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Overlapping functions of bone sialoprotein and pyrophosphate regulators in directing cementogenesis

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Abstract

Although acellular cementum is essential for tooth attachment, factors directing its development and regeneration remain poorly understood. Inorganic pyrophosphate (PP_i), a mineralization inhibitor, is a key regulator of cementum formation: tissue-nonspecific alkaline phosphatase (*Alpl*/TNAP) null mice (increased PP_i) feature deficient cementum, while progressive ankylosis protein (*Ank*/ANK) null mice (decreased PP_i) feature increased cementum. Bone sialoprotein (*Bsp*/BSP) and osteopontin (*Spp1*/OPN) are multifunctional extracellular matrix components of cementum proposed to have direct and indirect effects on cell activities and mineralization. Studies on dentoalveolar development of *Bsp* knockout (*Bsp*^{−/−}) mice revealed severely reduced acellular cementum, however underlying mechanisms remain unclear. The similarity in defective cementum phenotypes between *Bsp*^{−/−} mice and *Alpl*^{−/−} mice (the latter featuring elevated PP_i and OPN), prompted us to examine whether BSP is operating by modulating PP_i-associated genes. Genetic ablation of *Bsp* caused a 2-fold increase in circulating PP_i, altered mRNA expression of *Alpl*, *Spp1*, and *Ank*, and increased OPN protein in the periodontia. Generation of a *Bsp* knock-out (KO) cementoblast cell line revealed significantly decreased mineralization capacity, 50% increased PP_i in culture media, and increased *Spp1* and *Ank* mRNA expression. While addition of 2 μg/ml recombinant BSP altered *Spp1*, *Ank*, and *Enpp1* expression in cementoblasts, changes resulting from this dose were not dependent on the integrin-binding RGD motif or by genetic ablation of *Ank* on the *Bsp*^{−/−} MAPK/ERK signaling pathway. Decreasing PP_i mouse background

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reestablished cementum formation, allowing more than 3-fold increased acellular cementum volume compared to WT. However, deleting *Ank* did not fully compensate for the absence of BSP. *Bsp*^{-/-}; *Ank*^{-/-} double-deficient mice exhibited mean 20–27% reduced cementum thickness and volume compared to *Ank*^{-/-} mice. From these data, we conclude that the perturbations in PP_i metabolism are not solely driving the cementum pathology in *Bsp*^{-/-} mice, and that PP_i is more potent than BSP as a cementum regulator, as shown by the ability to override loss of BSP by lowering PP_i. We propose that BSP and PP_i work in concert to direct mineralization in cementum and likely other mineralized tissues.

MeSH key words

Extracellular Matrix; Tooth Calcification; Dental Cementum; Periodontium; Odontogenesis; Bone

1. Introduction

The periodontal complex functions to attach and support the tooth, and includes cementum, periodontal ligament (PDL), and alveolar bone [1]. Cementum is a thin mineralized tissue on the root surface that is present as acellular cementum on the cervical root and cellular cementum around the root apex. Acellular cementum is essential for anchoring PDL collagen fibers to the root surface. Cementum and bone share regulatory pathways and key developmental influences as evidenced by mouse genetic studies and human disease phenotypes [2–4]. However, accumulated evidence over the past two decades has revealed that acellular cementum is hypersensitive to regulators of mineralization, apparently more so than other hard tissues of the dentition and skeleton [5–7].

The first developmental disease understood to affect cementum was hypophosphatasia (HPP; OMIM 241500, 241510, 146300), an inherited error-of-metabolism caused by loss-of-function mutations in *ALPL*, the gene that encodes tissue-nonspecific alkaline phosphatase (TNAP) [8, 9]. In HPP, loss of TNAP function results in increased concentrations of inorganic pyrophosphate (PP_i) that is a potent inhibitor of physiological and pathological mineralization. HPP causes disturbed skeletal mineralization, including rickets and osteomalacia. In the teeth, HPP appears to disturb dentin and enamel mineralization, but more severely blocks formation and function of acellular cementum in both human subjects and mouse models, leading to PDL detachment and premature tooth loss [2, 10–16].

While TNAP functions to decrease extracellular PP_i concentrations, other proteins counteract this activity. The progressive ankylosis protein (*Ank*/ANK) regulates transport of PP_i out of the cell to the extracellular space [17–19], while extracellular pyrophosphatase/phosphodiesterase 1 (*Enpp1*/ENPP1) hydrolyzes extracellular nucleotide triphosphates to produce PP_i [20, 21]. Mice lacking either ANK or ENPP1 activity (*Ank*^{-/-} and *Enpp1*^{-/-} mice, respectively) feature decreased extracellular PP_i production/secretion and are predisposed to ectopic calcification [17, 19, 22, 23]. In the absence of ANK or ENPP1, acellular cementum is dramatically increased, pointing to PP_i as a central regulator tuning cementum growth and mineralization [5–7]. Additional examples of the sensitivity of acellular cementum to disturbances in mineralization come from studies of endocrine

disorders like X-linked hypophosphatemia (XLH; OMIM 307800) [24, 25] and autosomal recessive hypophosphatemic rickets (ARHR; OMIM 241520) [26], transgenic mouse models over-expressing mineralization inhibitors like matrix gla protein [27], or administration of pharmacologic agents such as etidronate, a synthetic PP_i analog [28–30], where acellular cementum formation and function are negatively affected.

The extracellular matrix (ECM) of cementum is rich in phosphoproteins, including bone sialoprotein (BSP/*Bsp*) and osteopontin (OPN/*Spp1*), both members of the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family, ECM phosphoproteins associated with mineralized tissues [31–33]. *Bsp* mRNA is expressed constitutively by cementoblasts during root formation and BSP protein has been localized throughout the thickness of the acellular and cellular cementum layers [6, 34–38]. While BSP has been used as a marker of cementoblasts and cementum, its importance was not appreciated until detailed studies of dentoalveolar development in *Bsp*^{−/−} mice revealed significantly reduced and nonfunctional acellular cementum, resulting in dysfunctional PDL attachment and periodontal breakdown [34, 39, 40]. While the dependence of proper cementum formation on BSP is apparent, the underlying mechanisms remain elusive. Like other SIBLINGs, BSP is a multifunctional protein with several highly conserved functional motifs, including an N-terminal collagen and matrix metalloproteinase 2 (MMP2) binding domain, two to three (depending on species) highly negative polyglutamic acid (polyE) stretches thought to nucleate hydroxyapatite crystal formation, and a C-terminal arginine-glycine-aspartic acid (RGD) integrin-binding domain that can promote cell signaling, attachment, and migration [33, 41, 42]. BSP undergoes post-translational modifications including serine/threonine phosphorylation, N- and O-linked glycosylation, and tyrosine sulfation, however, is not known to undergo proteolytic processing [41, 43–45]. Phosphorylation of a serine residue adjacent to one of the polyE domains (Ser136 in rat BSP) was found to be important in the nucleation of hydroxyapatite crystal by BSP [45]. OPN, a protein closely related to BSP, has been shown to have direct inhibitory effects on mineralization, as well as indirect effects by regulating PP_i metabolism through modulation of expression of *Ank*, *Enpp1*, and *Alpl*, and by being a PP_i -responsive gene [23, 46–52].

The demonstrated potency of BSP polyE domains to promote hydroxyapatite crystal nucleation and growth [50, 53–57], coupled with the sensitivity of cementum to mineralization disturbances, suggested as one hypothesis that BSP may be directly involved in initiation and/or growth of hydroxyapatite mineral in the cementum ECM. The similarity in defective cementum phenotypes between *Bsp*^{−/−} mice and *Alpl*^{−/−} mice, that feature elevated PP_i and OPN, prompted us to examine whether BSP may be operating indirectly by modulating genes that control PP_i metabolism. We used multiple approaches to test the effects of BSP on systemic and local PP_i regulatory factors. These included *Bsp*^{−/−} mice, as well as mice deficient for both *Bsp* and *Ank*, to test whether decreased PP_i would restore cementogenesis. A novel *Bsp* knock-out cementoblast cell line was employed to analyze mechanisms in a controlled *in vitro* environment.

2. Results

2.1. Altered pyrophosphate metabolism in *Bsp*^{-/-} mice

Bsp^{-/-} mice feature acellular cementum deficiency, though not a complete absence, resulting in periodontal ligament (PDL) detachment and periodontal destruction, as we have reported previously [34, 39, 40] (Figure 1-D). To identify the effects on the periodontal structures associated with changes in P_i/PP_i regulators and other factors, we first analyzed gene expression in tooth-associated periodontal tissues by quantitative PCR (QPCR) array. At 5 dpn, out of 84 genes included in the array, no mRNA changes in *Bsp*^{-/-} vs. WT periodontia reached the threshold of ± 2 -fold difference and $p < 0.05$ (Appendix Table 1). At 14 dpn, only *Sp7* (Osterix) and *Runx2* showed significant differences in *Bsp*^{-/-} vs. WT, with 2.7-fold ($p = 0.02$) and 2.9-fold ($p = 0.02$) decreases vs WT, respectively (Appendix Table 2). By 26 dpn (at completion of root length and during cellular cementum formation), 15 genes in *Bsp*^{-/-} were significantly up-regulated and 7 genes were significantly down-regulated (>2 -fold) in comparison to age-matched WT tissues (Table 1 and Appendix Table 3). At this age, transcription factors *Runx2* and *Sp7* were both increased 3 to 6-fold ($p < 0.01$) in *Bsp*^{-/-} over WT tissues. Several altered genes indicated dysregulation in Wnt pathway signaling in *Bsp*^{-/-} mice, including *Ctnnb1* (3.8-fold), *Lrp6* (3.2-fold increase), and *Dkk1* (12.2-fold decrease). A 2.9-fold increase in *Smad4* and 6-fold increase in *Bgn* (Biglycan) implicated increased TGF- β signaling in *Bsp*^{-/-} vs. WT periodontia. Two integrins and potential ligands for the BSP RGD motif, *Itgav* and *Itga4*, were significantly down-regulated (2- and 21-fold, respectively) in *Bsp*^{-/-} compared to WT tissues.

Especially notable, 3 regulators of PP_i in bones and teeth were significantly altered ($p < 0.05$) in *Bsp*^{-/-} vs. WT periodontal tissues by 26 dpn (Figure 1E), though none were significantly altered at earlier time points. *Alpl* was increased 4.6-fold, mirroring increased plasma ALP reported previously [34]. *Spp1*, encoding osteopontin (OPN), and *Ank*, were increased 4.3-fold and 3-fold, respectively, in 26 dpn *Bsp*^{-/-} periodontal tissues. *Enpp1* did not show a significant change in expression over WT.

Plasma PP_i concentrations were significantly increased (2-fold) in *Bsp*^{-/-} compared to WT mice at 14 dpn, with no difference at 26 dpn (Figure 1F, G). ELISA performed on plasma indicated no significant difference in mean circulating OPN in *Bsp*^{-/-} vs. WT mice at both ages (Figure 1G). However, IHC showed that at both early (14 dpn) and later (26 dpn) stages of root development, OPN protein was deposited on molar root surfaces where lack of BSP prompted an acellular cementum defect (Figure 1H–K). While no differences were noted in OPN expression patterns at day 14, by 26 dpn, *Bsp*^{-/-} PDL and alveolar bone exhibited locally increased OPN immunologic staining when compared with WT tissues.

2.2. Defective mineralization by *Bsp* knock-out cementoblasts

In order to further define the mechanism(s) by which BSP deficiency promotes defective periodontal mineralization, we generated *Bsp* knock-out (KO) cementoblasts using a CRISPR nuclease approach (Figure 2A) on an established line of OCCM.30 immortalized murine cementoblasts [6, 58]. An ossicle implant assay was used to evaluate 5 *Bsp* KO clones (Appendix Figure 1A) compared to WT OCCM.30 parent cells. After 8 wks within

collagen sponges implanted subcutaneously in mice, WT cells promoted large ossicles, while *Bsp* KO cells uniformly produced much smaller ossicles (Appendix Figure 1B). Radiography and high resolution micro-CT indicated *Bsp* KO ossicles to be less mineralized than WT (Figure 2B, C), and H&E staining revealed that compared to WT, *Bsp* KO cells produced extensive lightly stained regions suggestive of the appearance of unmineralized cementoid/osteoid (Figure 2D), though other ECM components were not assayed. Quantitative analyses of micro-CT data (Figure 2E, F) confirmed that WT cells promoted a mean 15-fold larger ossicle volume with 13-fold more hydroxyapatite than *Bsp* KO cells, both statistically significant differences ($p < 0.01$).

2.3. Altered pyrophosphate metabolism and related gene expression in *Bsp* knock-out cementoblasts

Based on *in vivo* mineralization results and complete ablation of the targeted *Bsp* region in *Bsp* KO clone 2, this clone was selected for further analysis *in vitro*. Analysis of proliferation in WT and *Bsp* KO cells confirmed similar cell growth patterns up to day 6, with some alterations at later days 8–12 (Appendix Figure 1C). Further *in vitro* experiments described below were performed in the first 6 days of cell culture where no differences were observed in WT and *Bsp* KO cell numbers.

To further explore potential changes in PP_i and P_i metabolism, OCCM.30 parent cementoblasts and *Bsp* KO clone 2 (hereafter referred to as *Bsp* KO) cells were analyzed *in vitro*. Analysis of cell culture media at confluence revealed that *Bsp* KO cell media contained 50% increased mean PP_i concentrations ($p < 0.05$) and no significant change in P_i concentrations (Figure 3A, B). The P_i/PP_i ratio, sometimes used as a surrogate indicator of the favorability for mineralization, was therefore decreased ~50% in *Bsp* KO vs. WT cementoblasts (33 vs. 58, respectively), a significant difference ($p < 0.05$) (Figure 3C).

Compared to WT cementoblasts, *Bsp* KO cells exhibited the expected significant decrease ($p < 0.01$) in *Bsp* mRNA expression to nearly undetectable levels (data not shown). At baseline (DMEM with 2% FBS, 50 μ g/ml AA, and 10 mM BGP, with no recombinant proteins added), *Bsp* KO cells expressed significantly increased *Spp1* and *Ank* mRNA compared to WT, as well as significantly decreased *Enpp1* and no change in *Alpl* (Figure 3D–G). These significant differences in expression of PP_i modulating genes in *Bsp* KO vs. WT cells prompted us to further define signaling effects by treating WT and *Bsp* KO cells with 2 μ g/ml recombinant BSP (rBSP) compared to the same dose of rBSP-KAE, a recombinant protein featuring a knock-in KAE mutation to replace and inactivate the RGD integrin-binding motif implicated in cell signaling [59–61]. For further comparison, cells were also treated in parallel with a related protein, OPN, which also harbors an RGD motif [62]. This protein was added as rOPN or the mutated rOPN-KAE version. Addition of rBSP or rBSP-KAE to WT cells increased *Spp1*, *Ank*, and *Enpp1* mRNA, with no effect on *Alpl* (Figure 3D–G). Conversely, addition of rBSP or rBSP-KAE to *Bsp* KO cells effectively decreased *Spp1* and *Ank*, with no effects on *Enpp1* or *Alpl*. Addition of rOPN or rOPN-KAE to WT cells had no effect on any gene except for *Enpp1*, which posted small but significant increases. Addition of rOPN or rOPN-KAE to *Bsp* KO cells had mixed effects, decreasing *Spp1* and *Ank* (as rBSP did), with no effect on *Enpp1* or *Alpl*. Surprisingly, no differences in

treatment effects were found between the normal RGD-harboring proteins and mutated KAE proteins, regardless of cell type.

BSP signaling via the RGD domain has been reported to function via integrin induction of mitogen-activated protein kinase (MAPK) pathway [59]. Western blot of cellular proteins was employed to determine if rBSP activated MAPK signaling by analysis of phosphorylated ERK1/2 and downstream kinase p90^{RSK}. Addition of 2 µg/ml rBSP yielded no change in phosphorylation state for ERK1/2 or p90RSK between WT or *Bsp* KO cells over the course of 120 min after treatment (Figure 4).

2.4. Ablation of *Ank* incompletely rescues the cementum phenotype in *Bsp*^{-/-} mice

Ank mRNA was increased in periodontia of *Bsp*^{-/-} mice (Figure 1E). To directly test *in vivo* the question of whether reduced cementum in *Bsp*^{-/-} mice was caused by increased PP_i concentrations, we generated mice lacking both *Bsp* and *Ank*. Inactivating mutation or genetic deletion of *Ank* reduced circulating PP_i by more than 50% [46] and reduced *in vitro* concentrations of extracellular PP_i in osteoblasts or fibroblast cultures by 25–50% [19, 23]. Loss of ANK function and the resulting significant decrease in PP_i encouraged rapid acellular cementum formation compared to WT mice [6, 7].

Genetic ablation of *Ank* on the *Bsp*^{-/-} mouse background proved capable of restoring acellular cementum formation on the molar tooth root (Figure 5A–D and Appendix Figure 2). By 9 dpn, increased acellular cementum deposition was visible in *Bsp*^{-/-}; *Ank*^{-/-} mice (Figure 5E and Appendix Figure 2A–D). At 26 dpn, double-deficient *Bsp*^{-/-}; *Ank*^{-/-} mice exhibited significantly reduced buccal (cheek side) acellular cementum (47%, *p* < 0.05) compared to *Ank*^{-/-} mice (Figure 5F). Likewise, at 60 dpn, *Bsp*^{-/-}; *Ank*^{-/-} mice featured significantly reduced buccal acellular cementum (31%, *p* < 0.001) compared to *Ank*^{-/-} mice (Figure 5G). However, measurements on lingual (tongue side) molar aspects at 26 and 60 dpn did not indicate differences. Because 2D histological measurements did not provide clear and conclusive results, high resolution micro-CT 3D analysis on 60 dpn first mandibular molars (Figure 5H–M) was performed. *Bsp*^{-/-} mouse acellular cementum could not be analyzed in this fashion due to its severe deficiency, which was well below the working resolution of the micro-CT scanner. Analysis of acellular cementum (segmented to separate it from underlying dentin) confirmed that compared to *Ank*^{-/-} mice, molars from *Bsp*^{-/-}; *Ank*^{-/-} mice had a significant 27% reduction in acellular cementum thickness (*p* < 0.05) and 20% reduction in acellular cementum volume (*p* < 0.01) (Figure 5N, O). Mean acellular cementum mineral density was increased in *Ank*^{-/-} mice vs. WT (6%) and *Bsp*^{-/-}; *Ank*^{-/-} mice (4%), though these differences were not significant (Figure 4P). Cellular cementum volume was significantly increased in both *Ank*^{-/-} (77%) and *Bsp*^{-/-}; *Ank*^{-/-} (64%) mice compared to WT (*p* < 0.001) (Figure 5Q).

Ank^{-/-} mice feature extensive “cementicles,” mineralized nodules extending from the normally smooth cervical cementum surface into PDL space, on the buccal aspect and sometimes extending further into spaces between molars when visualized in 3D by micro-CT (Appendix Figure 3A–C). While *Bsp*^{-/-}; *Ank*^{-/-} mice featured a 73% reduction in mean cementicle volume vs. *Ank*^{-/-} mice, this difference did not reach statistical significance due to high variation and small sample sizes (Appendix Figure 3D).

Other parameters were compared by micro-CT analysis in WT, *Ank*^{-/-}, and *Bsp*^{-/-}; *Ank*^{-/-} mouse molars. There were no differences between any groups in cellular cementum thickness or density (Appendix Figure 4A, B). No differences in dentin or enamel volumes or densities were noted in *Ank*^{-/-} or *Bsp*^{-/-}; *Ank*^{-/-} vs. WT mice (Appendix Figure 4C–F).

Notably, the periodontal destruction that manifests in *Bsp*^{-/-} mice was averted in double-deficient mice where PDL attachment was reestablished by restoring a layer of functional acellular cementum (Figure 6A–P) which was significantly thicker than acellular cementum in WT mice. This was not likely due to direct effects on osteoclasts, as numbers of tartrate-resistant acid phosphatase (TRAP) positive osteoclast-like cells on alveolar bone surfaces were not different between *Bsp*^{-/-} and *Bsp*^{-/-}; *Ank*^{-/-} mice at the time points tested (Figure 6Q).

3. Discussion

Acellular cementum is essential for proper attachment and function of teeth. Bone sialoprotein (BSP) is a major component of the extracellular matrix (ECM) of cementum and has been shown to be essential for acellular cementum growth and function in mice [34, 39, 40]. Here we demonstrate that genetic ablation of *Bsp* in mice causes perturbations in plasma levels of the mineralization inhibitor, inorganic pyrophosphate (PP_i), and alters mRNA expression of PP_i regulators, *Alpl*, *Spp1*, and *Ank*, within periodontal tissues. Generation of a *Bsp* knock-out (KO) cementoblast cell line revealed significantly decreased mineralization capacity in these cells, alterations in PP_i levels in culture media, and changes in PP_i regulators, *Spp1* and *Ank*, that are consistent with changes observed in *Bsp*^{-/-} mice *in vivo*. While addition of 2 µg/ml recombinant BSP altered *Spp1*, *Ank*, and *Enpp1* in WT cementoblasts, gene changes at this dose were not dependent on the RGD integrin-binding domain or MAPK/ERK signaling. Decreasing PP_i levels by genetic ablation of *Ank* on the *Bsp*^{-/-} mouse background reestablished acellular cementum formation to root surfaces and prevented the periodontal destruction found in between 14 and 26 days post birth in *Bsp*^{-/-} mice. However, in terms of cementum thickness and volume, reducing PP_i below normal levels by ablation of *Ank* did not fully compensate for the absence of BSP. This novel finding on the antagonistic functions of PP_i and BSP provides two important insights. First, though there is a transient perturbation in PP_i metabolism in *Bsp*^{-/-} mice, reversal of this disturbance coincides with lack of complete correction of cementum, supporting a separate role for BSP other than primarily as a driver of PP_i. Second, PP_i is more potent than BSP as a cementum regulator, as shown by the ability to override loss of BSP by lowering PP_i through *Ank* knock-out. Based on these observations, we propose that BSP and PP_i work in concert in directing mineralization of cementum and, therefore, possibly other mineralized tissues.

3.1. Intersecting roles of PP_i and BSP in mineralization of cementum

Bsp^{-/-} mice feature deficient and defective acellular cementum, resulting in PDL detachment and periodontal breakdown [34, 39, 40]. The demonstrated function of BSP polyE domains to promote hydroxyapatite crystal nucleation and growth [50, 53–57], coupled with the previously established sensitivity of cementum to mineralization

disturbances [2, 5–7, 10–16, 24–30], suggested as one hypothesis that BSP may be directly involved in initiation and/or growth of hydroxyapatite mineral in the cementum ECM. The cementum defect in *Bsp*^{−/−} mice strongly resembles a similar deficiency in the *Alpl*^{−/−} mouse model of HPP, prompting an alternative hypothesis, that like fellow SIBLING OPN, BSP could be signaling via its RGD motif to affect changes in PP_i metabolism. Feedback links between P_i, PP_i, and SIBLINGs have been reported previously. Extracellular P_i regulates *Spp1*/OPN expression in osteoblast and cementoblasts [63–66], while PP_i also increases *Spp1*/OPN in osteoblasts [22, 52], in part through MAPK signaling. Administration of P_i was also reported to affect *Bsp*, dentin matrix protein 1 (*Dmp1*), and numerous other genes in cementoblasts [66, 67]. An expanded role for P_i signaling in dental and skeletal mineralization has been further suggested by recent work showing that P_i induced formation and influenced protein composition of matrix vesicles in odontoblasts and osteoblasts [68]. *Enpp1* has been linked to differentiation in osteoblasts, including association of RUNX2 with the *Bsp* promoter [69]. In addition to its role in decreasing PP_i, TNAP was also shown to dephosphorylate OPN, rendering it less able to inhibit mineralization [51].

We previously found elevated ALP in *Bsp*^{−/−} mice during development [34, 40]. In the present study, we found further evidence that BSP is involved in regulating expression of PP_i-associated genes. First, circulating levels of PP_i showed disruptions in *Bsp*^{−/−} mice at some ages. Second, local expression of *Alpl*, *Spp1*, and *Ank* were disrupted in periodontal tissues. Third, PP_i levels and gene regulators were similarly disrupted in *Bsp* KO cementoblasts, *in vitro*. Fourth, addition of recombinant BSP (rBSP) affected gene levels of PP_i regulators in both control and *Bsp* KO cementoblasts. Despite these affirmative findings, two important observations generated in this study argue against PP_i being the primary driver for cementum pathology. First, *in vivo* alterations in *Alpl*, *Spp1*, and *Ank* were not found by 14 dpn, a time point when acellular cementum is already significantly decreased in *Bsp*^{−/−} mice [34]. Second, reduction in PP_i through *Ank* ablation allowed reestablishment of cementum formation in mice, but with a sustained and significant deficit in cementum thickness and volume in the absence of BSP (compared to *Ank*^{−/−} controls). These findings support a model where PP_i and BSP may work in concert directing cementogenesis. While we draw the conclusion that PP_i is not directly responsible for all cementum defects in *Bsp*^{−/−} mice, the observed disturbances in PP_i metabolism cannot be wholly ruled out as contributing to phenotypic changes in dentoalveolar and skeletal tissues, such as hypomineralization of alveolar and long bones.

Could BSP be affecting cementum and other tissues by alternative signaling mechanisms? Use of QPCR array identified alterations in tooth and bone-associated transcription factors (*Runx2*, *Sp7*). The potential role of *Runx2* in cementogenesis remains unclear [70], while *Sp7*/osterix was shown to be involved in cellular cementum formation, but knock-out or over-expression had no measureable effect on acellular cementum formation [71]. *Phospho1* mRNA was significantly down-regulated in *Bsp*^{−/−} mouse periodontia at 26 dpn. PHOSPHO1 is a phosphatase indicated to be important in matrix vesicle initiated mineralization of bone and mantle dentin [72–77]. In recent studies on periodontal development in *Phospho1*^{−/−} mice, we found that while alveolar bone and cellular cementum displayed mineralization defects, acellular cementum development appeared unperturbed

and no periodontal dysfunction was evident up to 3 months of age [78]. The implications of down-regulated *Phospho1* mRNA remain unclear, though it is intriguing that loss of BSP causes increased expression of *Alpl*, a critical positive regulator of acellular cementum formation [6, 15, 79].

QPCR array identified numerous alterations in genes associated with Wnt and TGF β signaling pathways (*Ctnnb1*, *Lrp6*, *Dkk1* and *Smad4*, *Bgn*, respectively) in *Bsp*^{-/-} vs. WT periodontal tissues. These changes are intriguing, especially because Wnt and TGF β signaling have both been implicated in cementogenesis [80–84]. However, like the changes in PP_i-associated genes, these were not identified until 26 dpn, after the primary cementum defect in *Bsp*^{-/-} had been evident for some time. In fact, by this age, *Bsp*^{-/-} mice exhibit PDL detachment and disorganization, junctional epithelium down growth, alveolar bone resorption, and beginnings of periodontal breakdown [34]. It is entirely possible that these transcriptional changes are in response to periodontal tissue changes rather than the cause of changes- a feedback loop reflecting tissue damage or attempts at healing.

Additional *in vivo* and *in vitro* findings can be considered in this context. Addition of 2 μ g/ml of rBSP to OCCM.30 cementoblasts altered gene expression of target genes, however signaling resulting from this dose did not appear to be through RGD-integrin binding (due to lack of phosphorylation of ERK and P90RSK), as has been demonstrated previously in osteoblasts [85]. In this context and at the dose used, the MAPK signaling pathway was not implicated in BSP signaling. Previous studies have also indicated that BSP can influence cell attachment and migration through RGD-independent mechanisms [86, 87].

3.2. Evolving roles of SIBLING proteins in cementogenesis

The SIBLING family comprises ECM phosphoproteins associated with mineralized tissues of the skeleton and dentition, including BSP, OPN, dentin matrix protein 1 (DMP1), dentin sialoprotein (DSP), dentin phosphoprotein (DPP), and matrix extracellular phosphoprotein (MEPE) [31–33]. DMP1, DSP, and DPP are important for dentinogenesis [88, 89], and DMP1 was also shown to regulate mineral metabolism via fibroblast growth factor 23 (FGF23)-mediated effects on P_i handling [90–92]. Loss of either *Dspp* or *Dmp1* in mice causes changes in acellular cementum, however it remains unclear if this is due to a direct role of the associated proteins in cementogenesis, or indirect effects of wider changes in the periodontium or mineral metabolism [26, 93–95]. While MEPE has been shown to regulate skeletal mineralization, its role in dental development has only been recently investigated, implicating the protein in dentinogenesis and amelogenesis, whereas its role in cementum formation remains undefined [96].

BSP and OPN have long served as markers for cementoblasts and cementum [35–37, 97–99], and the potential link between BSP and related protein OPN deserves special mention. OPN has been shown to have direct inhibitory effects on mineralization via the negative charges from its many phosphorylated serine residues [50, 100–102]. OPN also exerts indirect effects on mineralization by regulating PP_i metabolism through expression of *Ank*/ANK, *Enpp1*/ENPP1, and *Alpl*/TNAP [46, 47], and has been implicated in cell migration, attachment, and signaling through its RGD domain [103–108]. Loss of BSP increased *Spp1*/OPN in the periodontium and addition of rOPN affected expression of *Ank* in *Bsp* KO

cells and *Enpp1* in WT cementoblasts, *in vitro*. The potential role of OPN in directly inhibiting cementum mineralization, or itself regulating PP_i-associated genes, is worth considering but difficult to evaluate in the context of *Bsp*^{-/-} mice. Expression of *Spp1* did not increase until 26 dpn, though OPN protein was present on root surfaces at earlier ages. Crossing of *Bsp*^{-/-} and *Spp1*^{-/-} mice to create double-deficient mice is not possible due to the close proximity of these genes on mouse chromosome 5 [33]. Therefore, we are currently evaluating tooth development in *Spp1*^{-/-} mice, where we will also genetically alter PP_i by creating *Spp1*^{-/-}; *Alpl*^{-/-} and *Spp1*^{-/-}; *Ank*^{-/-} mice to test for OPN functions in periodontal tissues in normal, high, and low PP_i conditions (manuscript submitted). Potential interactions, compensations, or antagonistic functions of BSP and OPN may be addressed by other approaches in the future.

3.3. Conclusions

The *Bsp* KO cementoblasts created in this study appear to recapitulate effects of developmental *Bsp* ablation in mice, namely, significantly reduced capacity for mineralization similar to defects and delays in cementum and bone [34, 39, 40]. Here we have analyzed one of these clones (*Bsp* KO clone 2) in depth in terms of gene expression and response to rBSP and rOPN. In ongoing work following on the QPCR gene expression changes noted *in vivo*, the *Bsp* KO cementoblast lines will serve as valuable tools to identify alternative mechanisms for BSP signaling, such as by identifying transcriptomic changes by RNA sequencing. This approach will enable further studies on the potential feedback loop between BSP and PP_i-associated and other factors. The possibility that BSP is directly modulating cementum mineralization is also part of an ongoing study that will employ *in vivo* and *in vitro* transgenic approaches.

While the data generated here demonstrate overlapping roles for BSP and classic PP_i regulators in guiding cementum formation, and add new insights to the previous literature on links between P_i, PP_i, and SIBLING proteins, several limitations and unanswered questions remain. These include not yet identifying a specific signaling mechanism for BSP on cementoblasts, use of a single *Bsp* KO clone for *in vitro* signaling studies, and limitations inherent in cell culture approaches for understanding complex developmental processes. Additional studies are warranted to confirm whether feedback between BSP and PP_i regulators are direct, indirect, or coincidental, and identify more precisely the functions(s) of BSP in dental and skeletal development. Continued studies targeting the functional domains of BSP or conditionally ablating *Bsp* in tissue or time-specific fashion are anticipated to elucidate the mechanism(s) underlying the BSP-PP_i link and the importance of BSP in cementogenesis.

4. Experimental procedures

4.1. Animals

Animal procedures were performed in accordance with guidelines of the Animal Care and Use Committee (ACUC), National Institutes of Health (NIH; Bethesda, MD, USA). Preparation and genotyping of *Bsp* knock-out (*Bsp*^{-/-}) and *Ank*^{-/-} mice were described previously [6, 34, 109]. Mice were maintained on a mixed 129/CD1 background.

Heterozygote breeding pairs were employed to prepare homozygous *Bsp*^{-/-} and *Ank*^{-/-} mice, and double heterozygotes were used to generate mice ablated for both *Ank* and *Bsp* (*Bsp*^{-/-}; *Ank*^{-/-} mice), and littermate wild-type (WT) controls. After weaning, mice were provided a soft gel diet (Diet Gel 31M, Clear H₂O, Portland, ME, USA) to reduce incisor malocclusion noted in homozygous *Bsp*^{-/-} mice. Mandibles were harvested for analysis at ages 5, 9, 14, 26, and 60 days postnatal (dpn) with n=3–9 mice per genotype, unless otherwise noted.

4.2. *In vivo* gene expression analyses

For quantitative polymerase chain reaction (QPCR) array analysis of periodontia from *Bsp*^{-/-} and WT mice (5, 14, and 26 dpn, n=3 per genotype), first molars were removed from mandibles using 15c scalpel blades under a dissecting microscope. Full thickness mucoperiosteal flap was reflected to permit visualization of bone. Alveolar bone was removed to expose periodontal ligament (PDL) surrounding the root surface. Collected PDL samples were stored in *RNAlater* (Thermo Fisher Scientific, Waltham, MA, USA) at -80°C. Total RNA was extracted from the PDL using the RNeasy Micro kit (Qiagen, Germantown, MD, USA) and cDNA was synthesized by RT² PreAMP cDNA Synthesis Kit (Qiagen). Samples were analyzed for expression of 84 genes involved in differentiation, ECM, adhesion, and mineralization using a designer PCR array platform (#CAMP12364F) from SABioscience/Qiagen. Target gene expression was normalized to 5 housekeeping genes, and positive and negative PCR controls were included in each PCR plate. PCR array reactions were performed on the LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) following manufacturer's recommendations. Analysis of fold-changes in gene expression was performed as described previously [39], using RT² Profiler PCR array web portal (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

4.3. Blood biochemistry and assays

Mice (n=4–8 per genotype) were anesthetized with ketamine-xylazine and blood was collected in lithium heparin gel tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) by cardiac puncture. Plasma were separated by centrifugation at G-force for 10 min at room temperature, aliquoted and were stored at -80°C. Measurement of plasma pyrophosphate (PP_i) concentration followed the protocol described previously [110, 111]. In brief, plasma were heated at 65°C for 10 min, then 10 µl of plasma (in triplicate) were diluted 4 times with autoclaved distilled water and each sample was prepared in triplicate. Samples were assayed for PP_i by differential absorption on activated charcoal of UDP-D-[6-3H] glucose (Amersham Pharmacia, Piscataway, NJ, USA) by measuring the radioactivity in Ecolume (MP Biomedicals, Solon, OH, USA) [112]. Measurement of plasma OPN concentration employed a mouse/rat specific osteopontin ELISA kit (R&D Systems, Minneapolis, MN, USA) that was used according to manufacturer's instructions.

4.4. Targeted knock-out of *Bsp* in cementoblasts

OCCM.30 immortalized murine cementoblasts have been well-characterized for their gene expression profile and ability to promote mineralization *in vitro* and *in vivo* [6, 58]. Genetic deletion of *Bsp* in OCCM.30 cells was achieved using CRISPR/Cas9 system (Applied StemCell, Menlo Park, CA, USA). The deletion region was designed to cover 8 bp on exon

1, and the entirety of exons 2 (51 bp) and 3 (78 bp), generating a frame-shift deletion by precise end joining (Figure 2A). OCCM.30 parent cells displayed 50–70% transfection efficiency and were transfected with a dual gRNA vector containing mIbsp.g17 and mIbsp.g33, known to exhibit a low off-target profile. After puromycin (5 µg/ml) selection, paternal and maternal alleles were separated using TOPO vector cloning. Five independent *Bsp* knock-out (KO) clones were identified and confirmed by sequencing.

4.5. *In vivo* ossicle implant model

An ossicle implant model was employed to assay the effect of *Bsp* ablation on the ability of OCCM.30 cells to promote mineral nodule formation, *in vivo*. Parent OCCM.30 (WT) cells and n=5 *Bsp* KO clones were used. Procedures for seeding and implantation have been reported previously [113, 114]. In brief, collagen-elastin sponges (Zimmer, Carlsbad, CA, USA) were cut into 5 × 5 × 5 mm blocks and incubated in Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L glucose, L-glutamine, 110 mg/L sodium pyruvate, with phenol red dye; 11995-065, Invitrogen, Carlsbad, CA, USA) media for 30 min. Media were removed and 2×10⁶ cells (WT or *Bsp* KO) were suspended in 15 µl DMEM, seeded into each collagen block, and implanted into subcutaneous pouches along the back of SCID Hairless Congenic (SHCTM) mice (n=3 per cell genotype). Control implants included cell-free collagen sponges. Implants were retrieved at 8 wks and fixed overnight in 10% neutral buffered formalin.

4.6. Histology

Tissues were fixed in Bouin's solution and demineralized in AFS (acetic acid, formaldehyde, sodium chloride), as previously described [35]. Mandibles were processed and embedded for paraffin serial sectioning to collect coronal (buccal-lingual) sections of 5 µm in thickness. Fixed ossicle implants were demineralized in 10% neutral buffered formalin and hemisected for paraffin serial sectioning in 5 µm as well. Hematoxylin and eosin (H&E) staining was performed for morphological observations and histomorphometry.

Immunohistochemistry (IHC) was performed as described previously [34] using an avidin-biotinylated peroxidase (ABC) based kit (Vectastain Elite, Vector Labs, Burlingame, CA, USA) with a 3-amino-9-ethylcarbazole (AEC) substrate to produce a red-brown product. Primary antibody included rabbit polyclonal LF-175 rabbit anti-mouse osteopontin (OPN), which was used at 1:200 dilution with no antigen retrieval [35]. Secondary antibody (included in the ABC kit) was goat anti-rabbit IgG. Negative controls included slides processed through the same protocol except lacking primary antibody addition.

4.7. Histomorphometry

Static histomorphometry was used to measure acellular cementum thickness in coronal sections at 9, 26, and 60 dpn (n=3–9 per genotype, unless otherwise noted). From serial sections, central sections of the mesial root of the first mandibular molar were used for measurement. Leica digital imaging hub (version 4.0.9; Leica Biosystems, Buffalo Grove, IL, USA) or imageJ (version 1.51k; National Institutes of Health, Bethesda, MD, USA) was used to make calibrated measurements. Measurements of acellular cementum were made at fixed distances of 100 µm from the cementum-enamel junction (CEJ) for 14 dpn tissues and

400 μm from CEJ for 26 and 60 dpn tissues, on both buccal and lingual aspects of the tooth root.

4.8. Radiography and microcomputed tomography (micro-CT)

Implants were scanned in a cabinet x-ray (Faxitron Bioptics, LLC, Tucson, AZ, USA) at 30 kV for 40 sec for qualitative analysis, and scanned for quantitative analysis in a μCT 50 (Scanco Medical, Bassersdorf, Switzerland) at 70 kVp, 76 μA , 0.5 Al Filter, 300 ms integration time, and 10 μm voxel size. DICOM images were uploaded to AnalyzePro 1.0 (AnalyzeDirect, Overland Park, KS, USA) and calibrated to a standard curve of 5 hydroxyapatite (HA) standards of known densities ($\text{mg HA}/\text{cm}^3$). Implants were segmented into 4 density groups: 200–400, 400–600, 600–800, and greater than 800 mg/cm^3 HA. These were analyzed for average volume and density as a group and individually. Fixed hemi-mandibles from 60 dpn mice were scanned in 70% ethanol on μCT 50 using the same parameters described above, except for 2 μm voxel size. DICOM images were uploaded to AnalyzePro 1.0 (AnalyzeDirect) and oriented to show 3D surface views or 2D cut planes. Dentin was segmented at 650 mg/cm^3 HA, and enamel was segmented at 1600 mg/cm^3 HA. For segmentation of cementum, a median filter with a kernel size of 11 was employed. Cementum was segmented between 400–950 mg/cm^3 HA with manual corrections to exclude predentin and less dense dentin in regions away from dentin borders. The median filter tracing layer was used as a mask and final segmentation of the cementum was defined as above 650 mg/cm^3 HA on the original object. For volume analysis, each root (mesial and distal) was divided into thirds based on the total length measured from cementum-enamel junction (CEJ) to the most apical tip. The most cervical 2/3 of the root length was defined as acellular cementum and the most apical 1/3 of the root length was defined as cellular cementum. This ratio was guided by previous histological reports as well as pilot studies comparing cementum histology to micro-CT images, using landmarks including cementocyte lacunae and the expanded contour of cellular cementum on root surfaces. To measure acellular cementum thickness, 50 slices (100 μm) were selected 1/3 of the CEJ-apex distance. To measure cellular cementum thickness, 50 slices (100 μm) were selected 100 μm from the root apex. These regions were chosen for being well within the borders of the respective acellular and cellular cementum layers, with consistent landmarks used between samples. Thickness was determined using cortical thickness algorithms.

4.9. Cell culture

WT and *Bsp* KO OCCM.30 cells were maintained in DMEM with 10% v/v fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (all reagents from Invitrogen, Carlsbad, CA, USA). For *in vitro* gene expression experiments, cells (8×10^4 cells/well, 12-well tissue culture treated plate) were seeded in standard media as described above. After 24 hrs, media were changed to DMEM with 2% v/v FBS and 50 $\mu\text{g}/\text{ml}$ ascorbic acid (AA). At confluence (day 0), media were changed to include 10 mM β -glycerophosphate (BGP) for the remainder of the experiment.

4.10. Cell proliferation assay

For proliferation experiments, 1×10^4 cells/ml were plated in DMEM with 10% v/v FBS, antibiotics, and L-glutamine. After 24 hrs, media were changed to DMEM with 2% v/v FBS

and antibiotics and L-glutamine for the remainder of the experiment, and media were changed every 2–3 days. Cell proliferation was analyzed using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] based colorimetric assay, following manufacturer's instructions (Promega, Madison, WI, USA). Absorbance was measured at 490 nm using a 96-well plate reader.

4.11. Inorganic phosphate and pyrophosphate analysis, *in vitro*

For inorganic phosphate (P_i) and inorganic pyrophosphate (PP_i) assays, cells were plated as described above until they reached confluence. Media were removed, and cells were rinsed with Opti-MEM (Invitrogen) and plated in Opti-MEM (approximately 1.05 mM sodium phosphate monobasic) without added FBS, antibiotics, or L-glutamine, for 24 hrs before collection of media for analysis. Phosphate assay colorimetric kit (ab65622) and pyrophosphate assay fluorometric kit (ab112155) were used to measure P_i and PP_i concentrations, respectively, in culture media following manufacturer's directions (Abcam, Cambridge, MA, USA). Absorbance for P_i was recorded at 650 nm and fluorescence for PP_i was measured at excitation/emission of 316/456 nm using a 96-well plate reader. Concentrations were calculated using standard curves ($r > 0.98$).

4.12. Addition of recombinant proteins, *in vitro*

Experiments were performed with or without addition of recombinant BSP or OPN proteins (rBSP, rOPN), or with rBSP-KAE or rOPN-KAE (RGD integrin-binding sequence mutated to KAE). Recombinant proteins were purified from conditioned media of human bone marrow stromal cells infected with adenoviruses, as described previously [115, 116]. Doses for recombinant BSP proteins were based on dose ranges used in previous reports [87, 115, 117] and included 0.02, 0.2, and 2 $\mu\text{g/ml}$ rBSP, where all doses showed biological activity on expression of target genes (data not shown). Experiments with rBSP, rBSP-KAE, rOPN, and rOPN-KAE were performed with a dose of 2 $\mu\text{g/ml}$ recombinant protein for all treatments. Recombinant proteins were added for 24 hrs prior to RNA isolation.

4.13. Quantitative polymerase chain reaction

Isolation of total RNA, synthesis of cDNA, and performance of QPCR was performed as previously described [39]. PCR primers used included: *Alpl* (NM_007431.3): F- GGGGACATGCAGTATGAGTT and R- GGCCTGGTAGTTGTTGTGAG; *Spp1* (NM_001204201.1): F- TTTACAGCCTGCACCC and R- CTAGCAGTGACGGTCT; *Ank* (NM_020332): F- GAATCAGTCGGCCCAT and R- GTTCGCCAGTTTATTGCT; *Enpp1* (NM_001308327): F- CGCCACCGAGACTAAA and R- TCATAGCGTCCGTCAT; *Gapdh* (NM_001289726): F- ACCACAGTCCATGCCATCAC and R- TCCACCACCCTGTTGCTGTA.

4.14. Western blot analysis

For cellular protein analysis by Western blot, WT and *Bsp* KO cells were treated with rBSP for up to 120 min. Total cellular proteins were extracted from washed cells using M-PER mammalian protein extraction kit (#78501, Thermo Scientific, MA, USA) according to the manufacturer's recommendations. EDTA-free HaltTM protease inhibitor cocktail (Thermo

Scientific) was included to prevent protein degradation during the extraction process. Protein concentration was determined by the Bradford colorimetric assay (Thermo Scientific). 20 µg of total protein was used for SDS-PAGE in a 4% to 12% (w/v) polyacrylamide gel (Invitrogen, Grand Island, NY, USA), then transferred to a nitrocellulose membrane. An antibody cocktail targeted to the AKT/MAPK signaling pathway, including rabbit anti-mouse/human ERK1/2 and p-P90RSK, was used according to manufacturer directions (ab151279, Abcam, MA, USA) at a dilution of 1:250. IRDye secondary antibodies (LI-COR, Lincoln, NE, USA) were used. Detection was performed using a digital imaging system (ODYSSEY CLx-LI-COR).

4.15. Statistical analysis

Results for histomorphometry, *in vivo* ossicle assay, *in vitro* assays, and micro-CT analyses are expressed as mean ± standard deviation (SD). Data were analyzed using Student's (independent samples) *t*-test or one-way analysis of variance (ANOVA) with post-hoc Tukey test for multiple comparisons, where *p*-values < 0.05 were considered statistically significant. Statistical analyses were computed using GraphPad Prism 6.00 (GraphPad Software, La Jolla, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SIBLING	Small Integrin-Binding Ligand N-linked Glycoprotein
BSP	bone sialoprotein
OPN	Osteopontin
TNAP	tissue-nonspecific alkaline phosphatase
PP_i	inorganic pyrophosphate
ANK	progressive ankylosis protein
ENPP1	ectonucleotide pyrophosphatase phosphodiesterase 1
KO	knock-out
PDL	periodontal ligament
HPP	hypophosphatasia

ECM	extracellular matrix
RGD	arginine-glycine-aspartic acid
Poly-E	polyglutamic acid
DMEM	Dulbecco's Modified Eagle Medium
qPCR	quantitative polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay

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Highlights

- Bone sialoprotein knockout (*Bsp*^{-/-}) mice feature a 2-fold increase in circulating pyrophosphate (PP_i) and altered mRNA expression of PP_i regulators *Alpl*, *Spp1*, and *Ank* in the periodontia
- *Bsp* knock-out cementoblasts exhibit significantly decreased mineralization capacity and increased PP_i in culture media
- Addition of recombinant BSP altered *Spp1*, *Ank*, and *Enpp1* mRNA expression in cementoblasts, *in vitro*
- Decreasing PP_i by genetic ablation of *Ank* on the *Bsp*^{-/-} mouse background reestablished cementum formation but did not fully compensate for the absence of BSP
- Perturbations in PP_i metabolism are not solely driving the cementum pathology in *Bsp*^{-/-} mice and PP_i is more potent than BSP as a cementum regulator

Figure 1. Altered gene expression and pyrophosphate metabolism in *Bsp*^{-/-} mice
 Compared to normally developing mandibular first molar acellular cementum (AC) in (A, B) WT mice, molars in (C, D) *Bsp*^{-/-} mice exhibit AC deficiency (red *) and periodontal ligament (PDL) detachment (red #). (E) QPCR array performed on (n=3) periodontal tissues from *Bsp*^{-/-} vs. WT mice at 26 dpn identifies increased ($p < 0.05$ by *t*-test) *Alpl*, *Spp1*, and *Ank* mRNA. (F) Plasma pyrophosphate (PP_i) levels are increased significantly ($p < 0.05$ by *t*-test) in (n=4–8) *Bsp*^{-/-} vs. WT mice at 14 but not 26 dpn. (G) No significant differences are observed in circulating OPN levels in (n=5) *Bsp*^{-/-} vs. WT mice at 14 and 26 dpn ($p > 0.05$ by *t*-test). Immunohistochemistry for OPN reveals normal distribution in (H, I) WT AC, PDL, and alveolar bone (AB) at 14 and 26 dpn, while (J, K) *Bsp*^{-/-} mouse molars feature OPN at the sites of defective AC formation (compare insets in H and J) and AB resorption. DE = dentin.

Figure 2. *Bsp* knock-out cementoblasts exhibit defective mineralization

(A) *Bsp* knock-out (KO) OCCM.30 immortalized cementoblasts were created using a CRISPR nuclease approach targeting exons 1–3 (E1–E3) in the mouse *Bsp* sequence. Compared to WT OCCM.30 parent cells at 8 weeks, *Bsp* KO cementoblasts produced smaller and less mineralized implants as assessed by (B) radiography and (C) micro-CT. Shown here is one representative implant from $n=3-4$ per cell line. In 3D micro-CT images shown in (C), gray indicates less mineralized volumes (200–800 mg/cm³ HA) while blue indicates highly mineralized volumes (greater than 800 mg/cm³ HA). (D) Unlike WT implants, H&E stained histological sections reveal regions that appear hypomineralized in *Bsp* KO implants. Quantitative micro-CT analysis shows a (E) 15-fold significantly greater mean volume and (F) 13-fold significantly greater mean mineral content in WT vs. *Bsp* KO implants ($p < 0.05$ by *t*-test). In panels E and F, color coded volumes indicate densities of 200–400 mg/cm³ HA (red), 400–600 mg/cm³ HA (orange), 600–800 mg/cm³ HA (green), or greater than 800 mg/cm³ HA.

Figure 3. Altered pyrophosphate metabolism and related gene expression in *Bsp* knock-out cementoblasts

(A) Analysis of cell culture media 4 days after confluence reveals 50% significantly increased ($p < 0.05$ by t -test) PP_i concentrations in ($n=3$) *Bsp* KO vs. WT OCCM.30 cells. (B) No significant differences are observed in media P_i concentrations in ($n=3$) *Bsp* KO vs. WT OCCM.30 cells ($p > 0.05$ by t -test). (C) P_i/PP_i ratio is decreased nearly 50% in ($n=3$) *Bsp* KO vs. WT cementoblasts, a statistically significant difference ($p < 0.05$ by t -test). Administration of 2 μg/ml rBSP, mutated rBSP (rBSP-KAE), recombinant OPN (rOPN), or mutated rOPN (rOPN-KAE) was performed with WT and *Bsp* KO cells, measuring mRNA at 24 hrs after treatment. Altered mRNA expression of (D) *Spp1*, (E) *Ank*, (F) *Enpp1*, and (G) *Alpl* without and with protein treatments are indicated by different uppercase letters (intragroup differences within WT or KO) or lowercase letters (intergroup differences in WT vs. KO), ($p < 0.05$ by one-way ANOVA).

Figure 4. Recombinant BSP does not affect pP90RSK and pERK levels in cementoblasts
 Western blot of cellular proteins was employed to determine if rBSP activated MAPK signaling by analysis of phosphorylated ERK1/2 and downstream kinase p90^{RSK} in (A) WT or (B) *Bsp* KO cells. Addition of 2 μ g rBSP fails to phosphorylate either ERK1/2 or p90^{RSK} in either cell type over the course of 120 minutes.

Figure 5. Ablation of *Ank* incompletely rescues the cementum phenotype in *Bsp*^{-/-} mice
 Compared to (A) WT buccal side acellular cementum (AC) at 26 dpn, (B) *Bsp*^{-/-} mice feature reduced AC (red *) and (C) *Ank*^{-/-} mice feature increased AC. (D) Genetic ablation of *Ank* on the *Bsp*^{-/-} mouse background is capable of restoring AC formation on the molar tooth root. Representative histology from 9 and 60 dpn is shown in Appendix Figure 2. Histological measurements of buccal and lingual AC thickness at (E) 9, (F) 26, and (G) 60 dpn reveal significant differences between *Ank*^{-/-} and *Bsp*^{-/-}; *Ank*^{-/-} buccal AC at 26 dpn (* p < 0.05) and 60 dpn (***) p < 0.001), but no differences in lingual AC measurements. High resolution micro-CT analysis of 60 dpn first mandibular first molars was used to segment AC (yellow), as shown in (H–J) 3D surface models and (K–M) 2D cut planes in sagittal, coronal, and transverse sections. Quantitative analyses of AC data from *Bsp*^{-/-}; *Ank*^{-/-} vs. *Ank*^{-/-} mice reveal significantly decreased (N) AC thickness (* p < 0.05) and (O)

AC volume (* $p < 0.05$), (**P**) but no significant differences between groups in AC mineral density ($p > 0.05$ by one-way ANOVA). (**Q**) Both transgenic lines show significantly increased cellular cementum volume compared to WT controls (*** $p < 0.001$ by one-way ANOVA).

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Figure 6. Periodontal function is corrected in *Bsp*^{-/-}; *Ank*^{-/-} double-deficient mice
 High resolution micro-CT scanning of (n=3–4) 60 dpn first mandibular first molars (M1) was used to analyze periodontal status of WT, *Bsp*^{-/-}, *Ank*^{-/-}, and *Bsp*^{-/-}; *Ank*^{-/-} mice based on appearance of alveolar bone (AB). Compared to (A) sagittal, (B) coronal, and (C) transverse 2D cut planes, and (D) 3D views of the lingual aspect of the mandible from a representative WT mouse, (E–H) *Bsp*^{-/-} mice exhibit severe loss of AB (yellow * and arrows in 2D images and red arrows in 3D image) and widening of PDL space due to aggressive bone resorption following loss of tooth-PDL attachment. (I–L) *Ank*^{-/-} mice feature a flared root shape from expanded cementum and periodontal tissues are intact and functional. (M–P) *Bsp*^{-/-}; *Ank*^{-/-} mice display largely corrected periodontal status compared to *Bsp*^{-/-} mice, including normal AB in interproximal and furcation regions, and more even PDL-bone borders and AB ridge. (M) Tartrate-resistant acid phosphatase (TRAP) staining and osteoclast-like cell enumeration of (n=3–4) *Bsp*^{-/-} and *Bsp*^{-/-}; *Ank*^{-/-} mice at 26 and 60 dpn indicates no differences (p > 0.05 by one-way ANOVA) in osteoclasts/mm. Based on one-way ANOVA and post-hoc Tukey test, experimental groups are indicated to

not be different from one another by sharing the same capital letter, or to be significantly different ($p < 0.05$) from one another by being marked with different capital letters.

Table 1
Quantitative PCR comparing gene expression in periodontia of *Bsp*^{-/-} versus WT mice at 26 dpn

PCR array was performed on total RNA isolated from periodontal tissues of *Bsp*^{-/-} and WT mouse molars (n=3 each) at 26 dpn. This table shows relative expression levels (*Bsp*^{-/-} vs. WT, both normalized to housekeeping genes, in descending order of fold-change) for all genes showing 2-fold or more difference that were significantly up- or down-regulated (p < 0.05). Shading indicates the portion of the table that includes down-regulated factors. The entire gene list and calculated fold changes can be found in Appendix Table 3.

Gene symbol	Fold-change	p-value
<i>Osterix/SP7</i>	6.4464	0.004928
<i>Bgn</i>	6.1981	0.008007
<i>Periostin</i>	5.1047	0.016595
<i>Alpl</i>	4.6006	0.001835
<i>Spp1</i>	4.2628	0.025887
<i>Ctnnb1</i>	3.7803	0.024016
<i>Runx2</i>	3.6769	0.00001
<i>Lrp6</i>	3.2456	0.034124
<i>Ank</i>	3.135	0.015848
<i>Dmp1</i>	2.9454	0.000892
<i>Smad4</i>	2.8782	0.03149
<i>RANKL</i>	2.6979	0.003363
<i>Vdr</i>	2.5289	0.020998
<i>Pthr1</i>	2.2374	0.032302
<i>Abcc6</i>	2.0731	0.022166
<i>Itgav</i>	-2.0823	0.032629
<i>Phospho1</i>	-2.2839	0.005087
<i>Nt5e</i>	-2.5051	0.01441
<i>Galnt3</i>	-5.0918	0.025407
<i>Ocn</i>	-5.9033	0.007059
<i>Dkk1</i>	-12.2512	0.024818
<i>Bsp</i>	-19.0033	0.002106
<i>Itga4</i>	-21.2322	0.010895