Discussion

Perinatal HPP is the most severe form of HPP with an autosomal recessive mode of inheritance and is more common in Japan than in other countries (Satoh *et al.*, 2009). We have recently determined the prevalence of c.1559delT in *ALPL*, a common mutation resulting in perinatal HPP in Japanese, and the carrier frequency was 1/480 (Watanabe *et al.*, 2010). It is possible that some perinatal HPP patients are passed over as stillborn babies with unknown cause of death. On the other hand, with developments in perinatal care, the chance of diagnosis of perinatal lethal HPP during the fetal period is increasing. This indicates the need for fetal gene therapy, which was advocated in the report entitled "Prenatal Gene Transfer: Scientific, Medical, and Ethical Issues" by the Recombinant DNA Advisory Committee (Recombinant DNA Advisory Committee, 1999).

Several advantages of fetal gene therapy have been proposed for the treatment of genetic diseases. Immunological tolerance to the viral vector or transgenic proteins would be induced by fetal gene delivery, and it can achieve long-term effective expression of the transgene (Lipshutz *et al.*, 2000; Waddington *et al.*, 2003). Another advantage of fetal gene therapy is that gene delivery to tissues that are difficult to penetrate, such as the central nervous system, may be possible not only because of immaturity of the blood–brain barrier but also the abundance of stem cell populations in the fetus (Lipshutz *et al.*, 2000). Furthermore, fetal gene therapy requires smaller amounts of the vector compared to gene therapy in infants or adults, because of the small size of the fetus.

Enzyme replacement therapy for HPP, using a mineral-targeting recombinant form of TNALP has been shown to be effective for the prevention of all the skeletal and dental abnormalities of HPP (Millán *et al.*, 2008; McKee et al., 2011; Yadav et al., 2011). Those data indicate that expression of TNALP in the cells lacking TNALP activity is not absolutely required for the treatment of HPP. Furthermore, evidence is mounting indicating that a continuous supply of soluble TNALP from the circulation might be sufficient to improve mineralization. Indeed, our previous studies showed that systemic injection of lentiviral vector or AAV8 vector harboring either mineral-targeting as well as soluble non-targeted TNALP into neonatal mice resulted in sustained expression of TNALP in plasma and successful treatment of HPP, although transduction into the bone was very low (Yamamoto *et al.*, 2010; Matsumoto *et al.*, in press). Our present study demonstrates high ALP activity in the bones of treated mice. Real-time PCR and immunohistochemical analysis indicates that AAV9 was directly transferred into chondrocytes after intraperitoneal injection during the fetal period. Therefore, ALP activity in bones of fetal treated mice should be derived from the circulation and the resident chondrocytes. This approach would seem to be more effective than previous therapeutic strategies based on enzyme replacement by injection of either protein or vector in the neonatal period. Fetal injection of AAV9 vector represents a potential breakthrough for gene delivery into bone cells to treat systemic skeletal diseases, such as osteogenesis imperfecta and mucopolysaccharidosis.

It is interesting that chondrocytes were efficiently transduced with AAV vector after fetal gene transfer. Recently, Roybal et al., also reported that prenatal systemic administration of lentiviral vector resulted in efficient gene transfer into chondrocytes (Roybal *et al.*, 2011).

Therefore, chondrocytes or their stem cells appear to be preferentially susceptible to viral infection in utero. There are several possible explanations for the developmentally dependent distribution patterns of viral vector. Stem cells and progenitor cells of chondrocytes exists at higher frequency in fetal bones. These cells are more accessible to viral vectors than differentiated chondrocytes and provide a large pool of genetically modified chondrocytes. The structure of blood vessels is developmentally regulated (Herbert *et al.*, 2011) and unstable immature vessels at the early prenatal period should be more permeable to viral vectors. Changes in blood flow patterns in developing bones may also affect exposure of chondrocytes to viral vector in the circulation (Schachtner *et al.*, 1999; Mescher, 2009). In this study, two of the *Akp2*$^{-/-}$ fetuses failed to respond to treatment. These two newborn mice showed failure to thrive and developed seizures after birth. The reason for this failure is not clear. One possibility is that sufficient amounts of vector were not delivered to the fetus because of technical failure. It is sometime difficult to inject intrperitoneally without leakage through the semitranparent uterine wall.

Perinatal lethal and infantile forms of HPP are often associated with epileptic seizures. One of the vitamin B6 forms, pyridoxal-5'-phosphate (PLP) is the co-factor of numerous enzymes, including neurotransmitter-synthesizing enzymes such as gamma-aminobutyric acid (GABA), dopamine and serotonin (5-HT), which TNALP regulates via PLP in the neuropil (Negyessy *et al.*, 2011). TNALP is present widely throughout the human neocortex (Negyessy *et al.*, 2011). Consequently, deficiency of TNALP is thought to lead to epileptic seizures in HPP patients. Clinically, vitamin B6 is usually administered to HPP patients with epileptic seizures, but the efficacy has not yet been validated. Here, we

chose AAV9, which has advantages with regard to efficient transduction into the CNS, and furthermore the blood–brain barrier of the fetus or neonate is immature, which may permit vectors to pass through (Foust *et al.*, 2009; Miyake *et al.*, 2011). AAV9 vector-mediated replacement of TNALP within the brain would normalize the levels of neurotransmitters, including GABA synthesis, 5-HT, and dopamine, which are also PLP-dependent (Dolphin *et al.*, 1986; Hartvig *et al.*, 1995) and have efficacy in controlling seizures. Moreover, normalizing TNALP level in the brain from the fetal period was estimated to lead to sufficient development of the neocortex.

Safety and ethical problems are major concerns of fetal gene therapy. The safety of gene delivery into the immature tissues has not yet been established, and it will be necessary to accumulate much more evidence from animal trials to evaluate the risks and benefits of fetal gene therapy prior to use in humans. A high incidence of liver tumorigenesis in mice was reported following fetal injection of lentiviral vector (Themis *et al.*, 2005) and neonatal injection of AAV vector (Donsante *et al.*, 2007). As the fetal vasculature may be more permeable than that in adults, germline transmission is a serious ethical problem of fetal gene transfer. In mouse experiments, vector sequence was occasionally detectable in the gonads of fetal treated animals, but gene transfer into spermatozoa or in the offspring has not been found (Tenenbaum *et al.*, 2003; Waddington *et al.*, 2003). We also examined the germline and our data also confirmed the lack of transduction into the germline of treated mice. This may be because fetal gene therapy requires only small amounts of the vector. However, evidence of very low efficiency germline transmission into the sperm cells of sheep (Porada *et al.*, 2005) and gonadal cells of rhesus monkeys (Lee *et al.*, 2005) was recently reported after fetal gene

transfer. The timing of fetal gene therapy should be also considered because of the difference in development of the animal model and human being. It has been reported that day 16-17 of gestation for mice corresponds approximately to 15-20 weeks of gestation for human (Larsen et al., 1997). However, the developmental stages of each organ should be different widely between mouse and human. If fetal gene therapy becomes applicable to human in the future, the timing of treatment must be carefully determined by the systemic experiments using large animal models including non-human primates.

In conclusion, we demonstrate that lethal murine HPP mice can be treated by fetal gene therapy. A single injection of AAV vector expressing bone-targeted TNALP *in utero* resulted in long-term expression and systemic replacement of TNALP. ALP activities were detected in various systemic organs including bones. Treated animals showed good weight gain, normal mineralization, and seizure-free survival until at least 8 weeks when sacrificed for analysis. Although long-term follow-up is necessary for further evaluation of efficacy and safety of fetal gene therapy, this is the first report of successful gene therapy *in utero* for lethal murine HPP. The incidence of HPP diagnosis during the perinatal period will increase in Japan due to developments in perinatal care. Therefore, fetal gene therapy will become an important form of therapy for perinatal lethal 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.

References

Dolphin, D., Poulson, R., and Avramovi, O. (1986). Vitamin B6 pyridoxal phosphate. Chemical, biochemical and medical aspects. (John Wiley and Sons, NY).

Donsante, A., Miller, D.G., Li, Y., Vogler, C., Brunt, E.M., Russell, D.W., and Sands, M.S. (2007). AAV vector integration sites in mouse hepatocellular carcinoma. Science 317, 477.

Fedde, K.N., Blair, L., Silverstein, J., Coburn, S.P., Ryan, L.M., Weinstein, R.S., Waymire, K, Narisawa, S., Millán, J.L., Macgregor, G.R., and White, M.P. (1999). Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. J. Bone Miner. Res. 14, 2015-2026.

Foust, K.D., Nurre, E., Montgomery, C.L., Hermandez, A., Chan, C.M., and Kasper B.K. (2009). Intravascular AAV9 preferentially targets neonatal-neurons and adult-astrocytes in CNS. Nat. Biotechnol. 27, 59-65.

Goseki, M., Oida, S., and Sasaki, S. (1988). Detection of minor immunological differences among human "universal-type" alkaline phosphatases. J Cell Biochem. 38, 155-163.

Hartvig, P., Lindner, K.J., Bjurling P, Laengstrom B, and Tedroff J. (1995). Pyridoxine effect on synthesis rate of serotonin in the monkey brain measured with positron emission tomography. J. Neural Transm. Gen. Sect. 102, 91-97.

Herbert, S.P., and Stainier, D.Y. (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nat. Rev. Mol. Cell Biol. 12, 551-564.

Hermens, W.T., ter Brake, O., Dijkhuizen, P.A., Sonnemans, M.A., Grimm, D., Kleinschmidt,

J.A., and Verhaagen, J. (1999). Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. Hum Gene Ther. 10, 1885-1891.

Iwamoto, N., Watanabe, A., Yamamoto, M., Miyake, N., Kurai, T., Teramoto, A., and Shimada, T. (2009). Global diffuse distribution in the brain and efficient gene delivery to the dorsal root ganglia by intrathecal injection of adeno-associated viral vector serotype 1. J. Gene Med. 11, 498-505.

Larson, J.E., Morrow, S.L., Happel, L., Sharp, J.F., and Cohen, J.C. (1997). Reversal of cystic fibrosis phenotype in mice by gene therapy in utero. Lancet 349, 619-620.

Lee, C.C., Jimenez, D.F., Kohn, D.B., and Tarantal, A.F. (2005). Fetal gene transfer using lentiviral vectors and the potential for germ cell transduction in rhesus monkeys (Macaca mulatta). Hum. Gene Ther. 16, 417-425.

Lipshutz, G.S., Flebbe-Rehwaldt, L., and Gaensler, K.M. (2000). Reexpression following readministration of an adenoviral vector in adult mice after initial in utero adenoviral administration. Mol. Ther. 2, 374-380.

McKee, M.D., Nakano, Y., Masica, D. L., Gray, J.J., Lemire, I., Heft, R., Whyte, M.P., Crine, P., and Millán, J.L. (2011). Enzyme replacement prevents dental defects in a mouse model of hypophosphatasia. J. Dental Res. 90, 470-476.

Matsumoto, T., Miyake, K., Yamamoto, S., Orimo, H., Miyake, N., Odagaki, Y., Adachi, K., Iijima, O., Narisawa, S., Millán, J.L., Fukunaga, Y., and Shimada, T. (in press). Rescue of Severe Infantile Hypophosphatasia Mice by AAV Mediated Sustained Expression of Soluble Alkaline Phosphatase. Hum. Gene Ther.

McKee, M.D., Nakano, Y., Masica, D. L., Gray, J.J., Lemire, I., Heft, R., Whyte, M.P., Crine, P., and Millán, J.L. (2011). Enzyme replacement prevents dental defects in a mouse model of hypophosphatasia. J. Dental Res. 90, 470-476.

Mescher, A.L. (2009). Bone. In *Junqueira' s Basic Histology (12th edition).* (Mc Graw Hill, NY) *pp.* 121-139.

Millán, J. L., Narisawa, S., Lemire, I., Loisel, T.P., Boileau, G., Leonard, P., Gramatikova, S., Terkeltaub, R., Camacho, N.P., McKee, M.D., Crine, P., and Whyte, M.P. (2008). Enzyme replacement therapy for murine hypophosphatasia. J. Bone Miner. Res. 23, 777-787.

Miyake, N., Miyake, K., Yamamoto, M., Hirai, Y., and Shimada, T. (2011). Global gene transfer into the CNS after neonatal systemic delivery of single-stranded AAV vectors. Brain Res. 1389, 19-26.

Mornet, E., (2007). Hypophosphatasia. Orphanet. J. Rare Dis. 2, 40.

Nakamura-Utsunomiya, A., Okada, S., Hara, K., Miyagawa, S., Takeda, K., Fukuhara, R., Nakata, Y., Hayashidani, M., Tachikawa, K., Michigami, T., Ozono, Y., and Kobayashi, M. (2010). Clinical characteristics of perinatal lethal hypophosphatasia: a report of 6 cases. Clin. Pediatr. Endocrinol. 19, 7-13.

Narisawa, S., Frohlander, N., and Millán, J.L. (1997). Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Dev. Dyn. 208, 432-446.

Negyessy, L., Xiao, J., Kantor, O., Kovacs, G.G., Palkovits, M., Doczi, T.P., Renaud, L., Baksa, G., Glasz, T., Ashaber, M., Barone, P., and Fonta, C. (2011). Layer-specific activity of

tissue non-specific alkaline phosphatase in the human neocortex. Neuroscience 172, 406-418.

Recombinant DNA Advisory Committee, National Institutes Of Health. (1999). Prenatal Gene Transfer: Scientific, Medical, and Ethical Issues. Available at http://oba.od.nih.gov/oba/rac/gtpcreport.pdf (accessed July 2011)

Roybal, J.L., Endo, M, Zoltick, P.W., and Flake, A.W. (2011). Early gestational gene transfer of IL-10 by systemic administration of lentiviral vector can prevent arthritis in a murine model. Gene Ther. 18,719-726.

Porada, C.D., Park, P.J., Tellez, J., Ozturk, F., Glimp, H.A., Almeida-Porada, G., and Zanjani, E.D. (2005). Male germ-line cells are at risk following direct-injection retroviral-mediated gene transfer in utero. Mol. Ther. 12, 754-762.

Salvetti, A., Oreve, S., Chadeuf, G., Favre, D., Cherel, Y., Champion-Arnaud, P., David-Ameline, J., Moullier, P. (1998). Factors influencing recombinant adeno-associated virus production. Hum. Gene Ther. 9, 695-706.

Satoh, N., Murotsuki, A., and Sawai, H. (2009). The birth prevalence rates for skeletal dysplasia in the registration system of the Japan Forum of Fetal Skeletal Dysplasia. J. Jan. Perinat. Neonat. Med. 45, 1005-1007. (In Japanese)

Schachtner, S.K., Buck, C.A., Bergelson, J.M., and Baldwin, H.S. (1999). Temporally regulated expression patterns following in utero adenovirus-mediated gene transfer. Gene Ther. 6, 1249-1257.

Sugiyama, O., Orimo, H., Suzuki, S., Yamashita, K., Ito, H., and Shimada, T. (2003). Bone formation following transplantation of genetically modified primary bone marrow

stromal cells. J. Orthop. Res. 21, 630-637.

Tenenbaum, L., Lehtonen, E., and Monahan, P.E. (2003). Evaluation of risks related to the use of adeno-associated virus-based vectors. Curr. Gene Ther. 3, 545-565.

Themis, M, Waddington, S.N., Schmidt, M., Von Kalle, C, Wang, Y., Al-Allaf, F, Gregory, L.G., Nivsarkar, M., Holder, M.V., Buckley, S.M., Dighe, N., Ruthe, A.T., Mistry, A., Bigger, B., Rahim, A., Nguyen, T.H., Trono, D., Thrasher, A.J., and Coutelle, C. (2005). Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. Mol. Ther. 12, 763-771.

Waddington, S.N., Buckley, S.M., Nivsarkar, M., Jezzard, S., Schneider, H., Dahse, T., Kemball-Cook, G., Miah, M., Tucker, N., Dallman, M.J., Themis, M., and Coutelle, C. (2003). In utero gene transfer of human factor IX to fetal mice can induce postnatal tolerance of the exogenous clotting factor. Blood 101, 1359-1366.

Watanabe, A., Karasugi, T., Sawai, H., Naing, B.T., Ikegawa, S., Orimo, H., and Shimada, T. (2010). 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. J. Hum. Genet. 56, 166-168.

Whyte, M.P. (2010). Physiological role of alkaline phosphatase explored in hypophosphatasia. Ann N.Y. Acad. Sci. 1192, 190-200.

Yadav, M.C., Lemire, I., Leonard, P., Boileau, G., Blond, L., Beliveau, M., Cory, E., Sah, R.L., Whyte, M.P., Crine, P., and Millán, J.L. (2011). Dose response of bone-targeted enzyme replacement for murine hypophosphatasia. Bone 49, 250–256.

Yamamoto, S., Orimo, H., Matsumoto, T., Iijima, O., Narisawa, S., Maeda, T., Millán, J.L.,

and Shimada, T. (2010). Prolonged survival and phenotypic correction of *Akp2*$^{-/-}$ hypophosphatasia mice by lentiviral gene therapy. J. Bone Miner. Res. 26, 135-142.

Figure legends

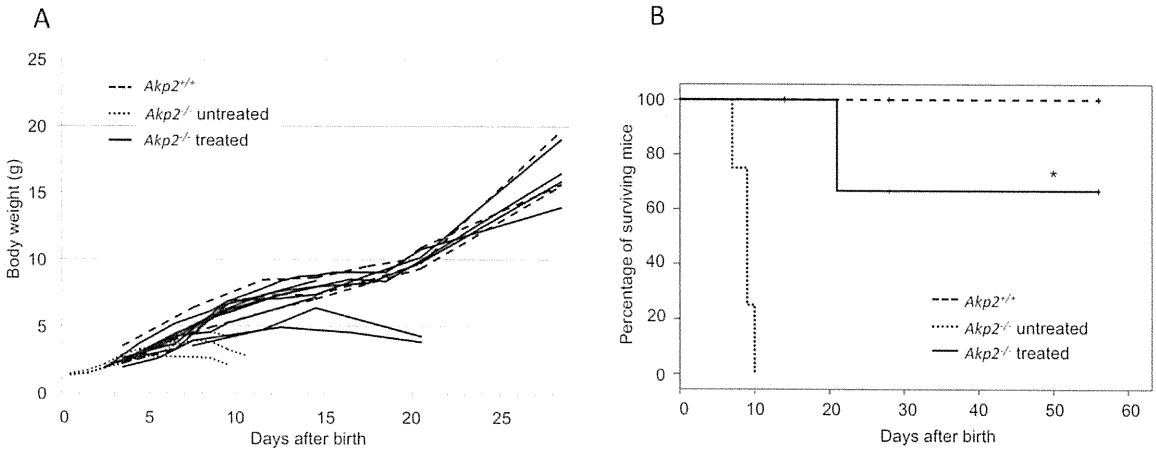
Figure 1. Therapeutic effects of fetal gene therapy. (A) Growth curves of *Akp*$^{+/+}$ mice (n = 4, long-dashed lines), untreated *Akp*$^{-/-}$ mice (n = 4, short-dashed lines), and treated *Akp*$^{-/-}$ mice (n = 9, continuous lines). (B) Survival curves of *Akp*$^{+/+}$ mice (n = 8, long-dashed line), untreated *Akp*$^{-/-}$ mice (n = 4, short-dashed line), and treated *Akp*$^{-/-}$ mice (n = 9, continuous line). Statistical analysis revealed that proportion of surviving of treated *Akp*$^{-/-}$ mice was significantly elevated compared to that of untreated *Akp*$^{-/-}$ mice (*$P < 0.001$).

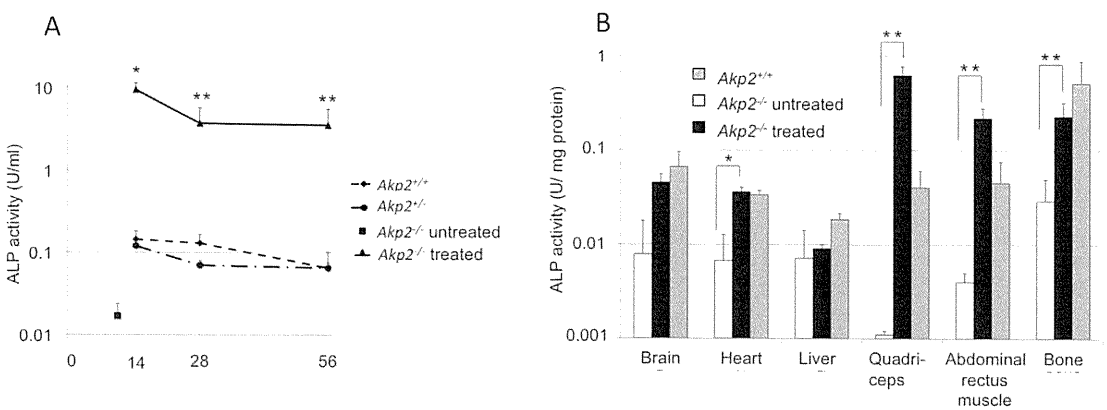
Figure 2. ALP activity in the plasma and tissues. (A) Concentration of plasma ALP activity of *Akp*$^{+/+}$ mice (n = 4, short-dashed line), *Akp*$^{+/-}$ mice (n = 4, long-dashed line), and treated *Akp*$^{-/-}$ mice (n = 3, continuous line) at 14, 28, and 56 days after birth. ALP activity in the plasma of untreated *Akp*$^{-/-}$ mice (n = 4) at 10 days after birth is indicated as a square marker. (B) ALP activity in the tissues of *Akp*$^{+/+}$ mice (day 14; n = 2 – 3, gray bars), untreated *Akp*$^{-/-}$ mice (day 10; n = 3, white bars) and treated *Akp*$^{-/-}$ mice (day 14; n = 3, black bars). Data are presented as means and SD. Student's t test was employed for comparisons between the two groups (*$P < 0.01$, **$P < 0.05$).

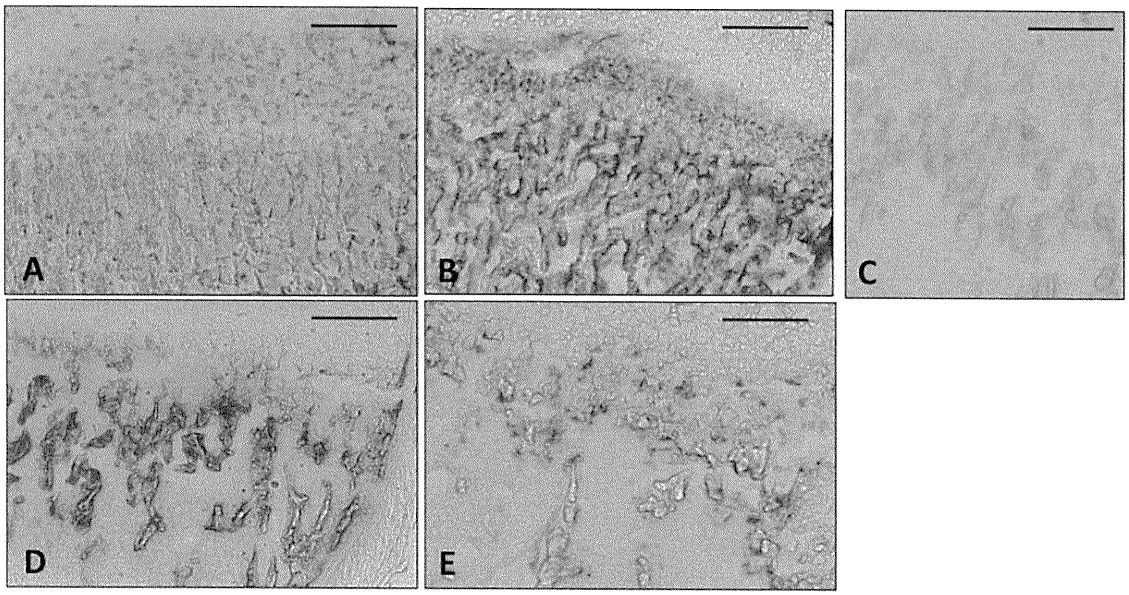
Figure 3. Histochemical staining for ALP activity. ALP activity is shown as purple staining. The pictures show the tibial bones of 14-day-old (A) and 56-day-old (B) *Akp*$^{+/+}$ mice, 10-day-old untreated *Akp*$^{-/-}$ mouse (C), 14-day-old (D) and 56-day-old (E) treated *Akp*$^{-/-}$ mice. Bars = 250 μm.

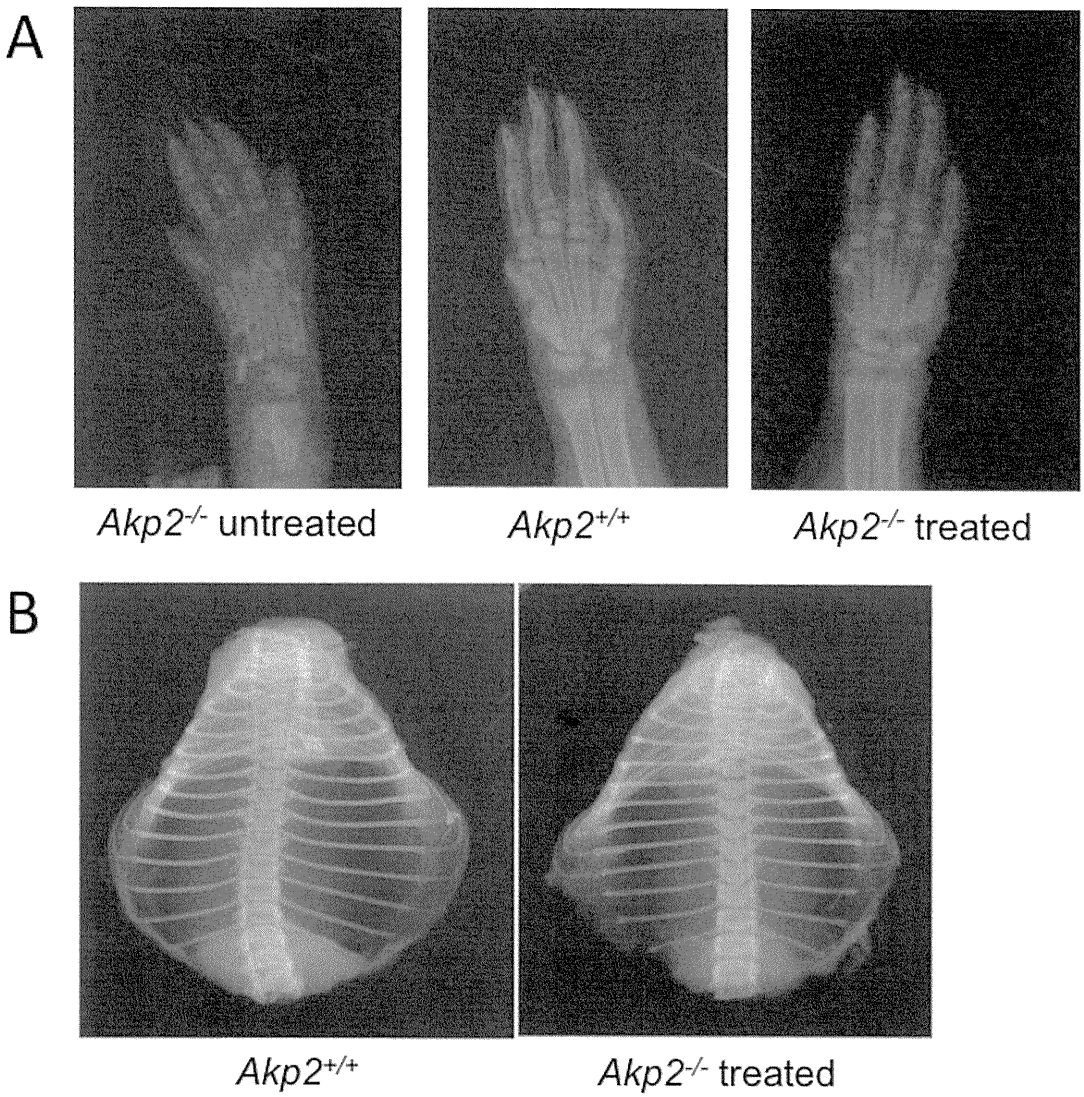
Figure 4. X-ray images. (A) Forepaw of 10-day-old untreated *Akp*$^{-/-}$ mouse, *Akp*$^{+/+}$ mouse, and treated *Akp*$^{-/-}$ mouse. (B) Thoracic cage of 56-day-old *Akp*$^{+/+}$ mouse and treated *Akp*$^{-/-}$ mouse.

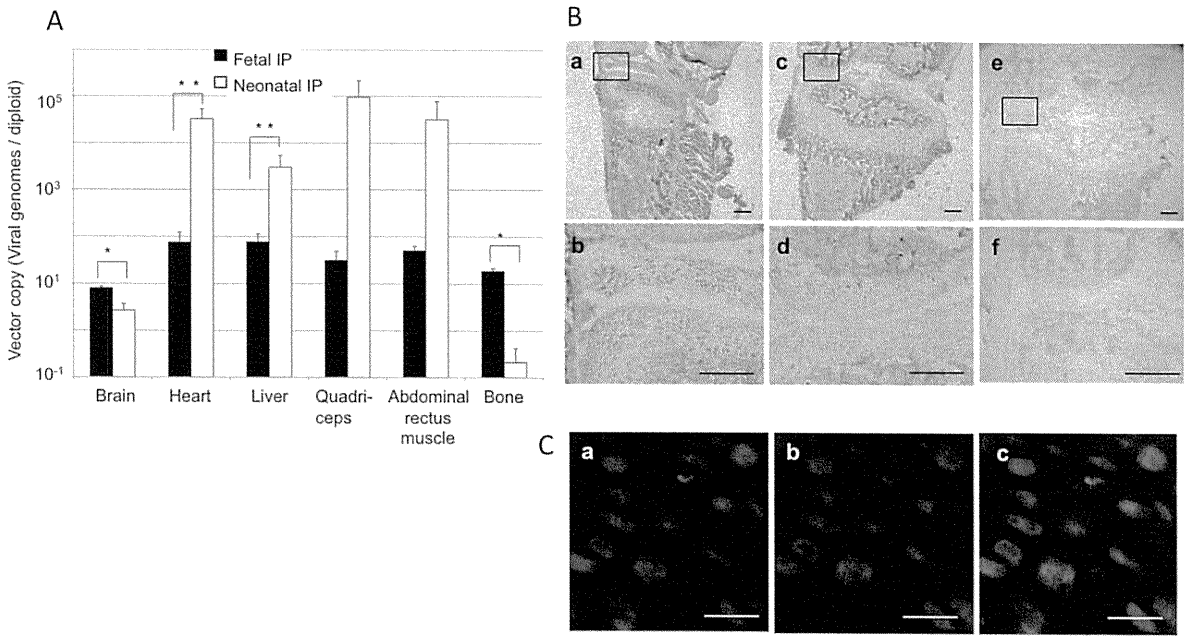
Figure 5. Biodistribution of AAV9-EGFP (real-time PCR). (A) Vector copy number in each tissue of *Akp*$^{+/+}$ mice after fetal (n = 3, black bar) or neonatal (n = 3, white bar) IP injection of AAV9-EGFP (real-time PCR). Data are presented as means and SD. Student's t test was employed for comparisons between the two groups (*$P < 0.01$, **$P < 0.05$). (B) DAB staining of tibial bone after fetal IP injection (a, b) and neonatal IP injection (c, d). Negative controls (e, f) were mice without vector injection. (b), (d), and (f) High-resolution photographs of the frames in (a), (c), and (e), respectively. Bars = 250 μm. (C) Immunostaining of tibial bone with anti-GFP (a), anti-collagen type II (b), and the merge of anti-GFP and anti-collagen type II (c). Bars = 50 μm.











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Rescue of Severe Infantile Hypophosphatasia Mice by AAV-Mediated Sustained Expression of Soluble Alkaline Phosphatase

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Abstract

Hypophosphatasia (HPP) is an inherited disease caused by a deficiency of tissue-nonspecific alkaline phosphatase (TNALP). The major symptom of human HPP is hypomineralization, rickets, or osteomalacia, although the clinical severity is highly variable. The phenotypes of TNALP knockout (*Akp2*$^{-/-}$) mice mimic those of the severe infantile form of HPP. *Akp2*$^{-/-}$ mice appear normal at birth, but they develop growth failure, epileptic seizures, and hypomineralization and die by 20 days of age. Previously, we have shown that the phenotype of *Akp2*$^{-/-}$ mice can be prevented by enzyme replacement of bone-targeted TNALP in which deca-aspartates are linked to the C-terminus of soluble TNALP (TNALP-D10). In the present study, we evaluated the therapeutic effects of adeno-associated virus serotype 8 (AAV8) vectors that express various forms of TNALP, including TNALP-D10, soluble TNALP tagged with the Flag epitopes (TNALP-F), and native glycosylphosphatidylinositol-anchored TNALP (TNALP-N). A single intravenous injection of 5×10^{10} vector genomes of AAV8-TNALP-D10 into *Akp2*$^{-/-}$ mice at day 1 resulted in prolonged survival and phenotypic correction. When AAV8-TNALP-F was injected into neonatal *Akp2*$^{-/-}$ mice, they also survived without epileptic seizures. Interestingly, survival effects were observed in some animals treated with AAV8-TNALP-N. All surviving *Akp2*$^{-/-}$ mice showed a healthy appearance and a normal activity with mature bone mineralization on X-rays. These results suggest that sustained alkaline phosphatase activity in plasma is essential and sufficient for the rescue of *Akp2*$^{-/-}$ mice. AAV8-mediated systemic gene therapy appears to be an effective treatment for the infantile form of human HPP.

Introduction

Hypophosphatasia (HPP) is an inherited systemic skeletal disease characterized by a deficiency in tissue-nonspecific alkaline phosphatase (TNALP), which is attached to the outer cell membrane of many cell types via a glycosylphosphatidylinositol (GPI) anchor (Millán, 2006). The major symptom of human HPP is hypomineralization, rickets, or osteomalacia, although the clinical severity is highly variable and ranges from a lethal perinatal form to mild odontohypophosphatasia that manifests only dental abnormalities (Whyte, 2002; Mornet, 2007). Patients with infantile HPP may appear normal at birth, but they gradually develop rickets before 6 months of age. Pyridoxine-responsive seizures often occur in severe forms of infantile HPP (Mornet, 2007).

Several approaches have been tried in an effort to treat patients with HPP. These treatments include transplantation of bone marrow cells (Whyte *et al.*, 2003; Tadokoro *et al.*, 2009), transplantation of bone fragments and cultured osteoblasts (Cahill *et al.*, 2007), the administration of parathyroid hormone (Whyte *et al.*, 2007; Camacho *et al.*, 2008), enzyme replacement therapy (ERT) with alkaline phosphatase (ALP)-rich serum from patients with Paget's disease of the bone (Whyte *et al.*, 1984), and the infusion of plasma from healthy individuals (Whyte *et al.*, 1986) or ALP purified from liver (Weninger *et al.*, 1989). These treatments have provided limited clinical and radiographic improvements in a few patients.

The phenotypes of TNALP knockout (*Akp2*$^{-/-}$) mice mimic those of severe infantile HPP; therefore, these *Akp2*$^{-/-}$ mice

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have been used as an animal model for HPP (Narisawa *et al.*, 1997). They appear normal at birth, but they rapidly develop growth failure, epileptic seizures, and hypomineralization and die by 20 days of age. We reported that the phenotype of *Akp2*$^{-/-}$ mice could be corrected by daily subcutaneous injections of a bone-targeted form of TNALP, which is created by linking a bone-targeting deca-aspartate sequence to the C-terminus of soluble TNALP (TNALP-D10) (Millán *et al.*, 2008). Based on these data, new clinical trials of ERT for patients with HPP have been initiated (http://clinicaltrials.gov).

Gene therapy has proven to be a useful tool for the treatment of several inherited diseases. Recently, we demonstrated that a single injection of lentiviral vector expressing TNALP-D10 resulted in sustained expression of TNALP and phenotypic correction of *Akp2*$^{-/-}$ mice (Yamamoto *et al.*, 2011). This gene therapy approach is referred to as viral vector–mediated ERT, and it is more practical than classical ERT, which requires repeated injections. One way to develop this approach is *in vivo* gene therapy with adeno-associated virus (AAV) vector. In the present study, we evaluated AAV vectors expressing various forms of TNALP. We found that AAV-mediated expression of soluble TNALP resulted in prolonged survival and the phenotypic correction of *Akp2*$^{-/-}$ mice.

Materials and Methods

Cell culture

The C2C12, HEK 293, and U2OS (human osteosarcoma HTB-96; ATCC, Manassas, VA) cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin under an atmosphere enriched with 5% CO_2.

Plasmid construction and vector production

The plasmids containing cDNA for native GPI-anchored TNALP (TNALP-N) (Goseki-Sone *et al.*, 1998), TNALP-D10 (Yamamoto *et al.*, 2011), and soluble TNALP with the flag epitope at the C-terminus (TNALP-F) (Di Mauro *et al.*, 2002) were used as templates to generate the *Eco*RI-*Not*I fragments of three forms of TNALP cDNA by PCR cloning. Primers were designed to introduce an *Eco*RI or *Not*I recognition site at each end. These cDNA fragments were subcloned into the *Eco*RI and *Not*I sites of pcDNA (Invitrogen, San Diego, CA) AAV vector plasmid, pAAV.CAαGBE containing the CAG promoter and the globin polyadenylation signal (Takahashi *et al.*, 2002). AAV-GFP was used as a control AAV vector (Noro *et al.*, 2004). Recombinant AAV serotype 1 or 8 (AAV1 or AAV8) vectors were generated using the triple transfection method (Salvetti *et al.* 1998) and purified as described previously (Hermens *et al.*, 1999; Kurai *et al.*, 2007). The titer of each AAV vector was determined by real-time PCR (7500 Fast; Applied Biosystems, Tokyo, Japan).

Vector characterization

C2C12 cells were plated on 24-well plates at a density of 2×10^4 cells/well and transduced with three different AAV1-TNALP vectors [1.5×10^{11} vector genomes (vg)/well]. The medium was changed a day after transduction. Ninety-six hours after transduction, the transduced cells and the supernatants were separately collected and assayed for ALP activity.

Enzyme activity and protein assay

ALP activity was determined as previously described (Sogabe *et al.*, 2008). One unit was defined as the amount of enzyme needed to catalyze production of 1 μmol of *p*-nitrophenol per minute. ALP activity in plasma and cell culture supernatants was calculated as units per milliliter. Those of cells and organs were assayed with supernatants of homogenized cells and organs standardized by milligrams of protein. Protein concentration was assayed by the DC protein assay kit (Bio-Rad, Tokyo, Japan).

Mineralization assay

C2C12 cells were plated on six-well plates at a density of 4×10^4 cells/well and transduced with AAV1-TNALP-D10, AAV1-TNALP-F, or AAV1-GFP (control) vector. The medium was changed on the day after transduction. Seventy-two hours later, the cells were approximately 90–100% confluent, and the supernatants were collected for use in the mineralization assays. U2OS cells were plated on 96-well plates at a density of 1×10^4 cells/well and cultured for 48 hr to reach confluency. Their medium was then replaced with the conditioned medium from the C2C12 transductants. At the same time, 10 m*M* β-glycerophosphate (Sigma-Aldrich, St. Louis, MO) was added to verify the tendency for calcification. Five days after the medium change, cell calcification was evaluated as described previously (Johnson *et al.*, 2001; Orimo *et al.*, 2008).

Binding assay

ALP binding to hydroxyapatite (Sangi, Tokyo, Japan) was assayed as described previously with some modification (Nishioka *et al.*, 2006). Aliquots of each supernatant, adjusted based on ALP activity to 0.1 U/ml, were added to hydroxyapatite suspended in 25 m*M* Tris-buffered saline (TBS; 0.6 mg/500 μl) and incubated for 30 min at 37°C. Then the mixture was centrifuged to separate the unbound and bound enzyme. The difference in ALP activity was assayed before and after binding to hydroxyapatite. The percentage binding of the ALP activity was calculated as follows: % binding = [(ALP activity before binding) − (ALP activity after binding)]/(ALP activity before binding) $\times$100.

Inorganic pyrophosphate (PPi) assay

PPi of plasma was assayed as described previously (Lust and Seegmiller, 1976). Initially, protein in plasma was removed by centrifugation at 14,000 *g* for 25 min at 4°C with a Microcon-30 (Millipore, Bedford, MA). Thereafter, aliquots of the resultant solution were added to the wells of an OptiPlate-96 F black plate (PerkinElmer, Waltham, MA), and the fluorescent signals were read using a microplate reader (ARVO-MX 1420 multilabel counter; PerkinElmer) at 460 nm. Each sample was analyzed in triplicate.

Animal experiments

The generation and characterization of the *Akp2*$^{-/-}$ mice were described previously (Narisawa *et al.*, 1997). Genotyping was performed by PCR with primers 5′-AGTCCGTGG GCATTGTGACTA-3′ and 5′-TGCTGCTCCACTCACGTC GAT-3′. All animal experiments were performed according

to protocols approved by the Nippon Medical School Animal Ethics Committee. A 29-gauge insulin syringe was used to inject AAV8-TNALP and control AAV8-GFP vectors into the external jugular veins of 1-day-old mice (Ogawa *et al.*, 2009). Blood samples were collected from cut tails using heparinized capillaries on day 10 after birth and from the orbital sinus of anesthetized animals after day 14. We checked physical activity and healthy appearance together with the seizures in untreated ($n=9$), control AAV8-GFP–injected ($n=4$), and treated ($n=32$) mice every morning for about an hour per day for at least 1 month. During the observation periods, the behaviors as described on the Racine scale (Racine, 1972) were counted as convulsions. At 56 days of age, treated mice were sacrificed under deep anesthesia by perfusion with 20 ml of PBS containing 10 U/ml heparin, followed by an additional 20 ml of PBS to wash out the blood. Then the organs were harvested.

X-ray analysis

X-ray analysis was performed as previously described (Yamamoto *et al.*, 2011). In brief, radiographic images were obtained on μFX1000 film (Fujifilm Corp., Tokyo, Japan) that was set up for the analysis of small animals at an energy level of 25 kV and an exposure time of 90 sec for 10-day-old mice and 10 sec for 56-day-old mice.

Bone mineral density (BMD)

After skin and muscle were removed, the whole bones were stored in 75% ethanol until analysis. The right femur was dislocated and an X-ray image was taken to an IP plate using μFX1000 (Fujifilm Corp.), which is set up for the analysis of small animals. Each scan included a phantom (Kyoto Kagaku, Kyoto, Japan). This phantom contained six densities, from 0 to 100 mg/cm^2 by 20 mg/cm^2 K_2HPO_4; it was ordered to fit adult mice BMD. The BMD of each image was obtained (Typhoon FLA 7000; GE Healthcare Japan, Tokyo, Japan) with a personal computer and analyzed with Multi Gauge ver. 3.0 software (Fujifilm Corp.). BMD values were expressed in milligrams per square centimeter of K_2HPO_4. The region of interest for analysis was the whole femur.

Biodistribution of AAV vector

Genomic DNA was extracted from seven main organs (heart, lung, liver, spleen, kidney, muscle, bone) of AAV8-TNALP-D10–injected mice by using QIAamp DNA mini kit (QIAGEN, Valencia, CA). The DNA was subjected to real-time PCR to detect the copy number of AAV vector. In short, genome copy titers were quantified by TaqMan polymerase chain reaction (Applied Biosystems) using primers (forward: 5′-CGTCAATGGGTGGAGTATTTA-3′; reverse: 5′-AGGTCA TGTACTGGGCATAATGC-3′) and a probe designed against the cytomegalovirus primer. Genomic DNA spiked with AAV vector plasmid DNA was used as a standard, and average copy number per diploid was determined.

Histological examination

The bone was directly stained without fixation and decalcification. The knee joints were removed and embedded in SCEM compound (Leica Microsystems, Tokyo, Japan) without fixation. Sections (10 μm thick) were cut with the Kawamoto film method (Leica Microsystems) and washed with ethanol followed by dH_2O. ALP activity was assayed by incubating the tissue with 0.1 mg/ml naphthol AS-MX phosphate as a substrate and 0.6 mg/ml fast blue salt as dye in 20 ml of 0.1 *M* Tris-HCl buffer (pH 8.5) for approximately 15 min at 37°C, as described previously (Li *et al.*, 2007). The tissue sections were then mounted on silane-coated slides (Muto Pure Chemicals, Ltd., Tokyo, Japan) and examined under a light microscope (BX60; Olympus Ltd., Tokyo, Japan) (Sogabe *et al.*, 2008).

Statistical analysis

Data from *in vivo* and *in vitro* experiments are expressed as means ± SD. Differences between groups were tested for statistical significance using Student's *t* test. *P* values less than 0.05 were considered statistically significant. The survival rate was analyzed by the Kaplan–Meier method, and differences in the survival rates were assessed by the Wilcoxon test.

Results

Characterization of soluble TNALP in vitro

We generated AAV1 vectors expressing various forms of TNALP (Fig. 1A). After transduction of C2C12 cells with these vectors, the distribution of TNALP was examined. ALP activities from AAV1 vector containing TNALP-N were detected mainly in the cell lysate fraction (90.4 ± 1.4%), whereas the activities from AAV1 vectors with TNALP-F and TNALP-D10 were recovered only in the supernatant fraction (96.4 ± 0.4% and 96.6 ± 1.3%) ($n=4$ each). These results suggest that most of TNALP-N is anchored to the cell membrane like endogenous TNALP, and only a small portion (less than 10%) is solubilized by enzymatic cleavage. In contrast, TNALP-F and TNALP-D10 are synthesized as soluble enzymes. These soluble forms of TNALP were further characterized by an *in vitro* mineralization assay and binding assay.

In vitro *mineralization and affinity for hydroxyapatite*

To analyze the function and specificity of soluble forms of TNALP, we assessed its ability to mediate mineralization and to bind hydroxyapatite. Osteoblastic U2OS cells incubated with conditioned medium containing TNALP-F or TNALP-D10 were strongly stained by alizarin red S (0.83 ± 0.28 and 0.80 ± 0.49 U/mg protein vs. 0.26 ± 0.20 U/mg protein in mock control) (Fig. 1B). This strong staining indicates significantly accelerated mineralization.

The affinity of TNALP-F for hydroxyapatite mineral was studied by the *in vitro* binding assay. TNALP-D10 showed a stronger affinity to hydroxyapatite (88.3 ± 0.5 % bound) as compared with TNALP-F (13.9 ± 2.0 % bound), confirming the binding ability of deca-aspartate to hydroxyapatite described previously (Millán *et al.*, 2008) (Fig. 1C). The flag epitope has a stretch of four aspartates (Fig. 1A), but TNALP-F did not show any significant affinity to hydroxyapatite.

Sustained expression of TNALP-D10 and a prosurvival effect were detected in AAV8-TNALP-D10–treated mice

First, we evaluated the therapeutic efficacy of AAV-mediated expression of TNALP-D10 in *Akp2*$^{-/-}$ mice. We used