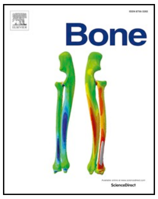
Bone 125 (2019) 8–15

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone



Full Length Article

Antagonistic interactions between osterix and pyrophosphate during cementum formation

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ARTICLE INFO

Keywords:
Cementogenesis
Cementum
Tooth
Pyrophosphate
Osterix
FGF

ABSTRACT

During cementum formation, the key roles of osterix (Osx) and inorganic pyrophosphate (PPi), mainly controlled by nucleotide pyrophosphatase 1 (Npp1; encoded by the *Enpp1* gene) and progressive ankylosis protein (Ank), have been demonstrated by animal models displaying altered cementum formation. In this study, we analyzed the relationship of Osx and local PPi during cementum formation using compound mutant mice with their wildtype and corresponding single gene mutants. Importantly, functional defects in PPi regulation led to the induction of Osx expression at the cervical cementum as demonstrated by *Enpp1* mutant mice and cementoblasts with the retroviral transduction of small hairpin RNA for *Enpp1* or *Ank*. Conversely, cementoblasts exposed to inorganic PPi or with the enforced expression of *Enpp1* or *Ank* reduced Osx expression in a concentration-dependent manner. Furthermore, the loss of *Osx* induced the higher expression of Npp1 and Ank at the apical region of the developing tooth root as observed in *Osx*-deficient mice. The activity of PPi-generating ectoenzymes (nucleoside triphosphate pyrophosphohydrolase, NTPPPHase) and the level of extracellular PPi were significantly increased in *Osx*-knockdown cementoblasts. However, the formation of ectopic cervical cementum was not completely diminished by inactivation of *Osx* in *Enpp1* mutant mice. In addition, fibroblast growth factor (FGF) receptor 1 (Fgfr1) was strongly localized in cementoblasts lining the acellular cementum and involved in the inhibitory regulation of matrix accumulation and further mineralization by supporting PPi production. Taken together, these results suggest that local PPi suppresses matrix accumulation and further mineralization through an antagonistic interaction with Osx under the synergistic influence of FGF signaling during cementum formation.

1. Introduction

Cementum, anchoring the tooth to surrounding alveolar bone through the periodontal ligament, is composed of two distinct acellular and cellular types with different locations and functions. Acellular cementum is developmentally formed first on the cervical tooth root while cellular cementum is formed later around the root apex. Acellular cementum is critical for tooth attachment and periodontal function while cellular cementum encases the apical portion of the root and provides an adaptive role in maintaining the tooth in its occlusal position [1]. This is especially important with regard to how acellular and cellular cementum differs developmentally, and how they interact with each other and with other involved regulatory factors. To date, several signaling molecules have been reported to be associated with the site-specific regulation of cementum formation. Cementum formation and its physiological maintenance appear very sensitive to disturbances in the inorganic phosphate (Pi) and pyrophosphate (PPi) ratio as shown in the animal model [2,3]. However, the developmental regulation of extracellular PPi through the balance effects of cytokines and other factors influencing acellular *versus* cellular cementum remains poorly understood.

Inorganic PPi, recognized as a key inhibitor of mineralization, is an important regulator for acellular cementum. Membrane-bound nucleotide pyrophosphatase 1 (Npp1) encoded by *Enpp1* and progressive ankylosis protein (Ank) have been identified as primary regulators of extracellular PPi concentrations around mineralizing cell types. Extracellular levels of PPi are normally held in check by balances in PPi generation by phosphodiesterase (NTPPPH) activity relative to PPi degradation by pyrophosphatases, by the balance effects of cytokines and growth factors, and by the transport of PPi from the cell interior

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https://doi.org/10.1016/j.bone.2019.05.001
Received 20 March 2019; Received in revised form 1 May 2019; Accepted 1 May 2019

involving the multiple-pass transmembrane protein Ank [4]. The *in vivo* role and regulation of PPi has been demonstrated by animal models. Both knock-out mice of *Ank* and *Enpp1* featuring PPi dysregulation resulted in a hypercementosis phenotype, indicating that both PPi regulators function in controlling cementum formation [2].

Osterix (Osx), a zinc finger-containing transcriptional factor, is an important regulator for cellular cementum together with Wnt/β-catenin signaling [5–7]. As demonstrated with an animal model, excessive cellular cementum is formed around tooth roots including the cervical region through the constitutive expression of stabilized *β-catenin* in cementoblasts [5]. It has also been shown that the local activation of the canonical Wnt signaling pathway using genetic or chemical methods can induce the *in vivo* formation of new cementum, particularly the cellular type [8]. Recently, we have demonstrated a reciprocal interaction between β-catenin and Osx in cementogenesis. Wnt/β-catenin signaling regulates Osx expression for cementoblast differentiation and cementum matrix secretion. Moreover, Osx reciprocally regulates Wnt/β-catenin activity by controlling Tcf/Lef expression [9], implying the Wnt/β-catenin/Osx signaling axis in cellular cementum formation.

In this study, we used transgenic and conditional knockout mutant mice displaying altered cementum formation and cementoblast cell lines to clarify the mechanisms of those key developmental factors and further investigate the relationship between local PPi and Osx during cementum formation. Our findings support the concept that extracellular PPi highly maintained in cervical cementum suppresses the Wnt/β-catenin/Osx signaling axis during cementum formation and inhibits matrix accumulation and mineralization across the periodontal ligament in the fibrous connection of the tooth to the alveolar bone.

2. Materials and methods

2.1. Mice

All the experimental procedures were approved by the Animal Welfare Committee of Chonbuk National University. All the mice were housed in a temperature-controlled environment with 12 h light/dark cycles. *Enpp1*^{asj} and *Axin2*$^{LacZ/+}$ mice as previously described were used in the procedures [10,11]. *Osx*-floxed (*Osx*$^{fl/fl}$) and *Wntless*-floxed (*Wls*$^{fl/fl}$) mice were crossed with *Osteocalcin-Cre* (^{OC}Cre) mice to inactivate the floxed genes conditionally in differentiating cementoblasts and cementocytes of the developing tooth [5,12,13]. Compound mutant mice of the experimental group were generated and genotyped by polymerase chain reaction analysis using previously described primers [14–16]. Age-matched wild type (WT) mice were used as the control. At least three independent littermates were used for each experimental group.

2.2. Tissue preparation, immunohistochemistry (IHC), and histomorphometry

For histologic analysis, the dissected mandibles were fixed in 4% paraformaldehyde and decalcified in 10% EDTA for 4 weeks at 4 °C. The decalcified tissues were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5-μm thickness. Slides were stained with hematoxylin and eosin (H-E). For immunostaining, the sections were treated with 3% hydrogen peroxide and incubated with rabbit polyclonal antibodies as described in the appendix. The Histostain Plus Rabbit Primary (DAB) kit (Zymed Laboratories, San Francisco, CA, USA) was used following the manufacturers' instructions. Cervical cementum thickness was measured as the shortest vertical distance at a site 100 μm apical from the cemento-enamel junction in the mid-sagittal section of the mandibular first molar using the AnalySIS Pro imaging system (Soft Imaging System, Münster, Germany) as described [9].

2.3. β-Galactosidase activity of *Axin2*$^{LacZ/+}$ mice

For the analysis of Wnt activity *in vivo*, 6-week-old *Axin2*$^{LacZ/+}$ mice were fixed in 0.25% glutaraldehyde, passed through a sucrose series, embedded in Negative-50 freezing medium (Richard Allan), and sectioned at 14 μm thickness using a cryostat microtome. The X-gal staining of the sections was performed as described previously [17]. The slides were then counterstained with eosin.

2.4. Cell culture, transfection, and retroviral transduction

OCCM-30, a mouse cementoblast cell line, was provided by Dr. Martha J. Somerman (National Institutes of Health, Bethesda, MD, USA) and cultured as described previously [9,18]. Cells at 95% confluency were treated with inorganic pyrophosphate (sodium pyrophosphate tetrabasic, Sigma Aldrich, St. Louis, MO, USA) or recombinant murine FGF2 (Peprotech, Rocky Hill, NJ, USA) in growth media for 24 h or osteogenic media (OM) for 4 days. To generate retroviral particles, short hairpin RNA (shRNA) against mouse *Osx* (TG514041), *Ank* (TR500093), and *Enpp1* (TG501613), or control shRNA (TR30013) were purchased from OriGene Technologies (Rockville, MD, USA). The establishment of stable cell lines by viral transductions were performed as described [9]. The luciferase *Osx* promoter plasmid −1269/+91 (a gift from Dr. Mark Nanes, Emory University, Atlanta, GA, USA) or TOPflash/FOPflash reporter (Addgene #12456 and #12457, respectively) constructs for Tcf/Lef binding activity and flag-tagged mouse *Ank* (MR207888), *Enpp1* (MR227498) or *Osx* (Sp7; MR226379, OriGene Technologies) constructs in the pCMV6 backbone were transfected as described [9].

2.5. Quantitation of extracellular PPi

Cells were incubated with or without recombinant murine FGF2 (Peprotech) in serum- and phenol red-free medium for 24 h. For the quantitation of extracellular PPi, cell culture supernatants were used with a PPiLight™ inorganic pyrophosphate assay kit (Lonza, Mapleton, IL, USA) according to the manufacturer's protocol.

2.6. Statistical analysis and supplemental information

Data are presented as mean ± standard error of the mean (SEM) of three or more separate experiments. Normal data with equal variance were analyzed using Student's *t*-test and $p < .05$ was considered statistically significant. Detailed descriptions for other experimental materials and methods are provided in the Supplementary information.

3. Results and discussion

3.1. PPi suppresses Osx and β-catenin in cementoblasts

It has been previously reported that extracellular PPi deficiency in *Enpp1* functional mutants (*Enpp1*^{asj}) or knockout mice of *Enpp1* or *Ank* contributes to increased cementogenesis [2,19]. Since Osx and β-catenin have been reported to be important regulators in cementum formation [5,6], we hypothesized that, to drive excessive cervical cementum formation, the expression of Osx and β-catenin might be induced by extracellular PPi deficiency in *Enpp1*^{asj} mice. *Enpp1*^{asj} mice at postnatal 28 days (P28) have an increased expression of Osx and β-catenin with lower Npp1 expression in cervical cementum (Fig. 1A). The increased activity of β-catenin at cervical cementum of these mice was confirmed with increased Lef1 expression as a downstream target of β-catenin and Axin2 β-galactosidase activity at a periodontal ligament (PDL)-faced side of ectopic cervical cementum in *Enpp1*^{asj} mice (Fig. 1A). Cellular cementum of *Enpp1*^{asj} mice was not changed at the apical region while excessive cementum was formed at cervical region (Supplementary Fig. 1A). In addition, the expressions of Npp1 and Osx

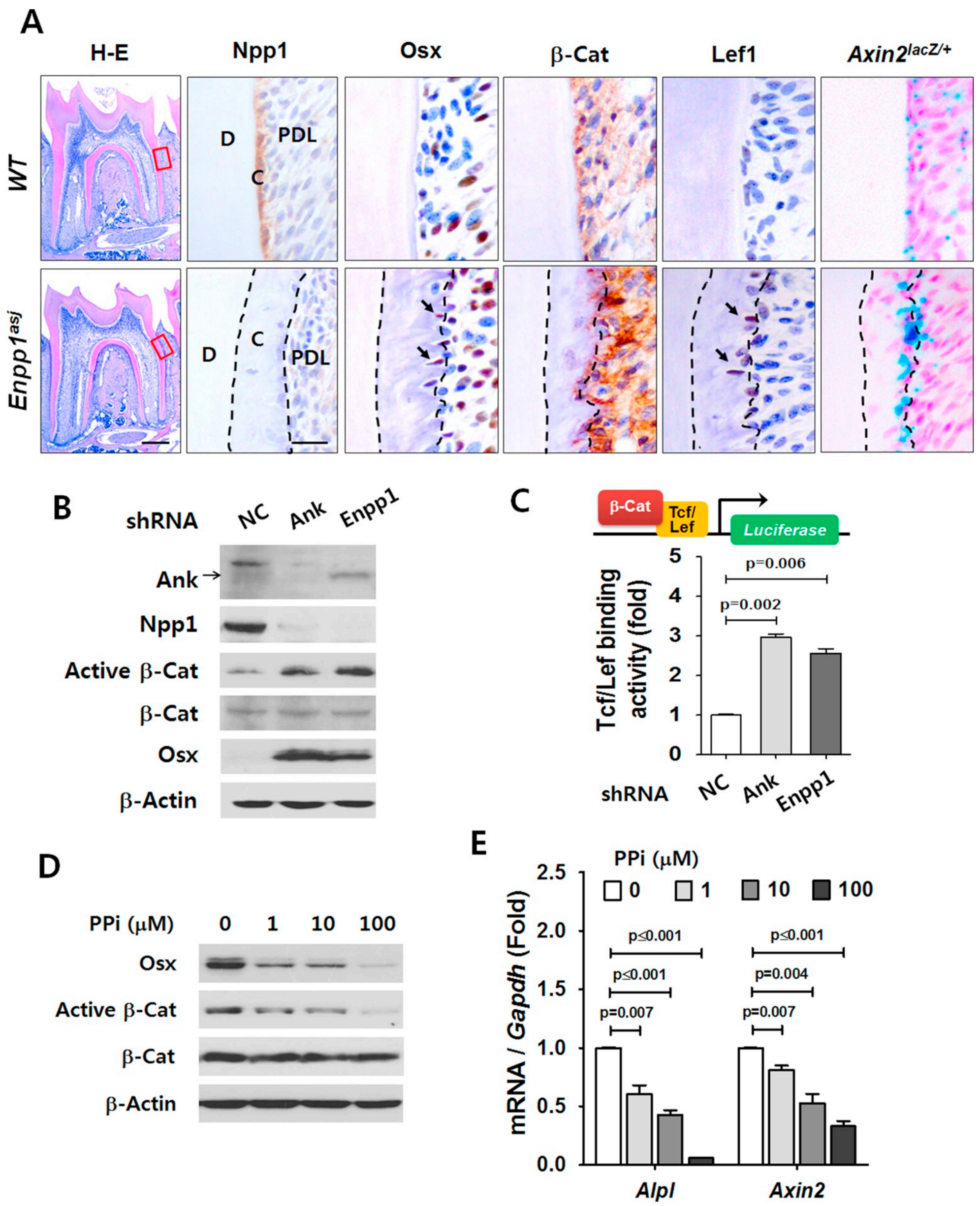

Fig. 1. PPi suppress Osx and β-catenin signaling in cementoblasts. (A) Molecular changes in the cervical cementum of WT and *Enpp1*^{asj} mutant mice were detected by immunohistochemical staining with the distal root of the mandibular first molar at P28. Red boxed areas of H-E stained sections show the cervical cementum of WT and excessive cervical cementum (indicated by dotted lines) of *Enpp1*^{asj} mice for immunostaining of Npp1, Osx, β-catenin, and Lef1 and Axin2 β-galactosidase activities, respectively. Arrows show the increased nuclear expression of Osx and Lef1, respectively. D, dentin; PDL, periodontal ligament; C, cementum. Scale bars: 400 μm (H-E) and 10 μm (IHC and β-galactosidase). (B) OCCM-30 cells were transduced with retroviruses harboring small hairpin RNA (shRNA) specific for *Ank*, *Enpp1*, and non-specific negative control (NC). The protein levels were analyzed by Western blotting. (C) Tcf/Lef binding activities of β-catenin were analyzed by TOPflash/FOPflash luciferase reporters in OCCM-30 cells expressing shRNAs. (D) OCCM-30 cells were treated with inorganic PPi for 24 h. The protein levels were analyzed by Western blotting. (E) The transcript levels of *Alpl* and *Axin2* were analyzed by real-time qPCR. RNA was isolated from OCCM-30 cells treated with inorganic PPi for 24 h. Samples shown in Western blotting are from the same experiment, and the gels/blots were processed under the same experimental conditions. β-Actin was used as a loading control. Significance was assigned for *p*-values as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were not changed remarkably at apical cementum (Supplementary Fig. 1B). Cementoblasts with stable expression of small hairpin RNA (shRNA) specific for *Ank* and *Enpp1* also exhibited the largely increased expression of Osx and non-phosphorylated active β-catenin with the reduced expression of Ank and Npp1 compared to the control (Fig. 1B). As analyzed by using FOPflash/TOPflash luciferase reporters, the Tcf/Lef binding activities of β-catenin in both *Ank* and *Enpp1*-knockdown cementoblasts were significantly elevated compared to the control (Fig. 1C). The treatment of inorganic PPi reduced the protein levels of Osx and non-phosphorylated active β-catenin and mRNA levels of their downstream target genes, *Alpl* and *Axin2*, respectively, in a concentration-dependent manner (Fig. 1D and E). The treatment of inorganic PPi also reduced the activity of the Osx promoter and the level of phosphorylated GSK3α/β (p-GSK3α/β; α^{Ser21} and β^{Ser9}), indicating an increase in total GSK3β activity and β-catenin degradation [20] (Supplementary Fig. 2A and B). Inorganic PPi also significantly induced the expression of *Ank*, implying a positive feedback regulation (Supplementary Fig. 2C and D). The levels of extracellular PPi in *Ank*- and *Enpp1*-knockdown cementoblasts were analyzed and compared to that of *Osx*-knockdown cells (Supplementary Fig. 2E). The level of extracellular PPi from *Ank*-knockdown cementoblasts was slightly reduced while largely increased in *Osx*-knockdown cementoblasts at the same condition. The level of extracellular PPi from *Enpp1*-knockdown cementoblasts was not reduced. It is likely that there is a compensatory regulation among PPi regulators for the global level of extracellular PPi in cementoblasts. In accordance with a suppressive effect of inorganic PPi to Osx and β-catenin, the overexpression of *Ank* and *Enpp1* significantly reduced the Tcf/Lef binding activity of β-catenin and the activity of the Osx promoter (Supplementary Fig. 2F and G).

To further define the relationship between Osx and PPi in cementum formation, we analyzed the abilities of extracellular matrix secretion and mineralization with *Enpp1*^{asj} mutant mice. *Enpp1*^{asj} mutant mice at P28 exhibited excessive cervical cementum with the stronger expression of matrix secretion such as Dmp1 and Bsp, but lower expression of Fgf23, a mineralization inhibitor, compared to the WT control (Fig. 2A). The transcripts of all the genes tested for differentiation and extracellular matrix secretion were remarkably upregulated by the stable knockdown of *Ank* and *Enpp1* in cementoblasts (Fig. 2B). Accordingly, alkaline phosphatase (ALP) activity and mineralization ability proven by Alizarin red staining were significantly upregulated in *Ank* and

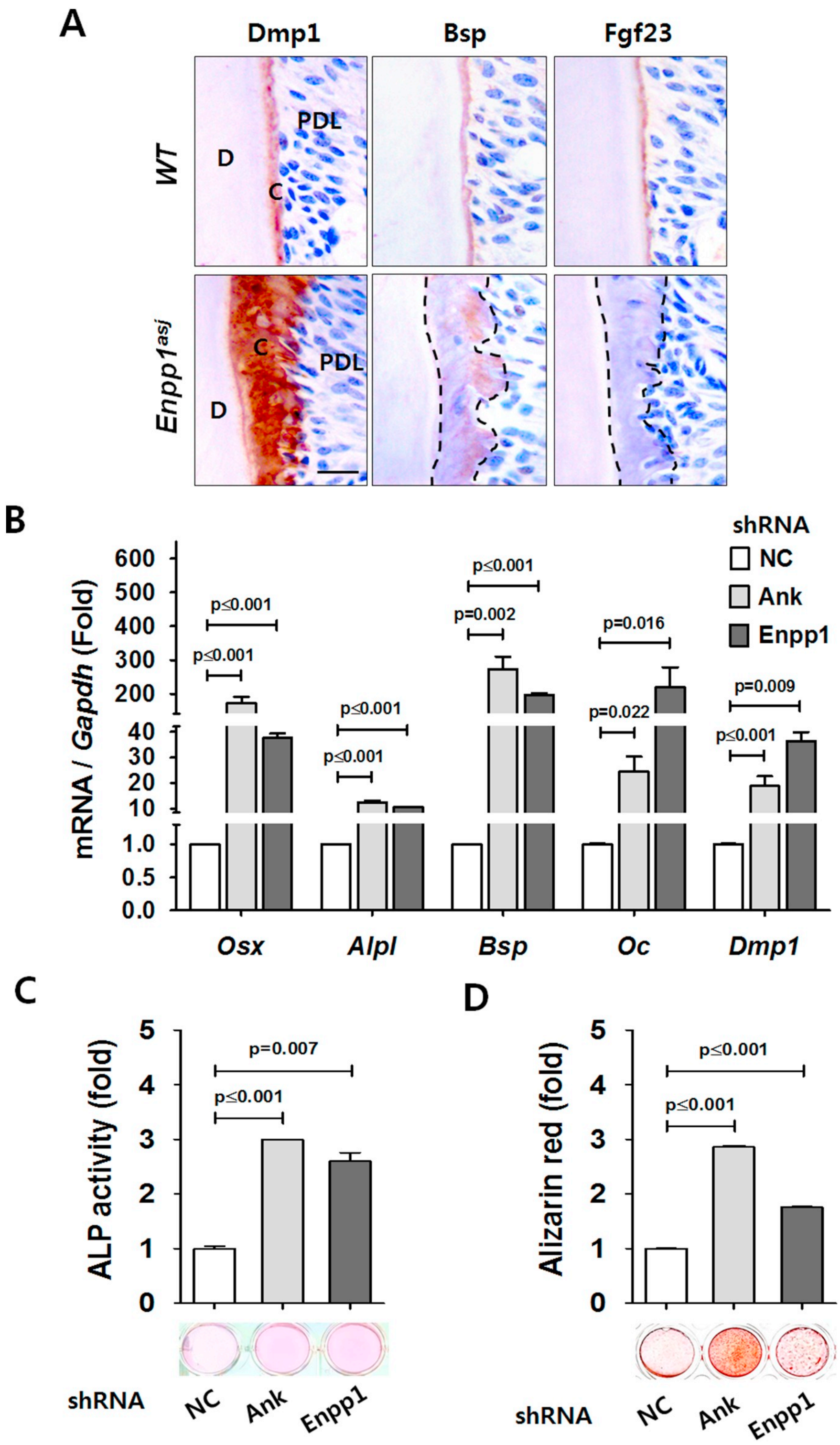

Fig. 2. PPi regulators suppress matrix secretion and mineralization in cementoblasts. (A) Molecular changes in the cervical cementum of WT and *Enpp1*^{asj} mutant mice were detected by immunohistochemical staining with the distal root of the mandibular first molar at P28. D, dentin; PDL, periodontal ligament; C, cementum. Scale bars: 10 μm. (B) The transcript levels of cementogenesis-associated genes were analyzed by real-time qPCR. RNA was isolated from OCCM-30 cells transduced with retroviruses harboring shRNA specific for *Ank*, *Enpp1*, and the negative control (NC). Alkaline phosphatase activity (C) and mineralization ability by Alizarin red S staining (D) were analyzed with OCCM-30 cells transduced with retroviruses harboring shRNA specific for *Ank*, *Enpp1*, and the negative control (NC). ALP activity was normalized to total protein content and expressed using the fold change.

Enpp1-knockdown cementoblasts (Fig. 2C and D). Cumulatively, these observations demonstrate that PPi suppresses Osx and β-catenin and the following processes of matrix secretion and mineralization in cementogenesis.

3.2. Osx suppresses Ank and Npp1 in cementoblasts

Previously, the positive roles of Osx and β-catenin in cellular cementum formation were well-demonstrated [5,6,9,10]. To maintain acellular cementum as a thin tissue on the root surface, cementoblasts modulate PPi production to inhibit apposition by increasing local PPi through Ank and Npp1. Therefore, we hypothesized some suppressive roles of Osx and β-catenin on PPi regulators such as Ank and Npp1 during cementum formation. To verify our hypothesis, we analyzed the molecular and physiological changes of *Osx*-knockdown cementoblasts and further cementum formation with *Osx*-deficient mice (*Osx*^{OC}). *Osx*-knockdown cementoblasts exhibited higher levels of Ank and Npp1 expression during differentiation as demonstrated by the *in vitro* treatment of osteogenic media (OM) compared to the control (Fig. 3A). The transcripts of *Ank* and *Enpp1* were largely upregulated while of *Osx*, *Alpl*, and the downstream

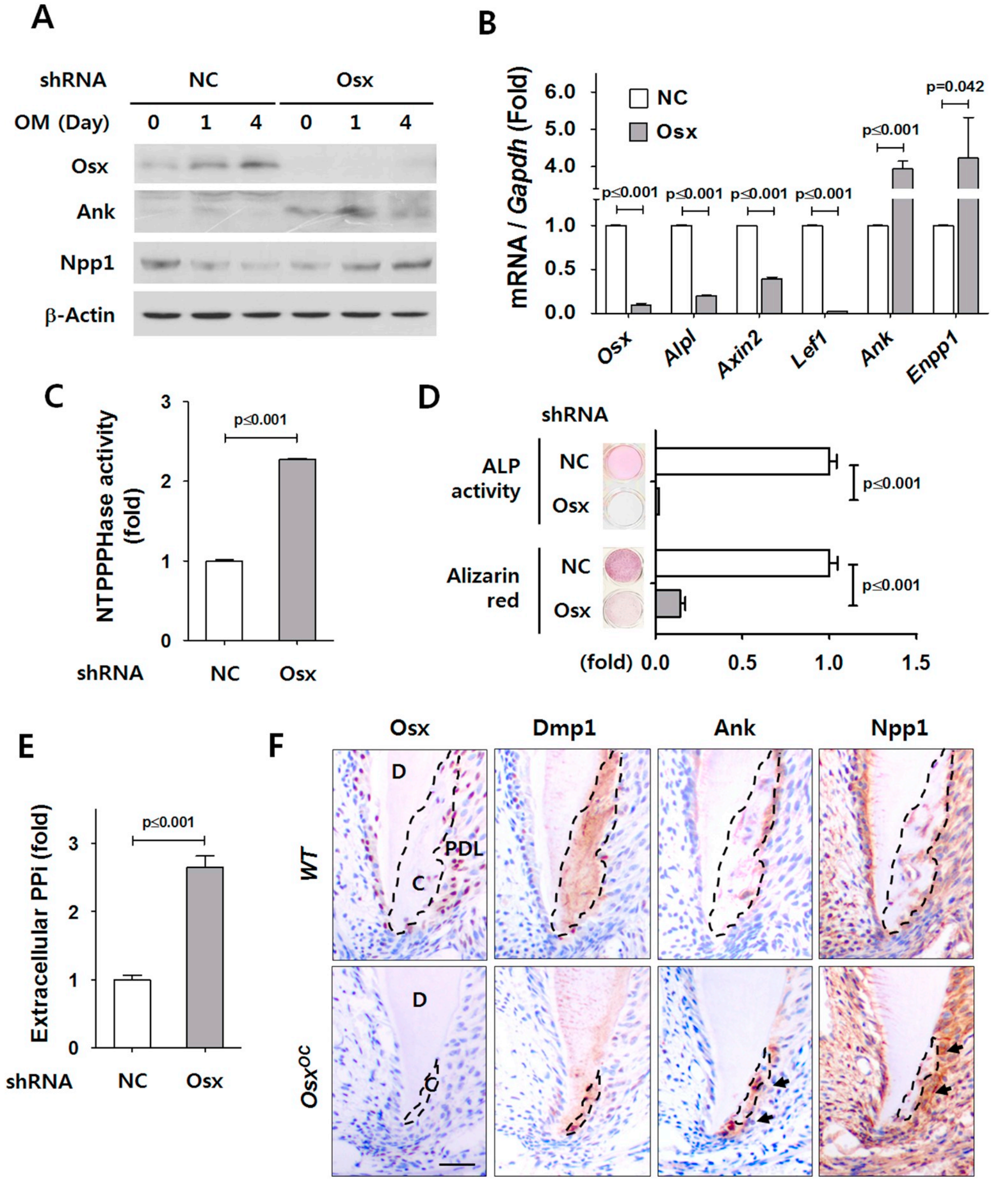

Fig. 3. Osx suppresses Ank and Npp1 in cementoblasts. (A) OCCM-30 cells were transduced with retroviruses harboring shRNA for *Osx* and the negative control (NC). The protein levels of OCCM-30 cells treated with OM for the indicated duration were analyzed by Western blotting after transduction. Samples shown in Western blotting are from the same experiment, and the gels/blots were processed under the same experimental conditions. β-Actin was used as a loading control. (B) The mRNA transcript levels were analyzed by real-time qPCR. RNA was isolated from OCCM-30 cells transduced with retroviruses harboring shRNA for *Osx* and the negative control (NC). NTPPPHase activity (C) and the levels of extracellular PPi (E) were analyzed with OCCM-30 cells transduced with retroviruses harboring shRNA specific for *Osx* and the negative control (NC). (D) ALP activity and mineralization ability by Alizarin red S staining were analyzed with OCCM-30 cells treated with OM for 4 days after transduction. ALP activity was normalized to total protein content and expressed using the fold change. (F) Molecular changes in the apical cementum of the mandibular first molar from WT and Osx^{OC} mutant mice were detected by immunohistochemical staining with the distal root of the mandibular first molar at P28. Dotted areas were indicated for apical cellular cementum. Arrows indicate increased immunoreactivities of Ank and Npp1 in apical cementoblasts of Osx^{OC} mutant mice. D, dentin; PDL, periodontal ligament; C, cementum. Scale bars: 20 μm. Significance was assigned for *p*-values as indicated.

target genes of β-catenin such as *Axin2* and *Lef1* were largely downregulated in *Osx*-knockdown cementoblasts (Fig. 3B). In accordance with this, the level of extracellular PPi analyzed with cell culture supernatant and the activity of PPi-generating ectoenzymes (nucleoside triphosphate pyrophosphohydrolase, NTPPPHase activity) were significantly increased in *Osx*-knockdown cementoblasts (Fig. 3C and E). As expected, the ALP activity and mineralization ability proven by Alizarin red staining were also remarkably decreased in *Osx*-knockdown cementoblasts (Fig. 3D). The molars of Osx^{OC} mice had a dramatic change in the volume of the apical cellular cementum layer, but no remarkable change in the phenotype and molecular levels of Npp1 and Ank at acellular cementum, implying the dispensable roles of Osx on Npp1 and Ank at cervical cementum (Supplementary Fig. 3A and B). Therefore, to check the roles of Osx on PPi regulation in cementogenesis, we noticed molecular changes of Ank and Npp1 expression at the apical region of the tooth root with the molars of Osx^{OC} mice. Through the immunohistochemical staining of molars from Osx^{OC} and WT mice at P28, concentrated higher immunoreactivities of Ank and Npp1 were detected around diminished cellular cementum in Osx^{OC} mice compared to WT (Fig. 3F). At postnatal week 6 (P6W), similar results were observed with the extended immunoreactivity of Fgf23, a mineralization inhibitor [21], at the cementum line to the apex of molars in Osx^{OC} when compared to WT (Supplementary Fig. 3C). Consequently, the loss of *Osx* in cementoblasts resulted in the inhibition of matrix accumulation and further mineralization through the higher expression of PPi regulators and, as the final outcome, the loss of cellular cementum apposition in the developing tooth root. Furthermore, the chemical inactivation of Wnt/β-catenin signaling by C59, an inhibitor of Wnt/β-catenin, induced a considerable induction of NTPPPHase activity *in vitro* (Supplementary Fig. 3D). Taken together with previous reports, these results suggest that Osx and β-catenin must be suppressed for adequate PPi regulation and the proper formation of acellular cementum. The expression of Tnap (ALP), an additional PPi regulator, was hardly detected and not changed at cervical region but reduced in cementocytes at apical cementum of Osx^{OC} mice compared to control WT. In $Enpp1^{asj}$ mice, the expression of Tnap was slightly increased in the cells faced to PDL at cervical region but not changed in cementocytes at apical region (Supplementary Fig. 4B). We found that the transcript level of *Alpl* is in a trend of following the level of Osx by real-time qPCR analysis with various cementoblasts, which is consistent with the pattern of immunohistochemical staining (Supplementary Fig. 4C).

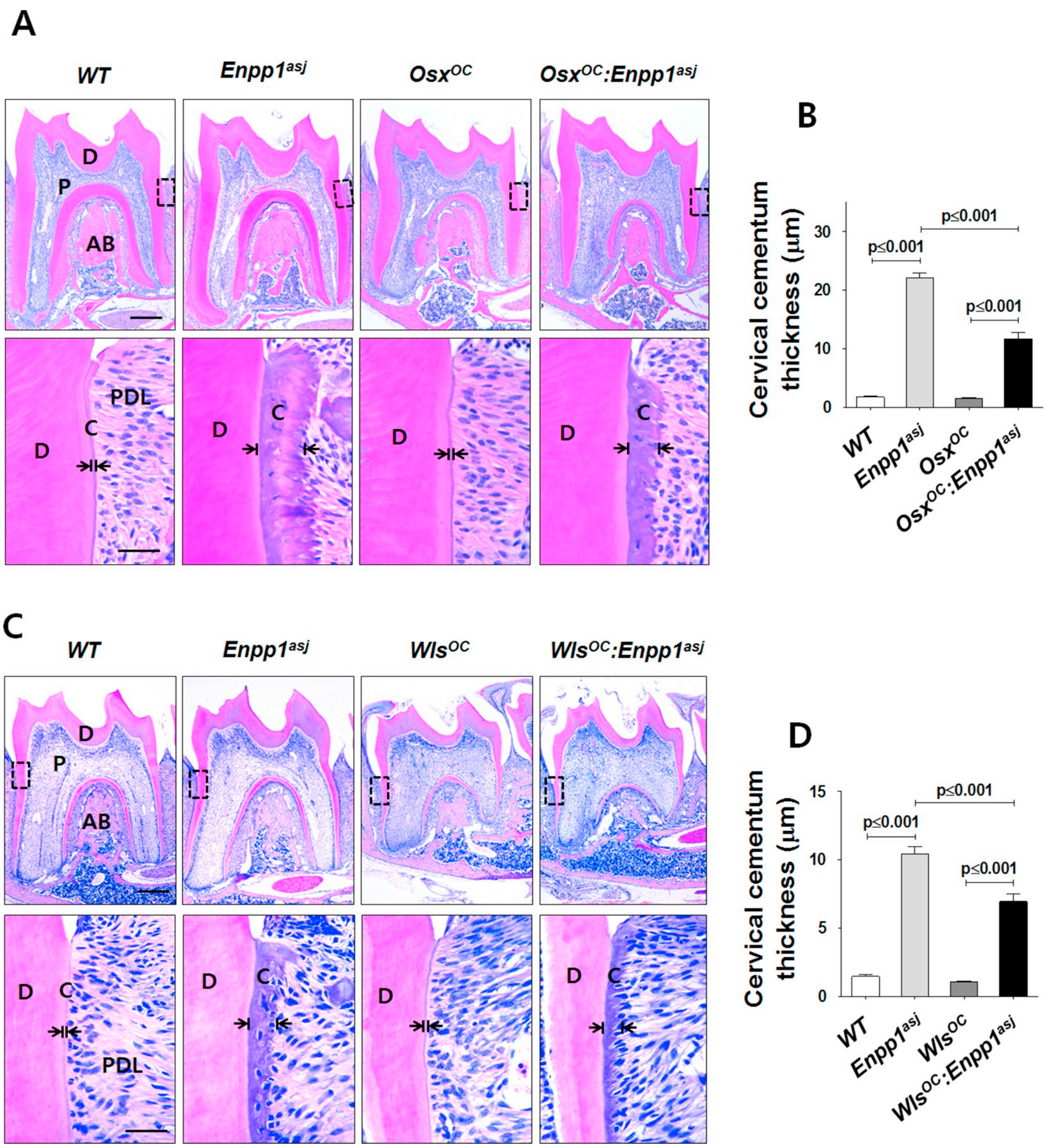

Fig. 4. Histologic changes in the cervical cementum by inactivation of *Enpp1*, *Osx*, and *Wls* and their double mutations. (A) The sections of the mandibular first molar from WT, $Enpp1^{asj}$, Osx^{OC}, and Osx^{OC}:$Enpp1^{asj}$ mutant mice at P28 were stained with H-E. (B) The thickness of cervical cementum of A was quantitated. (C) The sections of the mandibular first molar from WT, $Enpp1^{asj}$, Wls^{OC}, and Wls^{OC}:$Enpp1^{asj}$ mutant mice at P21 were stained with H-E. Below are higher-magnification views of cervical cementum in the boxed area of the upper images. Arrows indicate the thickness of cervical cementum. (D) The thickness of cervical cementum of C was quantitated. Significance was assigned for *p*-values as indicated. P, pulp; D, dentin; AB, alveolar bone; PDL, periodontal ligament; C, cementum. Scale bars: 400 μm (top of A and C) and 20 μm (bottom of A and C).

3.3. Inactivation of Wls and Osx partially rescued ectopic cervical cementum formation of $Enpp1^{asj}$ *mice*

As mentioned above, the functional defect of PPi regulation in cementoblasts led to the induction of Osx expression at the cervical cementum with *Enpp1* mutant mice and *Enpp1*-knockdown cementoblasts. Since extracellular PPi deficiency utilizes Osx and β-catenin to drive excessive cementum formation, we analyzed the thickness of cervical cementum with the molars of Osx^{OC}:$Enpp1^{asj}$ and Wls^{OC}:$Enpp1^{asj}$ mice, which correspond to $Enpp1^{asj}$ mice with the inactivation of *Osx* and *Wls*, respectively. Due to the lack of tooth roots in $Ctnnb1^{OC}$ mice [22], we inactivated *Wls* instead of *Ctnnb1* to block autologous canonical Wnt/β-catenin signaling. When comparing the single mutants, *i.e.*, Osx^{OC} and Wls^{OC}, with their corresponding WT, the roles of *Osx* and *Wls* were mainly detected in dentinogenesis by malformed dentin formation, but were not prominent in acellular cementum formation [12,13]. Compared to the ectopic cervical cementum layer of the molars of $Enpp1^{asj}$ mice, the cervical cementum of Osx^{OC}:$Enpp1^{asj}$ (Fig. 4A) and Wls^{OC}:$Enpp1^{asj}$ mice (Fig. 4C) were partially diminished (Fig. 4B and D). These results strongly suggest the possibility of other signaling routes to form ectopic cementum or additional factors conflicting with Osx and Wnt/β-catenin signaling to fine-tune PPi production.

3.4. Crosstalk of PPi regulators with FGF signaling

The presence of additional factors was predicted to include multiple, redundant negative regulators of mineral growth that inhibit the mineralization of cervical cementum as well as PDL space [2]. The development of a mineral-free PDL region between mineralized bones and teeth requires the 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 [23]. We observed that the immunoreactivity of fibroblast growth factor receptor 1 (Fgfr1) is dominant in the PDL space and overlapped with Ank along

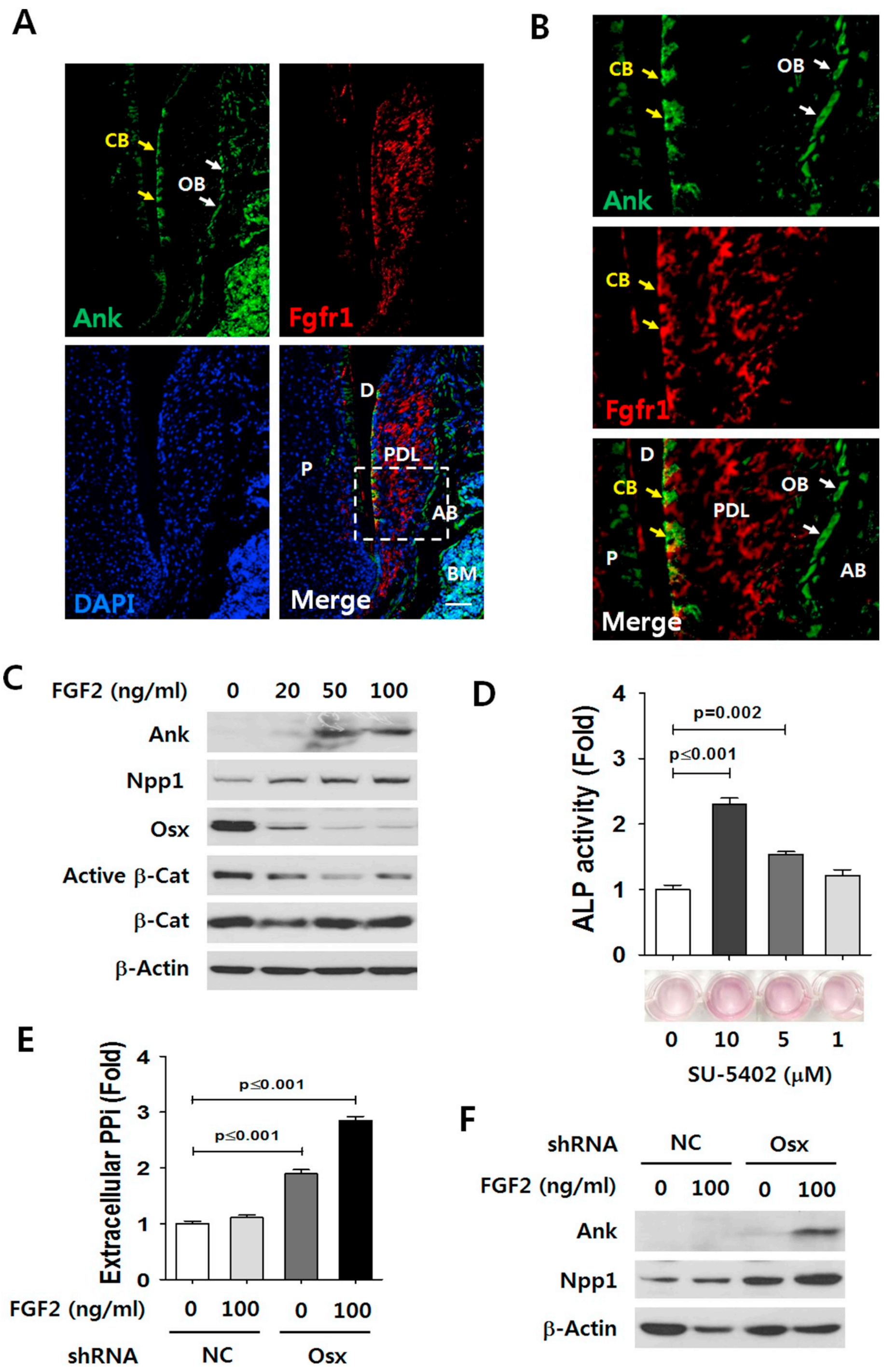

Fig. 5. Crosstalk between PPi regulators with FGF signaling in cervical cementum formation. (A) The sections of the mandibular first molar from WT mice at P14 were stained with immunofluorescence. (B) Higher-magnification views of cervical cementum and PDL in the boxed area of A. Yellow arrows indicate cementoblasts (CB) and white arrows indicate osteoblasts (OB). P, pulp; D, dentin; AB, alveolar bone; PDL, periodontal ligament; BM, bone marrow. Scale bars: 50 μm. (C) OCCM-30 cells were treated with the indicated concentration of recombinant murine FGF2 with treatment of OM for 24 h. The protein levels were analyzed by Western blotting. (D) ALP activity was analyzed with OCCM-30 cells treated with the indicated concentration of SU-5402 (an FGF receptor 1 inhibitor) and OM for 4 days. ALP activity was normalized to total protein content and expressed using the fold change. (E) The levels of extracellular PPi were analyzed with OCCM-30 cells treated with recombinant murine FGF2 after transduction with retroviruses harboring shRNA for *Osx* and the negative control (NC). (F) OCCM-30 cells were treated with recombinant murine FGF2 with treatment of OM for 4 days after transduction with retroviruses harboring shRNAs. The protein levels were analyzed by Western blotting. Samples shown in Western blotting are from the same experiment, and the gels/blots were processed under the same experimental conditions. β-Actin was used as a loading control. Significance was assigned for *p*-values as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cervical cementum line of the developing tooth root (Fig. 5A). Intriguingly, Fgfr1 was positive only in cementoblasts, but not in osteoblasts lining the outer border of alveolar bone at a side to the PDL space while Ank was positive in both cell types, but negative in the PDL space, strongly suggesting a specific role of FGF signaling in the PDL space and cervical cementum (Fig. 5B). Based on this observation, we hypothesized that FGF signaling might crosstalk with PPi regulators, Ank and Npp1, to control cementum formation. The expression of Ank and Npp1 were definitely increased by treatment with recombinant FGF2 to OCCM-30 cells while those of Osx and β-catenin were decreased in a concentration-dependent manner (Fig. 5C). The transcripts of *Ank* and *Npp1* were also changed similarly (Supplementary Fig. 5A). The Tcf/Lef binding activity of β-catenin and ALP activity also decreased with FGF2 in a concentration-dependent manner (Supplementary Fig. 5B and C). Inversely, ALP activity was increased by blocking FGF signaling with treatment of SU-5402, an FGF receptor 1 inhibitor, in a concentration-dependent manner (Fig. 5D). Next, we analyzed the additive effect of FGF2 on the protein levels of Ank and Npp1 and extracellular PPi level in *Osx*-knockdown OCCM-30 cells to evaluate the role of FGF signaling at the cervical region of the tooth root. As expected, the highest expression of Ank and Npp1 and the most elevated level of extracellular PPi were observed by the treatment of FGF2 in *Osx*-knockdown OCCM-30 cells than other control groups (Fig. 5E and F). These results suggest that, in addition to Npp1 and Ank, FGF signaling is also involved in PPi regulation and has inhibitory roles for matrix accumulation and further mineralization by supporting PPi production at the cervical region of the tooth root across the PDL space, suggesting cementum formation at the cervical region is governed under the influence of enriched FGF signaling during tooth root development. Nevertheless, it is likely that the development of cellular cementum as an adoptive tissue is controlled by distinct regulatory mechanisms with postnatally activated factors. In this respect, Wnt/β-catenin signaling is a powerful factor postnatally activated for cellular cementum development. Wnt/β-catenin activity is correlated to cellular cementum formation postnatally [10], and the endogenous activation of β-catenin in cementoblasts led to profound consequences of ectopic formation of cellular cementum through Osx expression [5,6].

4. Conclusions

Our findings indicate that local PPi suppresses matrix accumulation and further mineralization through an antagonistic interaction with Osx under the synergistic influence of FGF signaling during cementum formation and imply that cementum type might be determined by a balance between Osx and local PPi. These findings provide an insight into the developmental differences in acellular *versus* cellular cementum regulated by different mechanisms in the developing tooth root and suggest a novel therapeutic approach to control the reattachment of periodontal ligaments to tooth root surfaces in addition to the regeneration of new cementum in various periodontal diseases.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (Nos. 2017R1D1A1A09000545, 2017R1A5A2015061, and 2018R1A2A3074639). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.05.001.

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