensity of the mineralization was variable. Ossification of the carpal bones also was improved. All the untreated HPP mice died, with an average survival of 18.1 days. No differences in skeletal structure and mineralization were observed between the long survivors after treatment and the WT mice at 100 days of age. These results indicate that mineralization defects in HPP mice can be corrected efficiently by gene therapy.

Histochemical examination of the bone

The proximal tibias were analyzed histochemically for ALP activity using the azo-dye technique with methyl green counter-staining (Fig. 3). Strong ALP activity was detected in both the bone and hypertrophic cartilage zones of the WT mice (Fig. 3*A*), whereas no ALP signal was observed in the epiphysis of the HPP mice (Fig. 3*B*). After treatment with lentiviral vector, faint ALP staining was observed on the surface of the endosteal bone (Fig. 3*C*, *D*).

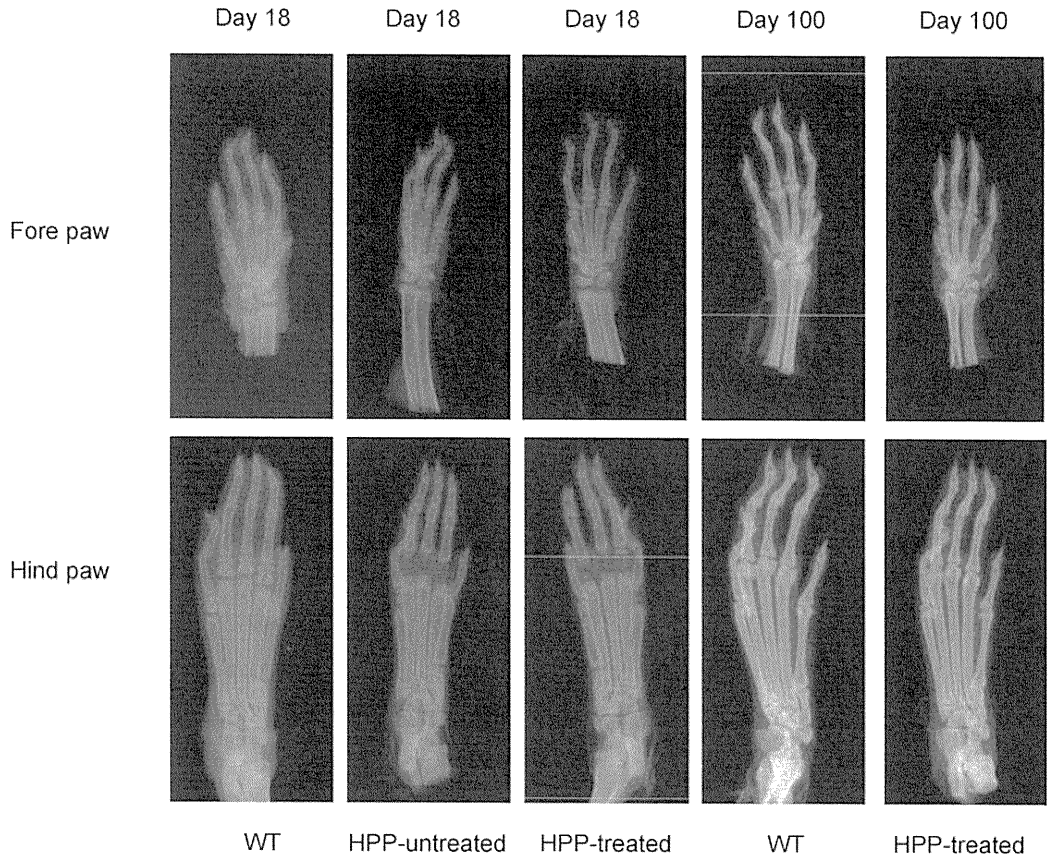

Fig. 2. X-ray images of the feet. Secondary ossification centers in the hind paws were absent in untreated HPP mice but were detectable in the treated mice at 18 days after birth. No differences in skeletal mineralization were observed between treated long survivors and WT mice at 100 days of age.

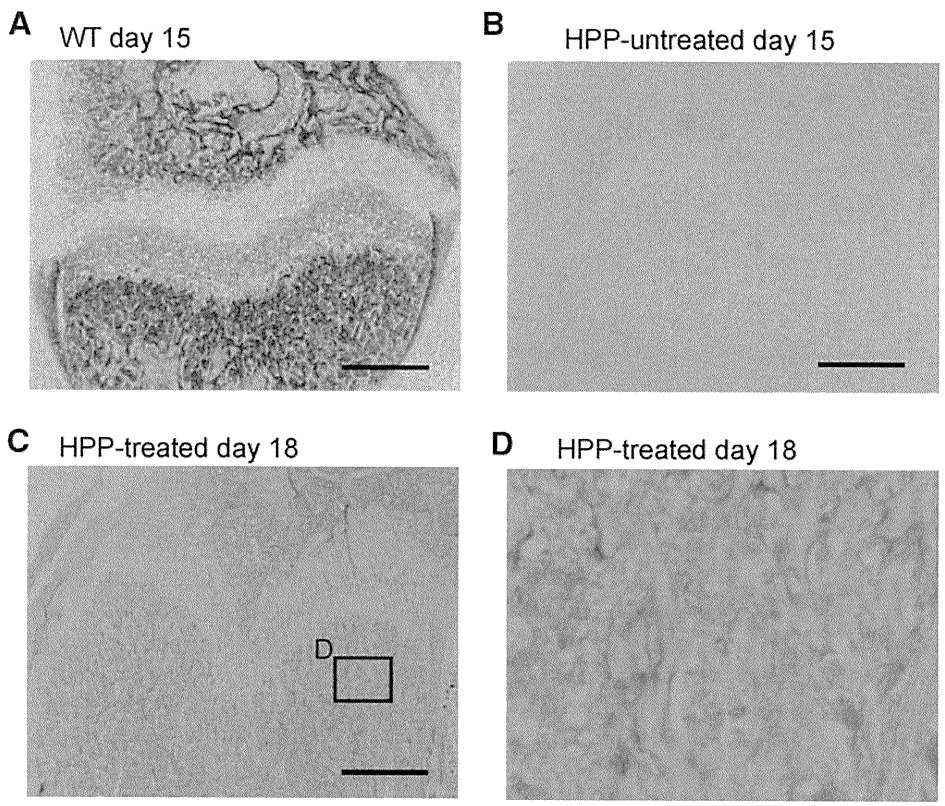

Fig. 3. Histochemical staining of ALP activity in the tibias. ALP activity was detected in WT (*A*) but not HPP mice (*B*) at 15 days after birth. Following treatment with lentiviral vector, (*C*) ALP activity was detected on the surface of the endosteal bone at 18 days after birth. (*D*) Magnified image of the square in panel *C*. Bars = 1 mm.

Discussion

TNALP is an ectoenzyme that is known to be particularly abundant on the cell surfaces of osteoblasts and hypertrophic chondrocytes, including their shed matrix vesicles.[6,39] Since ALP functions on the exterior of the cell, enzyme replacement following repeated administration of soluble ALP has been hypothesized as a potential approach to treat ALP deficiencies. However, the outcomes of previous clinical trials of enzyme-replacement therapy have proven disappointing. Intravenous infusions of ALP-rich serum from patients with Paget disease[13,14] and purified soluble ALP from human liver[16] and placenta[17] have shown no significant clinical benefits in patients with HPP. Recently, Millán and colleagues[25] demonstrated that daily injections of high-dose bone-targeted TNALP significantly extended the lifespan and corrected the abnormal phenotypes of HPP mice, suggesting that HPP could be treated by enzyme replacement if sufficient amounts of TNALP were able to reach the sites of skeletal mineralization. Based on these data, new clinical trials involving enzyme-replacement therapy for HPP patients have been initiated.[28]

A general problem of enzyme-replacement therapy is the short half-life of the administered protein in patients. A pharmacokinetic study showed that the half-life of bone-targeted TNALP is 34 hours in the plasma of adult mice, but in bone tissue the half-life is extended to more than 300 hours.[25] Nevertheless, repeated administration of large amounts of the enzyme is required for long-term correction. In the initial clinical trials, HPP patients received subcutaneous injections of bone-targeted TNALP three times weekly.[28] The preparation of adequate amounts of clinical-grade purified enzyme is a limitation, and repeated injection is highly invasive and not optimal for small children. In this study we demonstrated that a single injection of lentiviral vector resulted in sustained expression of ALP and phenotypic correction in HPP neonatal mice. As such, viral vector–mediated enzyme replacement may prove to be more practical than classic enzyme replacement by repeated injection.

One of the concerns of gene therapy is the safety of the viral vector. We used an HIV-1-based lentiviral vector in this study.[30] Lentivirus-mediated gene transfer has proven to be effective for long-term expression of transgenes in nondividing cells. Although the pathogenicity of HIV-1 was almost negligible in the current modified version of lentiviral vector, insertional mutagenesis is still a major concern for all integrating vectors.[40] To minimize the possibility of protooncogene activation, our novel self-inactivating lentiviral vector contains the insulator element from the chicken β-globin locus.[30] So far, lymphoproliferative complications owing to insertional mutagenesis have been detected in ex vivo hematopoietic stem cell gene therapy only. For the treatment of HPP, lentiviral vector was injected directly into the circulation of neonatal mice. After this in vivo systemic gene therapy, the lentiviral sequence was detected in the liver, lung, and heart. The oncogenicity of the integrated lentiviral vector in these differentiated tissues requires further examination in a long-term follow-up study.

We also found that the epileptic seizures were completely inhibited and the lifespan was significantly extended in the treated HPP mice. Without treatment, HPP mice died by 20 days of age.[22,35] The major cause of death in the untreated HPP mice was apnea, most likely resulting from their severe epileptic convulsions.[21] Pyridoxine-responsive seizures in HPP patients and HPP model mice are thought to be caused by reduced levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the brain.[21,35] PLP is an essential cofactor of glutamate decarboxylase, which is responsible for the synthesis of GABA.[41] Diminished hydrolysis of extracellular PLP in HPP causes decreased intracellular pyridoxal levels in cells. This results in a reduction in the rephosphorylation of pyridoxal to PLP, and thus biosynthesis of GABA within the brain cells is reduced.[10] The seizure phenotype can be rescued in part by administration of pyridoxal.[23] We demonstrated that epileptic seizures were efficiently inhibited by either systemic infusion of TNALP[25] or viral vector–mediated expression of TNALP, suggesting that the defective metabolism of PLP in the brain could be corrected by replacement of soluble TNALP.

Although epileptic seizures are observed in some severely affected patients, the major clinical complications in human HPP patients are directly related to defective skeletal mineralization.[3] Patients with severe infantile HPP usually die from respiratory failure caused by skeletal diseases in the chest, such as flail chest, rachitic deformity, and rib fractures.[3] Compared with severe infantile HPP in human patients, bone defects in our mouse model are relatively mild. The *Akp2*$^{-/-}$ HPP mice were born with a normal appearance and bone mineral deposition. Hypomineralization becomes apparent after around 10 days of age, although the severity of mineralization defects varies widely.[22] Lentivirus-mediated expression of bone-targeted ALP efficiently prevents the progressive skeletal demineralization, as well as the lethal epilepsy.

Since enzyme replacement also was effective,[25] the major mechanism of successful gene therapy for HPP mice seems to be due to continuous supply of bone-targeted TNALP from the vector-infected liver to the circulation. Another possibility is that osteoblasts and chondrocytes may be directly transduced with lentiviral vector. However, since the copy number of integrated vector in the whole bone tissue was very low, the ALP staining in treated mice is mainly due to the circulating TNALP-D10 in the bone matrix. The contribution of in situ expression of TNALP in bone cells to bone mineralization is not likely to be significant.

A major physiologic role for TNALP has been shown to be the restriction of the extracellular pool of PP_i, which is a strong inhibitor for mineralization.[24,39] Localization of TNALP to the skeleton should be important for the treatment of HPP. TNALP with a repetitive C-terminal extension of 10 Asp was shown to display high affinity for bone tissue both in vitro[26] and in vivo.[25] The use of the bone-targeted TNALP construct in a clinical setting is currently under investigation in clinical trials.[28]

The efficacy of gene therapy to correct hypomineralization was evaluated by radiographic examination. The major problem is that the severity of the mineralization defects in untreated infantile *Akp2*$^{-/-}$ mice is highly variable. In addition, X-rays of infantile mice could be taken only after euthanization. The time course of mineralization in a single animal could not be

examined under the condition used. Further studies using more reliable histomorphometric and micro-computed tomographic (μCT) techniques may be required to optimize the gene therapy protocol to rescue the skeletal phenotype.

In conclusion, we found that severe infantile HPP in *TNALP* knockout mice can be treated with a single injection of lentiviral vector during the neonatal period. Lentiviral-mediated gene therapy may prove to be an important option in the treatment of human hypophosphatasia.

Disclosures

JLM is a consultant for Enobia Pharma, Inc. All the other authors state that they have no conflicts of interest.

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SHORT COMMUNICATION

Prevalence of c.1559delT in *ALPL*, a common mutation resulting in the perinatal (lethal) form of hypophosphatasia in Japanese and effects of the mutation on heterozygous carriers

Atsushi Watanabe[1,2], Tatsuki Karasugi[3], Hideaki Sawai[4], Banyar Than Naing[2], Shiro Ikegawa[3], Hideo Orimo[2] and Takashi Shimada[1,2]

Hypophosphatasia (HPP) is an inherited disorder caused by mutations in *ALPL* that encodes an isozyme of alkaline phosphatase (ALP), TNSALP. One of the most frequent *ALPL* mutations is c.1559delT, which causes the most severe HPP, the perinatal (lethal) form (pl-HPP). c.1559delT has been found only in Japanese and its prevalence is suspected to be high; however, the allele frequency of c.1559delT in Japanese remains unknown. We designed a screening system for the mutation based on high-resolution melting curve analysis, and examined the frequency of c.1559delT. We found that the c.1559delT carrier frequency is 1/480 (95% confidence interval, 1/1562–1/284). This indicates that ~1 in 900 000 individuals to have pl-HPP caused by a homozygous c.1559delT mutation. In our analysis, the majority of c.1559delT carriers had normal values of HPP biochemical markers, such as serum ALP and urine phosphoethanolamine. Our results indicate that the only way to reliably detect whether individuals are pl-HPP carriers is to perform the *ALPL* mutation analysis.
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Keywords: *ALPL*; c.1559delT; perinatal form of hypophosphatasia; serum alkaline phosphatase; skeletal dysplasia; urine phosphoethanolamine

INTRODUCTION

Hypophosphatasia (HPP) is an inherited disorder characterized by defective mineralization of the bone and low activity of alkaline phosphatase (ALP; EC 3.1.3.1).[1,2] HPP is a clinically heterogeneous disease and classified into five forms according to severity and age of onset: perinatal (lethal), infantile (OMIM 241500), childhood (OMIM 241510), adult (OMIM 146300) and odontohypophosphatasia.[1] All forms of HPP display reduced activity of unfractionated serum ALP and the presence of either one or two pathologic mutations in *ALPL*, the gene encoding an ALP isozyme (TNSALP).

The perinatal (lethal) form of HPP (pl-HPP) is the most severe HPP with an autosomal recessive mode of inheritance. pl-HPP is more common in Japan than in other countries.[3] Parents of pl-HPP are heterozygous carriers of *ALPL* mutations. They show no clinical symptoms, but have reduced serum ALP activity and increased urinary phosphoethanolamine (PEA).[4–8]

ALPL is the only gene known to be associated with HPP.[1] More than 200 *ALPL* mutations have been described, accounting for most phenotype variabilities.[9] HPP is frequently caused by p.E191K and p.D378V in Caucasians,[1] whereas p.F327L[10] and c.1559delT[10,11] are more common in Japanese.[1] To date, c.1559delT has only been found in Japanese.[11] Some patients with pl-HPP are homozygous for c.1559delT, with parents who are heterozygous carriers for the mutation but with no evidence of consanguinity.[12,13]

To identify c.1559delT genotype and to examine its frequency in Japanese, we designed a screening system based on a high-resolution melting curve analysis.[14] In addition, we examined serum ALP activity and urine PEA in heterozygous c.1559delT carriers to determine whether these markers can identify the HPP carriers.

MATERIALS AND METHODS

This study was approved by the Institutional Genetic Research Ethics Committee at Nippon Medical School and RIKEN, Center for Genomic Medicine. Blood samples were collected under written informed consents from 3844 healthy Japanese without HPP and its related findings confirmed by orthopedic surgeons. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. The c.1559delT genotype screening was performed by the small amplicon genotyping method based on high-resolution melting curve

[1]Division of Clinical Genetics, Nippon Medical School Hospital, Tokyo, Japan; [2]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan; [3]Laboratory for Bone and Joint Diseases, Center for Genomic Medicine, RIKEN, Tokyo, Japan and [4]Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan
Correspondence: Dr A Watanabe, Division of Clinical Genetics, Nippon Medical School Hospital, 1-1-5 Sendagi Bunkyo-ku, Tokyo 113-8603, Japan.
E-mail: aw3703@nms.ac.jp
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analysis.[14] PCR primers for c.1559delT were designed to flank the mutation leaving only single base, including the mutation between the primers: 5′-TT TAAATTCTCGCGCTGGCCCTCTACCCC-3′ (forward) and 5′-TTTAAATTCC CTCAGAACAGGACGCTC-3′ (reverse). PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 45 cycles at 94°C for 30 s and annealing at 67°C for 30 s. After PCR, high-resolution melting was performed in a 96-well plate LightScanner (Idaho Technology, Salt Lake City, UT, USA), which collected data from 55°C to 97°C at a ramp rate of 0.10°C sec^{-1}. The observed number of c.1559delT carriers was divided by the total number of individuals tested to determine the carrier frequency. Serum ALP activity and urine PEA were measured in c.1559delT-heterozygous parents of pl-HPP patients.

RESULTS

Three *ALPL* c.1559delT genotypes (wt/wt, wt/c.1559delT and c.1559delT/c.1559delT) were distinguished by the modified small amplicon genotyping method (Figure 1). A heterogeneous c.1559delT mutation (wt/c.1559delT) was detected in 8 of 3844 healthy Japanese subjects, indicating a carrier frequency of 1/480 in the Japanese population (95% confidence interval, 1/1562–1/284).

The numerical value of ALP activity and urinary PEA varied in heterozygous c.1559delT carriers in parents of perinatal HPP patients. The majority of heterozygous c.1559delT carriers had normal levels of both ALP activity (five out of six males and three out of four females) and urinary PEA (three out of six males and four out of five females) (Figure 2).

DISCUSSION

Based on our results, we estimated the frequency of c.1559delT-homozygous individuals (for example, those with pl-HPP) to be 1/900 000. Previous studies showed that all Japanese pl-HPP patients carried the c.1559delT mutation in at least one allele; half (10/20) were homozygous for c.1559delT and half (10/20) were compound heterozygous for c.1559delT,[9–13,15] which gives a pl-HPP prevalence of 1/450 000 for patients that are homozygous or compound heterozygous for c.1559delT mutation. The other common mutation on *ALPL* in Japan, p.F327L, is a mild allele whose product retained ~70% of its enzymatic activity. Patients compound heterozygous for c.1559delT and p.F327L are not associated with pl-HPP.[10]

Biochemical markers, serum ALP activity and urinary PEA levels fell within their normal ranges in the majority of the c.1559delT carriers examined in this paper, whereas heterozygous carriers of the severe forms in other *ALPL* mutations were reported to have reduced serum ALP activity and increased urinary PEA.[4–8] Some possible reasons why c.1559delT carriers display normal marker levels are as follows: the first is the protein properties caused by the different mutation positions. The c.1559delT mutation causes a frameshift downstream of codon L503, resulting in the elimination of the termination codon at 508 and the addition of 80 amino acids at the C-terminus. The mutant protein forms an aggregate that is polyubiquitinated and then degraded in the proteasome. However, the aggregates possess enzyme activity, and may, therefore, influence physiological processes before their destruction.[16] Second, serum ALP activity is affected by some other factors. The genetic modifier of ALP is reported to have a potential influence on serum ALP activity.[17] Total ALP value is also elevated by some environmental factors, in vitamin D deficiency[2] or in the third trimester of gestation by the increasing placental ALP, which is not affected by TNSALP.[18] Recently, it was shown that patients who are homozygous for the c.1559delT mutation differed in the severity of HPP, including both their symptoms and serum ALP activity.[15]

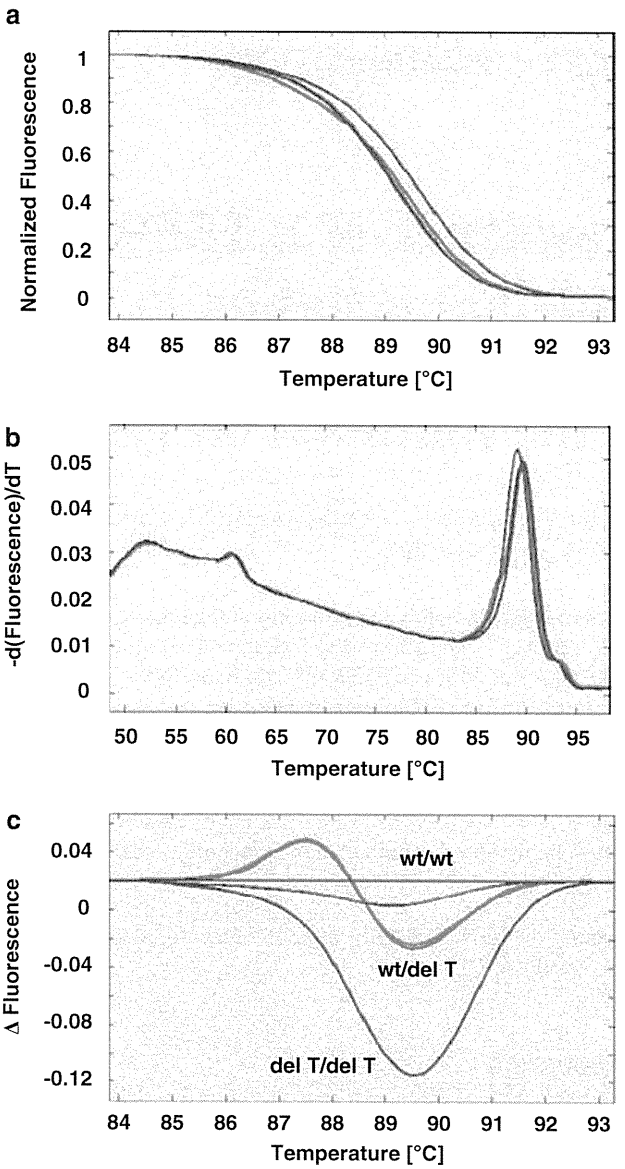

Figure 1 Identification of c.1559delT mutation in *ALPL* by small amplicon genotyping (SAG) method. (**a**) Normalized fluorescence plots. (**b**) d(fluorescence)/dT plot. (**c**) The corresponding fluorescence difference plots. Wild-type (wt/wt) samples are in gray; samples heterozygous for c.1559delT (wt/c.1559delT) are in red; and samples homozygous for c.1559delT (c.1559delT/c.1559delT) are in blue. The three genotypes were clearly distinguishable in the SAG method.

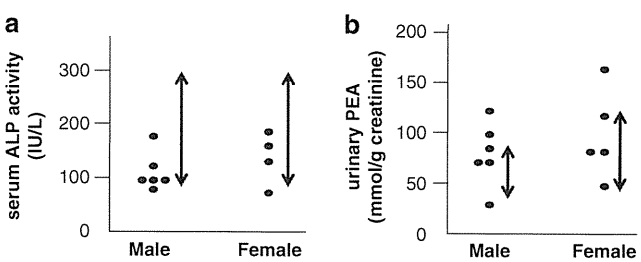

Figure 2 Biochemical marker levels in heterozygous carriers of the *ALPL* c.1559delT mutation. The serum ALP activity (**a**) and urinary PEA (**b**) levels in the majority of heterozygous carriers (wt/c.1559delT) fell within normal ranges (indicated by arrows).

Thus, the only way to reliably detect the pl-HPP carriers is to perform the *ALPL* mutation analysis. The small amplicon genotyping method in this study using the high-resolution melting curve analysis is a one-step, single-tube method for detection of specific mutations and faster, simpler and less expensive than the approaches requiring separations or labeled probes.[19]

The screening for c.1559delT in *ALPL* may be useful for diagnosis of pl-HPP in Japanese to provide optimum genetic counseling for fetal skeletal dysplasia. pl-HPP occasionally could not be diagnosed with sonographic examination in the first trimester because incomplete ossification is an usual finding at this stage of development.[20] To diagnose pl-HPP, collaborations between obstetricians and clinical geneticists are important and could provide support for parents of prenatal patients suspected of having skeletal dysplasia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Circulating Levels of Soluble α-Klotho Are Markedly Elevated in Human Umbilical Cord Blood

Yasuhisa Ohata,* Hitomi Arahori,* Noriyuki Namba, Taichi Kitaoka, Haruhiko Hirai, Kazuko Wada, Masahiro Nakayama, Toshimi Michigami, Akihiro Imura, Yo-ichi Nabeshima, Yuji Yamazaki, and Keiichi Ozono

Department of Pediatrics (Y.O., H.A., N.N., T.K., H.H., K.W., K.O.), Osaka University Graduate School of Medicine, Suita 565-0871, Japan; Bone and Mineral Research (Y.O., T.M.) and Clinical Laboratory Medicine and Anatomic Pathology (M.N.), Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi 594-1101, Japan; Department of Tumor Biology (A.I., Y.N.), Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan; Core Research for Evolutional Science and Technology (A.I., Y.N.), Science and Technology Corporation, Kawaguchi 332-0012, Japan; and Antibody Research Laboratories (Y.Y.), Kyowa Hakko Kirin Co., Ltd., Tokyo 194-8533, Japan

Context: Fetal serum levels of calcium and phosphate are higher than those in the maternal levels. Although α-Klotho is known to participate in calcium and phosphate metabolism in adults, its role in the perinatal period remains unknown.

Objective: This study aimed to determine the baseline levels of soluble α-Klotho in fetuses and compare them with those in neonates, mothers, and adults to clarify whether α-Klotho is involved in the fetal-specific regulation of calcium and phosphate metabolism.

Design and Setting: We conducted a cross-sectional evaluation of healthy babies (at birth and/or at 4 d after birth), their mothers, and adult volunteers at one hospital.

Participants: Twenty-one healthy mothers, their babies (23 in total, including two pairs of twins), and 25 adult volunteers participated in the study.

Main Outcome Measures: We measured the serum levels of soluble α-Klotho and fibroblast growth factor 23 (FGF23).

Results: In cord blood, the level of α-Klotho was markedly higher (3243 ± 1899 pg/ml) than levels in neonates at d 4 (582 ± 90 pg/ml), mothers (768 ± 261 pg/ml), and adult volunteers (681 ± 140 pg/ml) ($P < 0.001$), whereas the fetal level of FGF23 was lower than levels in the other subjects. The levels of soluble α-Klotho were negatively correlated with those of FGF23 in cord blood. Immunohistochemistry demonstrated that α-Klotho was predominantly expressed in syncytiotrophoblasts in normal term placenta.

Conclusion: Levels of soluble α-Klotho are markedly elevated in cord blood and might be useful as a biomarker for mineral metabolism in the fetus. **(*J Clin Endocrinol Metab* 96: E943–E947, 2011)**

Fetal mineral homeostasis is regulated differently from adult homeostasis. The levels of serum calcium and phosphate in the fetus are higher than the maternal levels during late gestation. PTH and PTHrP are known to be involved in calcium homeostasis (1, 2). On the other hand, the regulatory mechanism of the fetal phosphate level is poorly understood (3).

The *α-Klotho* gene encodes a single-pass transmembrane protein, which was originally identified as an aging-related gene (4). In adults, α-Klotho contributes to the

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* Y.O. and H.A. contributed equally to this work.

Abbreviations: FGF23, Fibroblast growth factor 23; 25-OHD, 25-hydroxyvitamin D; TRPV, transient receptor potential vanilloid.

regulation of calcium and phosphate homeostasis. In the parathyroid, α-Klotho binds to Na^+/K^+-ATPase to regulate PTH secretion and is involved in transepithelial calcium transport (5). α-Klotho is also involved in the activation of transient receptor potential vanilloid (TRPV) 5 in the kidney (6), indicating its central role in the maintenance of calcium homeostasis. In addition, α-Klotho participates in phosphate homeostasis by cooperating with fibroblast growth factor 23 (FGF23) and the FGF receptor (7). FGF23 reduces the serum phosphate level both by suppressing phosphate reabsorption and activating vitamin D in the proximal tubules (8–10).

Although *α-Klotho* is predominantly expressed in the kidney, parathyroid, and choroid plexus, it is also expressed in other tissues including the placenta (4). Its expression in the placenta has led us to hypothesize that α-Klotho might play a role in fetal mineral homeostasis as well as in postnatal homeostasis.

In addition to the transmembrane form, α-Klotho also exists in a soluble form. The soluble form, which is produced by the shedding of the transmembrane protein, is detectable in serum, cerebrospinal fluid, and urine (6, 11). Although soluble α-Klotho is considered to be a humoral factor (12), its regulatory mechanisms and functions are largely unknown, and it is considered that FGF23 signaling requires the transmembrane form of the protein.

Recently, a sandwich ELISA for soluble α-Klotho has been established (13). In the present study, we used this assay to measure the serum levels of soluble α-Klotho in cord blood at birth and compared them to the levels in neonates, mothers, and adults. We found that high levels of soluble α-Klotho are present in cord blood and analyzed the relationship between those of soluble α-Klotho and FGF23 in cord blood. To the best of our knowledge, this is the first report on the measurement of soluble α-Klotho levels in perinatal blood samples.

Subjects and Methods

Study participants

We recruited healthy pregnant women, their babies, and adult volunteers and obtained informed consent from all participants or their legal guardians. The institutional review board of Osaka University Hospital approved this study. The inclusion criteria were an unremarkable medical history, physical examination, and screening laboratory test results for endocrine and metabolic function. The exclusion criteria were premature or postmature infant delivery (gestational age under 37 wk and over 42 wk, respectively), and the neonate being light or heavy for their delivery date (birth weight under −1.5 SD and over 1.5 SD, respectively).

Twenty-one mothers and their babies (n = 23, including two pairs of twins) were enrolled. For comparison, 25 healthy adult volunteers ranging in age from 27 to 48 yr (11 males and 14 females) were also enrolled.

Blood analyses

After the delivery of the neonate, we immediately obtained cord blood samples from the umbilical vein, before the placenta was delivered. We also collected blood from the neonates at d 4 after birth in the morning and fasting morning blood from their mothers and the adult volunteers. The maternal blood was obtained within 24 h of the delivery. The participants were under no dietary restrictions during the study.

After we had collected the blood samples, we separated the serum instantly and stored it at −80 C until analysis. We measured the levels of serum soluble α-Klotho and intact FGF23 in all samples. The serum soluble α-Klotho levels were measured using an ELISA kit provided from Kyowa Hakko-Kirin (Tokyo, Japan) (13). The intra- and interassay coefficients of variation ranged from 2.7 to 9.8% (13). The serum levels of intact FGF23 were determined using a commercial sandwich ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan) (14). Serum calcium, phosphate, intact PTH, 25-hydroxyvitamin D (25-OHD), albumin, and creatinine levels were also measured in the samples except for those from neonates. We corrected the levels of calcium in the samples displaying hypoalbuminemia (albumin < 4.0 g/dl) as reported previously (15).

Immunohistochemistry

Normal human placenta (gestational age, 38 wk) was obtained from an uncomplicated pregnancy. The specimen was fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4-μm-thick sections. Antigen retrieval was performed using 10 mM citrate buffer (pH 5.9) for 15 min at 98 C. The sections were stained using anti-α-Klotho antibody (sc-22218; Santa Cruz Biotechnology, Santa Cruz, CA) and goat ImmunoCruz Staining System (Santa Cruz Biotechnology). The slides were counterstained with hematoxylin. Normal goat IgG was used as a negative control.

Statistical analyses

The results are expressed as the mean ± SD. We compared biochemical parameters and soluble α-Klotho and FGF23 levels among the groups by ANOVA, followed by the Tukey-Kramer method. The relationship between soluble α-Klotho and FGF23 in cord blood samples was analyzed using Pearson's correlation test.

All statistical analyses were conducted using JMP software version 8.0.1 (SAS Institute Inc., Cary, NC).

Results

Levels of soluble α-Klotho in cord blood are higher than those in neonates, mothers, and adults

The biochemical findings are shown in Table 1. Serum calcium, phosphate, intact PTH, 25-OHD, albumin, and creatinine levels were within the normal range in both the mother and adult groups (16). As previously reported, serum calcium and phosphate levels were significantly higher,