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Osteopontin regulates dentin and alveolar bone development and mineralization

B.L. Foster [a,*], M. Ao [b], C.R. Salmon [c], M.B. Chavez [a], T.N. Kolli [a], A.B. Tran [b], E.Y. Chu [b], K.R. Kantovitz [d], M. Yadav [e], S. Narisawa [e], J.L. Millán [e], F.H. Nociti Jr [c], M.J. Somerman [b]

[a] Division of Biosciences, College of Dentistry, The Ohio State University, Columbus, OH, USA
[b] National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), National Institutes of Health (NIH), Bethesda, MD, USA
[c] Department of Prosthodontics and Periodontics, Division of Periodontics, Piracicaba Dental School, University of Campinas, São Paulo, Brazil
[d] Department of Dental Materials, São Leopoldo Mandic Research Center, Campinas, São Paulo, Brazil
[e] Sanford Children's Health Research Center, Sanford Burnham Prebys Medical Research Institute, La Jolla, CA, USA

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ABSTRACT

The periodontal complex is essential for tooth attachment and function and includes the mineralized tissues, cementum and alveolar bone, separated by the unmineralized periodontal ligament (PDL). To gain insights into factors regulating cementum-PDL and bone-PDL borders and protecting against ectopic calcification within the PDL, we employed a proteomic approach to analyze PDL tissue from progressive ankylosis knock-out ($Ank^{-/-}$) mice, featuring reduced PP_i, rapid cementogenesis, and excessive acellular cementum. Using this approach, we identified the matrix protein osteopontin (*Spp1*/OPN) as an elevated factor of interest in $Ank^{-/-}$ mouse molar PDL. We studied the role of OPN in dental and periodontal development and function. During tooth development in wild-type (WT) mice, *Spp1* mRNA was transiently expressed by cementoblasts and strongly by alveolar bone osteoblasts. Developmental analysis from 14 to 240 days postnatal (dpn) indicated normal histological structures in $Spp1^{-/-}$ comparable to WT control mice. Microcomputed tomography (micro-CT) analysis at 30 and 90 dpn revealed significantly increased volumes and tissue mineral densities of $Spp1^{-/-}$ mouse dentin and alveolar bone, while pulp and PDL volumes were decreased and tissue densities were increased. However, acellular cementum growth was unaltered in $Spp1^{-/-}$ mice. Quantitative PCR of periodontal-derived mRNA failed to identify potential local compensators influencing cementum in $Spp1^{-/-}$ vs. WT mice at 26 dpn. We genetically deleted *Spp1* on the $Ank^{-/-}$ mouse background to determine whether increased *Spp1*/OPN was regulating periodontal tissues when the PDL space is challenged by hypercementosis in $Ank^{-/-}$ mice. $Ank^{-/-}$; $Spp1^{-/-}$ double deficient mice did not exhibit greater hypercementosis than that in $Ank^{-/-}$ mice. Based on these data, we conclude that OPN has a non-redundant role regulating formation and mineralization of dentin and bone, influences tissue properties of PDL and pulp, but does not control acellular cementum apposition. These findings may inform therapies targeted at controlling soft tissue calcification.

1. Introduction

The periodontium is the attachment complex for the tooth, and includes cementum, PDL, and alveolar bone. Cementum is a mineralized tissue covering the tooth root that serves as an interfacial connective tissue between the tooth root dentin and the PDL [1,2]. While alveolar bone is continually remodeled by osteoblast, ostecyte, and osteoclast activities to adapt to mechanical loading associated with tooth function, cementum does not undergo physiological remodeling, but forms by continual slow apposition throughout life, mediated by activities of cementoblasts. The acellular cementum of the cervical tooth root is marked by concentrated depositions of ECM proteins including bone sialoprotein (BSP) and osteopontin (OPN), multifunctional phosphoproteins thought to regulate biomineralization and contribute to cell

Abbreviations: ECM, extracellular matrix; OPN, osteopontin; BSP, bone sialoprotein; PDL, periodontal ligament; PP_i, inorganic pyrophosphate; TNAP, tissue-nonspecific alkaline phosphatase; ANK, progressive ankylosis protein; ENPP1, ectonucleotide pyrophosphatase phosphodiesterase 1; LCM, laser capture microdissection; LC-MS/MS, liquid chromatography-tandem mass spectrometry; micro-CT, micro-computed tomography; CEJ, cemento-enamel junction; H&E, hematoxylin and eosin; TB, toluidine blue; ABNFR, alcian blue/nuclear fast red; PR, picrosirius red; IHC, immunohistochemistry; ISH, in situ hybridization; WT, wild-type; FMOD, fibromodulin; SLRP, small leucine-rich proteoglycan; COL1A1, collagen type 1; COLXIIA, collagen XII; DCN, decorin; POSTN, periostin; SIBLING, small integrin-binding ligand N-linked glycoprotein.

* Corresponding author at: Division of Biosciences, College of Dentistry, The Ohio State University, 4163 Postle Hall, 305 W. 12th Avenue, Columbus, OH, USA.
E-mail address: foster.1004@osu.edu (B.L. Foster).

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signaling [3–9]. The apposition and mineralization of acellular cementum is strongly regulated by local mineral metabolism, especially levels of pyrophosphate (PP_i), a potent inhibitor of hydroxyapatite crystal growth. PP_i levels are controlled by tissue-nonspecific alkaline phosphatase (*Alpl*/TNAP), progressive ankylosis protein (*Ank*/ANK), and ectonucleotide pyrophosphatase phosphodiesterase 1 (*Enpp1*/ENPP1), and genetic ablation of any of these factors has dramatic effects on acellular cementum formation [10–14]. Cellular cementum surrounds the apical root and its formation has been reported to be less influenced by mineral metabolism [11,13].

The "sandwich" arrangement of cementum/PDL/bone maintains flexibility of the tooth in the socket, provides a means for distribution of the forces from occlusion, allows for mechanoresponsive cells to direct remodeling and repair, and maintains vascular, lymphatic, and nerve supply to the periodontium. For proper maintenance of periodontal function, the PDL must be maintained as an unmineralized fibrous tissue, though it lies between two mineralized tissues (bone and cementum), harbors osteo- and cemento-progenitor cells, and is composed of a fibrillar collagenous matrix rich in pro-mineralization factors, such as the enzyme TNAP [1,13,15,16]. While regulators of cementum and bone mineralization have been partially defined, it remains unclear what factors are essential for maintaining the cementum-PDL and bone-PDL borders, and for preserving the PDL in its unmineralized state. It has been hypothesized that a balance of locally expressed regulators allow for continued cementum growth and adaptation of alveolar bone, while the PDL space is maintained. This question, while both clinically relevant and critical to understanding the basic biology of the periodontium, may also lie at the heart of the evolution of the periodontium (tooth in socket, or gomphosis joint), as the ability to promote and restrict mineralization in a site-specific fashion would be key for creating and maintaining these hard-soft interfaces. This evolutionary concept is supported by studies pointing to an ancient origin of cementum and documenting variable regulation of periodontal mineralization in extinct and extant species [17–21].

Our goal in this study was to identify factors contributing to the regulation of mineralization in the periodontium. As a first approach, we performed proteomic analysis on PDL from $Ank^{-/-}$ mice to identify factors altered (compared to normal controls) under conditions of rapid cementogenesis and subsequent PDL-bone remodeling. From this screening, we identified the protein OPN as a factor of interest. We then analyzed developmental expression of *Spp1*/OPN in the periodontium, effects of *Spp1* ablation on dental and periodontal formation, and potential in vivo functional role(s) of OPN in hypermineralization of cementum.

2. Materials and methods

2.1. Mice

Animal experiments complied with ARRIVE guidelines [22]. All animal experiments were approved by the Animal Care and Use Committee (ACUC) of the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS, Bethesda, MD) the Institutional Animal Care and Use Committee (IACUC) of the Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA), or the IACUC of The Ohio State University (Columbus, OH). Previous publications described the generation, colony maintenance, and genotyping of mice null for the progressive ankylosis protein gene ($Ank^{-/-}$) on a mixed C57BL/6 and 129S1/SvlmJ background [11,23], mice null for the osteopontin gene ($Spp1^{-/-}$) on a C57BL/6 background [24,25], mice null for tissue-nonspecific alkaline phosphatase ($Alpl^{-/-}$) on a mixed C57BL/6 and 129S1/SvlmJ background [26,27], and mice null for both *Spp1* and *Alpl* ($Alpl^{-/-}$; $Spp1^{-/-}$) on the same mixed background [24,27]. Mice deficient for both *Ank* and *Spp1* ($Ank^{-/-}$;$Spp1^{-/-}$) were created by breeding double heterozygote males and females on a mixed C57BL/6, 129S1/SvlmJ, and 129/CD1 background. Three to six mice were analyzed per genotype (unless otherwise noted) at ages including 10, 14, 26, 30, 60, 90, and 240 days postnatal (dpn). Male and female mice did not show significant differences from one another, therefore, both were included in analyses. Mice were housed in standard mouse cages in a 12-hr light-dark cycle and with access to standard rodent chow and water ad libitum.

2.2. Laser capture microdissection and protein extraction

Tissues from 60 dpn wild-type (WT) control and $Ank^{-/-}$ mice ($n = 5$ and 6, respectively) were used for laser capture microdissection (LCM). Mandibles fixed in Bouin's solution, decalcified in AFS (acetic acid, formalin, and sodium chloride) solution, and embedded in paraffin were serial sectioned around the first molar region [4]. Tissues were deparaffinized by submerging twice in xylene for 2 min, followed by an additional xylene wash for 5 min. Tissues were then air dried and immediately microdissected. LCM of PDL was performed using combined infrared (IR) and ultraviolet (UV) laser cutting on an Arcturus XT Microdissection Instrument (Applied Biosystems, Waltham, MA, USA) with adjusted settings: UV cutting speed of 300 mm/s; IR laser power of 70–80 mW, duration of 20 ms, and spot size of 30 μm. The total microdissected areas of WT and $Ank^{-/-}$ PDL samples were calculated (average of 302 ± 123 μm^2) and compared ($p = 0.66$, *t*-test). The captured area was used to normalize the amount of tissue/protein for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Caps containing the captured tissues were incubated with 30 μL of 8 M urea for 30 min at room temperature for protein extraction. Samples were sonicated and centrifuged briefly. Whole protein extracts were reduced, alkylated, and trypsin digested as described previously [28–30]. The resulting tryptic peptide samples were dried in a vacuum concentrator and reconstituted in 0.1% formic acid for analysis.

2.3. LC-MS/MS and bioinformatics analysis

Peptide mixtures were analyzed in an electron transfer dissociation (ETD)-enabled LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to the EASY-nLC system (Proxeon Biosystem, West Palm Beach, FL, USA) through a Proxeon nanoelectrospray ion source in a data-dependent mode. Peptides were separated by a 2–90% acetonitrile gradient in 0.1% formic acid using a PicoFrit analytical column (20 cm × 75 μm internal diameter, 5 μm particle size; New Objective, Woburn, MA), at a flowrate of 300 nL/min for 45 min. The nanoelectrospray voltage was set to 1.7 kV with the source temperature at 275 °C. All instrumental parameters were set up in data-dependent acquisition mode. The Orbitrap analyzer acquired the full scans of MS spectra (m/z 300–1600) after accumulation to a target value of 1e6, with a resolution setting of $r = 60{,}000$. The 5 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 5000 and fragmented in the linear ion trap by low energy collision induced dissociation (CID; normalized collision energy of 35%). The signal threshold to trigger an MS/MS event was set to 500 counts. Dynamic exclusion was enabled with an exclusion size list of 500, exclusion duration of 60 s, and repeat count of 1. An activation $q = 0.25$ and time of 10 ms were used.

Protein identification was performed with the Sequest search engine (Thermo Finnigan, San Jose, CA, USA; version 1.4.0.288) employing Proteome Discover version 1.3 (Thermo Fisher Scientific). The MS/MS spectra (msf) generated from raw files were searched against the UniProt Mouse Protein Database (released 22 January 2014; 51,116 entries) with parameters set to a maximum of one missing cleavage, with a parent ion tolerance of 10.0 ppm for MS search and a fragment ion mass tolerance of 1.0 Da. Oxidation of methionine (+16 Da) was set as a variable modification, and carbamidomethylation in cysteine residues (+57 Da) was set as a fixed modification. For label-free protein quantification, the data files were analyzed in Scaffold Q+ (version Scaffold_4.6.1, Proteome Software, Inc., Portland, OR, USA). The quantitative