Musculoskeletal Pathology

Sustained Osteomalacia of Long Bones Despite Major Improvement in Other Hypophosphatasia-Related Mineral Deficits in Tissue Nonspecific Alkaline Phosphatase/Nucleotide Pyrophosphatase Phosphodiesterase 1 Double-Deficient Mice

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We have shown previously that the hypomineralization defects of the calvarium and vertebrae of tissue nonspecific alkaline phosphatase (TNAP)-deficient (*Akp2*^{-/-}) hypophosphatasia mice are rescued by simultaneous deletion of the *Enpp1* gene, which encodes nucleotide pyrophosphatase phosphodiesterase 1 (NPP1). Conversely, the hyperossification in the vertebral apophyses typical of *Enpp1*^{-/-} mice is corrected in [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-knockout mice. Here we have examined the appendicular skeletons of *Akp2*^{-/-}, *Enpp1*^{-/-}, and [*Akp2*^{-/-}; *Enpp1*^{-/-}] mice to ascertain the degree of rescue afforded at these skeletal sites. Alizarin red and Alcian blue whole mount analysis of the skeletons from wild-type, *Akp2*^{-/-}, and [*Akp2*^{-/-}; *Enpp1*^{-/-}] mice revealed that although calvarium and vertebrae of double-knockout mice were normalized with respect to mineral deposition, the femur and tibia were not. Using several different methodologies, we found reduced mineralization not only in *Akp2*^{-/-} but also in *Enpp1*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}] femurs and tibias. Analysis of calvarial- and bone marrow-derived osteoblasts for mineralized nodule formation *in vitro* showed increased mineral deposition by *Enpp1*^{-/-} calvarial osteoblasts but decreased mineral deposition by *Enpp1*^{-/-} long bone marrow-derived osteoblasts in comparison to wild-type cells. Thus, the osteomalacia of

***Akp2*^{-/-} mice and the hypomineralized phenotype of the long bones of *Enpp1*^{-/-} mice are not rescued by simultaneous deletion of TNAP and NPP1 functions. (*Am J Pathol* 2005, 166:1711–1720)**

Physiological biomineralization is a tightly controlled process that is initiated within osteoblast and chondrocyte-derived matrix vesicles (MVs). MVs are submicroscopic, extracellular, membrane-invested bodies containing calcium and inorganic phosphate (P_i). The first crystals of hydroxyapatite (HA) bone mineral are generated within MVs in growth plate cartilage,¹ developing bone,² and dentin.³ This initial phase of HA mineral deposition occurs within the protected microenvironment provided by the membrane of MVs,¹ and is followed by a second phase of mineral propagation in which HA protrudes into the matrix surrounding individual MVs.³ Inorganic pyrophosphate (PP_i) antagonizes the ability of P_i to crystallize with calcium to form HA and thereby inhibits HA deposition.^{4–6} Therefore for normal mineralization to proceed, a balance is required between levels of P_i and PP_i.

Several molecules are important in the regulation of P_i and PP_i levels, including tissue nonspecific alkaline phosphatase (TNAP) and nucleotide pyrophosphatase phosphodiesterase 1 (NPP1). Deletion of the TNAP gene (*Akp2*) in mice results in a model of infantile hypophosphatasia,^{7,8} characterized by rickets, osteomalacia, spontaneous bone fractures, and increased PP_i levels.⁹ TNAP is highly expressed on the outer surfaces of

Supported by the National Institutes of Health (grants DE05262, AR47908, AG07996, and DE12889) and the Veterans Administration Research Service.

Accepted for publication February 8, 2005.

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MVs,^{10,11} and there is considerable experimental evidence indicating that TNAP can stimulate mineral deposition *in vitro* by preparations of isolated MVs,^{12,13} or in slices of rachitic rat growth plate.¹⁴ In *Akp2*^{-/-} mice, and also in human hypophosphatasia, the initial stages of biomineralization are normal, ie, MVs deposit calcium phosphate internally which is rapidly converted to crystalline HA.^{15,16} However, the propagation phase in which mineral bursts from MVs into the surrounding matrix is defective in the absence of TNAP, leading to an overall decrease in the amount of perivesicular mineral deposited.^{15,16} Thus, in bone, the hydrolytic activity of TNAP restricts the concentration of the mineralization inhibitor PP_i while simultaneously contributing to the pool of P_i available for propagation of HA deposition.

In contrast, genetic ablation of a molecule that generates PP_i, ie, NPP1 (previously termed PC-1, *npps*, and *ttw*) causes a hypermineralized phenotype of both soft tissues and certain sites in the skeleton. Mice lacking the NPP1 gene (*Enpp1*) show decreased levels of PP_i and exhibit abnormalities including ankylosing intervertebral and peripheral joint hyperostosis and articular cartilage calcification. *Enpp1*^{-/-} mice also display arterial calcification and NPP1 deficiency has recently been linked to a syndrome of infantile arterial and periarticular calcification.¹⁷⁻¹⁹ Thus NPP1 serves as a physiological inhibitor of mineral deposition, at least in part, by generating PP_i.

We have previously shown that the abnormal PP_i levels and mineralization deficits of calvarium and spine associated with TNAP or NPP1 deficiencies are reversed and normalized in mice lacking both TNAP and NPP1, ie, [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-knockout mice.²⁰ These studies have indicated that both TNAP and NPP1 are potential therapeutic targets for the treatment of mineralization disorders. Interestingly, the so-called tiptoe walking mice (*ttw/ttw*), due to a NPP1 truncation mutation, are nearly identical to *Enpp1*^{-/-} mice, and have been documented to display trabecular bone loss in addition to spontaneous mineralization of soft tissue.^{17,21} In this article, we have performed a systematic study to fully document the extent of mineral deposition in bones of *Enpp1*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}] mice, to ascertain whether the *Enpp1*^{-/-} mice also display trabecular bone loss and whether correction of mineralization abnormalities in the [*Akp2*^{-/-}; *Enpp1*^{-/-}] mice extends to the entire skeleton.

Materials and Methods

Reagents

All routine chemicals were of an analytical grade from Sigma (St. Louis, MO), unless otherwise indicated.

Generation and Maintenance of *Akp2*^{-/-}, *Enpp1*^{-/-}, and [*Akp2*^{-/-}; *Enpp1*^{-/-}] Mice

The generation and characterization of *Akp2*^{-/-}, *Enpp1*^{-/-}, and [*Akp2*^{-/-}; *Enpp1*^{-/-}] mice have been reported previously.^{7,8,20,22} To determine genotypes,

genomic DNA was isolated from tails and analyzed using polymerase chain reaction protocols as described.²⁰

Whole Mount Skeletal Analysis

Whole mount skeletal preparations were prepared by removal of skin and viscera of mice followed by a 1-week immersion in 100% ethanol, followed by 100% acetone. Samples were then transferred to a 100% ethanol solution containing 0.01% alizarin red S, 0.015% Alcian blue 8GX, and 0.5% acetic acid for 3 weeks. Samples were destained with 1% (v/v) KOH/50% glycerol solution. Cleared samples were stored in 100% glycerol.

Microcomputerized Tomography (microCT)

The distribution and relative density of bone mineral was imaged and measured by microCT, performed by Enhanced Vision Systems Corp. (now GE Medical Systems) of London, Ontario, Canada. Specimens for microCT analysis consisted of sagittally-sectioned, 2.5% paraformaldehyde-fixed, upper tibias and attached femurs, dissected free of soft tissue. Volume cone-beam CT imaging was used to create 14- μ m resolution volume data sets from wild-type (WT) (*n* = 7), *Akp2*^{-/-} (*n* = 4), *Enpp1*^{-/-} (*n* = 4) and [*Akp2*^{-/-}; *Enpp1*^{-/-}] (*n* = 3) mice, all 10 days of age. The microCT images were analyzed for bone volume fraction, reported as percent of total bone volume, bone mineral density in mg/ml, mean trabecular bone thickness (Tb.Th) in mm, and trabecular number (Tb. N) per mm² of bone surface.

Light Microscopy and Alizarin Red Calcium Stains

Nondecalcified upper tibial growth plates and subjacent metaphyseal and cortical bone were dissected from 10-day-old WT (*n* = 7), *Akp2*^{-/-} (*n* = 4), *Enpp1*^{-/-} (*n* = 4), and [*Akp2*^{-/-}; *Enpp1*^{-/-}] (*n* = 3). Bones were fixed in 2.5% glutaraldehyde, buffered with cacodylate,¹ and then embedded in Spurr epoxy resin. Standard light microscopy was performed on 1- μ m-thick sections of the Spurr-embedded bones. Sections were also stained for calcium, using the alizarin red method,²³ which specifically stains calcium red in 1- μ m-thick bone sections without prior deplastization to remove the epoxy resin.

Fourier Transform-Infrared Imaging Spectroscopy (FT-IRIS)

FT-IRIS was performed on 1- μ m-thick unstained Spurr-embedded sections from the same blocks as used for light microscopy and alizarin red staining. Sections were cut onto BaF₂ infrared windows and a Bio-Rad (Cambridge, MA) FTS-60A step-scanning Stingray 6000 FTIR spectrometer with a UMA 300A FTIR microscope and a 64 × 64 MCT FPA detector was used to acquire spectra at 8 cm⁻¹ resolution under N₂ purge. Data were collected from 400 × 400 μ m² regions at 64 × 64 individual points

of 7 μm diameter, resulting in 4096 individual spectra. Infrared vibrations of both the mineral and matrix phases were monitored simultaneously. The ratio of the area of the mineral phosphate V_1 , V_3 absorbance from 900 to 1200 cm^{-1} to the area of the protein amide I absorbance from 1590 to 1720 cm^{-1} was calculated to obtain the relative amounts of mineral and protein present (mineral:matrix ratio). The ratio of the intensities of the phosphate contour at 1030 and 1020 cm^{-1} was calculated as an indicator of crystallinity. IR images were then created based on mineral:matrix ratio and crystallinity.

Transmission Electron Microscopy

Ultrathin sections were taken from the sectioned surfaces of Spurr-embedded blocks of upper tibial growth plates and metaphysis used to produce 1- μm -thick sections for light microscopy and alizarin red staining. Transmission electron microscopy sections were stained with lead citrate and uranyl acetate as previously described.¹ The sections were examined and photographed using a Zeiss EM10A electron microscope (Oberkochen, Germany).

Immunohistochemistry

WT mice, both day 10 and day 20, were euthanized and bone tissue was harvested. Bones were fixed in 4% paraformaldehyde/phosphate-buffered saline and processed and sectioned as previously described.²⁴ Paraffin-embedded sections were dewaxed and rehydrated before antibody staining. Antibody staining was performed using a Histomouse-Max kit, according to the manufacturer's instructions. The monoclonal antibody to NPP1²⁵ was used at a 1:50 dilution. Negative controls were performed using either mouse IgG at the same dilution as the primary antibody, or with no primary antibody present.

Primary Osteoblast Isolation and Differentiation

Primary cultures of osteoblasts were isolated from calvaria of 3-day-old WT and *Enpp1*^{-/-} mice by sequential collagenase digestion, as described.²⁴ An enriched cell population of osteoblastic phenotype was plated at a density of approximately 4×10^4 cells/ cm^2 in osteogenic medium, ie, α -minimal essential medium supplemented with 10% fetal calf serum, ascorbic acid (50 $\mu\text{g}/\text{ml}$), and β -glycerophosphate (10 mmol/L). To isolate osteoblast-like cells from bone marrow, whole bone marrow was flushed from the long bones of 3-month-old WT and *Enpp1*^{-/-} mice. The marrow suspension was dispersed to attain a homogenous cell suspension and was then centrifuged at 1100 rpm for 5 minutes. The cell pellet was resuspended in α -minimal essential medium supplemented with 10% fetal calf serum and was filtered through a 70- μm sieve. Cells were plated at a density of 4×10^6 cells/well in a six-well plate. After 5 days the nonadherent cell population was removed and cells were refed in osteogenic medium as for calvarial cells. For both systems the medium was completely replaced every third day. After 14 to 21 days of culture in osteogenic

medium, the cells were fixed in 4% paraformaldehyde/phosphate-buffered saline and alkaline phosphatase activity followed by von Kossa staining, performed as previously described.²⁴ The nodules formed were quantified as previously described, using the point-counting method, and expressed as the percentage area mineralized.²⁴

Western Blot Analysis and NPP1 and TNAP Activity Assays

Three-day-old WT mice ($n = 6$) were euthanized and calvaria were cut into two pieces, long bones (tibia and femur) were isolated from each leg resulting in two samples/tissue type/animal. All tissue was minced with a scalpel, sonicated, and mixed for 1 hour (at 4°C) in 0.5 mol/L Tris, pH 8.0, 1.6 mmol/L MgCl_2 , and 1% Triton X-100. NPP1 and alkaline phosphatase assays were performed as described in Vaingankar and colleagues.²⁶ Western blot analysis for ANK, TNAP, and NPP1 was performed as described.^{27,28} Briefly, primary antibody diluted at a concentration of 3 $\mu\text{g}/\text{ml}$ in SuperBlock containing 5% normal goat serum, was incubated with one membrane strip at room temperature for 2 hours. After washing, all membrane strips were incubated with peroxidase-conjugated anti-mouse immunoglobulins (Cappel, West Chester, PA) in 5% normal goat serum-Tris-buffered saline/Tween 20. Immunoreaction was detected by the ECL-plus system (Amersham Bioscience, Piscataway, NJ).

Statistical Analysis

All data were analyzed by one-factor analyses of variance or two-factor analysis of variance when appropriate. After statistically significant effects in the analyses of variance, posthoc comparisons among means were conducted with Fisher's LSD test. The level of significance was set at 0.05.

Results

Whole Mount Analysis of Mineral Deposition in WT, *Akp2*^{-/-}, and [*Akp2*^{-/-}; *Enpp1*^{-/-}] Mice

We have previously reported that the vertebral apophyses and calvaria from mice with null mutations in both the *Akp2* and *Enpp1* genes display a dramatic amelioration of the hypomineralized phenotype of mice deficient in *Akp2* alone.²⁰ In this study we have further analyzed the [*Akp2*^{-/-}; *Enpp1*^{-/-}] skeletons to assess the effects of loss of NPP1 activity on the hypophosphatasia phenotype of *Akp2*^{-/-} mice. A comparison of the amount of mineralized bone between WT, *Akp2*^{-/-}, and [*Akp2*^{-/-}; *Enpp1*^{-/-}] whole mount skeletal specimens, as measured by alizarin red and Alcian blue staining, revealed a differential rescue of the hypomineralized phenotype with respect to bone site in double-knockout mice (Figure 1). It is important to note that in these studies, Alcian blue is used at pH 5.5 and therefore stains unmineralized os-

Figure 1. Whole mounted calvaria, spine, metatarsals, and tibias, stained for calcium with alizarin red and unmineralized osteoid with Alcian blue from WT, *Akp2*^{-/-}, and [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-knockout mice. Although double deficiency of TNAP and NPP1 restored mineralization in the calvaria and spine, and to a lesser extent in the metatarsal bones of the feet, the phalangeal bones and the tibias of double-knockout mice still displayed moderate to severe hypomineralization in comparison to WT controls.

teoid rather than cartilage.²⁰ The calvarial bones and the vertebral apophyses of the [*Akp2*^{-/-}; *Enpp1*^{-/-}] mice displayed a clearly normalized morphology (Figure 1). A partial correction is observed in the metatarsal bones, but not in the phalanges. Both the femur and tibia from these double-knockout mice do not appear to have rescue in comparison to either the vertebrae or calvaria, and display a residual abnormality in mineral deposition, with the degree of positive alizarin red staining not restored to WT levels in the double-knockout mice. These data suggest that the simultaneous ablation of the *Akp2* and *Enpp1* genes, while affecting a dramatic amelioration of mineralization deficits in certain bones, does not show signifi-

cant rescue of mineralization abnormalities in the long bones.

MicroCT and Alizarin Red Staining of Long Bones

To further quantify the amount of mineral present, and the extent to which [*Akp2*^{-/-}; *Enpp1*^{-/-}] long bones are normalized, we performed a detailed examination of these bones using microCT and high-resolution alizarin red stains. MicroCT analysis of the upper tibial growth plate, cortex, and metaphysis of WT, *Akp2*^{-/-}, *Enpp1*^{-/-}, and

Figure 2. MicroCT images of upper tibias from WT (A), *Akp2*^{-/-} (B), *Enpp1*^{-/-} (C), and [*Akp2*^{-/-}; *Enpp1*^{-/-}] (D) mice. Note: moderate deficiency of bone mineralization in NPP1 deficiency and moderate to severe mineralization in TNAP deficiency and TNAP/NPP1 double deficiency.

[*Akp2*^{-/-}; *Enpp1*^{-/-}] tibias revealed that in fact *Enpp1*^{-/-} tibias display a moderately to severely reduced mineral content in both the upper growth plate and subjacent bone (Figure 2). Given that *Akp2*^{-/-} mice display severe hypomineralization, and that *Enpp1*^{-/-} tibial growth plates are also under-mineralized it is not surprising that in [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-knockout tibias, mineral content was even more reduced in comparison to *Enpp1*^{-/-} single knockout tibias (Figure 2, C versus D). In addition the epiphyses (secondary ossification centers) were less well developed in both *Enpp1*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}] deficiencies in comparison to WT (Figure 2). To quantify these findings, bone mineral density, bone volume fraction, and average bone trabecular thickness, were measured by microCT densitometry. Bone mineral density, bone volume fraction, and trabecular thickness were all moderately reduced in NPP1-deficient tibias in comparison to WT. In the [*Akp2*^{-/-}; *Enpp1*^{-/-}] tibias there was an even more marked reduction in the bone mineral density, bone volume fraction, and trabecular thickness (Table 1).

To further investigate the pattern of mineral deposition in these mice, we performed histological analysis using nondecalcified tibias from each respective genotype and used alizarin red staining to detect calcium (Figure 3). These data confirmed the microCT data, ie, showing a decrease of calcification in lower hypertrophic zones of growth plate as well as in metaphyseal bone trabeculae and cortical bone in *Enpp1*^{-/-} mice (Figure 3). Mineral deposits in NPP1-deficient tibias were paler and less intensely red-brown stained suggesting a decreased concentration of CaPO₄ in these animals. Also, there was an abnormal accumulation of uncalcified bone matrix

(osteoid) at surfaces of the cortex and metaphyseal bone trabeculae in *Enpp1*^{-/-} tibias that was more pronounced in [*Akp2*^{-/-}; *Enpp1*^{-/-}]-deficient mouse tibias (Figure 3). We confirmed our histological observations that *Enpp1*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}] tibia have less mineral than WT controls using FT-IRIS analysis, which also indicated reduced calcium phosphate mineral in NPP1-deficient tibias and markedly reduced mineral in the [*Akp2*^{-/-}; *Enpp1*^{-/-}] double deficiency. No differences in crystallinity of the mineral phase were detected in WT versus *Enpp1*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}]-deficient tibias (Figure 4).

Transmission Electron Microscopy

Transmission electron microscopy showed a decrease in overall mineral density in *Enpp1*^{-/-} growth plates and at surfaces of newly formed bone in the metaphysis. There was an apparent further decrease in overall mineral density in [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-knockout growth plates and bone (Figure 5; A to I). The fine structure of mineral was less needle-like and more granular in both *Enpp1*^{-/-} knockout and [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-knockout bones (Figure 5; D to I). As is the case at WT sites of initial mineralization, MVs contained needle-like apatite crystals in *Enpp1*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}]-deficient growth plates and bone (Figure 5; A, D, and G). In *Enpp1*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}]-deficient bones there was a thicker layer of unmineralized osteoid, characterized by more exposed type I collagen fibrils con-

Table 1. Micro-CT Comparison of Average Bone Mineral Density (BMD), Bone Volume Fraction (BVf), and Trabecular Thickness in Wild-Type versus TNAP, NPP1, and TNAP/NPP1-Deficient Upper Tibias

Genotype	BMD (mg/cc)	BVf (%)	Trabecular thickness (mm)	n
WT	174 ± 14	46 ± 5	53 ± 8	7
<i>Akp2</i> ^{-/-}	92 ± 8*	27 ± 5*	30 ± 3*	4
<i>Enpp1</i> ^{-/-}	137 ± 11	40 ± 5	41 ± 1	4
<i>Akp2</i> ^{-/-} ; <i>Enpp1</i> ^{-/-}	77 ± 19*	18 ± 5*	27 ± 3*	3

Data presented as mean ± SE.
 *P < 0.05, WT versus knockout.

Figure 3. Alizarin red staining for calcium in 1- μ m-thick sections from Spurr-embedded tibial growth plates, cortex, and metaphyses of WT (**A, B**), *Akp2*^{-/-} (**C, D**), *Enpp1*^{-/-} (**E, F**), and [*Akp2*^{-/-}; *Enpp1*^{-/-}] (**G, H**) upper tibias. Mineral was slightly to moderately reduced in growth plate and bone of *Enpp1*^{-/-} tibias and moderately to severely reduced in *Akp2*^{-/-} and [*Akp2*^{-/-}; *Enpp1*^{-/-}] tibias. Alizarin red stain with toluidine blue counterstain. Original magnifications, $\times 595$.

taining partly mineralized MVs (Figure 4; C, F, and I). The finding of more osteoid at primary sites of bone mineralization is an indication of osteomalacia, ie, inadequate mineralization.

In Vitro Analysis of Osteoblast Differentiation

To further examine the site-specific effects of NPP1 deficiency we performed *in vitro* analysis of osteoblast differ-

entiation from osteoprogenitor cells using both primary calvarial- and bone marrow-derived osteoblasts and compared the ability of these cells to differentiate into mineralizing osteoblasts using the well-established bone nodule assay.²⁹ As has been previously shown, nodule assays show that calvarial osteoblasts from *Enpp1*^{-/-} mice display increased mineral deposition in comparison to WT control cultures (Figure 6). Interestingly however, we observed the opposite phenotype in bone marrow-

Figure 4. **A:** An IR image based on the mineral/matrix ratio obtained in a Spurr-embedded section from an *Enpp1*^{-/-} mouse. Data were collected at 6.25- μ m intervals from a trabecular bone region as outlined on the IR image (regions of marrow were excluded from the analysis). **B:** Typical baselined infrared spectra from the WT (black), *Enpp1*^{-/-} (red), and *Akp2*^{-/-}; *Enpp1*^{-/-} (blue) are shown. The IR absorbances from the embedding media Spurr resin, the bone matrix protein (amide I), and mineral phosphate band are labeled. **C:** The mean mineral/matrix ratio was reduced in the *Enpp1*^{-/-} compared to the WT, and even further reduced in the *Akp2*^{-/-}; *Enpp1*^{-/-} compared to both the WT and the *Enpp1*^{-/-}. *, Significantly different ($P < 0.05$) compared to WT; #, significantly different compared to *Enpp1*^{-/-}.

Figure 5. Transmission electron microscopy of mineralization sites in growth plate and metaphyseal bone matrix of WT (A–C), *Enpp1*^{−/−} (D–F), and [*Akp2*^{−/−}; *Enpp1*^{−/−}] (G–I) mice. A, D, and G compare early mineralization in upper growth plates; B, E, and H compare the calcified zones of lower growth plates; and C, F, and I compare surfaces of newly formed bone trabeculae in the metaphyses. Note the granularity of mineral in *Enpp1*^{−/−} and *Akp2*^{−/−}; *Enpp1*^{−/−} bones plus markedly increased uncalcified bone osteoid in the non-WT bones. Arrows indicate partially or fully calcified MVs in growth plate cartilage (A, D, G) and partially or fully calcified osteoid (C, F, I). Original magnifications: ×151,000 (A); ×143,000 (D, G); ×37,000 (B); ×98,000 (E); ×49,000 (H); ×29,000 (C); ×33,000 (F); ×40,000 (I).

Figure 6. Calvarial and bone marrow-derived osteoblast differentiation assay. Osteoblast-like cells were isolated from WT and *Enpp1*^{-/-} calvaria and bone marrow as described in the Materials and Methods. Cells were cultured in the presence of ascorbic acid (50 μ g/ml) and β -glycerophosphate (10 mmol/L) for 21 days, cells were fixed and stained for alkaline phosphatase activity (red staining) followed by von Kossa staining for mineral (brown/black staining). The area of the culture well covered by alkaline phosphatase-positive, mineralized nodules was quantified using a point-counting method and was expressed as the percentage surface area mineralized. WT osteoblasts (white bars) and *Enpp1*^{-/-} osteoblasts (black bars) were tested. Data represent the mean $\pm$ SEM from triplicate wells. ***, $P < 0.001$ posthoc test after significant two-way analysis of variance.

derived osteoblast cultures from long bones. These cultures demonstrated that calcified nodule formation was in fact inhibited in *Enpp1*^{-/-} bone marrow cultures in comparison to WT cells and overall the amount of mineral deposited in these cultures was significantly decreased in comparison to WT levels (Figure 6). As is the caveat with all primary culture systems, cultures of osteogenic cells do not represent a pure population of osteoprogenitor cells and one must assume the presence of other cell types that are not of the osteoprogenitor lineage. We determined that the rate of proliferation between WT and *Enpp1*^{-/-} calvarial and bone marrow cells was equal (data not shown). Therefore, the difference we observe in the amount of mineral produced at the end of culture period is a function of the differentiation capacity of the cells, and not of the growth rate. Overall, given our *in vivo* analysis demonstrating an osteomalacic phenotype in tibia and our *in vitro* data, demonstrating that bone marrow-derived osteoblasts do not produce as much mineral as WT cells, demonstrate that loss of NPP1 activity affects skeletal sites in a site-specific manner.

Comparison of NPP1 versus TNAP Expression in Calvaria versus Long Bones

Overall, the immunohistochemical staining intensities for NPP1 were similar in both tibias and vertebrae of WT mice, with maximal staining intensity in prehypertrophic and hypertrophic chondrocytes of the growth plate and in

Figure 7. Protein and enzymatic characterization of TNAP and NPP1 in WT calvaria and long bones. Six WT mice were euthanized at 3 days of age. The calvaria were cut into two equal halves and the halves were pooled to constitute two equivalent samples (no. 1 and no. 2), representative of six animals each. The right-sided femurs and tibias of the six animals were pooled (sample no. 1) and compared to a separate pool of six left-sided tibias and femurs (sample no. 2). Comparison was by immunoblotting (A), analysis of enzymatic activity of NPP1 (B) and TNAP (C), and was performed using 5 μ g of total protein per sample in triplicate. *, $P < 0.05$.

osteoblasts of tibias versus vertebrae (data not shown). However, Western blots showed a reduced expression of NPP1 protein in femur/tibia bone samples versus calvarial bone samples (Figure 7A), while TNAP protein expression was approximately equivalent in femur/tibia versus calvarial bone samples (Figure 7A). In contrast to the Western blot data showing equivalent TNAP protein in calvaria and femur/tibia, the enzymatic activities of both TNAP and NPP1 were significantly increased in calvaria versus femur/tibia bone extracts (Figure 7B).

Discussion

In this study, we report that the effects of NPP1 deficiency on mineral deposition are site-specific. As previously reported, *Enpp1*^{-/-} mice display spontaneous calcification of soft tissues.^{21,24,30} However we document here that these mice also display moderate to severe hypomineralization in the long bones. We have previously shown that simultaneous deletion of *Akp2* and *Enpp1* dramatically normalizes the hypo- and hypermineralization defects in calvaria and spine of the *Akp2*^{-/-} and *Enpp1*^{-/-} mice, respectively. However, in this study we show that this rescue is site-specific and extends only to the calvaria, spine, and incompletely in the metatarsals, in [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-deficient mice. Hypomineralization of the long bones remains, despite the dramatic amelioration of the mineral deficits at other sites and the systemic correction of circulating PP_i levels.²⁰

Another mouse model of NPP1 deficiency has been described, the *ttw/ttw* tiptoe walking mouse (previously termed *twy/twy*), which is a naturally occurring mutant that, like the *Enpp1*^{-/-} mouse, exhibits pathological ossification of the spinal ligaments similar to ossification of the posterior longitudinal ligament.¹⁷ However, a state of osteopenia has also been demonstrated in the *ttw/ttw* mouse model of NPP1 deficiency,^{17,21,31} despite the marked spontaneous pathological soft tissue calcification

also present. In addition, abnormalities in matrix synthesis, reflected by heightened type XI collagen expression at calcifying ligaments and sustained elevation of collagen production by primary *ttw/ttw* calvarial osteoblasts have also been documented.³⁰ Given that the *ttw/ttw* mice are very similar to the *Enpp1*^{-/-} mice, these data support our finding that lack of NPP1-mediated PP_i production affects mineralization of skeletal sites in a selective manner.

Okawa and colleagues,²¹ described an amelioration of the osteopenic phenotype of *ttw/ttw* mice by administration of calcitonin. Calcitonin's main biological function is to inhibit osteoclast activity, and it has been successfully used in treatment of diseases caused by increased osteoclastic activity such as Paget's disease.³² The successful treatment of osteopenia in *ttw/ttw* mice by calcitonin suggests that in these mice, and perhaps also in *Enpp1*^{-/-} mice, a state of increased osteoclastic activity leads to bone loss, albeit only at certain sites. Maintenance of normal bone mass occurs due to a tight balance between bone formation via osteoblasts and bone resorption via osteoclasts, and these cells are tightly coupled to each other. In a NPP1-deficient state, increased osteoclast activity could occur in an attempt to balance the increased mineral deposition caused by NPP1 deficiency. Alternatively, PP_i may function to limit osteoclast activity, and in *Enpp1*^{-/-} mice abnormally low levels of PP_i may lead to increased osteoclastic bone resorption. PP_i is chemically analogous to the bisphosphonate family of drugs, which potently inhibit osteoclast activity by inducing apoptosis.^{5,33} However, it remains to be seen whether *Enpp1*^{-/-}-derived osteoclasts display increased number or resorptive activity.

We speculate that the hypermineralization in NPP1-deficient mouse calvaria and vertebrae is based on the known mineralization inhibitory effects of inorganic pyrophosphate (PP_i)^{4,6,5} versus the mineral promoting effects of orthophosphate (P_i).^{12,13} When present *in vivo* at supraphysiological concentrations, PP_i inhibits calcium phosphate mineral formation by coating HA crystals, thus preventing mineral crystal growth and proliferative self-nucleation.⁵ In tissues where high NPP1 enzyme activity releases more than sufficient PP_i to balance P_i generation, an overall balance of mineral formation rate would be expected in which TNAP stimulates mineral formation while NPP1 generation of high levels of PP_i inhibits and prevents excessive HA mineral deposition (Figure 8A). At sites such as calvaria and vertebrae, an abnormal reduction in NPP1 activity, as would occur in *Enpp1*^{-/-} mice, should result in unopposed P_i generation leading to excessive mineralization (Figure 8B). At these sites, a balancing deletion of *Akp2* would be expected to restore a more normal balance between local concentrations of PP_i and P_i, and, thus, should at least partially restore normal mineralization. This is what we have observed in calvaria and vertebrae of [*Akp2*^{-/-}; *Enpp1*^{-/-}] double-knockout mice.²⁰

However, in contrast to the mechanism discussed above, in which high endogenous PP_i levels in calvaria and vertebrae would have an inhibitory effect on mineralization, the ability of near physiological concentrations

Figure 8. Diagram outlining the differential effects of high and low extracellular PP_i concentrations on HA formation. ATP hydrolysis by NPP1 produces inorganic pyrophosphate (PP_i), which can be further hydrolyzed by TNAP to yield orthophosphate (P_i). The resulting P_i molecules are incorporated into HA mineral. **A:** High levels of nonhydrolyzed PP_i block the formation of calcium phosphate mineral.⁴⁻⁶ **B:** Instead, under low extracellular PP_i concentrations, the effect of PP_i is mostly stimulatory² as it is rapidly hydrolyzed by TNAP into P_i and incorporated into HA.

of PP_i, in the range of 0.1 mmol/L,³⁴ to stimulate mineralization has been observed in organ-cultured chick femurs.² Also, we have recently observed that near physiological concentrations of PP_i (0.01 up to 0.1 mmol/L) can stimulate HA deposition as initiated by isolated rat MVs, whereas higher concentrations more than 1 mmol/L inhibit mineral deposition.³⁵ Given that at physiological pH the *K_m* of TNAP for PP_i is ~0.5 mmol/L,³⁶ at this range of physiological extracellular PP_i concentrations³⁵ most PP_i would be hydrolyzed to orthophosphate (P_i) by TNAP (Figure 8B) thus providing P_i for incorporation into nascent mineral. The process of mineral formation would be further augmented by TNAP hydrolysis of any mild excess PP_i, thus removing persistent mineral inhibiting effects of higher PP_i concentrations. However, in tissues where there is a relatively high endogenous NPP1 activity, such as the cranium, vertebrae, and ligaments (Figure 7), higher endogenous levels of PP_i generation would exceed the ability of local TNAP and other endogenous pyrophosphatases to metabolize PP_i to P_i. The result would be persistently high local concentrations of PP_i, rising to levels more than 1 mmol/L. This would be inhibitory to mineral deposition. Because the PP_i-generating effect of NPP1 is augmented by ANK-mediated PP_i transport to the extracellular fluid, this could help explain the hypermineralization of ligaments and cartilage seen in humans and mice suffering from a genetic deficiency of ANK.^{24,37-39}

In summary, new data are presented showing hypomineralization of long bones in *Enpp1*^{-/-} mice and that this deficit is more severe in the [*Akp2*^{-/-}; *Enpp1*^{-/-}] double deficiency. The hypomineralization observed in long bones of *Enpp1*^{-/-} mice may be related to relatively lower levels of endogenous NPP1 expression throughout the long bones. Thus, in long bones, a major effect of complete deletion of NPP1 activity would be further reducing extracellular PP_i to abnormally low levels, ie, below levels at which TNAP would have sufficient PP_i substrate to generate P_i for normal mineral formation. Such a

P_i deficiency resulting from inadequate PP_i hydrolysis by TNAP would be made worse in the [*Akp2*^{-/-}; *Enpp1*^{-/-}] double deficiency in which not enough PP_i would be generated by NPP1 nor hydrolyzed by TNAP to support a normal mineralization rate.

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