Central Role of Pyrophosphate in Acellular Cementum Formation

Brian L. Foster1*, Kanako J. Nagatomo2, Francisco H. Nociti Jr.1,3, Hanson Fong4, Daisy Dunn2, Anne B. Tran1, Wei Wang5, Sonoko Narisawa5, Jose Luis Millán5, Martha J. Somerman1

1 Laboratory of Oral Connective Tissue Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), National Institutes of Health (NIH), Bethesda, Maryland, United States of America, 2 Department of Periodontics, University of Washington School of Dentistry, Seattle, Washington, United States of America, 3 Division of Periodontics, School of Dentistry at Piracicaba, State University of Campinas, Piracicaba, São Paulo, Brazil, 4 Materials Science and Engineering, University of Washington, Seattle, Washington, United States of America, 5 Sanford Children’s Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, California, United States of America

Abstract

Background: Inorganic pyrophosphate (PPi) is a physiologic inhibitor of hydroxyapatite mineral precipitation involved in regulating mineralized tissue development and pathologic calcification. Local levels of PPi are controlled by antagonistic functions of factors that decrease PPi and promote mineralization (tissue-nonspecific alkaline phosphatase, Alpl/TNAP), and those that increase local PPi and restrict mineralization (progressive ankylosis protein, ANK; ectonucleotide pyrophosphatase phosphodiesterase-1, NPP1). The cementum enveloping the tooth root is essential for tooth function by providing attachment to the surrounding bone via the nonmineralized periodontal ligament. At present, the developmental regulation of cementum remains poorly understood, hampering efforts for regeneration. To elucidate the role of PPi in cementum formation, we analyzed root development in knock-out (−/−) mice featuring PPi dysregulation.

Results: Excess PPi in the Alpl−/− mouse inhibited cementum formation, causing root detachment consistent with premature tooth loss in the human condition hypophosphatasia, though cementoblast phenotype was unperturbed. Deficient PPi, in both Ank and Enpp1−/− mice significantly increased cementum apposition and overall thickness more than 12-fold vs. controls, while dentin and cellular cementum were unaltered. Though PPi regulators are widely expressed, cementoblasts selectively expressed greater ANK and NPP1 along the root surface, and dramatically increased ANK or NPP1 in models of reduced PPi, output, in compensatory fashion. In vitro mechanistic studies confirmed that under low PPi mineralizing conditions, cementoblasts increased Ank (5-fold) and Enpp1 (20-fold), while increasing PPi inhibited mineralization and associated increases in Ank and Enpp1 mRNA.

Conclusions: Results from these studies demonstrate a novel developmental regulation of acellular cementum, wherein cementoblasts tune cementogenesis by modulating local levels of PPi, directing and regulating mineral apposition. These findings underscore developmental differences in acellular versus cellular cementum, and suggest new approaches for cementum regeneration.

Introduction

The mineralized tissues of the teeth and skeleton are subject to homeostasis of inorganic phosphate (Pi) for normal development and maintenance [1]. The hydroxyapatite (HAP) deposited to mineralize these hard tissues is a compound of Pi and ionic calcium. Pyrophosphate (PPi), composed of two molecules of Pi, functions as a pivotal regulator of physiological mineralization and pathologic calcification by acting as a potent inhibitor of HAP crystal precipitation [2–5]. Though the potential for PPi to inhibit biological mineralization is clear from in vitro experiments, the in vivo role and regulation of PPi, has been more difficult to elucidate. Through study of the heritable conditions such as hypophosphatasia (HPP), spontaneous mutations, and directed gene ablations in mouse models, the key regulators of PPi have been identified, and their roles in shaping mineralized tissues have been partially defined. As measurement of PPi in vivo at mineralization fronts is not possible, the analysis of cellular proteins that manufacture, transport, or degrade PPi has served to clarify the mechanisms for PPi modulation, in conjunction with in vitro experiments.

Local tissue concentrations of PPi are controlled by a number of regulatory enzymes and transporters. Tissue nonspecific alkaline phosphatase (TNAP) is an ectoenzyme capable of hydrolyzing PPi, and providing Pi [6]. TNAP is expressed by mineralizing cells of bones and teeth, and is critical for proper skeletal mineralization [5,7]. Hydrolysis by alkaline phosphatase activity (ALP) thus
Pyrophosphate and Acellular Cementum

In order to develop a comprehensive understanding of how PPi regulates tooth root development, we performed a detailed histological study of developing first mandibular molars and incisors of mice harboring homozygous knock-out (−/−) of Alpl (high PPi), Ank, or Enpp1 (low PPi), compared to age-matched homozygous wild-type (+/+) controls. Days were selected to capture developmental time points of interest during molar root formation, i.e., during acellular cementogenesis (14 days postnatal, dpn), at completion of the root and following cellular cementogenesis (26 dpn), and after more than a month in occlusion (60 dpn). Alpl−/− mice were limited to a maximum age of 21 dpn because of shortened lifespan. Morphological observations on H&E stained sections were paired with in situ hybridization (ISH) and immunohistochemistry (IHC) for selected mineralized tissue-associated factors.

Acellular cementogenesis requires diminution of pyrophosphate

In the infantile form of HPP, the skeleton is properly mineralized at birth, but postnatal skeletogenesis is compromised [7]. Alpl−/− mice phenocopy aspects of infantile HPP, where loss of TNAP was previously reported to have little effect on bone until postnatal day 6 [10,24]. At 14 dpn, the majority of alveolar and mandibular bone in Alpl−/− mice was well developed, though signs of hyperosteoidosis were noted in the bone adjacent to the molar root (Figure 2A and B). In Alpl+/+ molars, acellular cementum (AEFC) covered the root dentin as a thin and uniform basophilic layer. Alpl−/− molars were marked by disruption of acellular cementum, visible as reduction of the basophilic layer (cementum aplasia or severe hypoplasia) and direct contact of PDL cells and tissues with dentin. By 21 dpn this cementum defect was sometimes associated with tearing at the PDL-AEFC interface, suggesting poor integration of Sharpey’s fibers at the root surface (not seen at the PDL-bone interface) (Figure 2C and D) and consistent with HPP case reports observing premature tooth exfoliation. This is not likely to be a processing artifact, as infiltrating cells were present in the tear zone. These results agree with AEFC disruption described in this Alpl−/− model [23], as well as a different TNAP loss-of-function mouse [23].

To further investigate the mechanism for the cementum defect in Alpl−/− mice, IHC was performed for two cementum markers, extracellular matrix (ECM) proteins bone sialoprotein (BSP) and osteopontin (OPN), which are present at high concentrations in acellular cementum of controls (Figure 2E and G). Both BSP and OPN immune localization were disrupted on the Alpl−/− root surface (Figure 2F and H), compared to the strong, even staining on Alpl+/+ controls. Scanning electron microscopy (SEM) provided improved resolution to explore the root surface. While Alpl−/− molar displayed a cementum layer on the root dentin surface, this layer was absent in the Alpl−/− molar (Figure 3). The disruption of cementum initiation and concomitant lack of BSP and OPN localization supports the hypothesis that high PPi in Alpl−/− is acting to inhibit cementogenesis and HAP apposition on the root surface.

Attenuation of pyrophosphate increases acellular cementum

Both Ank and Enpp1−/− mice are deficient in extracellular PPi, though by different mechanisms. In molars of both null mice at
hypoplasia in 14 dpn compared to control supported histological observations of cementum markers bone sialoprotein (BSP) and osteopontin (OPN). At the completion of root development at 26 dpn, both Ank and Enpp1−/− mice featured a hypercementosis phenotype, indicating both PPi regulators function in controlling cementum formation. Comparative analysis between Ank and Enpp1−/− and their respective controls was accomplished by measuring the growth rate of cervical cementum over time. During early root formation between 14 and 26 dpn, Ank and Enpp1−/− molars featured at least 10-fold greater cementogenesis compared to controls (Figure 5A). Ank and Enpp1−/− cementum continued to increase at a rate of 0.2–0.7 μm/day from 26 to 60 dpn, while over the same period, controls featured tightly controlled apposition, growing at the much slower pace of 0.01–0.03 μm/day.

While cementum was dramatically affected by loss of ANK or NPP1, dramatic changes in other tissues were not observed. Histomorphometry at age 26 dpn was performed to measure cross-sectional widths to determine if PDL and alveolar bone were affected. Cementum was significantly increased in both null models, with Ank−/− at 14-fold and Enpp1−/− at more than 13-fold the width of age-matched controls (Figure 5B). A direct comparison of the two homozygous knock-out models revealed that Ank−/− featured slightly, but significantly, thicker cementum at the age sampled. Histomorphometry confirmed that PDL space was maintained in both null models, even significantly larger in Ank−/− vs. +/+ mice, despite exuberant cementogenesis. Alveolar bone on the lingual aspect tended towards reduced cross-sectional dimension in both Ank and Enpp1−/− models, though the effect was not statistically significant as measured here. Tartrate resistant acid phosphatase (TRAP) staining confirmed increased numbers of osteoclast-like cells (TRAP positive, multinucleated) on the bone surface adjacent to the tooth root in Ank−/− molars [26]. A modeling/remodeling of bone away from the root provides a mechanism for maintenance of the PDL in the face of expanding cementum.

One of the key functional characteristics of the cervical cementum is the extrinsic nature of the collagen fibers, which serve to anchor the tooth to surrounding alveolar bone. Picrosirius red staining in association with polarized light microscopy was used to visualize the birefringent collagen fibers of the periodontia [27]. The thick cementum of Ank and Enpp1−/− molars featured a high concentration of extrinsic collagen fibers, which were continuous with the fibers in the PDL proper (Figure 6B and D). As this thick cementum in the null molars features dense extrinsic collagen fibers, but also contains numerous cell inclusions, it could properly be labeled cellular extrinsic fiber cementum (CEF), a form of cementum not typical for cervical molar roots, and furthermore, not previously described in the cementum family. Importantly, the observation of an ongoing, progressive apposition on the root surfaces of Ank and Enpp1−/− mice confirms this is thickening of the normally present extrinsic fiber cementum, and is not likely to be a different type of ectopic calcification on the root surface. As a comparison, Alpl−/− molars were examined, and confirmed tearing at the root-PDL interface, osteoid invasion of the PDL space, and poorly organized and sparsely embedded collagen fibers at the cervical root (Figure 6F).

Cementum, bone, and dentin are also characterized by their extracellular matrix (ECM) protein composition, and these ECM proteins contribute to crystal growth and regulation, and affect mechanical properties of these tissues. Because of the dramatic changes in cementum apposition, we investigated the ECM profile similarly, with thickened cementum evident on all root surfaces compared to controls (Figure 4M–R). The fact that acellular cementum on all murine teeth was similarly affected by reduced PPi supports this as a central molecular regulator of cementogenesis which is not tooth- or stage-specific in its influence.
in PPi deficient mice. In the low PPi environment of the Ank and Enpp1−/− mice, the thick cervical cementum was marked by increased OPN and dentin matrix protein 1 (DMP1), proteins of the SIBLING family (Figure 7A–F and G–I). OPN staining strongly labeled control acellular cementum, and was intensely expressed in the corresponding Ank and Enpp1−/− cervical cementum and associated cementoblast cells. DMP1, a marker for osteocytes, odontoblasts, and cementocytes, was present at low or undetectable levels in acellular cementum in controls, in contrast to intense localization in expanded Ank and Enpp1−/− cementum. OPN and DMP1 levels were not changed in Ank or Enpp1−/− apical cementum, as well as in other dentoalveolar locations. The source of the increased OPN and DMP1 protein was confirmed, as cementoblast gene expression for both Opn and Dmp1 mRNA was increased in Ank and Enpp1−/− mice (Figure 8A–C and D–F). OPN and DMP1 expression changes were not observed in other cell populations in the dentoalveolar complex in these mice. Another characteristic marker for cementum, BSP, was present in control and null cementum (Figure 7M–R), and where protein concentration was diluted in the larger cementum volume of the Ank and Enpp1−/− mice, mRNA levels in cementoblasts were unaltered (Figure 8G–I). Thus, increased cementogenesis in Ank and Enpp1−/− teeth was linked to increased OPN and DMP1 specifically in cervical cementum. It is notable that OPN was increased in cementum as a result of reduced extracellular PPi. This change is opposite to the cementum. It is notable that OPN was increased in cementum as a linked to increased OPN and DMP1 specifically in cervical cementoblasts were unaltered (Figure 8G–I).

Cementoblasts express pyrophosphate regulators in a time and space restricted manner

Acellular cementum was shown to be exceptionally sensitive to regulation by PPi; with increased PPi, (as in Alpl−/− mice) AEFC was severely inhibited, and under reduced PPi conditions (as in Ank and Enpp1−/− mice) cementum thickness increased significantly, a trend not reflected in other dental hard tissues. In order to understand the sensitivity of acellular cementum to PPi metabolism, we mapped the expression of TNAP, ANK, and NPP1 during tooth root formation. We also assayed these factors in all of the null models to determine if there were compensatory or antagonistic expression changes that would contribute to phenotypes under PPi dysregulation.

TNAP was widely expressed during molar root formation, most strongly in mineralizing osteoblasts, odontoblasts, and cementoblasts (Figure 9A). As previously reported, TNAP was also strongly localized to the PDL region [28,29]. TNAP localization was not altered in developing Ank and Enpp1−/− molars (Figure 9B and C).

We previously reported wide expression of ANK gene and protein in the tooth and supporting tissues [26], paralleling previous findings that ANK is expressed in several tissues system-wide [12]. Using a refined immunohistochemistry technique, which allowed more sensitive identification of differential ANK protein localization, we discovered that after acellular cementum formed, ANK was labeled most intensely in cementoblasts lining the molar and incisor roots (Figure 9D). Developmental localization of NPP1 protein was similar to that of ANK, with most intense staining found in cementoblasts (Figure 9G). Both ANK and NPP1 stained weakly in other cells, including PDL cells, osteoblasts, and odontoblasts. Immunolocalization revealed compensatory up-regulation, where NPP1 was increased in Ank−/− and ANK was increased in Enpp1−/− (Figure 9F and H). Most interestingly, the observed increase was found only in cementoblasts, and not in other cell populations of the dentoalveolar region. These data suggested that ANK and NPP1 were differentially expressed by cementoblasts and employed to tightly regulate PPi, and developmental cementum apposition. However,
it still remained unclear by what mechanism PPi was controlling cementum apposition and ECM composition.

Pyrophosphate controls mineralization and coupled gene expression in cementoblast cultures

PPi regulators ANK and NPP1 were preferentially expressed by cementoblasts after initiation of cementogenesis, and their expression was modulated under conditions of low extracellular PPi and increased apposition. Expression levels of cementum ECM proteins OPN and DMP1 were also responsive to PPi deficiency, reflecting the altered homeostasis of P/Pi ratio or increased cementum apposition in Ank and Enpp1−/−. These data together suggested that cementoblasts associated with AEFC regulate PPi as a means to tightly control the process of apposition and related gene expression. In vitro experiments were performed to determine how these genes were regulated during mineral formation, and what potential role PPi played in their regulation. Because of the technical obstacles in isolating and identifying

Figure 4. Attenuation of pyrophosphate increases acellular cementum. The cervical cementum (c) is a thin, acellular layer in Ank; Enpp1+/+ control molars at (A) 14 dpn and (D) 26 dpn, while the (G) apical cementum is thicker and contains cementocytes. Knock-out of either Ank or Enpp1 results in expanded cervical cementum compared to control, visible by 14 dpn (B and C), and progressively thicker by 26 dpn (E and F). In contrast, the apical cementum in Ank and Enpp1−/− molars (H and I) was not different from +/+ control. (J–L) Acellular cementum of the incisor lingual root analog was similarly expanded in Ank and Enpp1−/− vs. control. (M–R) Hypercementosis resulting from loss of ANK was confirmed on all three mandibular molars. Abbreviations: d = dentin; c = acellular cementum; p = periodontal ligament; b = bone. Scale bar for A–L represents 200 μm, and for M–R represents 400 μm.
doi:10.1371/journal.pone.0038393.g004
primary cementoblasts, we opted to use an immortalized cementoblast cell line (OCCM.30) and modulate exogenously added PPi. OCCM.30 cells were cultured in control media or mineralization media where 5 mM β-glycerophosphate (BGP) was added. BGP served as an organic Pi source, mimicking similar sources in vivo and commonly used for in vitro mineralization experiments [30–33]. Cells receiving control media lacking BGP failed to mineralize during the course of the experiment. While cells cultured with BGP produced mineral nodules by day 6, with increased staining and calcium incorporation at day 8 (Figure 10A and B).

Cells were introduced to exogenous PPi to create culture conditions of low (10 μM) and high (100 μM) PPi. The lower dose of 10 μM PPi did not affect mineralization, while the higher dose of 100 μM was confirmed as an inhibitor of mineral nodule formation under these conditions. While PPi is an inhibitor of HAP crystal precipitation, it has also been reported to have cell signaling effects in osteoblasts [4,14,16]. Neither dose of PPi affected OCCM.30 cell proliferation, viability, or collagen synthesis compared to controls (Figure 11), therefore these processes were not indirectly affecting mineralization. Cementoblast ALP enzyme activity was uniform across treatments and times, and added 100 μM PPi did not appreciably affect ALP (Figure 10C), indicating the effect of PPi on mineralization was not by inhibition of TNAP. An enzymatic assay for 5’-nucleotide phosphodiesterase I and nucleotide pyrophosphohydrolase (NTPPPH) activity demonstrated significantly increased NPP1 function with mineralization at days 4, 6, and 8, while 100 μM PPi brought activity back to basal levels of non-mineralizing cultures (Figure 10D).

PPi associated and cementoblast marker genes were assayed by quantitative PCR. Under non-mineralizing conditions, Ank, Enpp1,

**Figure 5. Increased cementum apposition in Ank and Enpp1+/− teeth.** (A) During early root formation between 14 and 26 dpn, both Ank and Enpp1+/− molars featured at least 10-fold greater cementogenesis compared to controls. From 26 to 60 dpn, Ank and Enpp1+/− cementum continued to increase at a rate of 0.2–0.7 μm/day, while Ank and Enpp1+/− controls featured tightly controlled apposition at the pace of 0.01–0.05 μm/day. (B) Histomorphometry confirmed Ank or Enpp1+/− cervical cementum was significantly increased compared to controls, while PDL width was maintained and alveolar bone thickness tended towards reduction. Values with the same letter were not significantly different, while different letters indicate a statistically significant intergroup (genotype) difference (p<0.05) as tested by ANOVA followed by the Tukey test for direct pair-wise comparisons.

doi:10.1371/journal.pone.0038393.g005
Pyrophosphate and Acellular Cementum

Figure 6. Progressive mineralization of extrinsic collagen fibers in Ank and Enpp1<sup>−/−</sup> cervical cementum. Picrosirius red staining with polarized light microscopy was used to visualize birefringent collagen fibers of periodontal tissues in mandibular first molar roots. Histological sections of 60 dpn (A) control Ank; Enpp1<sup>−/−</sup> cut in a horizontal plane and (C) coronal plane revealed high density of embedded extrinsic fibers in the acellular cementum, where the high degree of birefringence (intense coloration) makes visible the organization and orientation of the major PDL collagen fibers. Observation of (B) Ank<sup>−/−</sup> and (D) Enpp1<sup>−/−</sup> expanded cervical cementum (yellow dotted outline, flanked by white arrows) in the same orientations revealed a similar high density of embedded fibers, continuous from PDL through the cementum. (E) Control Alpl<sup>+/−</sup> molars at 21 dpn cut in a coronal plane show an organized and attached PDL, while conversely, (F) Alpl<sup>−/−</sup> molar exhibited tearing at the root-PDL interface (#), osteoid invasion of the PDL space, and poorly organized and sparsely embedded collagen fibers at the cervical root. Abbreviations: d = dentin; c = acellular cementum; p = periodontal ligament; b = bone. Scale bar = 100 μm. doi:10.1371/journal.pone.0038393.g006

Discussion

These studies aimed to define the regulatory role of PP<sub>i</sub> in tooth root cementum development. We demonstrate here that PP<sub>i</sub> serves as an essential regulator of tooth root acellular cementum development, and a key determinant defining the hard-soft interface between the cementum and PDL. Dysregulation of PP<sub>i</sub>, resulting from loss of any of the central PP<sub>i</sub> controlling factors explored here had profound consequences on development of acellular extrinsic fiber cementum (AEFC), a tissue essential to tooth attachment and function. To wit, loss of TNAP caused severe underdevelopment or even absence of acellular cementum. Loss of either ANK or ENPP1 resulted in loss of control of cementum apposition, causing an exceptional hypercementosis. Because these three factors, TNAP, ANK, and NPP1, primarily adjust extracellular PP<sub>i</sub>, this strongly supports PP<sub>i</sub> as the key mechanistic factor uniting the cementum phenotypes in all three of these mouse models, prompting us to propose that PP<sub>i</sub> regulates acellular cementum in a molecular “rheostat” fashion, i.e. acellular cementum thickness relates inversely to PP<sub>i</sub> production.

Based on these collective data, we propose a model whereby PP<sub>i</sub> plays a central and novel role in acellular cementum formation (Figure 14). The periodontal region is extremely rich in ALP activity (reducing local PP<sub>i</sub>) and thus a permissive milieu for cementum formation on the root surface. In the course of normal development, cementoblasts modulate PP<sub>i</sub> to curb apposition (by increasing PP<sub>i</sub> via ANK and NPP1) to maintain AEFC as a thin tissue on the root surface. When one of these PP<sub>i</sub> factors is removed from the equation, apposition cannot be fully regulated and cementoblasts attempt to compensate by increasing expression of its counterpart PP<sub>i</sub> regulator. In addition to directly controlling cementum mineral apposition, these studies suggest PP<sub>i</sub> influences ECM protein composition; in the face of rapid cementogenesis, cementoblasts increased expression of OPN and DMP1. The increase in OPN, a negative regulator of HAP crystal growth, may be an additional mechanism cementoblasts employ to limit extent of cementum apposition. In vitro experiments support this interpretation of the role of PP<sub>i</sub> in controlling both mineral accumulation and cementoblast expression profile. What emerges is a portrait of acellular cementum as a mineralized tissue heavily governed by regulation of the physical-chemical process of mineral
precipitation, and the cementoblast as a cell capable of directing PPi metabolism to promote and restrain cementogenesis.

**On the role of pyrophosphate as a negative regulator of acellular cementum**

In these studies, we confirm that loss of TNAP function in the \( \text{Alpl}^{-/-} \) mouse causes aplasia or severe hypoplasia of the acellular cementum. This is in line with a previous report from this and another model of TNAP loss-of-function [23,25], as well as reports from human hypophosphatasia (HPP) subjects [21,34], who harbor a mutation in the human homologue, \( \text{Alpl} \) [7]. We extend previous analyses of the \( \text{Alpl}^{-/-} \) tooth cementum phenotype with gene and protein assays. Cementoblasts express similar levels of Bsp mRNA, while protein distribution of both Bsp and OPN appear disrupted. We interpret these results to mean that cementoblast phenotype is maintained in the face of loss of TNAP, but the disruption of AEFC synthesis prevents accretion of OPN and BSP proteins on the root surface. Loss of OPN protein under conditions where acellular cementum was inhibited has been reported previously [25,35], and this observation makes sense because BSP and OPN are both mineral-binding members of the SIBLING family which play a role in the mineralization process [36], and in the close relationship of AEFC cementogenesis with the act of mineralization.

We show strong TNAP localization in the developing root region, and ALP activity has been reported to be strong in the periodontium, with highest activity adjacent to the mineralizing bone and developing cementum surfaces [29]. Moreover, the same
Figure 8. Reduced pyrophosphate alters gene expression in cervical cementoblasts. Ocn mRNA is markedly increased in root-lining cementoblasts in both (B) Ank and (C) Enpp1–/–, compared to (A) Ank and Enpp1+/+ controls. Increased numbers of cells associated with the thick cervical cementum express Dmp1 in (E) Ank and (F) Enpp1–/– molar teeth, compared to (D) +/+ controls. Bsp gene expression was not different in cementoblasts in (H) Ank and (I) Enpp1–/– vs. (G) +/+ controls. Black arrowheads indicate regions of positively stained cells. All panels are samples from mice at 14 dpn. Abbreviations: d = dentin, c = (cervical) cementum; p = periodontal ligament; b = bone. Scale bar = 100 μm.

doi:10.1371/journal.pone.0038393.g008

Figure 9. Cementoblasts express pyrophosphate regulators in a time and space restricted manner. TNAP was expressed strongly in all the periodontal tissues in (A) Ank; Enpp1+/+ control as well as both (B, C) Ank and Enpp1–/– models. Loss of ANK or NPP1 did not alter cementoblast TNAP expression. ANK was localized selectively to cementoblasts in (D) control, and was increased when (F) Enpp1 was ablated. Like ANK, NPP1 was found at selectively greater concentrations in cementoblasts in (G) controls, and was increased upon (H) Ank–/–. Specificity of antibody staining was confirmed in null mice in (E) and (I). All panels are mandibular first molar teeth at 26 dpn. Abbreviations: d = dentin; c = acellular cementum; p = periodontal ligament; b = bone. Cervical cementum is indicated by opposing black arrows. Scale bar = 100 μm.

doi:10.1371/journal.pone.0038393.g009
study identified a significant correlation between measured TNAP activity and acellular cementum thickness. The critical influence of TNAP on cementum apposition is likely by clearance of mineralization inhibitor PPi, rather than by providing local Pi for HAP precipitation, for several reasons. First, circulating PPi is in the micromolar range, while Pi is much higher in the millimolar range, so in the highly vascular periodontal region hydrolysis of PPi is not likely to appreciably increase Pi available for mineralization, though local, compartmentalized ionic dynamics in vivo are difficult to predict. Secondly, mouse models of hypophosphatemia described to date tend to feature bone, dentin, and cellular cementum disorders, while acellular cementum is less

Figure 10. Pyrophosphate regulates cementoblast mineralization and nucleotide pyrophosphohydrolase (NTPPPH) activity, in vitro. (A) By von Kossa staining, OCCM.30 cells cultured with 5 mM BGP produced mineral nodules by days 6 and 8, while cells receiving only AA did not mineralize. The low dose of 10 μM PPi did not affect mineral nodule precipitation, however, the higher dose of 100 μM was a potent inhibitor of mineral nodules. (B) Quantitative calcium assay performed on days 6 and 8 confirmed visual mineral nodule staining by von Kossa. (C) Relative ALP enzyme activity was not affected by inhibition of mineralization by 100 μM PPi. (D) NTPPPHase activity was increased under mineralizing conditions, but inclusion of 100 μM PPi brought activity back to basal levels of non-mineralizing cultures. Graphs show mean +/- SD for n = 3 samples. Lowercase letters indicate treatment comparison at each time point, where different letters indicate a statistically significant intergroup difference. Uppercase letters indicate comparisons over time in the same treatment group, where different letters indicate a statistically significant intragroup difference. Values sharing the same uppercase or lowercase letter in were not significantly different. Means were compared by ANOVA (p<0.05) followed by the Tukey test for direct pair-wise comparisons. 

doi:10.1371/journal.pone.0038393.g010
affected [37,38]. Thirdly, in studies employing a PPi analog, 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP), it was found that HEBP inhibited formation of acellular cementum entirely, while cellular cementum and bone matrices were produced, but remained unmineralized [35,39,40]. A parallel pattern emerged when mineralization inhibitor matrix gla protein (MGP) was ectopically expressed in bones and teeth; bone, dentin, and cellular cementum matrices were produced yet remained unmineralized, while AEFC was absent [41]; MGP and PPi may have parallel functions as mineral regulators throughout the body. These studies indicate that cementogenesis depends heavily on creation of a physicochemical environment conducive for apposition, such as by PPi clearance.

Diminished pyrophosphate relieves the negative regulation on cementogenesis

Further evidence for PPi, as a central regulator of cementum thickness was garnered from studying models with deficient PPi, the Ank and Enpp1/2 mice. In these mice, a progressive thickening of AEFC was found during root development, which corroborated previous findings in mice harboring suspected loss-of-function mutations in these genes [42,43]. By completion of root formation, these null models exhibited 12-fold or greater AEFC vs. controls, with a significantly increased rate of apposition over the developmental time period. Importantly, we have shown this expanded cervical cementum shares the same mineral and mechanical properties as WT controls [26,44,45]. This is strong evidence that PPi is a key factor controlling acellular cementum formation, for several reasons. Firstly, ANK and NPP1 are membrane-bound proteins, which have been identified as primary regulators of extracellular PPi concentrations around mineralizing cell types, as well as elsewhere in the body. However, they operate by different mechanisms, with ANK affecting PPi transport and NPP1 acting as an ectoenzyme, producing PPi through catalysis of trinucleosides. The common link in functions of both these proteins is extracellular PPi production. That nearly identical AEFC phenotypes result from ablation of either of these genes is potent evidence for the indispensable role of PPi in influencing acellular cementum formation. Though ANK and NPP1 share similarity in function by increasing extracellular PPi, loss of NPP1 causes a more severe skeletal hypermineralization phenotype in mice, a difference possibly related to inclusion of NPP1 in matrix vesicles, whereas ANK was found to be absent in matrix vesicles [16]. It is intriguing then that loss of ANK or NPP1 had nearly identical phenotypic results on acellular cementum, a tissue where there is no clear role of matrix vesicles in mineralization.
Pyrophosphate and Acellular Cementum

Day:

\[
\begin{array}{cccccc}
\text{1} & \text{3} & \text{5} & \text{7} \\
\text{Ank} & \text{Bb} & \text{Bc} & \text{Bb} & \text{Bb} \\
\text{Enpp1} & \text{Bb} & \text{Bc} & \text{Cc} & \text{Cc} \\
\text{Opn} & \text{Bb} & \text{Bc} & \text{Bb} & \text{Bb} \\
\text{Dmp1} & \text{Bb} & \text{Bc} & \text{Bc} & \text{Bc} \\
\text{Alpl} & \text{Ba} & \text{Ba} & \text{Ba} & \text{Ba} \\
\text{Bsp} & \text{Ba} & \text{Ba} & \text{Ba} & \text{Ba} \\
\text{Col1} & \text{Ba} & \text{Ba} & \text{Ba} & \text{Ba} \\
\end{array}
\]

Relative expression/Gapdh

\[
\begin{array}{cccccc}
\text{1} & \text{3} & \text{5} & \text{7} \\
\text{Ank} & \text{Bb} & \text{Bc} & \text{Bb} & \text{Bb} \\
\text{Enpp1} & \text{Bb} & \text{Bc} & \text{Cc} & \text{Cc} \\
\text{Opn} & \text{Bb} & \text{Bc} & \text{Bb} & \text{Bb} \\
\text{Dmp1} & \text{Bb} & \text{Bc} & \text{Bc} & \text{Bc} \\
\text{Alpl} & \text{Ba} & \text{Ba} & \text{Ba} & \text{Ba} \\
\text{Bsp} & \text{Ba} & \text{Ba} & \text{Ba} & \text{Ba} \\
\text{Col1} & \text{Ba} & \text{Ba} & \text{Ba} & \text{Ba} \\
\end{array}
\]
Secondly, ANK and NPP1 are expressed in the dentoalveolar region during tooth formation and cementogenesis. While both ANK and NPP1 are widely expressed throughout the body, both were found to be selectively more highly expressed in cementoblasts lining the tooth root. Also, a special importance for PP i production in regulating cementum was indicated indirectly by findings that human PDL tissue expresses significantly higher basal levels of TNA P, ANK, and NPP1 than pulp [21,36]. This hypothesis is supported by the finding that cementoblasts dramatically increased either ANK or NPP1 expression in response to loss of the other factor, likely an attempt to compensate for lack of extracellular PP i output in these mice. The nature of the interaction between ANK and NPP1 in tooth formation is currently the subject of study in a series of double-deficient mice. That expression of these PP i regulating factors is enriched in tooth root and they are inducible in each others’ absence supports a central physiologic function for PP i in normal control of cementogenesis. The possible involvement of other complementary and antagonistic factors in PP i homeostasis in the root region is an intriguing question currently being studied. One candidate is CD73, a cell surface protein operating downstream of NPP1 which may regulate Alp i expression, and that has been linked to vascular calcification [47].

This essential role of PP i, however, seems to be limited to the cervical acellular cementum. ANK and NPP1 were not as consistently localized to regions of apical cementum, did not exhibit compensatory up-regulation in the apical portion of knock-out molars, and loss of ANK and NPP1 did not impact the phenotype of CIFC. In this respect, the cellular cementum showed a clear difference in developmental regulation from AEFC and more similarity to alveolar bone. Similarly, loss of TNA P and the resulting increased PP i, affected cellular cementum and bone in similar ways.

On the influence of pyrophosphate metabolism on cementum extracellular matrix composition

Reduced PP i not only resulted in more rapid AEFC apposition, but also led to altered cementoblast gene expression and matrix composition. OPN and DMP1, both mineral-regulating ECM proteins from the SBLING family [36], were increased at the gene and protein level in ANk and Enpp1−/− cementoblasts. Expression of Bsp, a key cementoblast marker and SBLING family member was unaltered in ANk and Enpp1−/− teeth. Bsp immunostaining indicated a diffuse presence in the thick cementum, likely diluted in relatively greater volume of the mineralized cementum. Bsp expression in cementoblasts in vitro was unaffected during mineralization or its inhibition by PP i.

OPN is a multifunctional ECM protein and a marker for cementum [48–50]. OPN has been shown in vitro to be an inhibitor of hydroxyapatite mineral crystal growth [51,52], an observation supported by study of the Opn−/− (Spp1−/−) mouse [53], as well as other models where increased OPN was found to disrupt skeletal mineralization [16,54]. We suggest that increased expression of OPN by ANk and Enpp1−/− cementoblasts represents an additional mechanism for control of apposition; like NPP1, an attempt at normalization of the cementogenesis process. While previous studies using osteoblasts have cited PP i expression as a signal increasing Opn expression [4,16], we found here that cementoblasts exposed to PP i in vitro significantly reduced Opn expression during the mineralizing phase of the experiment. The cementoblast reaction to increase OPN in response to mineralization under low PP i conditions, is opposite to that of osteoblasts, which were found to reduce OPN in ANk and Enpp1−/− mice, contributing to the bone pathology. The divergent response underscores the unique mineral metabolism of cementum.

DMP1 is highly expressed in the osteocytes embedded in bone matrix, and is associated with maintenance of the lacunar-canalicular system of these cells [55–57]. DMP1 was increased in osteocytes in loaded bone, perhaps functioning in the mechanical response [58,59]. In the context of increased cementum apposition in ANk and Enpp1−/− mice, we hypothesize that induction of Dmp1 gene expression reflects rapid apposition and embedding of cervical root cementoblasts as cementocytes. The cementoblasts that direct AEFC normally remain as lining cells adjacent to this thin tissue. In vitro, mineralizing cementoblasts also increased Dmp1 gene almost 140-fold, paralleling other studies where DMP1 was induced in periodontal ligament cells in mineralizing 3-dimensional gels [60].

The mechanism for altered Opn and Dmp1 gene and protein expression is unknown, but under investigation. Based on in vivo and in vitro data, we propose an “outside-in” type of matrix-cell signaling mechanism whereby increased cementum apposition switches on expression of Opn and Dmp1, as well as ANk and Enpp1.

Cementoblasts as pyrophosphate sensitive cells

Localization of PP i, regulators over the course of tooth development supported a role for PP i in modulating cementogenesis. The developing periodontal region shows strong immunolocalization of TNA P and high ALP activity, in effect producing a highly pro-mineralization environment. These are favorable circumstances for apposition of the cementum layer on the root dentin surface. However, a question that arises is how cementum may remain a thin and slow growing mineralized layer in such an environment permissive for mineralization. ANK and NPP1 localized most strongly to the cementoblasts of the AEFC following cementum formation, suggesting initiation of cementogenesis occurs under the influence of TNA P activity, but that after cementum deposition (usually several μm in mice), cementoblasts increase ANK and NPP1 to restrict further cementum apposition. Thus, in a scenario where either ANK or NPP1 function is lost, cementum apposition is not adequately controlled and the other is up-regulated, along with increased OPN, in an attempt to regain homeostasis of cementum.

In vitro experiments employing a cementoblast cell line provided a mechanistic platform for probing these proposed roles of ANK, NPP1, and PP i, in cementoblast mineralization and gene expres-
Figure 13. Timing of pyrophosphate removal determines cementoblast mineralization and coordinated gene expression in vitro. (A) By von Kossa staining, OCCM.30 cells cultured with 5 mM BGP produced mineral nodules by days 4, 6, and 8, while inclusion of 100 μM Pi inhibited mineralization for the entire experiment. When PPi was removed after 4 days, OCCM.30 cells began mineralizing the matrix by days 6 and 8. (B)
Quantitative calcium assay performed on days 4, 6, and 8 confirmed visual mineral nodule staining by von Kossa. (C) Mineralizing cultures (AA + BGP) increased expression of Ank, Enpp1, Opn, and Dmp1 at day 3, concurrent with mineralization. Inclusion of 100 μM PPi significantly depressed expression of Ank, Enpp1, and Opn on day 3 compared to mineralizing cultures. Removal of PPi on day 4 led to increased Ank, Enpp1, Opn, and Dmp1 on day 5, coincident with mineralization. Graphs in (B) and (C) show mean +/- SD for n = 3 samples. Lowercase letters indicate treatment comparison at each time point, where different letters indicate a statistically significant intergroup difference. Uppercase letters indicate comparisons over time in the same treatment group, where different letters indicate a statistically significant intragroup difference. Values sharing the same uppercase or lowercase letter in were not significantly different. Means were compared by ANOVA (p<0.05) followed by the Tukey test for direct pair-wise comparisons.

doi:10.1371/journal.pone.0038393.g013

![Pyrophosphate and Acellular Cementum](image)

**Figure 14. Model for the hypothesized role of in formation of acellular cementum.** Cementum apposition depends on precipitation of calcium (Ca^{2+}) and phosphate (Pi) ions on the root surface, and pyrophosphate (PPi) acts as a potent inhibitor of hydroxyapatite crystal precipitation. Local pericellular PPi concentration is controlled primarily by three cellular factors: Tissue nonspecific alkaline phosphatase (TNAP; hydrolyzes PPi to Pi), progressive ankylosis protein (ANK; regulates transport of PPi from the intracellular to extracellular space), and ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1; produces PPi from hydrolysis of nucleotide triphosphates). Cementoblasts express TNAP, ANK, and NPP1 in order to regulate PPi concentrations and to create high PPi conditions for cells. Secondly, while circulating PPi is effective in blocking mineralization in these cells, it is insufficient in blocking mineralization in these cells. Mineralization is affected by multiple factors including cellular activities, cell culture media, Ca^{2+} and Pi availability, as well as known and unknown factors present in fetal bovine serum (FBS) supplemented to the media; therefore, it is difficult to directly compare doses across studies where one or more of these variables may differ, especially cell type and time points examined [4,16]. Based on these criteria, we employed the higher dose of 100 μM PPi to create high PPi conditions for cells. Secondly, while circulating PPi levels are reported, it remains unknown how local, pericellular concentrations of PPi may vary. Biomineralization is well known to be a process dependent on compartmentalization, i.e. creation of localized, protected regions conducive to mineral precipitation. Therefore, we reasoned that cementoblasts, cells that show high in vivo and in vitro expression of PPi regulators ANK and NPP1, could potentially create localized, high PPi conditions to mediate biomineralization-related activities. This hypothesis supported using a higher exogenous dose of PPi, in order to effectively regulate OCCM.30-mediated mineralization in vitro.

**Insights into acellular and cellular cementum development and regeneration.** In mammals, the fibrous connection of the tooth to the bony socket is classified as a gomphosis, or fibrous joint, and is unique in the body in that the periodontal ligament joins bone on one side to a non-bone substance on the other side, in this case the tooth root cementum [61]. The gomphosis attachment is unique to mammals and crocodilian reptiles, having developed from more ancient forms of tooth attachment such as direct ankylosis to bone [62–65]. This interposed ligament was made possible likely by a combination of events such as alterations in the HERS during root development, changes in the supporting bone (“bone of attachment”), and the rise of a unique tissue, the cementum, as well as diminution of mineralization in the region between bones and teeth [66]. The development of a mineral-free PDL region between mineralized bones and teeth requires localized expression of factors to establish a mineralization boundary at the hard-soft tissue interface and continue to maintain PDL space throughout the life of the tooth. In these studies, we demonstrate that maintenance of that hard-soft interface at the tooth root surface...
depends on finely tuned PPi homeostasis. Intriguingly, unlike the ectopic calcification found in the joints, the PDL remained nonmineralized in the face of cementum expansion in Ank and Enpp1−/− mice, indicating the presence of additional factors that inhibit mineralization across the PDL space. These likely include multiple, redundant negative regulators of mineral growth, as well as factors that indirectly prevent mineralization by influencing cell differentiation [67].

These studies lend support to the idea that there are key differences influencing acellular versus cellular cementum development. Namely, acellular cementum is dependent on precise modulation of local PPi, whereas cellular cementum is much less sensitive to fluctuations in local PPi. The morphology, speed of formation, and ECM protein composition of acellular cementum were altered dramatically by disruption of local PPi homeostasis. Conversely, cellular cementum remained unaffected in all of these aspects. The primary cementoblasts of the AEFC adapted their expression of ANK, NPP1, and OPN in attempts to compensate for altered PPi modulation for cementum regeneration. It is especially attractive to consider such a novel approach when growth and differentiation factors used to date have been limited in terms of true cementum regeneration, PDL integration, and/or predictability [68–70]. In a preliminary proof-of-principle study, we employed the Ank−/− mouse, featuring deficient extracellular PPi, and increased cementogenesis, to analyze tissue repair and regeneration in a periodontal fenestration model [45]. Importantly, we found that Ank−/− mice featured significantly greater new cementum vs. controls, more organized mineral deposition on the root surface in the defect areas, and recapitulated expression of ANK, NPP1, and OPN in attempts to compensate for altered PPi modulation for cementum regeneration. It is especially attractive to consider such a novel approach when growth and differentiation factors used to date have been limited in terms of true cementum regeneration, PDL integration, and/or predictability [68–70].

This finding provides insight into the origins of the two main types of cementum, and also informs clinical regenerative therapies. The profound influence of PPi metabolism on acellular cementum development immediately suggests the concept of PPi modulation for cementum regeneration. It is especially attractive to consider such a novel approach when growth and differentiation factors used to date have been limited in terms of true cementum regeneration, PDL integration, and/or predictability [68–70]. In a preliminary proof-of-principle study, we employed the Ank−/− mouse, featuring deficient extracellular PPi, and increased cementogenesis, to analyze tissue repair and regeneration in a periodontal fenestration model [45]. Importantly, we found that Ank−/− mice featured significantly greater new cementum vs. controls, more organized mineral deposition on the root surface in the defect areas, and recapitulated expression patterns mapped during cementum development, including strong OPN and DMP1 in the cementum matrix, and elevated NPP1 in associated cementoblasts. Thus, in this pilot study in mice we found that reduced local levels of PPi promoted increased cementum regeneration. There has been concern voiced about regenerated cementum being the cellular type in a majority of studies [as summarized in [69]], and thus not optimal for PDL attachment. Our findings support that the cellular or acellular nature may be a reflection of the speed of formation, and that both can support sufficient extrinsic PDL fiber insertion.

Materials and Methods

Ethics Statement

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and AVMA Guidelines on Euthanasia. The protocol for all animal studies was approved by the Institutional Animal Care and Use Committee (IACUC), University of Washington, Seattle, WA (Protocols 4010-01 and 4010-03).

Mouse strains

Preparation and genotyping of mouse models was previously described for Ank−/−, Alpl−/− (previously known as Alkp2−/−), and Enpp1−/− [10,14,16,54]. Ank and Alpl mice were maintained on a mixed background of 129S1/SvImJ and C57BL/6 strains, and Enpp1 mice were maintained on a mixed background of C57BL/6×129/SvFJ strains. Mice were housed in a specific pathogen free facility in 12 hr light-dark cycles with access to water ad libitum. Ank and Enpp1−/− mice were fed a standard rodent diet, while Alpl litters were provided a vitamin B6 enforced diet to reduce seizures and prolong lifespan (TestDiet, Richmond, IN). Heterozygote breeding pairs were employed to prepare homozygote −/− mice and age-matched +/+ controls at specific ages during tooth development. Heterozygotes were examined to determine any morphological tooth phenotype. Mice were sacrificed by cervical dislocation and mandible tissues harvested. At least three control (+/+ ) and null (−/−) mandibles were examined for each age of interest.

Histology

Mouse mandibles were harvested and prepared for histology as previously described [26]. Briefly, mandibles were sagittally hemisectioned and fixed in Bouin’s solution at 4°C overnight. Hemi-mandibles were demineralized [for tissues post 8 dpn] in acetic acid/formalin/sodium chloride (AFS) solution at 4°C. Tissues were paraffin embedded after standard histological processing. Five μm buccal-lingual (coronal) serial sections of the first mandibular molar or longitudinal (sagittal) sections of hemi-mandibles were prepared by rotary microtome and mounted on charged glass slides. Slides were deparaffinized in xylene for histological analyses, including hematoxylin and eosin (H&E) staining used for morphological characterization.

Growth measurement and histomorphometry

Cervical cementum on the lingual aspect of the mesial root of the mandibular first molar was measured at a fixed distance of 300 μm from the cementum-enamel junction (CEJ) in Ank and Enpp1 +/+ and −/− sections from 14–60 dpn, using two central sections from the set of serial sections. Static histomorphometry was used to measure cervical cementum, PDL, and alveolar ridge bone width on the lingual aspect of mesial roots of mandibular first molars at the age 26 dpn. Calibrated measurements were made using SPOT software (Diagnostic Instruments, Sterling Heights, MI). ANOVA followed by the Tukey test for direct comparisons was employed for statistical testing of histomorphometric measurements (PASW (SPSS) Statistics software, version 19).

Picrosirius red stain for collagen in histological sections

Tissues processed for histology were stained with a picrosirius red staining kit according to manufacturer directions (Polysciences, Inc., Warrington, PA). Deparaffinized slides were immersed in 0.2% phosphomolybdic acid hydrate, rinsed in water, incubated in direct red 80 for 60 min, then 0.01 N HCl solution for an additional 2 min. Samples were rinsed in 75% ethanol for 45 sec, then dehydrated in xylene, cleared, and mounted with coverslips. Digital images were captured with an Optiphot-2 microscope (Nikon Instruments, Inc., Melville, NY) fitted with a light polarizer, using an EOS 5D Mark II digital camera (Canon U.S.A., Inc., Lake Success, NY).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on histological sections as previously described [26]. Primary antibodies were
used with biotinylated secondary antibodies (Vectastain Elite ABC, Vector Labs, Burlingame, CA) and color reactions were developed to a red product using a 3-aminophenyl-9-ethylcarbazole (AEC) substrate kit (Vector Labs). Positive controls included normal mouse tissues and negative controls were performed in the absence of primary antibody. Primary antibodies included: monoclonal rat anti-human ALPL/TNAP (R&D Systems, Minneapolis, MN); rabbit anti-mouse progressive ankylosis protein (ANK3) [12]; rabbit anti-mouse bone sialoprotein (BSP), (a gift from Dr. Kenny Franceschi, University of Michigan); rabbit anti-antigen [74]. Negative controls included sense probes.

SEM analyses were performed in sections from at least three (n = 3) animals for each age, with representative staining chosen for photographs shown in Results.

In situ hybridization

In situ hybridization was performed using a non-radioactive in situ hybridization (ISH) protocol employing a digoxigenin (DIG)-labeled cRNA probe for genes of interest, as described previously [26]. Linearized probes were cleaved by phenol-chloroform precipitation. Riboprobe synthesis was performed using a digoxigenin-UTP-labeled kit (Roche Applied Science, Indianapolis, IN). Probes were fractionated at 60°C and riboprobe concentrations were checked by dot blot on Hybond N+ nylon membrane (GE Healthcare, Piscataway, NJ). Messenger RNAs were labeled by incubation of depaffinized sections with NBT/BCIP (Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt). Probes used for ISH included: mouse Dmp1 plasmid (provided by Dr. Ann George, Northwestern University) [73]; mouse Dmp1 and Bsp probes (provided by Dr. Marian Young, NIH/NIDCR) [74]. Negative controls included sense probes.

Electron microscopy

Scanning electron microscopy (SEM) analyses were performed on hemi-mandibles from 20 dpn control and Alpl−/− mice as previously described [26]. Briefly, mandibles were sequentially dehydrated in aqueous ethanol solutions and mounted in room-temperature-cure epoxy (Allied High Tech Inc, Rancho Dominguez, CA). Specimens were cut using a precision wafering saw (Buehler Ltd, Lake Bluff, IL) to expose the mesial surface of the first molar. The cut surface was then ground further distally to expose the interior of the first molar using 600 then 1500 grit SiC papers, followed by smoothing via ultramicrotoming with a 45° angle diamond knife (Diatome, Inc, Hatfield, PA) fitted onto a MT 6000-XL ultra-microtome (Bal-Tec RMC, Inc, Tucson, AZ). Specimens were mounted on SEM stubs, sputter coated with 5 nm of Pt for electron conductivity (SPI Supplies Inc, West Chester, PA), and imaged by an JSM7000F (JEOL-USA, Inc., Peabody, MA) SEM operating at 15 kV in backscattering mode.

Cell culture and in vitro assays

Isolation and characterization of OCCM.30 murine cementoblasts has been previously described [75,76]. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% v/v fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin [75]. For gene expression and mineralization experiments, OCCM.30 cells were plated in standard media as described above, with media changed after 24 hrs to DMEM with 1% FBS with 50 µg/ml ascorbic acid (AA). Media were changed every 48 hrs for the remainder of the experiment. Inclusion of organic phosphate source β-glycerophosphate ([BGP; 5 mM] was used to create mineralizing conditions. Inorganic PPi (10 or 100 µM) was added to assay effects on cell function. Both BGP and PPi were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture experiments were performed at least three times in triplicate with representative results presented.

Cell proliferation was measured using a non-radioactive, MTS-based assay, following manufacturer’s directions (CellTiter 96® AQurer Proliferation Assay, Promega, Madison, WI). Absorbance was measured at 570 nm, with reference reading at 590 nm. Absorbance is proportional to the number of living cells in culture.

Production of collagen by cells in vitro was quantified by picrosirius red staining, using methods modified from previous reports [77,78]. Briefly, cells were rinsed with PBS and fixed in Bouin’s solution for 1 hr at room temperature. The fixative was removed and the plate rinsed several times in water to remove excess Bouin’s solution. The collagenous matrix in plates was stained by incubation with picrosirius red dye (Direct Red 80, Polysciences, Inc., Warrington, PA) while gently shaking. Unbound dye was removed by rinsing several times with 0.01 N HCl. Bound dye was removed by incubation and shaking with 0.1 N NaOH for at least 1 hr. Picrosirius red was quantified by reading the absorbance at 550 nm. Quantity of collagen was calibrated against a standard curve created by plating and eluting known concentrations of rat tail collagen.

Von Kossa staining for mineral nodule formation was performed using standard procedures [79]. Silver stain was visualized as black, and stain intensity indicated the amount of calcium phosphate precipitation in the cell matrix (silver ions react with phosphate). Cell mineralization in vitro was quantitatively assayed by measuring calcium deposits, using a method modified from a previous report [77]. To cell culture wells, 500 µl 0.5 N HCl was added and plates were agitated for 60 min to dissolve calcium-phosphate precipitations. Eluted calcium was measured using a calcium assay (Genzyme Diagnostics, Farmingham, MA). One µl of sample was added to 99 µl Arsenazo reagent and absorbance was read at 650 nm. Standard curves were prepared using a calcium stock solution.

A modified assay for measuring in vitro alkaline phosphatase activity (ALP) was used [80]. Briefly, cell cultures were rinsed with PBS and incubated with 200 µl p-Nitrophenyl phosphate (PNPP, Sigma) in the dark at ambient room temperature for 30 min. After incubation, 10 µl supernatant for each condition was transferred to a 96 well plate containing 90 µl of 3 N NaOH (stop solution) per well. Absorbance was recorded at a wavelength of 405 nm.

An enzymatic assay was employed to measure the activity of pyrophosphate-generating ectoenzymes (nucleoside triphosphate pyrophosphohydrolase, NTPPPHase activity), based on a previously described procedure [81]. Cells were rinsed with PBS and incubated for 2 hrs with 2.0 ml of 1 mM thymidine 5′-monophosphate p-nitrophenyl ester sodium (TMPNP) solution at 37°C and without CO2. After incubation, 20 µl supernatant for each condition was transferred to a 96 well plate containing 80 µl of 0.1 N NaOH (stop solution) per well. Absorbance was recorded at a wavelength of 410 nm.

RNA isolation and real-time quantitative RT-PCR

Isolation of RNA, synthesis of cDNA, and performance of real-time quantitative PCR was undertaken as previously described [26]. Total RNA from cells was isolated using the RNeasy Micro kit (Qiagen, Valencia, CA) and cDNA was synthesized from
1.0 μg RNA (Transcriptor kit, Roche Applied Science). PCR reactions were performed with DNA Master SYBR Green I kit (Roche Applied Science) on the Roche Lightcycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany) using intron-spanning primers (http://www.gene-expression-analysis.com/). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was employed as a housekeeping/reference gene for target gene normalization and relative quantification with amplification efficiency correction. Primer sequences used are listed in Table 1. PCR product identification was performed by post-amplification melting curve analysis. To detect intra- and intergroup gene expression differences, we employed a one-way ANOVA with post-hoc Tukey test, using PASW (SPSS) Statistics 19 software.

### References

1. Foster BL, Tompkins KA, Rutherford RB, Zhang H, Chu EY, et al. (2008) Phosphatase–known and potential roles during development and regeneration of teeth and supporting structures. Birth Defects Res C Embryo Today 84: 201–314.

2. Fleisch H, Bisaz S (1962) Mechanism of calcification: inhibitory role of pyrophosphate. Nature 195: 911–912.

3. Meyer JL (1984) Can biological calcification occur in the presence of pyrophosphate? Arch Biochem Biophys 231: 1–8.

4. Addison WN, Azati F, Sorensen ES, Kaartinen MT, McKee MD (2007) Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. J Biol Chem 282: 15872–15883.

5. Muredsh H, Harmey D, Millan JL, McKay MD, Karsenty G (2005) Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of EGM mineralization to bone. Genes Dev 19: 1093–1104.

6. Millan JL (2006) Mammalian Alkaline Phosphatases. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA. 322 p.

7. Whyte MP (1994) Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr Rev 15: 439–461.

8. Whyte MP (2002) Hypophosphatiasis Nature’s window to alkaline phosphatase in man. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of Bone Biology. 2nd ed. San Diego, CA: Academic Press. pp. 1229–1248.

9. Reibel A, Maniere M, Claus F, Droz D, Aebi M, et al. (2005) Oroental phenotype and genotype findings in all subtypes of hypophosphatiasis. Orphanet J Rare Dis 4: 4.

10. Narisawa S, Fröhlander N, Millan JL (1997) Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Dev Dyn 208: 432–446.

11. Millan J, Narisawa S, Lemire I, Loisel T, Boileau G, et al. (2008) Enzyme replacement therapy for murine hypophosphatasia. J Bone Miner Res 23: 777–787.

12. Ho AM, Johnson MD, Kingsley DM (2000) Role of the mouse ank gene in control of tissue calcification and arthritis. Science 289: 265–270.

13. Kingsley DM, Reiner RJ, Kingsley DM (2006) Biochemical and genetic analysis of ANK in arthritis and bone disease. Am J Hum Genet 79: 1017–1029.

14. Johnson K, Goding J, Van Etten D, Sali A, Hu SL, et al. (2005) Linked deficiencies in extracellular PIP5 and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. J Bone Miner Res 18: 994–1004.

15. Johnson K, Moffa A, Chen Y, Pritzker K, Goding J, et al. (1999) Matrix vesicle plasma cell membrane glycoprotein-1 regulates mineralization by murine osteoblastic MC3T3 cells. J Bone Miner Res 14: 803–892.

16. Harmey D, Heise L, Narisawa S, Johnson KA, Terkelnaa R, et al. (2004) Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: an integrated model of the pathogenesis of mineralization disorders. J Pathol 164: 1199–1209.

17. Heise L, Johnson K, Anderson H, Narisawa S, Sali A, et al. (2002) Tissue-specific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proc Natl Acad Sci U S A 99: 9445–9449.

18. Foster BL, Popowics TE, Fong HK, Somerman MJ (2007) Advances in defining regulators of cementum development and periodontal regeneration. Curr Top Dev Biol 78: 47–126.

19. Bosshardt D (2005) Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? J Dent Res 84: 390–406.

20. Diekwisch TG (2001) The developmental biology of cementum. Int J Dev Biol 45: 695–706.

21. van den Bos T, Handoko G, Nichol A, Ryan LM, Cohnb SP, et al. (2005) Cementum and dentin in hypophosphatasia. J Dent Res 84: 1021–1025.

22. Bruckner R, Riddles N, Porter D (1962) Hypophosphatiasis with premature shedding of teeth and aplasia of cementum. Oral Surg Oral Med Oral Pathol 15: 1351–1369.

23. Beertsen W, VandenBos T, Everts V (1999) Root development in mice lacking functional tissue non-specific alkaline phosphatase gene: inhibition of acellular cementum formation. J Dent Res 78: 1221–1229.

24. Feilke KN, Blair L, Silverstein J, Cohnb SP, Ryan LM, et al. (1999) Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. J Bone Miner Res 14: 2015–2026.

25. McKee MD, Nakano Y, Masica DL, Gray JJ, Lemire I, et al. (2011) Enzyme Replacement Therapy Prevents Dental Defects in a Model of Hypophosphatasia. J Dent Res.

26. Foster BL, Nagatomo KJ, Banaeimsho S, Tompkins KA, Fong H, et al. (2011) The Progressive Ankylosis Protein Regulates Cementum Apposition and Extracellular Matrix Composition. Cells Tissues Organs.

### Acknowledgments

A portion of this research was completed when BLF, FHN, ABT, and MJS were affiliated with the University of Washington School of Dentistry, Department of Periodontics (Seattle, WA, USA). The authors would like to thank Jirawan Wade for preparing histological sections, Casey Self for assistance with polarized light microscopy, and Lay Soon for assistance with histomorphometry. Thanks to Ann Rosenthal (Medical College of Wisconsin, Milwaukee, WI) for assistance with the NTUPPH activity protocol.

### Author Contributions

Conceived and designed the experiments: BLF MJS. Performed the experiments: BLF KJN HF DD ABT SN WW. Analyzed the data: BLF MJS FHN JLM. Contributed reagents/materials/analysis tools: JLM. Wrote the paper: BLF.

---

### Table 1. Real time quantitative PCR primer sequences.

| Gene Symbol | Gene Name                  | Forward (5′-3′)              | Reverse 5′-3′               | Gene ID   |
|-------------|----------------------------|-----------------------------|----------------------------|-----------|
| Apl         | Tissue nonspecific alkaline phosphatase | GGGGACATCGACATTAGTTG       | GGCCTGATTGTTGTTGAG         | 11647     |
| Ank         | Progressive ankylosis protein | GAATCGACCGGCCTCAT           | GTCGCCGATTTGTTGCT          | 11732     |
| Bsp         | Bone sialoprotein           | GAGACGGCGCAGATTTCC          | AGTGGCGCTAACTCAA           | 15891     |
| Cat1        | Collagen type 1 alpha 1     | CACCCCGGGCGGAAAAGATT       | GGGCCGGAAAGACAGGACT        | 12842     |
| Dmp1        | Dentin matrix protein 1     | GCCGGAGTAAAGAGTTA          | GTCCCCGTTGGCTACCTC         | 13406     |
| Enpp1       | Ectonucleotide pyrophosphatase phosphodiesterase | GCCACCGAGAAGACTAA        | TCATAGGGCCGCTCAT          | 18605     |
| Gapdh       | Glyceraldehyde-3 phosphate dehydrogenase | ACCACAGTTCCATGCCATCAC    | TTCACCACCGTTGGCTGTA        | 14433     |
| Ocn         | Osteopontin                | TTTACAGCCTGCACCC           | CTAGCAGTGACGGCT           | 20750     |

doi:10.1371/journal.pone.0038393.t001

---
53. Boskey AL, Spevak L, Paschalis E, Doty SB, McKee MD (1995) Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite mineralization by osteopontin. Bone Miner 22: 147–159.

54. Harrney D, Johnson KA, Zelenk J, Camacho NP, Hoylaerts MF, et al. (2006) Elevated skeletal osteopontin levels contribute to the hypophosphatemia phenotype in akp2(/−/−) mice. J Bone Miner Res 21: 1377–1387.

55. Toyosawa S, Shintani S, Fujiwara T, Oshima T, Sato A, et al. (2001) Dentin matrix protein 1 is predominantly expressed in chicken and rat osteocytes but not in osteoblasts. J Bone Miner Res 16: 2017–2026.

56. Gluhak-Heinrich J, Ye L, Bond, Eick D, Bonekamal E, et al. (2005) Osteopontin-induced formation and growth in a gelatin-gel. Bone Miner 38: 1310–1315.

57. Rios H, Ye L, Duschevich V, Eck D, Bonekamal E, et al. (2005) DMP1 is essential for osteocyte formation and function. J Musculoskeletal Neuronal Interact 5: 325–327.

58. Yang W, Lu Y, Kalajie I, Guo D, Harris M, et al. (2005) Dentin matrix protein 1 gene cis-regulation: use in osteocytes to characterize local responses to mechanical loading in vitro and in vivo. J Biol Chem 280: 20689–20690.

59. Giannobile WV, Somerman MJ (2003) Growth and amelogenin-like factors in periodontal ligament fibroblasts embedded in three-dimensional collagen gels. Cell Tissue Res 192: 116–124.

60. Ho SP, Marshall SJ, Ryder MM, Marshall GW (2007) The tooth attachment mechanism defined by structure, chemical composition and mechanical properties of collagen fibers in the periodontium. Biomaterials 28: 5238–5245.

61. Osathanon T, Giachelli C, Somerman M (2009) Immobilization of alkaline phosphatase on microporous nanofibrous fibrin scaffolds for bone tissue engineering. Biomaterials 30: 4513–4521.

62. McIntosh JE, Anderton X, Flores-De-Jacoby L, Carlson DS, Shuler CF, et al. (2002) Caiman periodontium as an intermediate between basal vertebrate ankylosis-type attachment and mammalian "true" periodontium. Microsc Res Tech 59: 449–459.

63. Abe T (2004) Observation on the ultrastructure of the cementum in a mosasaur (Caiman crocodilus). J Oral Biosci 46: 125–131.

64. Luan X, Dangar A, Ito Y, Dzurinsky R, et al. (2009) The mesosalven tooth attachment apparatus as a paradigm for the evolution of the gnathostome periodontium. Evol Dev 11: 247–259.

65. Luan X, Ito Y, Dickwisch TG (2006) Evolution and development of Hertwig’s epithelial root sheath. Dev Dyn 235: 1593–1599.

66. MacNeil R, Berry J, D’Errico J, Strayhorn C, Piotrowski B, et al. (1995) Role of osteopontin in tooth mineralization. J Dent Res 81: 817–821.

67. Fong H, Foster BL, Sarikaya M, Somerman MJ (2009) Structure and function of Ank/Ank mutant mouse dental tissues–an animal model for studying periodontal regeneration. Arch Oral Biol.

68. Giannobile WV, Somerman MJ (2003) Growth and amelogenin-like factors in periodontal wound healing. A systematic review. Ann Periodontol 8: 193–204.

69. Boshardt DD, Sculean A (2009) Does periodontal tissue regeneration really work? Periodontol 51: 208–219.

70. Esposito M, Gruven MG, Papanikolaou N, Couthard P, Worthington HV (2009) Enamel matrix derivative (Emdogain®) for periodontal tissue regeneration in intrabony defects. Cochrane Database Syst Rev: CD003875.

71. Saltveit ME, Bakketeig LS, Risnes K, et al. (2000) Mineralization of bone nodules formed in vitro: the role of alkaline phosphatase. J Dent Res 82: 315–323.

72. Takahashi N, Watanabe E, Takano Y (2002) On the origin and formation and growth in a gelatin-gel. Bone Miner 22: 147–159.

73. Ochiai Y, Iwamoto T, Koyama T, et al. (1996) Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite mineralization by osteopontin. Bone Miner 22: 147–159.

74. Yamagishi H, Sugiyama S, Takahashi N, Watanabe E, Takano Y, et al. (1996) Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite mineralization by osteopontin. Bone Miner 22: 147–159.

75. D’Errico JA, Berry JE, Ouyang H, Strayhorn CL, Windle JJ, et al. (2000) Modulation of phosphate/pyrophosphate metabolism to regenerate periodontium: a novel in vivo approach. J Periodontol.

76. Rodrigues TL, Foster BL, Silverio KG, Martins L, Casati MZ, et al. (2011) The mosasaur (Caiman crocodilus) as a model for studying periodontal regeneration. Arch Oral Biol.

77. Stüber JK, Knopf ML, Kats L, et al. (2001) Matrix Gla protein inhibition of tooth mineralization. J Dent Res 80: 839–844.

78. Tullberg-Reinert H, Jundt G (1999) In situ measurement of collagen synthesis by periodontal ligament fibroblasts embedded in three-dimensional collagen gels. Cells Tissues Organs 192: 116–124.

79. Junqueira L, Bignolas G, Brentani R (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 11: 447–455.

80. van den Bos T, Beertsen W (1998) Alkaline phosphatase activity in human periodontal ligament: age effect and relation to cementum growth rate. J Periodontal Res 33: 1–6.

81. Groeneveld MC, Everts V, Beertsen W (1995) Alkaline phosphatase activity in the periodontal ligament and gingiva of the rat molar: its relation to cementum formation. J Dent Res 74: 1374–1381.

82. Tenenbaum HC (1981) Role of organic phosphate in mineralization of bone formed in vitro. J Dent Res 60 Spec No C: 1586–1589.

83. Tenenbaum HC (1987) Levamisole and inorganic pyrophosphate inhibit beta-glyceraldehyde phosphate induced mineralization of bone formed in vitro. Bone Miner 3: 13–26.

84. Bellows CG, Heersche JN, Aubin JE (1992) Inorganic phosphate added exogenously or released from beta-glyceraldehyde phosphate initiates mineralization of osteoid nodules in vitro. Bone Miner 17: 13–29.

85. Bellows CG, Aubin JE, Heersche JN (1991) Initiation and progression of mineralization of bone nodules formed in vitro: the role of alkaline phosphatase. Organic phosphate. Bone Miner 14: 27–40.

86. Chappell IL (1993) Hypophosphatemia: dental aspects and mode of inheritance. J Clin Periodontol 20: 615–622.

87. Jayawardena CK, Takahashi N, Watanabe E, Takano Y (2002) On the origin and formation and growth in a gelatin-gel. Bone Miner 22: 147–159.