injection of either lentiviral or adeno-associated viral (AAV) vector expressing TNALP-D10 into postnatal HPP mice resulted in prolonged seizure-free survival and phenotypic correction (Yamamoto *et al.*, 2010; Matsumoto *et al.*, in press). This gene therapy approach is referred to as viral vector-mediated ERT, and it is more practical than classical ERT that requires repeated injections. Neonatal gene therapy may be an important option for treatment of severe HPP.

Due to the remarkable progress of prenatal diagnosis with clinical imaging, including echography and computed tomography, as well as molecular testing, the chance of diagnosis of perinatal lethal HPP during the fetal period is increasing. Fetal gene therapy may be the only choice options of treatment for perinatal HPP and severe infantile HPP in the future. In the present study, we evaluated the feasibility of gene therapy during the fetal period. Here, we report that systemic injection of AAV vector *in utero* is an effective strategy for treatment of perinatal orearly-onset lethal genetic diseases.

Materials and Methods

Construction and preparation of recombinant adeno-associated viral vector

The AAV vector plasmid containing cDNA for TNALP-D10 was described previously (Matsumoto *et al.*, in press). Recombinant AAV serotype 9 (AAV9) vector was generated by the triple transfection method (Salvetti *et al.*, 1998) and purified as described previously (Hermens *et al.*, 1999; Miyake *et al.*, 2011). The titer of each AAV vector was determined by real-time PCR (7500 Fast; Applied Biosystems, Tokyo, Japan). AAV9-EGFP (Miyake *et al.*, 2011) was used to analyze the biodistribution of the vector.

Animal procedures and vector injection

All animal procedures were performed in accordance with the guidelines approved by the Nippon Medical School Animal Ethics Committee. *Akp2*$^{-/-}$ mice were obtained by mating *Akp2*$^{+/-}$ mice with a mixed genetic background of 129J and C57Bl/6J, which were generated by the Millán laboratory (Fedde *et al.*, 1999; Narisawa *et al.*, 1997). AAV9 vector was intraperitoneally injected into fetal or postnatal mice. For fetal vector injection, pregnant dams on day 15 of gestation were anesthetized by intramuscular injection of 0.6 mg of pentobarbital in 100 μl of PBS and inhalation of isoflurane. A midline laparotomy was performed and the uterus was exposed. Vector (8.3×10^{10} viral genomes [vg] of AAV9-TNALP-D10 or AAV9-EGFP/body weight [g]) was injected transuterine, intraperitoneally into each fetus using a 33-gauge Ito syringe (Ito Corporation, Shizuoka, Japan). The amount of vector for fetal injection was 1.0×10^{11} vg/10 μl/body. After the

injections, the peritoneum was filled with 300 μl of PBS and the abdominal muscle layer and the skin layer were closed with 5-0 nylon sutures. After normal delivery, the newborn mice stayed for 1 month with the dam. The genotype of treated fetuses was determined after delivery. For neonatal injection, 2.0×10^{11} vg/20 μl/body was intraperitoneally injected into the mice on postnatal day 1.

ALP activity in plasma and organs

Blood samples were collected from the orbital sinus using heparinized capillaries on days 14, 28, and 56 after birth. After plasma separation, ALP activity in the plasma was quantified using a colorimetric assay for ALP as described previously (Goseki *et al.*,1988). ALP activity was described in units (U) defined as the amount of enzyme needed to catalyze production of 1 μmol p-nitrophenol formed per minute and calculated as U/ml.

Brain, heart, liver, quadriceps, abdominal rectus muscle, and tibial bone were dissected out under deep anesthesia following perfusion with 20 ml of PBS containing 10 U/ml of heparin followed by 20 ml of PBS. Bone marrow and soft tissues were carefully removed from the isolated bone. Each organ was homogenized with 500 μl of dH_2O using a Percellys 24 bead-beating homogenizer (Bertin Technologies, Paris, France) and centrifuged at 14000 × *g* for 5 min. ALP activity of the supernatant was analyzed as described above and standardized by 1 mg of protein. Protein concentration was determined using a DC protein assay kit (Bio-Rad, Tokyo, Japan).

Histochemical examination of bone

Knee joints were removed under deep anesthesia following perfusion with 20 ml of PBS containing 10 U/ml of heparin and 20 ml of PBS, followed by embedding in SCEM compound (Leica Microsystems, Tokyo, Japan) and freezing, without fixation or decalcification. Sections (14 μm thick) were cut with the Kawamoto film method (Leica Microsystems) and washed with 99.5% ethanol and 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 BB salt as dye in 20 ml of 0.1M Tris-HCl buffer (pH 8.5) for 15 min at 37°C, as described previously (Sugiyama *et al.*, 2003). After mounting on silane-coated slides (Muto Pure Chemicals Ltd., Tokyo, Japan), they were examined under a light microscope (BX 60; Olympus Ltd., Tokyo, Japan).

For immunostaining, sections (10 μm thick) of the knee joint were cut with the Kawamoto film method (Leica Microsystems) and fixed with 10% formarine. The sections were incubated with 1% skim milk in PBS for 60 minutes (blocking). After the blocking, the sections were incubated with primary antibodies for 60 minutes at room temperature followed by 48 hours at 4°C. The primary antibodies were rabbit anti-collagen II (1:100; Abcam, Cambridge, UK) and mouse anti-GFP (1:400; Invitrogen, OR). After washing with PBS, they were incubated with secondary antibodies, which were goat anti-rabbit IgG conjugated with alexa 568 (1:500; Invitrogen) and donkey anti-mouse IgG conjugated with alexa 488 (1:500; Invitrogen), at room temperature for 2 hours. After mounting on silane-coated slides (Muto Pure Chemicals Ltd.), they were examined with fluorescence microscopy (BX60; Olympus Ltd.).

X-ray analysis

Radiographic images were obtained using μFX-1000 (Fujifilm, Tokyo, Japan) with energy of 25 kV and exposure time of 10 s, and imaged with FLA-7000 (Fujifilm).

Biodistribution of AAV vector

Biodistribution of AAV9 vector was determined by real-time PCR of genomic DNA of *Akp2*$^{+/+}$ mice after intraperitoneal (IP) injection of AAV9-EGFP. Heart, liver, quadriceps, abdominal rectus muscle, tibial bone, brain, and gonads were removed under deep anesthesia following perfusion with 20 ml of PBS containing 10 U/ml of heparin followed by 20 ml of PBS at 14 days of age. Each tissue of the mice was homogenized using a Percellys 24 bead-beating homogenizer (Bertin Technologies) followed by DNA extraction using a Gentra Puregene kit (Qiagen Sciences, Germantown, MD). Real-time PCR was performed using primers designed to amplify part of the CMV promoter of AAV9 (sense, 5'-GACGTCAATAATGACGTATG-3'; antisense, 5'-GGTAATAGCGATGACTAATACG-3'). Reaction was carried out with 100 ng of template, 0.2 μmol/l of each primer, SYBR Premix Ex Taq (Perfect Real Time; Takara, Tokyo, Japan) and Rox Reference Dye II (Takara). The amplification conditions were 95°C for 10 s, followed by 40 cycles of 95°C for 15 s and 60°C for 34 s. Dissociation was performed at 95°C for 15 s, 60°C for 1 min, and 95 °C for 15 s. GFP expression in the tissues of the mice treated with AAV9-EGFP was visualized by immunohistochemical staining using anti-EGFP antibody (MBL, Aichi, Japan) and the avidin/biotinylated enzyme complex method using diaminobenzidine dihydrochloride (DAB) as described previously (Iwamoto *et al.*, 2009).

Statistical analyses

Differences between groups were tested for statistical significance using Student's *t* test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. The survival rate was analyzed by the Kaplan–Meier method, and differences in survival rate were assessed by the Wilcoxon's test.

Results

Fetal injection of AAV9-TNALP-D10 prolonged survival and improved development of Akp2$^{-/-}$ mice

Fetal mice were obtained by mating *Akp2*$^{+/-}$ heterozygous mice, which are healthy and have a normal lifespan. AAV9-TNALP-D10 (1×10^{11} viral genomes/10 μl) was injected intraperitoneally into a total of 88 fetuses on embryonic day 15 from 11 pregnant dams. Among the treated mice, 47 (53%) were live-born, while 41 (47%) did not live longer than half a day because they were eaten by the dam. Genotyping of live neonates showed that 9 of 47 were *Akp2*$^{-/-}$. Non-treated *Akp2*$^{-/-}$ mice were born with a normal appearance but showed growth failure with epileptic seizures and did not live longer than 11 days. In contrast, the weight and growth rate of treated *Akp2*$^{-/-}$ mice (7/9) were indistinguishable from those of wild-type littermates (17.1 ± 1.4 g [n = 3] vs. 16.9 ± 1.9 g [n = 3] on day 28 [Fig. 1A], 19.3 ± 2.8 g [n = 3] vs. 21.7 ± 1.7 g [n = 3] on day 56) and the lifespan was significantly extended up to at least 56 days (the time of sacrifice for analysis) (Fig. 1B). Seizures were not observed in these surviving mice throughout the experimental period. Two of the *Akp2*$^{-/-}$ fetuses failed to respond to treatment. They showed severe weight loss and convulsions, and died on postnatal day 21.

ALP activities were elevated in the fetal treated mice

To measure the levels of TNALP-D10 expression in the bloodstream and in the tissues after fetal gene therapy, ALP activity was analyzed by colorimetric assay. The plasma

ALP activities of the *in utero*-treated mice were markedly increased and significantly remained more than 10 times higher than those in wild-type mice ($Akp2^{+/+}$) during the observation period (9.3 ± 2.5 U/ml vs. 0.15 ± 0.085 U/ml [$n = 3$] on day 14, $P < 0.01$, 3.7 ± 2.0 U/ml vs. 0.13 ± 0.037 U/ml [$n = 3$] on day 28, $P < 0.05$, and 3.5 ± 1.5 U/ml vs. 0.065 ± 0.020 U/ml [$n = 3$] on day 56, $P < 0.05$) (Fig. 2A). Despite the super-physiologically high levels of ALP activity, no gross deformities of the bones or abnormal calcification were observed on X-ray images (data not shown).

ALP activities in the tissues of treated $Akp2^{-/-}$ mice were measured 14 days after birth (20 days after vector injection). ALP activity was elevated in heart, quadriceps, abdominal rectus muscle, and surprisingly in the bone (Fig. 2B), which was not observed in our previous study with neonatal injection of AAV vector.

Elevated ALP activities were detected in the bone after fetal gene therapy

The cartilage zone of the proximal tibia was analyzed histochemically for ALP activity using the azo-dye technique. In the epiphysis of $Akp2^{+/+}$ mice, strong ALP activity was observed in the hypertrophic chondrocytes and on the surface of the endosteal bones on days 14 (Fig. 3A) and 56 (Fig. 3B). The ALP staining in the bone of $Akp2^{+/+}$ mice appeared to be strengthened with age. In the corresponding area of the tibial bone of untreated $Akp^{-/-}$ mice on day 10, growth of the trabecula was significantly inhibited and no ALP signal was detected (Fig. 3C). After fetal gene therapy, strong ALP signals were detected mainly on the surface of the endosteal bones on day 14, although the density of the trabecula is still less than that of

Akp2$^{+/+}$ *mice*(Fig. 3D). Faint scattered ALP signals were also observed in the cartilage zone of fetal treated *Akp2*$^{-/-}$ mice on day 56 (Fig. 3E).

X-ray images revealed the skeletal phenotypic correction after fetal gene therapy

Mineralization of fetal treated *Akp2*$^{-/-}$ mice was evaluated by X-ray examination. The radiographic changes in *Akp2*$^{-/-}$ mice became apparent during the first 7 to 10 days of life, although the severity of the mineralization defects was highly variable. X-ray images of the most severely affected cases on day 10 showed that a lack of secondary ossification centers and reduced numbers of carpal bones compared to *Akp2*$^{+/+}$ mice, indicating ossification incompetence in HPP mice. In contrast, apparent ossification centers were detected in all treated mice on day 10 (n = 3, Fig. 4A). No differences in skeletal structure or mineralization, including the size of the thoracic cage, were observed between *Akp2*$^{+/+}$ and treated *Akp2*$^{-/-}$ mice on day 56 (n = 3, Fig. 4B).

Chondrocytes were transduced by fetal injection of AAV9

To confirm whether the elevated ALP activity in the tissues (especially bones) was derived from other tissues via the bloodstream or was expressed in the original tissues, the biodistribution of AAV9 vector was determined using real-time PCR on genomic DNA. AAV9-EGFP, which does not have special affinity to the bone, was used to confirm the vector biodistribution. DNA was isolated from each tissue of the *Akp2*$^{+/+}$ mice treated by fetal or neonatal IP injection of AAV9-EGFP. After neonatal IP injection, high copy numbers of AAV9 were observed in the heart and liver, whereas transduction into the bone was very low. This

distribution pattern was similar to that obtained after neonatal intravenous (IV) injection of AAV8 vector (Matsumoto *et al.*, in press). On the other hand, after fetal IP injection, AAV9 vector was widely distributed to all organs examined except the gonads. Importantly, AAV sequence was significantly elevated in brain and bone compared with neonatal IP injection (Fig. 5A, $P < 0.01$). No vector sequence was detected in the gonads of the pups (data not shown). AAV9 mediated transduction of the bone tissue *in utero* was confirmed by histochemical staining (Fig. 5B, 5C). EGFP expression detected by DAB staining was observed especially in chondrocytes in the growth cartilage area. The double-positive cells with EGFP and collagen II of the treated mice confirm that These data suggest that chondrocytes are susceptible to AAV9 transduction at least during the fetal period.

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.

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