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Enzyme replacement therapy on hypophosphatasia mouse model

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Abstract

Hypophosphatasia (HPP) is an inborn error of metabolism caused by deficiency of the tissue-nonspecific alkaline phosphatase (TNSALP), resulting in a defect of bone mineralization. Natural substrates for this ectoenzyme accumulate extracellularly including inorganic pyrophosphate (PPi), an inhibitor of mineralization, and pyridoxal 5-phosphate (PLP), a co-factor form of vitamin B6.

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Dr. Tomatsu, a principal investigator, was the former employee of Saint Louis University, while the experiments of the project have been conducted and completed.

Compliance with Ethics

Conflict of Interest:

All the authors contributed to "Original Article" have no conflict interest with any other party.

Hirotaoka Oikawa, Shunji Tomatsu, Bisong Haupt, Adriana M. Montaño, Tsutomu Shimada, and William S. Sly declare that they have no conflict of interest.

Informed Consent:

Not applicable for this manuscript.

This article does not contain any studies with human subjects performed by the any of the authors.

Animal Rights:

All institutional and national guidelines for the care and use of laboratory animals were followed.

Contribution:

Hirotaoka Oikawa: He has contributed to the planning, conduct of mouse experiments, and reporting of the work described in the article.

Shunji Tomatsu: He has contributed to the planning, conduct of the whole experiments, and reporting of the work described in the article.

Bisong Haupt: She has contributed to the planning, conduct of pathological studies, and reporting of the work described in the article.

Adriana M. Montaño: She has contributed to the planning, conduct of pathological studies, and reporting of the work described in the article.

Tsutomu Shimada: He has contributed to conduct data and statistical analyses and reporting of the work described in the article.

William S. Sly: He has contributed to the planning and reporting of the work described in the article.

Enzyme replacement therapy (ERT) for HPP by functional TNSALP is one of the therapeutic options. The C-terminal-anchorless human recombinant TNSALP derived from Chinese hamster ovary cell lines was purified. TNSALP-null mice (*Akp2*^{-/-}), an infantile model of HPP, were treated from birth using TNSALP and vitamin B6 diet. Long-term efficacy studies of ERT consisted of every 3 days subcutaneous or intravenous injections till 28 days old (dose 20 U/g) and subsequently every 3 days intravenous injections for 6 months (dose 10 U/g). We assessed therapeutic effect by growth and survival rates, fertility, skeletal manifestations, and radiographic and pathological finding.

Treated *Akp2*^{-/-} mice grew normally till 4 weeks and appeared well with a minimum skeletal abnormality as well as absence of epilepsy, compared with untreated mice which died by 3 weeks old. The prognosis of TNSALP-treated *Akp2*^{-/-} mice was improved substantially: 1) prolonged life span over 6 months, 2) improvement of the growth, and 3) normal fertility. After 6 months of treatment, we found moderate hypomineralization with abnormal proliferative chondrocytes in growth plate and articular cartilage.

In conclusion, ERT with human native TNSALP improves substantial clinical manifestations in *Akp2*^{-/-} mice, suggesting that ERT with anchorless TNSALP is also a potential therapy for HPP.

Keywords

hypophosphatasia; severity tissue-nonspecific alkaline phosphatase; enzyme replacement therapy; bone mineralization

Introduction

Hypophosphatasia (HPP) is a rare, heritable skeletal disorder, resulting from a deficiency of tissue nonspecific alkaline phosphatase (TNSALP) abundant in bone and teeth. The presentation of HPP is remarkably varied even within groups of patients diagnosed with the same clinical form (Whyte 2001). Perinatal HPP represents the most severe form of the disease, with almost no mineralization in the skeleton and death *in utero* or early after birth. Seizures have also been reported in patients with an infantile form (Baumgartner et al 2007, Whyte et al 1994, Whyte et al 1988). The most severe forms, perinatal and infantile HPP, arise from homozygosity or compound heterozygosity for null mutations, characterized by severe skeletal hypomineralization that leads to deformities of the limbs, skull, and ribcage. Most cases of perinatal HPP result in stillbirth or postnatal lethality, whereas the prognosis for an infantile form is unpredictable. When first diagnosed as an infantile form, around 50% of the patients die within the next several months; however, others can show remarkable spontaneous improvement over the ensuing months or years.

Two TNSALP knock-out mouse models (*Akp2*^{-/-}) were developed in two independent laboratories (Waymire et al 1995, Narisawa et al 1997). The mutant alleles of TNSALP have been confirmed to be null for alkaline phosphatase (ALP; EC. 3.1.3.1) activity in both mouse models. *Akp2*^{-/-} mutant mice complete embryogenesis and are born without any anatomical or radiographic abnormality (Waymire et al 1995, MacGregor et al 1995, Narisawa et al 1997). They appear normal for the first few days of life and are

indistinguishable from their littermate controls ($Akp2^{+/+}$ or $^{+/-}$). At approximately 6-8 days postnatally, the $Akp2^{-/-}$ mutant mice manifest growth impairment, display spontaneous seizures and apnoea, and die at about 10-14 days of age. Approximately 50% of the $Akp2^{-/-}$ mice display impaired mineralization of bone at the time of death (Narisawa et al 1997). $Akp2^{-/-}$ mice supplemented with exogenous pyridoxal phosphate lived longer than untreated mice, but their skeletal preparations showed no improvement compared with the untreated HPP mice. These skeletal problems worsened with their increasing life span, showing progressive hypomineralization with time (Waymire et al 1995, Narisawa et al 1997, Narisawa et al 2001).

Since HPP is caused by a deficiency of a single enzyme, TNSALP, this disorder is potentially amenable to enzyme replacement therapy (ERT) like lysosomal storage disorders (LSDs). Treating LSDs with ERT has been successful experimentally and clinically. ERTs have been approved for use in patients with Gaucher disease, Fabry disease, mucopolysaccharidosis I (MPS I), MPS II and MPS VI (Burrow et al 2007). Patients treated with ERT had clinical improvement of somatic manifestations and improved quality of life. However, the results of ERT with intravenous infusion of plasma ALP or purified liver ALP in patients with HPP have been disappointing (Whyte et al 1982). Continuous delivery of high doses of TNSALP to bone would be needed to induce physiological bone mineralization. Human TNSALP was bioengineered with the C terminus extended by the Fc region of human IgG for one-step purification and a deca-aspartate sequence (D10) for targeting to mineralizing tissue (sALP-FcD10). $Akp2^{-/-}$ mice were treated from birth using sALP-FcD10 and pyridoxal phosphate (Millan et al 2008). Efficacy studies consisted of daily subcutaneous injections with 1, 2, or 8.2 mg/kg sALP-FcD10 for 15, 19, and 15 or 52 days, respectively. $Akp2^{-/-}$ mice receiving high-dose sALP-FcD10 grew normally and no evidence of significant skeletal or dental disease was found till 52 days, suggesting that ERT using this recombinant form of human TNSALP prevents infantile HPP in $Akp2^{-/-}$ mice. The clear, positive correlation between dose and prevention of mineralization defects of bones was confirmed. There was also a positive relationship between dose and survival (Yadav MC et al 2011). ERT with sALP-FcD10 enzyme also proved improvement on skeletal radiographs and pulmonary and physical function in infants and young children with life-threatening HPP (Whyte et al 2012, Rodriguez et al 2012).

While sALP-FcD10 enzyme was confirmed effective, these reports did not describe any comparison with untagged native TNSALP enzyme, histopathology in long bones and a long-term efficacy of treatment. Thus, whether ERT by untagged native TNSALP is effective or not remains unanswered.

In this article, we have evaluated therapeutic efficacy by treating $Akp2^{-/-}$ mice for a long-term (6 months) with C-terminal-anchorless human native TNSALP.

Materials and Methods

Mouse model of infantile HPP

Heterozygous $Akp2^{+/-}$ mice (129S7- $Akp2^{tm1Sor}/J$) were purchased from Jackson Laboratories (Bar Harbor, ME) (Waymire et al 1995). Homozygous mice were generated on

the background of hybrid B6/129 heterozygous breeding pairs. All experiments were conducted with the highest standards of humane animal care approved by the local committee at Saint Louis University.

Enzyme purification

TNSALP enzyme was purified by a 2-step column procedure as described previously (Nishioka et al 2006). Briefly, the medium containing the enzyme was filtered through a 0.2 μ m filter, purified by columns of DEAE-Sepharose (Sigma, St. Louis, MO) and Sephacryl S-400-HR (Sigma). The active eluted fractions were pooled and dialyzed against Tris buffer containing 0.1 M NaCl by using ultrafiltration YM-30 filter (Millipore, Billerica, MA). The dialyzed fractions were then concentrated and stored at -80°C until use.

Measurement of ALP activity

A 50 μ l of volume of sample was combined with 250 μ l of 10 mM *p*-nitrophenyl phosphate (*p*NPP) (Sigma) as a substrate in 1 M diethanolamine, pH 9.8, containing 1 mM magnesium chloride, 0.02 mM zinc chloride, and incubated at 37°C . The time-dependent increase in absorbance at 405 nm was measured on a plate spectrophotometer (EL800, Bio-Tek Instrument, Inc., Winooski, VT). One unit of activity was defined as the quantity of enzyme that catalyzed the hydrolysis of 1 μ mol substrate in 1 min (Nishioka et al 2006).

Enzyme replacement therapy

Akp2^{-/-} mice were infused with purified TNSALP as follows: day 1 newborn *Akp2*^{-/-} mice were infused IC with 10 U/g TNSALP, and from day 3 to day 28, *Akp2*^{-/-} mice were infused SC with a dose of 20 U/g. After day 31, *Akp2*^{-/-} mice were treated IV with 10 U/g of TNSALP for 6 months every 3 days. The mice were fed 325 ppm of pyridoxine supplemental diet (Harlan Teklad, Madison, WI).

One week after the last infusion, tissues from TNSALP-treated and wild-type mice were perfused at necropsy with PBS, fixed in 10% formalin and embedded in paraffin. For evaluation of bone pathology by light microscopy, hematoxylin and eosin (HE) and toluidine blue (TB)-stained 4.0- μ m-thick sections of knee joints were assessed without knowledge of treatment group.

Fertile test

Over 8-week-old *Akp2*^{-/-} male and female mice treated by ERT in treatment protocol 2 were bred to evaluate the ability of fertility. After the newborn pups from *Akp2*^{-/-} cross mating, those pups were tested by to check the genotyping. The following primers were used: 5'-AGGGGGATGTGCAAGGCGATT-3', 5'-CTGGCACAAAAGAGTTGGTAAGGC-3', and 5'-GATCGGAACGTCAATTAACGTCAA-3' (wild-type allele: 160 bp, mutant allele: 195 bp).

Pharmacokinetic study by using 6-month-old *Akp2*^{-/-} mice

Six-month-old *Akp2*^{-/-} mice treated and wild-type were infused with 10 U/g body weight for pharmacokinetic study. TNSALP enzyme was infused intravenously (IV) and the blood samples were collected to measure the level of ALP activity with time course (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h). Consequently, pharmacokinetic parameters were obtained as described above.

X-ray examination

One-month and 6-month-old mice were euthanized using CO₂. Plain radiograms of the wild-type, heterozygous, and homozygous mice were taken. Measurements for humerus, ulna, femur and tibia were made using plain radiographs of the bones of each mouse. The mean length measurement and standard deviation were calculated.

Micro-CT analysis

A micro-CT scan was performed on bones using a Scanco μ CT40 system (Scanco Medical; Brüttisellen, Switzerland) according to manufacturer's instructions. Scans were focused on the knee joints and toes. A three dimensional reconstruction of each bone was made and bone mineral density (BMD) was calculated using the results of the micro-CT scan. The mean and standard deviation of the BMD for *Akp2*^{-/-} and wild-type mice were calculated and compared using unpaired Student's *t*-test.

Results

Enzyme replacement Therapy

Akp2^{-/-} mice were infused IV with 10 U/g body weight of TNSALP every 3 days (16 mg/kg body weight per 4 days). The life span of *Akp2*^{-/-} mice was markedly prolonged over 6 months (Fig. 1). Treated *Akp2*^{-/-} mice were grown up similar with wild-type and heterozygous mice until 1 month old. Difference in body weight between treated and wild-type mice were less than 5 g until 2.5 months of age. At age of 3 months, treated mice did not increase body weight, therefore, discrepancy of the body weight between treated *Akp2*^{-/-} mice and wild-type mice was increased. No adipose tissue was observed in treated *Akp2*^{-/-} mice at autopsy. These results suggest that this treatment protocol markedly prolonged life span of *Akp2*^{-/-} mice and that the body weight difference was caused mainly by absence of fat tissues. Absence of fat could come from the less diet volume, compared with the wild-type mice.

Morphological analysis

We characterized the therapeutic efficacy by using clinical observation and X-ray pictures of treated *Akp2*^{-/-} and wild-type mice. The treated *Akp2*^{-/-} mice showed normal length until 1 month old and normal teeth morphology without malocclusion (data not shown). X-ray picture of the whole body from treated *Akp2*^{-/-} mice showed hypomineralization to some extent compared with that of wild-type mice (data not shown). Hypomineralization was not marked in limbs and the bone lengths of any limb of treated *Akp2*^{-/-} mice were not shown significantly different compared with those of wild-type mice (data not shown). Thus, bone

growth of mice treated by new regimen was almost normal until 1 month of age. However, likewise the body weight curve, the body length of *Akp2*^{-/-} mice treated for 6 months became shorter than that of the age-matched wild-type mice (Fig. 2A), and moreover, X-ray picture of the treated *Akp2*^{-/-} mice showed appreciable bone dysgenesis in rib bone and the knee joint sections (Figs. 2b, m, n). Moderate hypomineralization was also observed in treated *Akp2*^{-/-} mice (Figs. 2b, g-n). Treated *Akp2*^{-/-} mice also showed malocclusion on incisor teeth (Fig. 2c-f). Bone length from each limb showed that humerus, ulna, radius, femur, and tibia of the treated *Akp2*^{-/-} mice were significantly shorter than those of wild-type mice (Fig. 2o), although mice could still walk without waddling gate.

Micro-CT Findings

Micro-CT analysis of the bones of the knee joint and hind limb in 2-3 week-old untreated *Akp2*^{-/-} mice already showed less ossification (lower BMD) compared with the age-matched wild-type mice (data not shown). At 1 month of age, bones in the knee joints of treated *Akp2*^{-/-} mice had slightly less ossified bone than those in age-matched wild-type mice (data not shown). Micro-CT examination of bones in the knee joint of *Akp2*^{-/-} mice treated for 6 months showed hypomineralization compared with wild-type mice. Bone dysgenesis was obvious (Fig. 3). Micro-CT scans of the hind limbs of 2-week-old untreated *Akp2*^{-/-} mice showed severe skeletal defects, including disappearance of secondary ossification centers (data not shown), whereas the skeleton of the hind limbs appeared normal with ERT at age of 6 months (Fig. 3).

Three-week-old untreated *Akp2*^{-/-} mice had a lower BMD (208.2 ± 10.0 mgHA/mL, $n = 4$) than comparable age-matched wild-type mice (263.5 ± 14.4 mgHA/mL, $p < 0.001$), consistent with the finding of less ossification on micro-CT images. At 1 month of age, BMD in treated mice was 247.3 ± 4.7 mgHA/mL ($n = 4$). At 6 month of age, *Akp2*^{-/-} mice had a significant decreased BMD (258.0 ± 8.5 mgHA/mL, $n = 4$), compared with that in wild-type mice (605.6 ± 32.1 mgHA/mL, $n = 4$, $p < 0.001$).

Fertility

We investigated the fertility of treated *Akp2*^{-/-} mice by crossing homozygous mice obtained in protocol 2. In this study, over 4-month-old treated *Akp2*^{-/-} male and female mice were used for the mating and these mating pairs were continuously treated with ERT. Homozygous mating mice provided the pups confirmed as homozygous *Akp2*^{-/-} (data not shown). These homozygous mice appeared normal at birth but within a few weeks all mice died of seizure and displayed premature growth.

Pharmacokinetic study in treated *Akp2*^{-/-} mice

We measured the pharmacokinetic parameters of TNSALP in 6-month-old treated *Akp2*^{-/-} and wild-type mice. Mice were injected IV with 10 U/g body weight TNSALP in *Akp2*^{-/-} ($n = 4$) and wild-type mice ($n = 4$). The baseline level of plasma ALP activity was decreased with age in wild-type mice. At 1-day-old, 1-month-old, and 6-month-old wild-type mice, the baseline level of the enzyme activity was 4.03 U/mL, 0.82 U/mL, and 0.27 U/mL, respectively. This finding suggests that a higher level of TNSALP could be required in early developmental stage of bone growth.

When we infused single IV injection of 10 U/g TNSALP in 6-month-old wild-type mice, the enzyme activity retained over the baseline level for 2 days. However, TNSALP pharmacokinetic parameters were not reproduced by using 6-month-old treated *Akp2*^{-/-} mice (Table 2). Unlike clearance in 6-month-old wild-type mice ($t_{1/2}$ of 30.59 h), the clearance of TNSALP in 6-month-old treated *Akp2*^{-/-} mice was greatly diminished ($t_{1/2}$ of 9.27 h). AUC and MRT were also reduced compared with those in wild-type mice, suggesting the rapid elimination. Plasma ALP activity was 1.1 U/mL in 24 h after infusion and 0.11 U/mL in 48 h after infusion in treated *Akp2*^{-/-} mice, suggesting that plasma ALP level after 48 h infusion is around 40% enzyme activity seen in 6-month-old wild-type mice (Table 2). Anti-ALP antibody by ELISA was measured in serum of treated mice, confirming that the antibody against ALP was raised (data not shown).

Histopathology

We studied the skeletal manifestation by using X-rays and micro-CT of treated *Akp2*^{-/-} mice and wild-type mice at ages of 6 months.

At the age of 6 months, articular, meniscal and epiphyseal cartilage showed marked abnormal chondrocyte proliferation with increased cellularity (Fig. 4a-i). The architecture of articular cartilage area was greatly distorted with abnormal hypertrophic chondrocytes. While secondary ossification centers were seen in wild-type mice, they were almost absent in treated *Akp2*^{-/-} mice (Fig. 4a-h). TB staining showed dark blue in the whole articular cartilage area (Fig. 4a-d). Delayed maturation and abnormal proliferation of articular chondrocytes seem to underlie the defective formation of secondary ossification.

The growth plate region in 6-month-old *Akp2*^{-/-} mice was thickened (Fig. 4c, d, i-l). The cells were swollen with increased gray or translucent matrix by HE staining, especially prominent in the proliferative and hypertrophic zones. The proliferative and hypertrophic zones with hypercellularity showed marked disorganization with a distorted arrangement of cells.

In 6-month-old *Akp2*^{-/-} mice, cortical bone also showed extensive osteoid deposition (Figs. 4o, p). The mineralization defects were noted in cortical bone and areas of radiolucency in long bones. The light microscopic views revealed a loss of the parallel order of the bone matrix with loss of the concentric arrangement of lamellae or haversian system formation.

Discussion

In this article, we have demonstrated 1) that ERT with native TNSALP preserved life span over 6 months and fertility, and improved the growth defect and skeletal disease, 2) that micro-CT and X-rays at 6-month-old treated mice presented hypomineralized and shortened bones, and 3) that histopathology showed abnormal proliferative chondrocytes, greatly reduced osteoblasts, and the resultant cumulative unmineralized osteoid.

Previous study showed that *Akp2*^{-/-} mice receiving once daily subcutaneous injections of high-dose bone-targeted ALP (8.2 mg/kg) grew normally and appeared well without skeletal and dental disease or epilepsy for 52 days, resulting in prevention of infantile HPP (Millan

et al 2008). Dose-dependent improvement of bone mineralization and skeletal dysplasia was observed (Yadav et al 2011, Yadav et al 2012, McKee et al 2011). However, these reports did not describe a long-term efficacy of ERT and histopathology in long bones, and did not compare with untagged native enzyme. Therefore, it was of great interest to know whether native enzyme can be effective in treating skeletal disease as observed with a targeted enzyme.

We have treated *Akp2*^{-/-} mice with every 3-day injections of native ALP (16 mg/kg in 4 days – half dose in previous report; Millan et al 2008) for 6 months. Although *Akp2*^{-/-} osteoblasts treated by ERT were somewhat capable of matrix mineralization in a short term (data not shown), osteoid accumulated progressively in subchondral and cortical bone, suggesting that mineralization process becomes impaired with time. The onset of unmineralization of osteoid during a high dose ERT treatment for a long term indicates that the cumulative effect of low TNSALP activity level prevents normal mineralization, leading to generalized skeletal abnormality. We performed pharmacokinetic study on 6-month-old treated *Akp2*^{-/-} mice. Forty-eight hours after IV infusion of native TNSALP into these treated *Akp2*^{-/-} and wild-type mice, the activity in plasma of *Akp2*^{-/-} mice was decreased to 40% of enzyme activity compared with that of wild-type mice. Half life of the infused enzyme was one-third compared with that seen in wild-type mice. Therefore, it could be difficult to retain sufficient enzyme to mineralize the bone in *Akp2*^{-/-} mice for a long term. One explanation why half life is shortened may result from raising antibody against ALP enzyme since a high dose of enzyme was infused continuously. Our preliminary data showed all mice treated for a long term elevated antibody against ALP (data not shown). It is worthwhile to assess whether introduction of immune tolerance model on *Akp2*^{-/-} mice could prevent shortened half life of infused enzyme, resulting in preservation of mineralization process and therapeutic efficacy (Sly et al 2001, Tomatsu et al 2003).

Furthermore, Narisawa et al. (2001) reported that the total number of mature osteoblasts was reduced in *Akp2*^{-/-} mice treated with vitamin B6 consistent with our finding that ERT-treated *Akp2*^{-/-} mice showed the substantial decrease of the number of osteoblasts. This reduction in cellularity was not due to increased apoptotic death of osteoblast precursor cells in the bone marrow (Narisawa et al 2001). Thus, mechanism of dysfunction and reduction of osteoblasts remained unknown although it is expected that sufficient ALP enzyme could be essential to maintain normal maturation and function of osteoblasts.

In *Akp2*^{-/-} mice treated with ERT, the slowing of endochondral growth and the gradual bone mineralization defect resemble the histopathology of the late-onset rather than the infantile form of the original untreated *Akp2*^{-/-} mice. The accumulation of subchondral and cortical osteoid caused by defective mineralization leads to distortion of architecture in bone. The gradual onset of skeletal abnormalities in patients with adult HPP is likely to be associated with the decreased synthesis of TNSALP, when the adolescent growth spurt ends. Hough et al (2007) described late-onset HPP murine model with notably defective endochondral ossification and bone mineralization as well as inappropriate proliferation of cartilage into menisci and joint capsules.

Marked abnormal proliferative chondrocytes in articular and epiphyseal cartilage were observed in *Akp2*^{-/-} mice treated in this study. The etiology of abnormal proliferation could be induced by feedback of unmineralized bone, up or down regulation of bone mineralization factors, inflammatory factors, and microfracture and repair (Hough et al 2007). Further studies on the etiology of abnormal proliferation are required to elucidate its mechanism.

In conclusion, these findings revealed 1) that administration of native TNSALP with the current regimen provide a great impact to improvements of clinical and pathological phenotypes at early stage, prolonged life span, and recovery of fertility, resulting in prevention of the early onset and lethal form of the disease, and 2) that sustained delivery of TNSALP with a high concentration could prevent a primary skeletal disease in a mouse model.

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Abbreviations

ALP	alkaline phosphatase
AUC	areas under the curve
BMD	bone mineral density
CL	total clearance
C_{max}	peak concentration
D10	a deca-aspartate sequence
ERT	enzyme replacement therapy
HE	hematoxylin and eosin
HPP	hypophosphatasia
IV	intravenously
LSDs	lysosomal storage disorders
MPS	mucopolysaccharidosis
MRT	mean residence time
PLP	pyridoxal 5-phosphate
pNPP	p-nitrophenyl phosphate
PPi	inorganic pyrophosphate
SC	subcutaneously
t_{1/2}	apparent elimination half-life

TB	toluidine blue
Tmax	concentration peak time
TNSALP	tissue-nonspecific alkaline phosphatase
Vd	steady-state distribution volume

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Figure 1. Growth curves in TNSALP treated HOMO mice

The open circular and square markers show the body weight of wild-type and heterozygous mice, respectively. The filled circular markers show the body weight of treated homozygous mice. The open triangle marker depicts the body weight of untreated mice (n=4).

Figure 2. Phenotype, X-ray pictures, and bone length of 6-month-old treated $Akp2^{-/-}$ mouse in comparison with the age-matched wild-type and heterozygous mice
a) Clinical picture of the whole body. **b)** X-ray picture of the whole body. The arrow and circles marker show bone dysgenesis with hypomineralization. **c-f)** Incisor teeth appearance. A treated $Akp2^{-/-}$ mouse has deformed teeth with a curved upper incisor and smaller lower incisor. **g-n)** X-ray images reveal occasional distortion of arm and leg and patchy areas of radiolucency in long bones of aged $Akp2^{-/-}$ mice. Chest, ribs and spine in treated mice are shorter and smaller with moderate hypomineralization. Ribs are moderately deformed. **o-s)** Lengths of humerus, ulna, radius, femur, and tibia were compared between wild-type, heterozygous and treated mice. *: 0.05 >, **: 0.01 >, ***: 0.0001 >

Figure 3. Three dimensional micro-CT reconstructions of knee joints and knuckle of 6-month-old wild-type and treated *Akp2*^{-/-} mice

Six-month-old ERT-treated mice had all bony structures but some defect of mineralization remained unfixed. Ossification was not completed.

Figure 4. Histopathology of HE and TB staining of knee joints

Left panels show the wild-type mice, while right panels show treated *Akp2*^{-/-} mice.

Original magnification (a-f: x 20, g, h, k-p: x40, i, j: x 10). T: tibia, GP: growth plate.

a-f) TB staining: Articular and epiphyseal cartilage from a 6-month-old *Akp2*^{-/-} mouse appears hyperplastic with distorted architecture compared with a wild-type control.

Cellularity of chondrocytes in hypertrophic and proliferative layers of growth plate region and articular cartilage increases markedly (open arrows). Cell size in hypertrophic cells greatly increases.

g-j) HE staining: Unusual growth caused by an excessive multiplication of articular chondrocytes is observed in a 6-month-old *Akp2*^{-/-} mouse. Cell size in chondrocytes with opaque matrix greatly increases (open arrows).

k-n) HE staining: Epiphyseal chondrocytes are hyperplastic and the column architecture is distorted and lost. The hypertrophic cells become bigger in size and increase markedly in number. The pale

staining tissue that accumulates subchondrally is noncalcified osteoid (open arrows). Fewer osteoblasts are present adjacent to trabeculae of 6-month-old *Akp2*^{-/-} mouse compared with a wild-type control. **o, p**) HE staining: Bone marrow shows the loss of osteoblasts along the trabeculae and osteoid increases abnormally (open arrows). **q, r**) HE staining: Excessive non-calcified osteoid deposition (open arrows) can be seen in cortical bone (tibia) of 6-month-old *Akp2*^{-/-} mouse. The parallel order of the bone matrix with concentric arrangement of lamellae or haversian system formation is lost

Table 1

Pharmacokinetic study (IV injection, n=4)

	TNSALP treated HOMO	WT
$t_{1/2}$ (h)	9.27±2.02	30.59±0.87
Tmax (h)	0.5	0.5
Cmax (U/mL)	135.14±16.08	154.41 ±6.64
AUC (U/mL*h)	352.48±57.47	1528.25±280.77
Bioavailability (%)	23.69±1.50	100
MRT (h)	3.54±0.85	9.44±0.50
Vd (mL/g)	0.100±0.015	0.070±0.015
CL (mL/h*g)	0.032±0.007	0.0078±0.0022