Characterization of SIBLING proteins in the mineralized tissues

by

Sandeep Dab

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Abstract

Characterization of SIBLING proteins in the mineralized tissues (2020)
Sandeep Dab
Supervisor: Dr. Monica Gibson

Introduction: The SIBLING family of proteins is a group of non-collagenous proteins(NCPs)previously thought to be expressed only in dentin but have been demonstrated in other mineralized and non-mineralized tissues. They are believed to play vital roles in both osteogenesis and dentinogenesis. In the past, there have been random reports of their expression patterns in some mineralized tissues. Since, both osteogenesis and odontogenesis are tightly regulated lifelong processes and involve a peak of mineralization we used three different age groups. We systematically analyzed the mandibles, teeth and tibias of 1-, 3- and 6-month old WT (wild type) mice by determining immunohistochemical expression of SIBLING proteins in these mineralized tissues.

Methods: 15 WT mice (C57BL/6J – The Jackson Laboratory; Bar Harbor, ME) were euthanized with CO_2 - at ages 1, 3 and 6 months. Five hemimandibles and 5 tibias each for 1, 3 and 6-month old mice were randomly selected from dissected samples for use in the experiments. They were immediately fixed in PFA, decalcified and then embedded in paraffin. The samples were serially sectioned and mounted on slides for histological and immunohistochemical analysis. Hematoxylin and Eosin staining was done to localize various microscopic structures in the mandible and tibia of 1-, 3- and 6-month-old mice. Immunohistochemistry was done using antibodies for Dentin sialoprotein (DSP), dentin matrix protein 1 (DMP1), bone sialoprotein (BSP) and osteopontin (OPN). The immunostaining pattern was observed and documented. Semi-quantitative analysis of the immunostaining intensity was

done using ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA] and compared between the three age groups by 2 investigators independently.

Results: Immunostaining of DSP in tibia showed its peak expression in the 3-month-age group and staining was especially concentrated along the calcification zone. In the molars, DSP was expressed in the dentin matrix surrounding the dentinal tubules, predentin, alveolar bone, cellular cementum, and PDL with highest expression at 1-month. A similar expression of DMP1 was seen in the tibia and dentin. BSP expression in the tibia not only increased from 1- to 3-months but also showed a broader distribution pattern in the growth plate with immunopositive staining in the resting, proliferating and hypertrophic zones. High positive staining for BSP was observed especially in the acellular cementum. BSP was also expressed in the predentin, cellular cementum, alveolar bone and periodontal ligament (PDL). OPN was mainly expressed in the bone although a lower level of OPN was observed at all age groups in the teeth. The immunostaining intensity was less for all proteins in the 6-month tibia samples.

Conclusion: This study elaborated on the expression profile of the four SIBLING proteinsat three different stages of development in the mice. Their expression showed variations in their staining intensity and temporospatial patterning concordant with skeletal and dental maturity.In the tibia, the proteins showed strong immunopositive staining in the destructionand calcification zonesof growth plate. The maximum intensity of expression was found at 3-months of age corresponding to the timing of greatest conversion of osteoid into bone in the tibia. The expression in the teeth was high at 1month corresponding to the relative time of greatest conversion of predentin into mineralized dentin. A rise in their expression at 6 months happened at the same time as increased rate of cuspal attrition and slow dentin remodeling. IHC findings for DMP1 reinforce previous studies showing increased expression with mechanical loading, and

localization in regions where DSP was expressed. BSP's broad range of expression profile in all zones of growth plate, predentin and acellular cementum which could possibly be explained byprevious study showing its ability to bind with both the collagen and hydroxyapatite in an age and/or function dependent fashion. OPN's overall expression showing a pattern consistent with the pace and extent of osteogenesis and dentinogenesis and may partake in controlled inhibition. Taken together, since the SIBLING proteins were expressed in all age groups albeit at varying levels in different tissues, these findings suggest some role in this tightly regulated mineralization process.

Preface

This thesis is an original work by Sandeep Dab. This project was approved by theUniversity of Alberta Ethics Board (AUP00002086) under the name "**Expression of SIBLING protein in dental and skeletal tissues**". This study was funded by "Fund for Dentistry grant".

No part of this thesis has been previously published.

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LIST OF ABBREVIATIONS	
ARHR	Autosomal Recessive Hypophosphatemic Rickets
BMP 1	Bone Morphogenic Protein - 1
BSP	Bone Sialoprotein
CEJ	Cemento-Enamel Junction
CZ	Calcification Zone
DAB	3,3'-Diaminobenzidine
DEJ	Dentino-enamel junction
DD	Dentin Dysplasia
DGI	Dentinogenesis Imperfecta
DSPP	Dentin Sialophosphoprotein
DSP	Dentin Sialoprotein
DSP-PG	Proteoglycan form of DSP
DPP	Dentin Phosphoprotein
DMP1	Dentin Matrix Protein
EDTA	Ethylenediaminetetraacetic Acid
ECM	Extracellular Matrix
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
H&E	Hematoxylin and Eosin
HA	Hydroxyapatite
HERS	Hertwig's Epithelial Root Sheath
IHC	Immunohistochemistry
MEPE	Matrix Extracellular Phosphoglycoprotein
MSC	Mesenchymal stem cells
NCPs	Non-Collagenous Proteins
OPN	Osteopontin
PDL	Periodontal Ligament
PD	Primary Dentins
PFA	Paraformaldehyde
SIBLING	Small Integrin-Binding Ligand N-Linked Glycoprotein
SPSS	Statistical Package for the Social Sciences
RD	Reactionary Dentin/Reparative Dentin
RGD	Arg-Gly-Asp
SD	Secondary Dentin
T	Tibia
F	Femur
Tb.N	Trabecular number
Tb.Th	Trabecular Thickness
BV/TV	Ratio of Bone volume to Total volume in the region of interest
POC	Primary Ossification Center
SOC	Secondary Ossification Center

Research Question:

Both bone and dentin formation involves laying down of collagen in the extracellular matrix (ECM) by precursor osteoblasts and odontoblasts respectively. Closer to the precursor cells an unmineralized zone is maintained but distally the matrix undergoes mineralization and maturation in a stepwise and highly controlled manner. This dynamic biomineralization system is precisely regulated by molecular crosstalk through expression of proteins and enzymes. Certain preprogrammed biochemical events which include activation of transcription factors regulate the gene expression, and protein secretion. Various moleculesincluding secreted proteins in the extracellular space then control mineral nucleation, orientation and organization of the crystals. Throughout this process the width ofunmineralized zone ismaintained indicating biomineralization to be an orderly process. Disruption in this strict molecular control has been shown to lead to a wide array of human disorders affecting the biomineralization of the craniofacial complex and skeleton. A vast majority of mechanisms involved in osteogenesis and dentinogenesis remain unexplored or undefined. Cumulative evidence suggests non-collagenous proteins (NCPs), particularly the SIBLING family (Small Integrin-Binding LIgand, N-linked Glycoprotein) role biomineralization. This calcium binding family of phosphoproteins is believed to play an important role in modulating key events in this process. These phosphoproteins share several common features including localization on chromosome 4 in humans and 5 on mice, RGD motif and similar modifications post-translation. While their specific roles are yet to be outlined their post-translational modifications have been shown to be important in the initiation, rate and extent of biomineralization. It has been demonstrated that they are expressed in various mineralized tissues. The distribution pattern of the SIBLING

proteins has not been compared in various mineralized tissues at ages corresponding to skeletal growth and maturity.

Research Objectives:

The research proposed in this thesis is as follows:

AIM 1: To determine the localization and expression of SIBLING proteins in dental and skeletal tissues of 1-, 3- and 6-month-old wild-type mice.

AIM 2: To quantify the relative expression pattern and intensity of four SIBLING family proteins in the alveolar bone, dentin, cementum, and tibia at 1-, 3- and 6-months.

Hypothesis:

$H_{0:}$ The SIBLING proteins are expressed in the mineralized tissues. Their intensity of expression changes at different time points in age.

H_a: The SIBLING proteins are not expressed in mineralized tissue and their intensity of expression does not change with time.

This research will broaden the understanding of SIBLING protein expression in dental and skeletal tissues.

Chapter 1. Background

Background

A. Macroscopic & Microscopic Structure of Mouse Long Bones

Morphologically and functionally, body is composed of a complex organization of specialized tissues working together for specific structures and functions. (1) Bone is one specialized connective tissue that provides structural strength and rigidity to the body. (1) Structurally and microscopically, bone can be identified as cortical or trabecular. (1) (**Figure 1.1**) Cortical bone is highly dense mineralized outer protective layer with high resistance to bending or torsion. (1) The trabecular bone, on the other hand, is highly porous and has bone marrow.(1)Cortical bone surrounds the trabecular bone like a shell in the shafts of long bones. This feature endows the bone high compressive strength. Based on cellular arrangement pattern, microscopically long bone it is subdivided into three regions: epiphysis, metaphysis, and diaphysis. (1) (16)

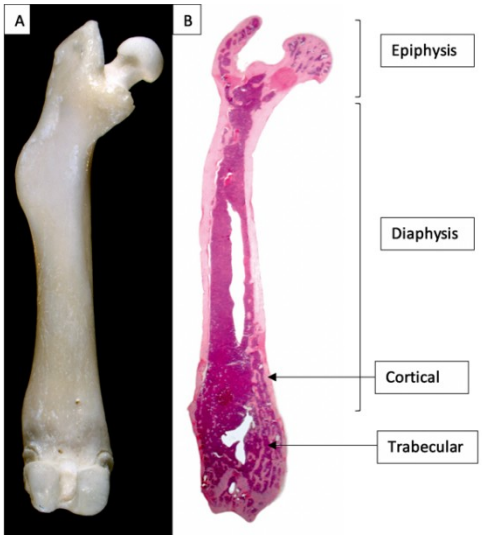

Figure 1.1.Macroscopic anatomy of a long bone (femur). A) Gross morphology of isolated femur Bone B) Histological longitudinal section. Hematoxylin-eosin stain. (Reprinted from - Morphological Mouse Phenotyping: Anatomy, Histology and Imaging, Jesus Ruberte, Ana Carretero, Marc Navarro, Osteology, page 12, Copyright © 2017 with permission from Elsevier; http://www.elsevier.com)

Bone matrix has both organic and inorganic components. The organic component of the bony extracellular matrix (ECM) comprises approximately 20% of the wet bone weight. Majority of the organic ECM (~90%) is composed of collagen type 1, providing flexibility to bone.(1) (4) The collagen fibers, secreted by specialized cells, form a scaffold which is

suspended in ground substance. The inorganic hydroxyapatite crystals which contribute 65-70% of bone wet weight surround the collagen fibers of organic matrix. (1)(16) A small percentage of this organic ECM represents highly multi-functional non-collagenous proteins (NCPs). (8)Controlled mineralization of the ECM components occurs under the influence of various molecules including NCPs.(3) (6) (16)

a. Mouse Tibia:

In the leg below the knee joint the tibia is larger of the two bones. This bone is involved in axial loading and has a substantial volume of cortical and cancellous/trabecular bone. The mouse tibia morphologically consists of a cortical shell encasing the cancellous marrow. Structurally, based on gross anatomy and pattern of arrangement of tissues, it has three regions: epiphysis, metaphysis, and diaphysis. (2-4) The tibial growth comprises processes occurring from early development through postnatal growth until achieving adult morphology. It is a combined product of interaction between various developmental units and the surrounding environment. (2-4) During embryonic development, mesenchymal stem cells condense and differentiate to form single anlagen of cartilage. This cartilaginous zone is divided into proximal and distal segments. As ossification begins, a collar of bone surrounds the anlagen at both ends establishing the growth plate. (2-4) The growth plate then lies sandwiched between the epiphysis on articular side and diaphysis on the shaft side of tibia. After this, the process of endochondral ossification in these growth plates lengthens the tibia. Chondrocyte proliferation and hypertrophy are the primary means of long bone lengthening. (3) The resultant cartilage model produced by growth plate is replaced by bone. This replacement process involves death of the hypertrophic chondrocytes, creating lacunae which are subsequently invaded by blood vessels, chondroclasts, osteoclasts and osteoblasts. The chondroclasts remove cartilage and osteoblasts in coordination with osteoclasts ultimately deposit bone at the previous site of cartilaginous model. (2-4)

There are4 discrete zones of chondrocytes in the growth plate: (3)(6)
Reserve zone, Proliferative zone, Pre-hypertrophic zone, and Hypertrophic zone. (**Figure 1.2**)
The reserve zone has small sized chondrocytes embedded in the matrix. It secures the growth plate to the secondary ossification center and do not directly contribute to bone growth. However, it supplies new chondrocytes via cell division at a rate that maintains pace with dying chondrocytes at metaphyseal end. (3) The chondrocytes in the proliferative zone are slightly larger and are stacked on each other.(3) (6) The zone of maturation and hypertrophy harbors

chondrocytes with accumulated glycogen and therefore are larger in size. With hypertrophy the source of nutrition (diffusion from periosteum) lies further away from hypertrophic chondrocytes to sustain their growth. Depletion of nutrition thus causes their degeneration leaving lacunar spaces. These spaces get invaded with blood vessels and osteoblasts which start depositing osteoid. (3) (4) (**Figure 1.3**)

This leads to growth in the length of the bone. (3)(6) This immature osteoid matrix is gradually converted into calcified tissue by organized mineral precipitation resulting in a zone of calcified matrix. (4) The growth of long bone stops with the cessation of chondrocyte proliferation in the growth plate. In mice, the epiphyseal plate persists throughout life after skeletal maturity, but does not contribute to the limb's growth.(5) The long bone growth is indirectly controlled by a complex interaction of cells, hormones, micro-molecules and host micro-environment. (3) (4)

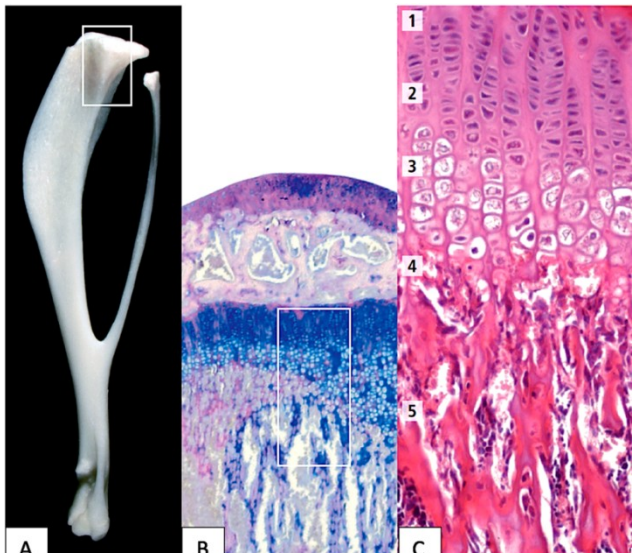

Figure 1.2.Structure of the tibia and tibial growth plate. A) Gross morphology of tibia B) Hematoxylin-eosin stain (5X). Figure 1.2 B is a magnified area representing the H&E-stained longitudinal section of the rectangle in Figure 1.2 A.C) Hematoxylin-eosin stain (200X).Figure 1.2 C represents the magnified area outlined in the center panel rectangle (growth plate) in Figure 1.2 B. 1: Zone of proliferation; 2: Mature zone; 3: Zone of hypertrophied chondrocytes; 4: Zone of destruction; 5: Zone of ossification/calcification (CZ).(Reprinted from - Morphological Mouse Phenotyping: Anatomy, Histology and Imaging, Jesus Ruberte, Ana Carretero, Marc Navarro, Osteology, page 16& 50, Copyright © 2017 with permission from Elsevier; http://www.elsevier.com)

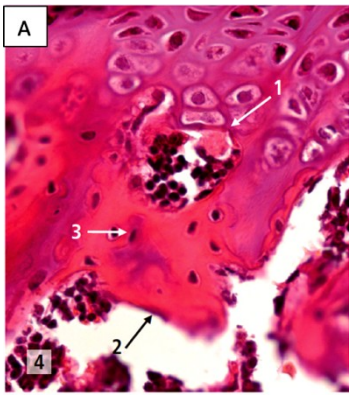

Figure 1.3.Zone of destruction and calcification in the growth plate. A) Hematoxylin-eosin stain (400X). (Source - Morphological Mouse Phenotyping: Anatomy, Histology and Imaging by Jesus Ruberte, Ana Carretero, Marc Navarro) 1: Osteoclast; 2: Osteoblast; 3: Osteocyte; 4: Bone marrow.(Reprinted from - Morphological Mouse Phenotyping: Anatomy, Histology and Imaging, Jesus Ruberte, Ana Carretero, Marc Navarro, Osteology, page 16, Copyright © 2017 with permission from Elsevier; http://www.elsevier.com)

b. Endochondral Osteogenesis from inception to skeletal maturity (C57BL/6 mice tibia):

The median life span of C57BL/6 mice has been shown to be on an average 29 months for females and 30 months for males. (5)(6) Postnatally, these mice grow to a considerable size until they attain sexual maturity. Endochondral ossification occurring at the epiphyseal ends of long bones facilitates longitudinal bone growth. During tibial growth, new cartilage is produced at the epiphyseal end of the growth plate (GP) while new bone replaces the previously made cartilage at the metaphyseal end of GP. Longitudinal bone growth depends mostly on the rate of proliferation and the size of the degenerating chondrocytes along the column of the growth plate. Peak longitudinal bone growth occurs between 3 and 6 weeks and therefore the length of the bone increases rapidly from 1 to 1.5 months. (5)(6) At this age, the thickness of the proximal growth plate is proportional to the rate of bone growth. The plate maintains the width during growth until 6 weeks, when the growth rate starts to decelerate, reaching near half of its maximum width at 3 months. There is a subsequent progressive decrease in the number of proliferative and hypertrophic chondrocytes with the lowest numbers at sexual maturity. (6) Once bone length, mass, and mechanical properties approach mature levels, longitudinal bone growth slows dramatically at puberty (between 1~3months) and appears to cease between 6 and 12 months of age in both sexes, even though the growth plate does not fuse. Bone marrow

cultures from 4 and 6-month-old mice show larger bone marrow cavities and reduced trabecular numbers with increasing age along with an increase in the number of bone marrow cells. (5)(3)

B. Macroscopic and Microscopic structure of Mouse Teeth:

The tooth is a highly calcified structure found in the jaws. It is a morphologically complex organization of enamel, dentin, cementum, and pulpal tissues. (8) (9) Anatomically, a tooth can be single rooted or multirooted and has a crown that is visible in the oral cavity. The crown of a tooth has three dental tissues of varying mineralization: a core of unmineralized pulp chamber surrounded by mineralized dentin which is further enclosed by highly mineralized enamel. (9) The root is composed of pulpal tissue in the root canal complex surrounded by dentin. The unmineralized pulp tissue in pulp chamber and root canal system is composed of connective tissue containing nerves, blood vessels and ground substance. (8) (10) The radicular/root portion of dentin from outside is covered by a thin layer of mineralized cementum. (**Figure 1.4**) The radicular portion of the tooth is surrounded by tissues with a specialized organization and architecture,known as the periodontium. (9)(95)

The periodontium of tooth is a complex of the gingiva, PDL, tooth cementum, and surrounding alveolar bone. The tooth is suspended in the alveolar socket by specialized connective tissue fibers called periodontal ligament fibers (PDL).The sharpey's fibers of PDL anchor into the mineralized cementum of root surface, on one side, and bundle bone of bony socket on the other. Adapting to the mechanical loading of tooth the alveolar bone undergoes continual remodeling by osteoblastic, osteoclastic, and osteocytic activity. (9) Whereas, cementum undergoes continuous slow apposition throughout life via cementoblasts. This arrangement of periodontium acts as a shock absorber by distributing forces of occlusion, maintaining flexibility of tooth in the socket, and transducing the mechanoreceptive cells in directing the remodeling and repair. The PDL must remain in an unmineralized state between the bone and cementum for proper functioning. This relationship is maintained by osteo- and cemento-progenitor cells under the strict molecular influence of micro-molecules and NCPs. (9)

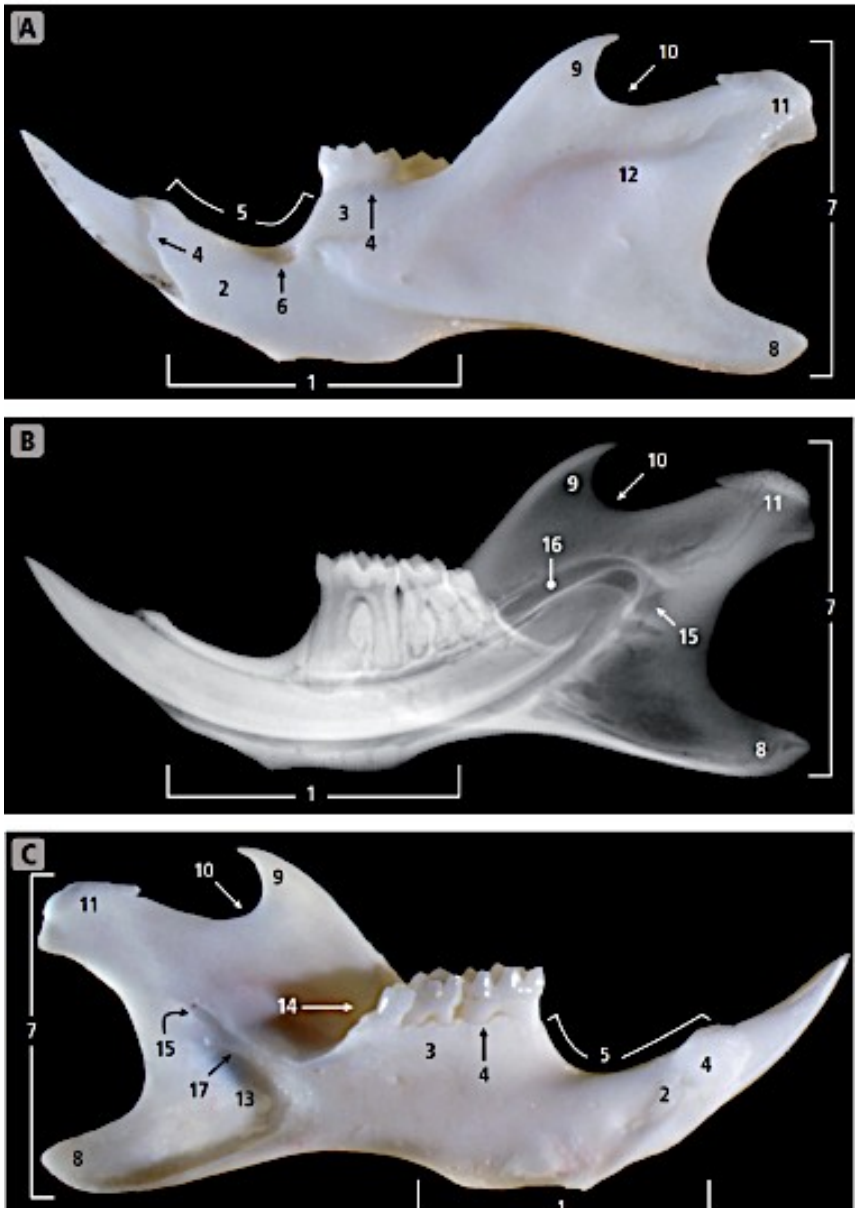

Figure 1.4. Gross morphology of Mandible. A) Lateral view. B) Mediolateral radiography. C) Medial view. 1: Body of mandible; 2: Incisive part; 3: Molar part; 4: Alveolar border; 5: Interalveolar margin; 6: Mental foramen; 7: Ramus of mandible; 8: Angular process; 9: Coronoid process; 10: Mandibular notch; 11: Condyloid process; 12: Masseteric tuberosity; 13: Pterygoid fossa; 14: Retromolar fossa; 15: Mandibular foramen; 16: Mandibular canal; 17: Mylohyoid groove. (Reprinted from - Morphological Mouse Phenotyping: Anatomy, Histology and Imaging, Jesus Ruberte, Ana Carretero, Marc Navarro, Osteology, page 27, Copyright © 2017 with permission from Elsevier; http://www.elsevier.com)

a. Dentin

Like the bone, dentin matrix is a composite of 70% inorganic substances, 20% organic substances and 10% water by weight. The dentinal tubules traverse the dentin from the pulp towards the dentino-enamel junction. (8)(9)(95) At the light microscopy level, the dentin can be morphologically distinguished into primary and secondary dentin. The primary dentin is further characterized into mantle dentin, circumpulpal dentin, and predentin. Located on the pulpal aspect of the dentin is a zone of nonmineralized predentin. Predentin is the initial dentin matrix that is laid down by odontoblasts prior to mineralization and is 10-40 μm wide. (8) Due to its unmineralized state, in H & E stained slides it is recognized by a pale colored region closely approximating the odontoblasts. The odontoblastic processes that closely approximate help to lay down dentinal matrix components. The remaining bulk of primary dentin is composed of circumpulpal dentin. The dentinal tubules are surrounded by a matrix of inter-tubular and peritubular dentin. The most prominent part of circumpulpal dentin is inter-tubular dentin which is secreted by the odontoblasts. The peritubular dentin is a thin, highly mineralized and well-demarcated sheath found around the lumen of dentinal tubules. (8)(95) In mice, peritubular dentin is usually absent in the continuously erupting incisors. Changes in the predentin at the mineralizing front lead to the formation of the inter-tubular dentin. The outermost layer of primary dentin (about 20μm thick) that is laid down first is called mantle dentin. It has fewer tubules with irregular organic matrix and is less mineralized. Odontoblasts lay down primary dentin through a highly orchestrated process until the tooth becomes functional. As soon as the teeth contact teeth in the opposing arch, formation of secondary dentin starts and continues throughout life. Primary and secondary dentin can be differentiated by the change in the curvature of the dentinal tubules from perpendicular in the primary dentin into more of an accentuated S-shape curvature in secondary dentin. Tertiary dentin can be reactionary or reparative depending on the intensity of the stimulus/injury and type of cells involved in repair. Reactionary dentin forms as a response to external stimuli (such as caries or tooth abrasion) by pre-existing odontoblasts, whereas reparative dentin is formed as a result of the activity of the newly differentiated pulp progenitor cells. (7)(8) (**Figure 1.5**)

As mice age, the teeth come into occlusion with subsequent loading. Due to compressive forces, the alveolar process remodels, and attrition of cusp tips commences. (7, 9) This leads to the reactionary dentin formation by the viable odontoblasts. These odontoblasts lay the dentin

down in an irregular fashion when compared to the primary dentin demonstrated by sharply contrasting incremental lines with abrupt changes. The remodeling of the dentin is a slow process while the root apex and alveolar bone undergo continual remodeling. (7)

The organic part of dentin matrix determines its morphology and is believed to play a key role in the formation of the mineralized phase. The organic matrix is dominated by a web of collagen type I fibers but as in bone, dentin harbors minor amounts of NCPs. (9)

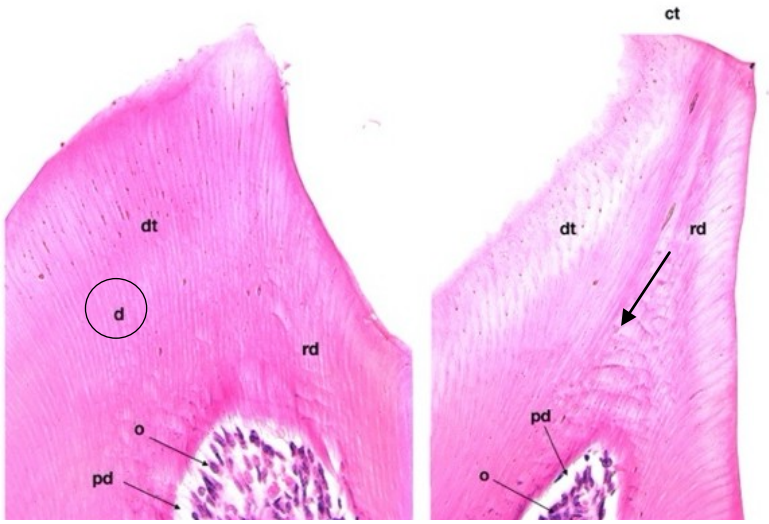

Figure 1.5. H&E staining of mesio-distal sections of mouse molarshowing primary dentin, and reactionary dentin. Cusp 40xH&E staining: the photomicrograph shows mandibular molars. (H&E 40x).; primary/secondary dentin (p) circle; reactionary dentin (rd) arrow; pd - predentin; o -odontoblastic layer; p - pulp; dt - dentinal tubules; ct - cusp tip attrition. (Source: H & E Dab)

(i) Dentinogenesis:

The proteoglycans along with type I collagen organize forming a fibrillar network near the dentin-predentin border to allow proper induction of the HA crystal deposition. (10) (11) Interestingly, odontoblasts are also known to directly secrete these proteoglycans into the mineralization front. (12) Within the predentin the glycosaminoglycans (chondroitin sulphate and keratan sulphate) are implicated to potentially play a role in inhibition of mineralization and show gradient changes. The keratan sulphate shows increasing while chondroitin sulphate shows a decreasing gradient from pulp towards mineralized front of dentin, (11) Since MMP-3 has been shown to be expressed at the junction of inner third and outer 2/3rd of the predentin, this shift in

the distribution gradient of proteoglycan chondroitin sulphate appears to be associated with changes in the activities of matrix metalloproteinase-3 (MMP-3). (15) Additionally, there is a concomitant increase in decorin from inner third to outer 2/3rd. (**Figure 1.6**) Both decorin, a proteoglycan, with 1 chondroitin sulfate chains and biglycan with 2 chondroitin sulfate chains are found in dentin. Dentinal proteoglycans, when compared to predentin proteoglycans, therefore contain higher amounts of chondroitin sulfate (13) (14) and thus have 19 times the binding affinity for hydroxyapatite. (14) Proteoglycans through their core proteins interact with collagen fibrils and their chondroitin sulfate chains possibly pierce into the collagen fibrils as needle-shaped structures. These structures thus capture calcium ions to bond to hydroxyapatite causing its nucleation around the collagen fibrils possibly under the influence of various molecules. (13) (14)

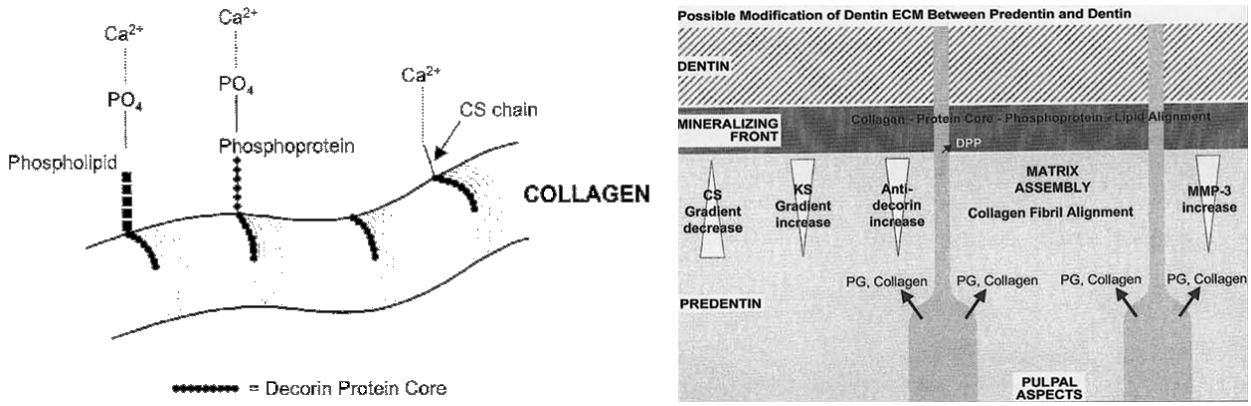

Figure 1.6. Known distribution of Some ECM components across predentin-dentin and suggested interactions of decorin at predentin-dentin interface. (Reprinted from Proteoglycans in Dentinogenesis. Embery, G; Pages 343-344; Critical Reviews in Oral Biology & Medicine, 12(4), 331–349.With permission from SAGE Publications)

b. Alveolar bone:

The teeth that are suspended through periodontal ligament and encased in the socket formed by alveolar bone.

(i) Intramembranous ossification:

Contrary to endochondral ossification of long bones like tibia, the alveolar bone develops by intramembranous ossification. It develops directly from mesenchyme without intermediary cartilaginous anlagen. The process involves condensation of mesenchymal stem cell (MSC) with

high cellular density that forms a template for future bone. The MSCs differentiate into osteoblasts forming an ossification center. Type-I collagen is laid down byosteoblasts and continues to be deposited forming scaffold for the alveolar bone. This is followed by organization and direct mineralization without intermediary cartilage formation. The aggregates of bony matrix form bony spicules and osteoblasts along the surface of the spicule secrete more osteoid with resulting increased size. Fusion of adjacent spicules leads to formation of trabeculae and its further growth leads to formation of woven bone. (16)

Root cementum, periodontal membrane, and the alveolar bone together constitute the *attachment apparatus* of the teeth. Its main function is the distribution of forces generated by mastication or occlusal contact. The alveolar bone may in response to functional demands and tooth cusp attrition undergo adaptive changes known as remodeling. (1) (16)

C. Biomineralization:

About 65% of human skeletal weight is attributed to the bones. Mineralized tissues of the skeleton possess biomechanical properties, allowing locomotion, and executing certain functions. For example, long bones provide rigidity and joints provide the range of motion and teeth withstand enormous amounts of chewing loads owing to high compressive strength of crystalline enamel and ductility of dentin. The size, shape, orientation and crystal order are orchestrated to provide rigidity and function. (17) This orderly process is known as mineralization.

Mineralization is a lifelong process which starts during embryonic stages and continues throughout in the form of bone remodeling. In most organisms, mineralization occurs within the organic matrix by well-coordinated biological processes. The organic matrix, a mainly type I collagen meshwork, forms the protein component that provides three-dimensional structure extending throughout bone. (18) At first, hydroxyapatite (HA) mineral in bone and dentin forms in a solution that is supersaturated with calcium and phosphate. During biomineralization under physiological conditions (a highly regulated process) these minerals precipitate into HA crystals. The crystal formation of bone and dentin apatite occurs primarily in two steps: nucleation and crystal growth. Nucleation is characterized by aggregation of ions into an ordered structure of few cells once reaching a critical size. Following nucleation, the process propagates by spontaneous precipitation of ions on the earlier deposited crystal structures known as crystal

growth.(18)The mineralization process is facilitated by this meshwork which acts as a fibrillar scaffold for mineral deposition.

a. Biomineralization of bone, dentin, and cementum

Bone, dentin and cementum share relatively similar composition and development mechanism. The precursor cells for bone, dentin and cementum produce a distinct set of extracellular matrix molecules composed predominantly of type I collagen fibrils. (19) Unique to the bone, cementum and dentin formation process is the biomineralization of the unmineralized proteinaceous matrix i.e. osteoid in bone, predentin in dentin, and cementoid in cementum, all of which lie close to their formative cells. These unmineralized precursors are readily transformed into mineralized matrix distally from the cells as the apatite crystals get deposited. This dynamic activity is influenced by the surrounding microenvironment that may act to enhance or inhibit mineralization of these precursors. (19)

Since both dentinogenesis (predentin to dentin) and odontogenesis (osteoid to bone) involve conversion of unmineralized structures to mineralized product, these processes strongly suggest resemblance of critical steps.(20) **(Figure 1.7)**

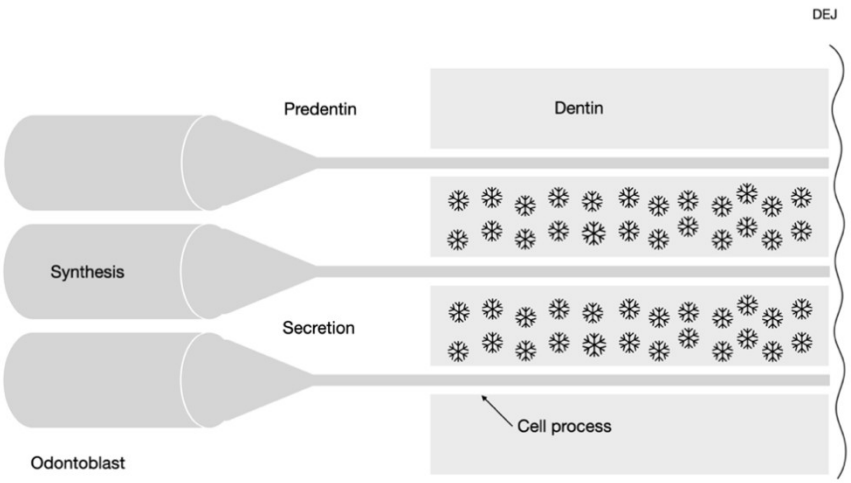

Figure 1.7. Schematic representation of the dentin cell matrix organization (Source: Dab)

Both transformations result in mineralized and unmineralized tissues of certain uniform thickness that are constantly maintained physiologically. Therefore, the mechanisms underlying the rate of synthesis must be closely regulated. The control of these mechanisms must involve processing and activation of certain molecules. (63) Pathological disruption of this regulation

may lead to changes in the mineralization of unmineralized matrices. This disruption of regulation is phenotypically exemplified inpathological conditions like dentinogenesis imperfecta and osteomalacia where mutations lead to dentin and bone mineralization defects. (24)

The aim of this study is to investigate the localization of SIBLING proteins expression pattern. These proteins have been shown to be involved in the regulation processes which control biomineralization. (10) (11) (15) (19) (21) (32) (37) (39) (42) (54) It also aims to compare their intensity variation across three age groups (1-, 3-, and 6-month old WT mice) and among the proteins. This will also help creating baseline data for better understanding of their dynamic interactions for future research in the field. (20)

(Sharing characteristics of bone and dentin)

D. Proteins expressed in the ECM of Bone and Dentin

Mouse and human genetic studies have confirmed that fully differentiated osteoblasts and odontoblasts are a prerequisite for laying down mineralized tissues. (21) Another, prerequisite for formation of dentin and bone is secretion of type – I collagen matrices (predentin and osteoid) by osteoblasts and odontoblasts, respectively. Consequently, these matrices serve as a scaffold for temporospatial deposition of mineralized (hydroxyapatite) in a precisely regulated manner. Therefore, this matrix would contribute to the mineralization together with highly specialized NCPs. Either the osteoblasts/odontoblasts synthesize NCPs or are derivatives of other tissues. Within the bone and dentin these NCPs then accumulate in relation to collagen or hydroxyapatite or both. They have various distinct functions including cell matrix adhesion and interprotein communications or collaborations. Their nucleation and inhibition activities control the mineral deposition and regulation of fibril formation following binding to collagen. Many proteins like NCPs and proteoglycans are suspected of having a direct role in biomineralization. (10)(11)(15)(25) One such NCP is SIBLING (Small-Integrin-Binding Ligand, N-linked Glycoprotein) family (a calcium binding family) which has 5 members:

i. Dentin sialophosphoprotein (DSPP),
ii. Dentin matrix protein 1 (DMP1),
iii. Bone sialoprotein (BSP),
iv. Osteopontin (OPN), and
v. Matrix extracellular phosphoglycoprotein (MEPE). (22) (**Figure 1.8**)

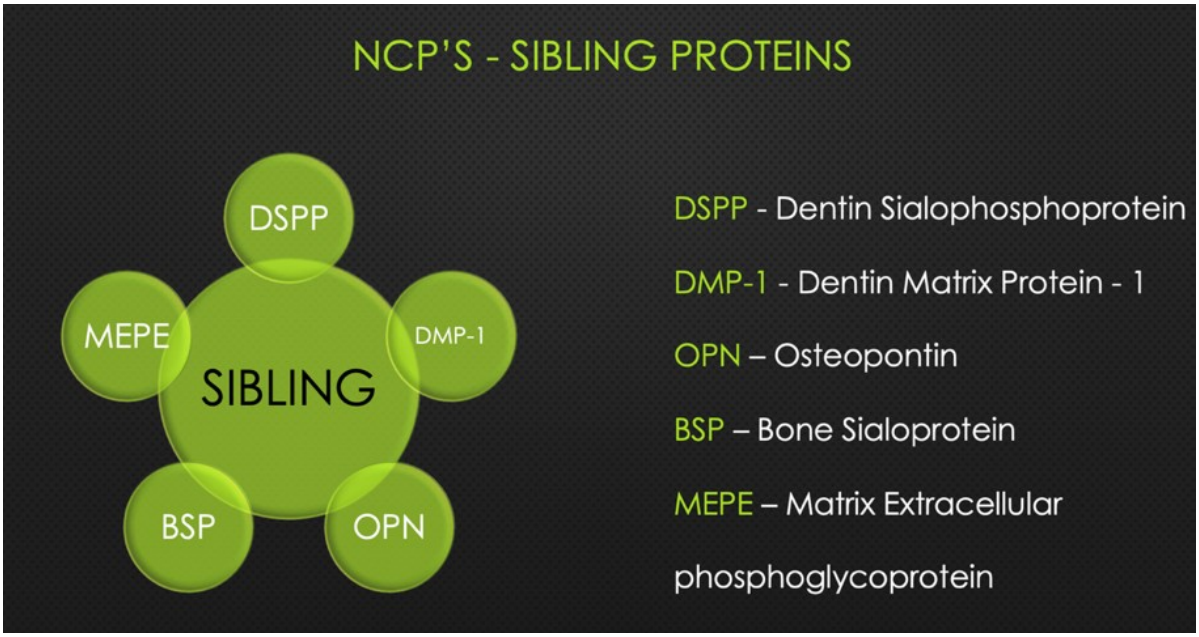

Figure 1.8. Schematic representation of the five known SIBLING proteins (Source: Dab)

Although, these proteins have little intrinsic homology in known sequences, they share similar developmental traits listed below: (23)

i. Located on chromosome 5q in the mouse and human 4q21-23 region.
ii. Similarity in exon structure.
iii. Have an RGD tripeptide motif (Arg-Gly-Asp) that mediates cell signaling via special integrins. and attachment.
iv. Undergo modifications post-translation e.g. glycosylation and phosphorylation.
v. In bone and dentin, are produced and extravasated into the ECM during formation and mineralization of these tissues. (23)(69)(71)
vi. All are acidic and contain a high degree of random coil structure.

The SIBLINGs in addition to other diverse functions, have been associated with biomineralization as enhancers and/or inhibitors. (45) Although their expression has been shown to be similar in quality, they may differ in quantity. (69) It has been previously reported in the scientific literature that mutations in the SIBLING family genes are associated with abnormalities in their phenotypic expression. Two members, DSPP and DMP1, have been

directly associated with some human genetic diseases. Phenotypically these diseases present as alterations like hypomineralization of bone and dentin. (35)(56)

The literature has shown that disruption of the biomineralization process leads to altered morphotype, phenotype together with involvement of SIBLING proteins. Although there is an abundance of scientific evidence in the literature, questions remain unanswered regarding the exact role of SIBLING proteins in both osteogenesis and odontogenesis. The SIBLING family of proteins display differential temporo-spatial expression profiles in tissues and functions that are highly dependent on post-translational modifications. Together, they are believed to actively orchestrate HA mineralization and crystal growth. The SIBLING proteins have been widely evaluated individually and their key roles in the process of biomineralization have been characterized. (10) (15) These pieces of evidence therefore direct us for the need to explore further pathways critical to biomineralization and their involvement. To elaborate on the role of SIBLINGs in biomineralization in my thesis, our research aims to investigate the expression of DSPP, BSP, DMP1 and OPN in these mineralized tissues.

E. SIBLING family of proteins and its correlation to Dentinogenesis and osteogenesis

a. Dentin sialophosphoprotein -(DSPP)

DSPP is the largest and most differentially expressed SIBLING protein in dentin ECM. (24) Although historically authors thought it to be expressed exclusively in dentin, DSPP has, in the last few decades, been shown to be expressed in bone, cementum, differentiating odontoblasts, pericytes of dental pulp and non-mineralizing tissues like lung and kidney (albeit in low amounts).(25)(26)(27)(28) It has also been demonstrated that its expression level in rat dentin is about 1/400th that of long bone. (25)Western blot experiments have confirmed detection of only trace amounts of inactive full-length form of DSPP is found in tooth.(29)It therefore must undergo proteolytic processing into its active form. Previous studies suggest possible role of BMP-1 in processing of DSPP into DSP and DPP fragments and further degradation of DSP by MMP - 20 and -2. (18) The dentin sialoprotein (DSP) (30)(31)(32) and dentin phosphoprotein (DPP) (33)(12)(34) originate from the amino and carboxy terminal regions, respectively. (35) **(Figure 1.9)** Earlier studies suggest DSP to be 1/10th of DPP in dentin extracts. (36)(37) Both the processed fragments DPP and DSP have been shown to play major roles in the nucleation of

hydroxyapatite crystals during the mineralization process. (32) (38) Accumulated *in vitro* data surrounding the dentin apatite crystal formation and growth suggests that DPP plays an important role of initiator and modulator. Due to its highly phosphorylated state it has a strong affinity to Ca^{2+} when attached to collagen fibrils. It has been shown that elimination of phosphate groups resulted in loss of its ability to nucleate apatite into collagen. (9)(39)(40) DSP is the second most abundant NCP in dentin ECM compared to DPP. (36)The exact role of DSP is unknown, while one study demonstrated its insignificant to no effect on *in vitro* mineralization (41), another study indicated that DSP was involved in regulating the initiation of dentin mineralization. (42) A previous *in vitro* study also indicated DSPs role as either an inhibitor of dentin mineralization or an antagonist of accelerating action of DPP thus preventing rapid mineralization of predentin. (15) Further, a report has suggested a proteoglycan (PG) form of DSP, is major component of dentin ECM proteoglycan. This PG form of DSP is more abundant than DSP and has a chondroitin-6-sulfate GAG (glycosaminoglycan) chain. (43) The exact nature, composition and biological function of this carbohydrate is largely unknown. (32) This form may be involved in nucleation of hydroxyapatite crystals by interactions between the proteoglycans and the collagen fibrils. (44)

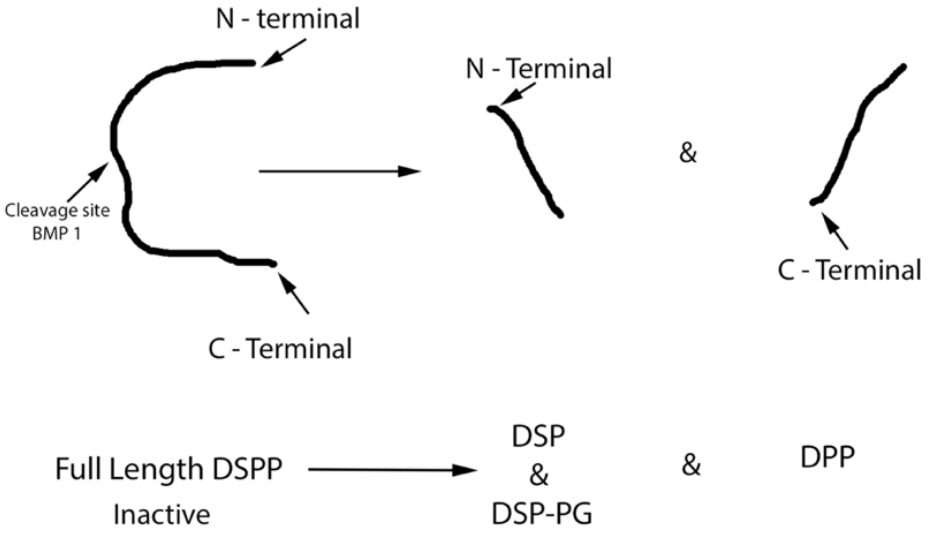

Figure 1.9. Full Length DSPP is an inactive precursor. It is proteolytically processed into DSP, DPP and DSP-PG. DSP is rich in carbohydrates whereas DPP contains large number of phosphates. (Source: Dab adapted and Reprinted from Qin, C. Critical Reviews in Oral Biology & Medicine, 15(3), 126–136.With permission from SAGE Publications)

DSP has also been shown to have a slight but concentration dependent ability to nucleate or inhibit HA crystal growth. An *in vitro* study reported DSP to act as a nucleator at low concentrations and inhibitor at higher concentrations. (39) DSP has also been shown to be primarily present in the organic (e.g. predentin) and not the mineralized extract. (45) Thus, dentinal DSP potentially plays a regulatory role in the matrix and assists hydroxyapatite formation along the collagen fibril.(42)

In humans, the pathogenesis of dentinogenesis imperfecta (**Figure 1.10**) (46) and dentin dysplasia type II (**Figure 1.11**) have been associated with mutations of DSPP gene. These patients present with hypomineralization defects of dentin, opalescent teeth with pulpal obliteration or thistle tube appearance clinically and disorganized dentinal tubules histologically. (47) Similarly, *Dspp* knockout mice ($dspp^{-/-}$) show expanded pulp chambers, alterations in predentin, and hypomineralization defects of dentin and bone.(48) (49) In addition, $dspp^{-/-}$ mice show changes in the bone structural properties at various ages. (50) This study thus indicates DSPP may play a key role not only in the initial mineralization (nucleation) but also continues to play role at various ages. Cumulative results from these studies in humans and mice have revealed mineralization defects in bone and dentin. The mechanisms by which DSP regulates osteogenesis or dentinogenesis, however, remain largely unclear.

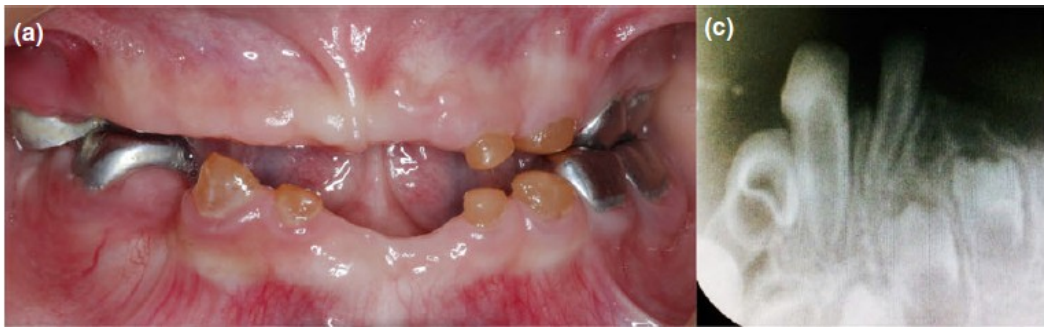

Figure 1.10. Clinical and radiographic features of a patient with DSPP mutation affected with dentinogenesis imperfecta – III showing amber discoloration of the teeth, severe attrition, enlarged pulp chambers and root canals. (Reprinted from Phenotype and genotype analyses in seven families with dentinogenesis imperfecta or dentin dysplasia H Feng, F Zhang, J Yang, et al, Page 363, Oral Diseases 2017, 23(3), 360-366. Copyright © 2017 With permission from JohnWiley and Sons, Oral Diseases)

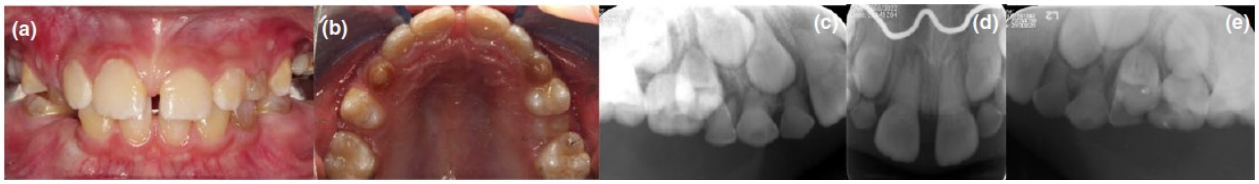

Figure 1.11. Clinical presentation, and radiographic findings for patient with mutation in DSPP affected with dentin dysplasia type - II showing opalescent yellow discoloration and attrition of primary canines. Radiographs show obliterated pulp chambers, root canals in both primary and permanent teeth. (Reprinted from Phenotype and genotype analyses in seven families with dentinogenesis imperfecta or dentin dysplasia H Feng, F Zhang, J Yang, et al, Page 363, Oral Diseases 2017, 23(3), 360-366.Copyright © 2017 With permission from John Wiley and Sons, Oral Diseases)

b. Dentin Matrix protein – (DMP1/DMP-1)

DMP1, a highly acidic phosphoprotein, was first identified in rat odontoblast by cDNA cloning of mRNA library.(51)Although it was thought to be dentin specific (52)it was later identified in various tissues including dentin, bone, (53) cementum and non-mineralized tissues.(54)(55) In contrast to DSPP, DMP1 is mainly expressed in developing bone and cartilage especially in osteocytes, osteoblasts and hypertrophic chondrocytes postnatally.(26) In teeth, it has been shown to be expressed in the odontoblasts, dental pulp cells, cementum, dentin, and predentin. (**Figure 1.12**) Within the dentin, it is localized predominantly in the peritubular region while, in cementum it is mainly found in cementocytes and the surrounding matrix. (56)(57) Like other SIBLING proteins, the proteolytic processing of DMP1 appears essential to its function and localization. Cleavage of DMP1 into C-terminal and N-terminal fragments in bone and dentin has been documented with protein chemistry studies. (58) The COOH (C-) terminal is consistently found in areas of vascular invasion and is considered an important step in matrix mineralization. (59)When phosphorylated (a post-translational modification), the N-terminal domain of DMP1 has been shown to inhibit the nucleation and growth of HA; however, C-terminal of DMP-1 is a well-established nucleators of HA formation in the presence of type 1 collagen. (60)(61)

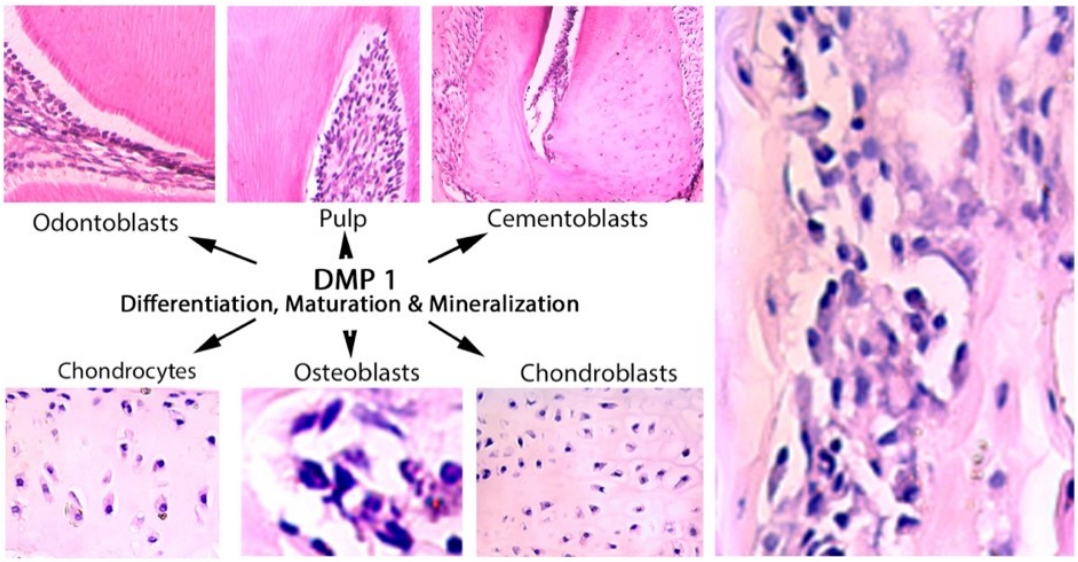

Figure 1.12. DMP1 is expressed in mineralized tissues and their precursors. The current hypothesis suggests its role in osteogenesis and dentinogenesis via cell differentiation, maturation and mineralization. (Source: *Dab &adapted and Reprinted from Qin C., J Dent Res 86(12):1134-1141, 2007. With permission from SAGE Publications)

Functionally, DMP1's potential for high calcium ion binding capacity is indicated by presence of unusually large number of acidic domains like phosphates. These phosphates possibly take part in calcium ions sequestration. A lattice like structural orientation of phosphate groups in DMP1 fits its supposed function of calcium phosphate crystal growth on itself. (31) **(Figure 1.13)** This property of protein that is considered for participation in mineralization, suggests its role in regulation of matrix mineralization. This calcium sequestering hypothesis is well supported by *in vitro* observations. (40) (54)

DMP1 has been shown to be expressed in both pulp and odontoblasts. (56)(63) DMP1 KO mice show odontogenic defects such as widening of predentin layer, enlarged pulp chambers, irregular maturation of predentin into dentin leading to thin dentin layer and abnormalities in dentinal tubules. (**Figure 1.14**) Analysis of the data suggests its key role in odontoblast differentiation, tubule formation and mineralization during odontogenesis.

DMP1 knockout mice have significantly lower mineral content with delayed conversion of unmineralized predentin or osteoid to dentin and bone. Re-expression of DMP1 in these mice rescues the skeletal defects.(62)(63) These mice have also been shown to develop abnormalities similarto rickets including enlarged growth plate with expansion of the chondrocyte zone, delays

in secondary ossification, osteomalacia and short limbs.(62)(64)Mice lacking DMP1 show similar phenotypic manifestations *in vivo*. (54) (**Figure 1.15**)

Osteocytes are considered essential for mechanosensation. (66) (56) Immunostaining experiments have shown a dramatic increase in the expression of DMP1 in the dendritic processes of osteocytes in response to mechanical stimulation. (65)Consequently, there is earlier onset of mineralization and accelerated differentiation in DMP1 overexpressing cells *in vitro*. (67) These studies therefore indicate a functional link of DMP1 with mineralization via mechanosensation by osteocytes.

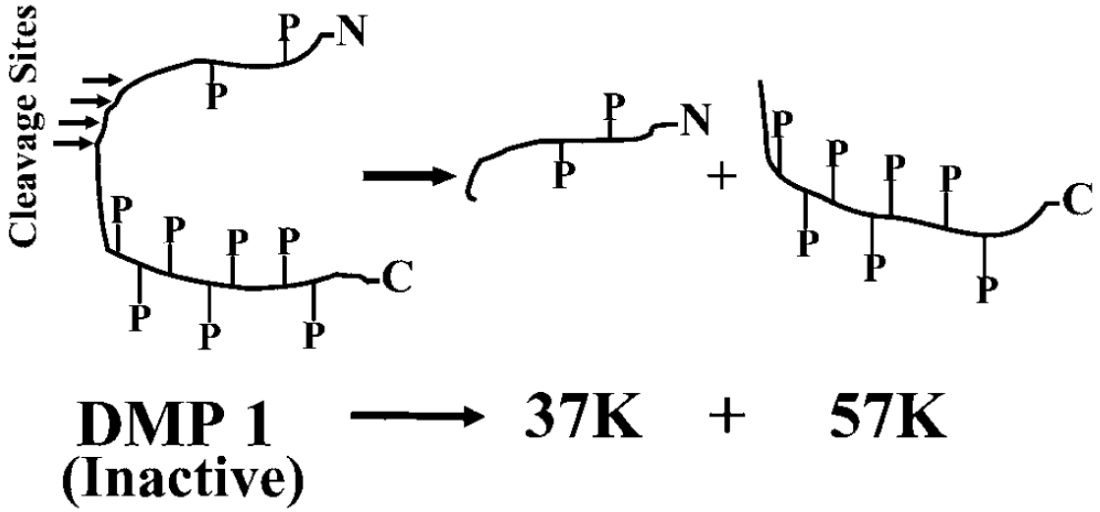

Figure 1.13. Full-length DMP1 is an inactive precursor.DMP1 is proteolytically processed into fragments originating from the amino- and carboxy-terminal regions of the DMP1 amino acid sequence, respectively. The carboxy terminal has more phosphate groups providing a lattice like arrangement. (Source: Reprinted from Qin, C. Critical Reviews in Oral Biology & Medicine, 15(3), 126–136.With permission from SAGE Publications)

Taken together, DMP1 appears to play a vital role in orchestrating the laying down of unmineralized precursors followed by mineralization. Defects in this process may possibly lead to changes in dentin and bone morphology. (**Figure 1.15**) It, therefore, primes us to study its distribution pattern in tissues where mineralization and remodeling takes place e.g. long bones, cementum, and dentin.

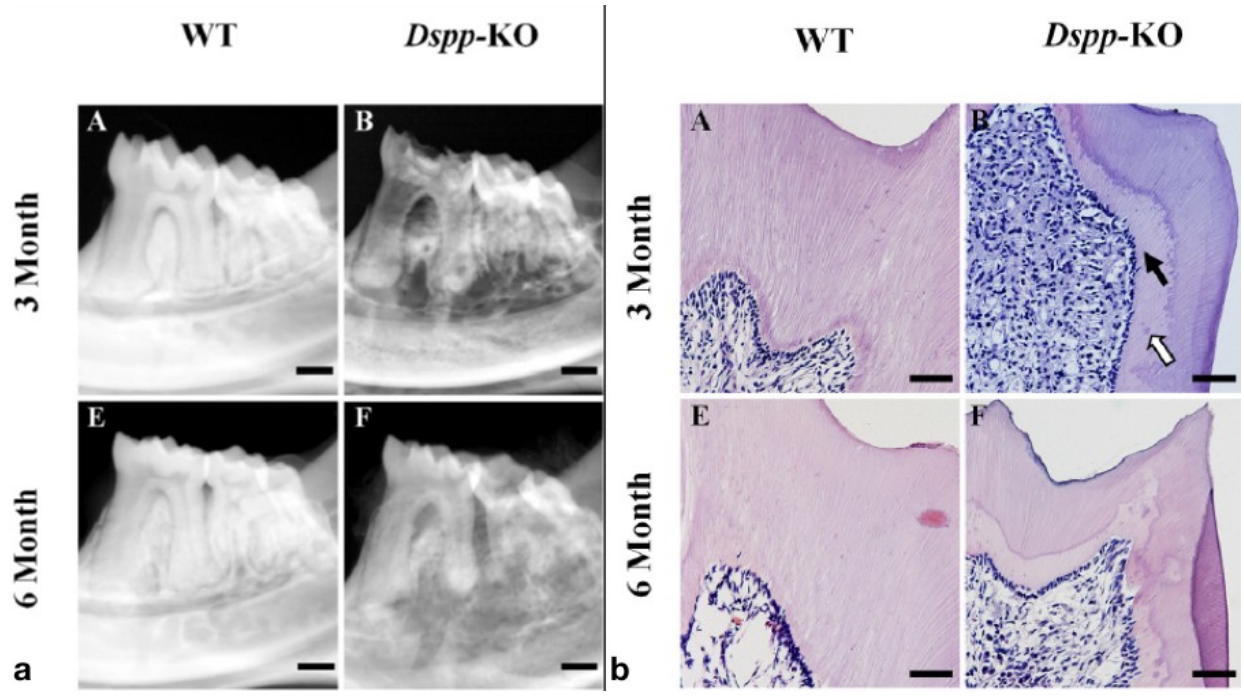

Figure 1.14. Plain x-ray analysis (a) shows at postnatal 3 and 6months.WT mice had evenly distributed and well mineralized dentin (A& E). The mandibular molars in the dspp-KO mice (B& F) had enlarged pulp chambers and thinner dentin compared to the WT mice. (b) H & E staining of the dentin-pulp complex in 3 and 6-month-old mice. At postnatal 3 and 6months, the dspp-KO mice (B, F) had wider predentin (solid arrow), uncoalescing calcospherites (hollow arrow) and an irregular dentin-predentin border compared with the WT mice (A& E). (Reprinted from "Proteolytic Processing of Dentin Sialophosphoprotein (DSPP) Is Essential to Dentinogenesis", Zhu et al., Page 30430-30431, Copyright © 2012, Journal of Biological Chemistry, 287(36), 30426-30435. With permission from American society for biochemistry & molecular biol).

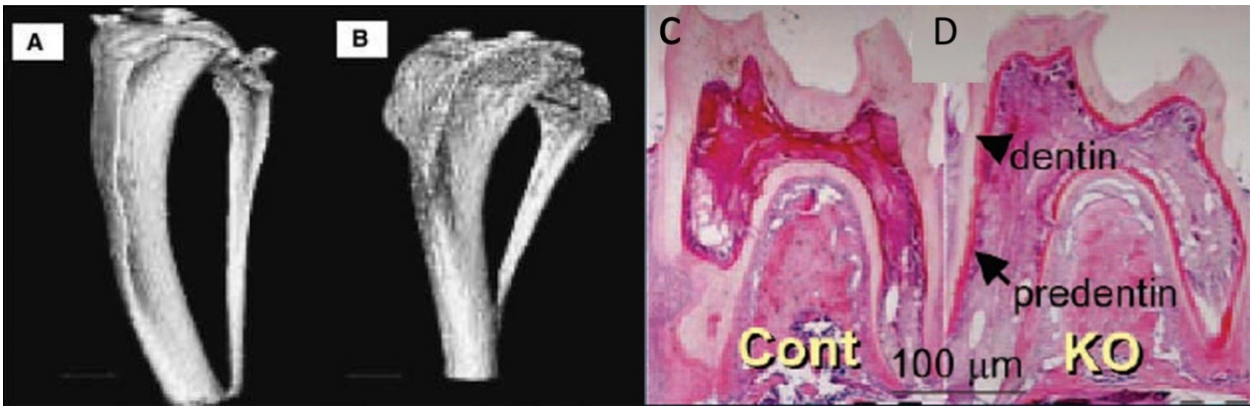

Figure 1.15. 3-D rendered µCT image of mice tibias Dmp1(A) WT tibia (B) KO tibia showed enlarged metaphysis, abnormal shape and shorter length. Deletion of Dmp-1 leads to defects in maturation of predentin to dentin. Representative hematoxylin/eosin-stained sections of molars show an extended predentin layer, enlarged pulp chamber and reduced dentin in Dmp-1 null mice (C) compared with the wild-type (D) mice, suggesting that DMP-1 is required for maturation of predentin into dentin. (Reprinted from"DMP1 Depletion Decreases Bone Mineralization In Vivo: An FTIR ImagingAnalysis"Ling J Bone Miner Res. 2005 December; 20(12): 2169–2177, Page 12, Copyright © 2009 and "Deletion of Dentin Matrix Protein-1Leads to a Partial Failure of Maturation of Predentin into Dentin, Hypomineralization, and Expanded Cavities of Pulp and Root Canal during Postnatal Tooth Development" Vol. 279, No. 18, Issue of April 30, pp. 19141–19148, 2004, page 19145,Copyright © 2004, Journal of Biological Chemistry, With permission from John Wiley and Sons and American society for biochemistry &molecular biol)

3. Bone Sialoprotein – (BSP)

BSP has been extensively reviewed and unlike OPN has been found exclusively in mineralized tissues. (7)(81)(82) It is a multifunctional protein that presents in bone, mineralizing cartilage, alveolar bone, reactionary dentin, acellular and cellular cementum and is strongly expressed in cementoblasts. (75)(83) Analysis of the *in vitro* data suggests BSPs role as a nucleator of the initial apatite crystals. With mineral growth on the collagen matrix, it is suggested to switch its function in directing the growth of crystals via inhibition.(84) (76) (85) Similarly, BSP has been shown to be expressed at the onset of the bone formation suggesting its

role in HA nucleation. (86) This nucleation potency is increased on BSP binding to collagen, suggesting they may have a mutually co-operative behavior. (84)

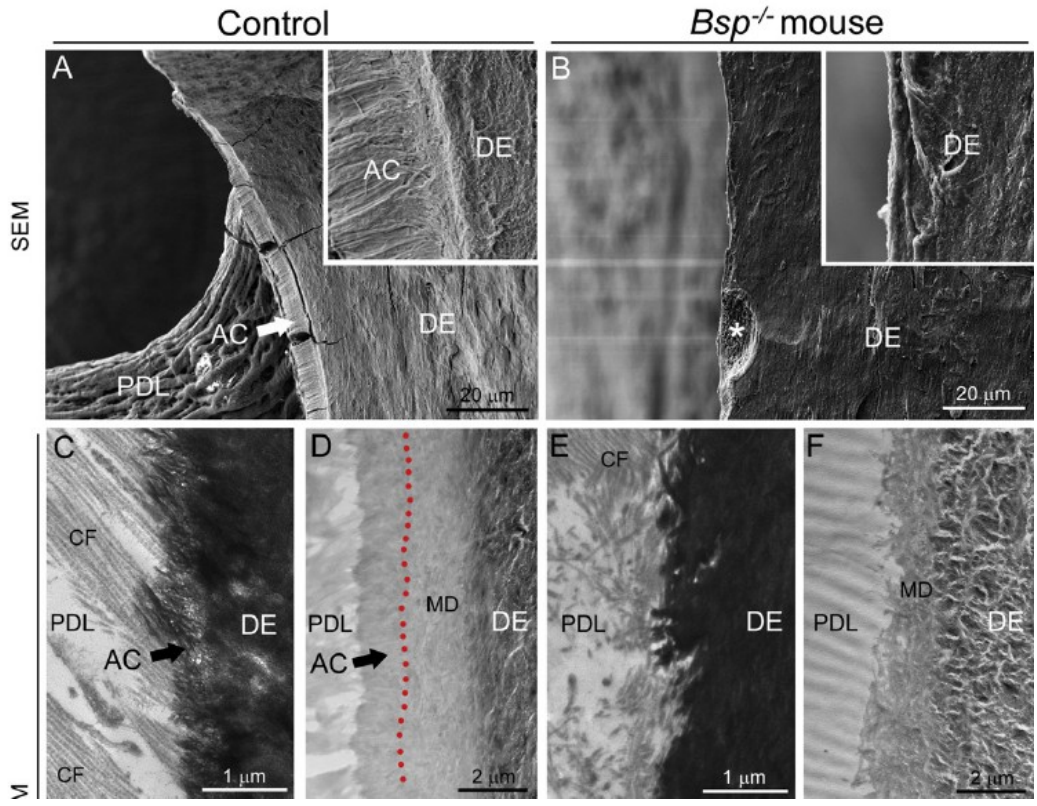

Figure 1.16. Defective cementum and periodontal ligament attachment in Bsp null mouse molars Compared to the well-developed acellular cementum (AC) in the (A) WT mouse molar at 30 dpn, SEM in back-scattered mode reveals that (B) Bsp−/− mouse molars do not feature a recognizable cementum layer on the dentin (DE) root surface, and no attached periodontal ligament (PDL) is observed on these molars. (C&E) TEM shows Bsp−/− mouse displaying a severely disrupted root surface topography, absence of sharpey's fiber development, and disarray in the adjacent PDL collagen fibers versus (D&F) WT mouse molar root. (Reprinted from "Mineralization defects in cementum and craniofacial bone from loss of bone sialoprotein" Foster, Bone. 2015 Sep; 78: 150–164., page 157,Copyright © 2015, with permission from Elsevier)

BSP null mice *(Bsp$^{-/-}$)* studies have shown defective cementum formation with progressive loss of periodontal attachment and bone loss. (**Figure 1.16**) (87)(88)(89) *Bsp$^{-/-}$* mice studies also report defects in the growth and repair of long bones. These also demonstrated

shorter hypomineralized bones, greater trabecular bone and lower turnover rate. (90) BSP also plays a role in upregulating osteoclastogenesis and therefore maintenance of homeostasis by remodeling. (91) Its role in osteoclastogenesis can be best seen in patients with Paget's disease, which reveal high levels of serum BSP. Following anti-resorptive treatment these patients showed a significant reduction in serum levels of BSP. (92)(93)

However, based on skeletal development to date the distribution pattern and localization of BSP has not been fully characterized in various mineralized tissues.

4. Osteopontin – (OPN)

Preliminary studies on OPN focused on its role in mineralization, bone homeostasis and remodeling. OPN is also known as secreted phosphoprotein 1 (SPP1) and was originally thought to serve as a bridge between cells and hydroxyapatite in the ECM of bone. Its mineral-binding ability is mediated through its phosphate groups and acidic domains. Persistent efforts of researchers have not only established its role in regulating mineralization of calcium phosphate but also in bone resorption and inflammatory processes. Also expressed by cells e.g. osteoblasts and in a variety of tissues including cartilage, cementum, mantle dentin, dentino-cemental junction, tertiary dentin and predentin, OPN is expressed in significant quantities in bone.(68)(7)(69) Particularly striking expression of this protein has been demonstrated at the matrix-matrix and matrix-mineral tissue interfaces e.g. cement line/resting line. These lines represent temporary pauses in the bone growth. (**Figure 1.17**) This expression along these lines is consistent with its function of inhibition of mineralization and or as a mediator of interfacial adhesion. (70) (71)(72)(73)

McKee and colleagues have demonstrated intense expression of OPN in primary spongiosa just below the epiphyseal growth plate – a site associated with vascular invasion and osteoblast differentiation. (74) OPN expression has also been shown in early bone formation and at sites of bone resorption facilitated by osteoclasts, suggesting its role in bone remodeling. In the periodontal complex, a dense OPN-rich network of afibrillar organic matrix embeds the sharpey's fibers into the cementum. (70) The acellular cementum of the tooth root has marked concentrations of OPN. (75)

There has been substantial documentation, both *in vitro*and *in vivo*, of the major inhibitory role of OPN in HA formation and growth. Additional studies have revealed that OPN

plays a regulatory role in both initiation and growth of hydroxyapatite crystals.(76)Boskey and colleagues showed inhibition of hydroxyapatite formation and growth *in vitro* and increased the mineral content and crystallinity with deficiency in mouse bone *in vivo*. (77)(78) The results of OPN knockout mice (*Spp*$^{-/-}$) also showed higher area/volume of cellular cementum with increases in mineral density, and size. (72) In addition, increased accumulation of OPN in X-linked hypophosphatemia has been shown to cause defective dental and skeletal mineralization. (79) It has been reported that in OPN-deficient mice the articular cartilage is affected due to loss of proteoglycan, which causes osteoarthritic structural changes. (80) The bioactivity of OPN is thus suggestive of numerous biological roles, not only in the regulation of calcium phosphate mineralization, but also in modulation of extensive biological events like arterial calcification, inflammation, bone resorption, cardiovascular disease and cancer. (66)

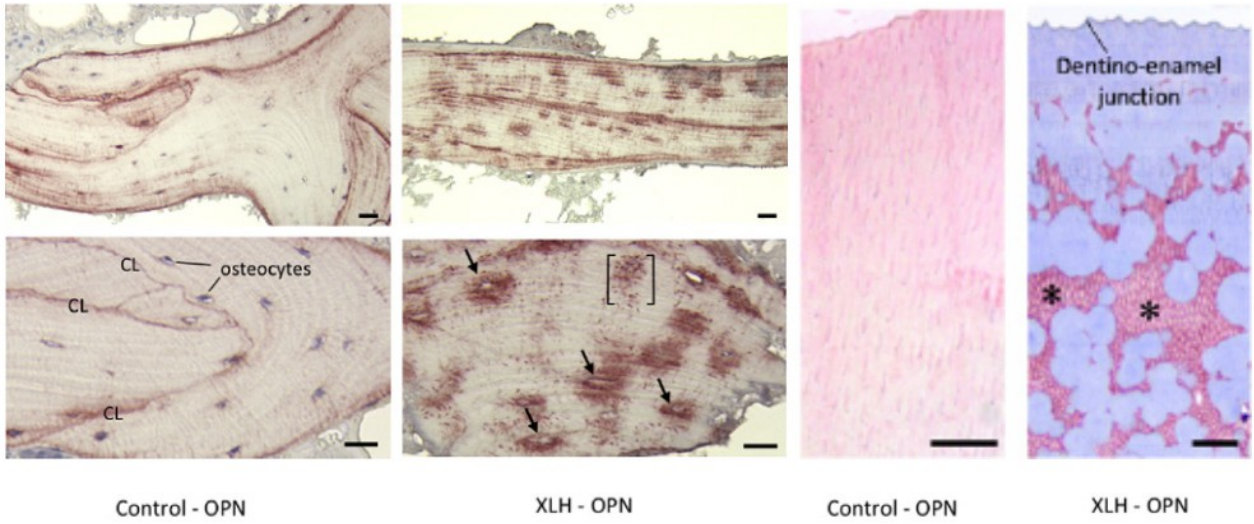

Figure 1.17. Immunohistochemical analysis of control versus X-Linked hypophosphatasia (XLH) patient. OPN stains strongest at cement lines in control while in XLH around hypomineralized regions. Similarly, dentin shows diffuse staining without prominent localization while XLH shows expression at interglobular spaces. (Reprinted from "Osteopontin and the dento-osseous pathobiology of X-linkedhypophosphatemia" Boukpessi, Bone 95 (2017) 151–161, page 156, Copyright © 2017, with permission from Elsevier)

F. SIBLING proteins distribution pattern in long bone and mandible

The articular surface, epiphysis, and metaphysis of the tibia and femur contribute to growth in length and width of the long bones. (6) This role is specifically displayed by the

growth plate, which harbors the hypertrophic chondroblasts responsible for the development, proliferation and production of ECM. The ECM in the growth plate is composed of collagen, proteoglycans and some non-collagenous proteins. (45) The hypertrophic chondroblasts lay down unmineralized matrix which undergoes mineralization under the influence of these NCPs. Previous studies have shown that some SIBLING protein members, such as BSP andDMP1, are expressed in cartilage. (56) Also, as mentioned earlier, it has been shown that structural changes in cartilage can be seen with OPN deficiency (80) and OPN's level is elevated in the cartilage of osteoarthritis patients. It has been suggested that DSPP and DMP1 may complement function acting in co-ordination within the complex milieu of bone matrix to regulate the formation and mineralization of hard tissues. (94) These proteins interact with other molecules and growth factors influenced by or influencing various known pathways thus contributing to the maintenance of homeostasis. (**Figure 1.19**) Previous studies also suggested that SIBLING family members may show varied distribution between inorganic and organic phases of bone and dentin. (**Table 1.1.**)

Substantial evidence in the literature supports SIBLING proteins important role in the mineralization during the growth and development of these tissues. However, until now, no methodical evaluation of the mineralized tissues based on skeletal development to maturity has been performed that demonstrates the distribution pattern and intensity of SIBLING proteins. This calls for research to elaborate individual and comparative staining patterns of mineral-modulating domains of the SIBLING proteins. (**Figure 1.18**) Therefore, to elucidate the molecular expression pattern, we systematically characterized the long bones and mandible to assess their distribution.

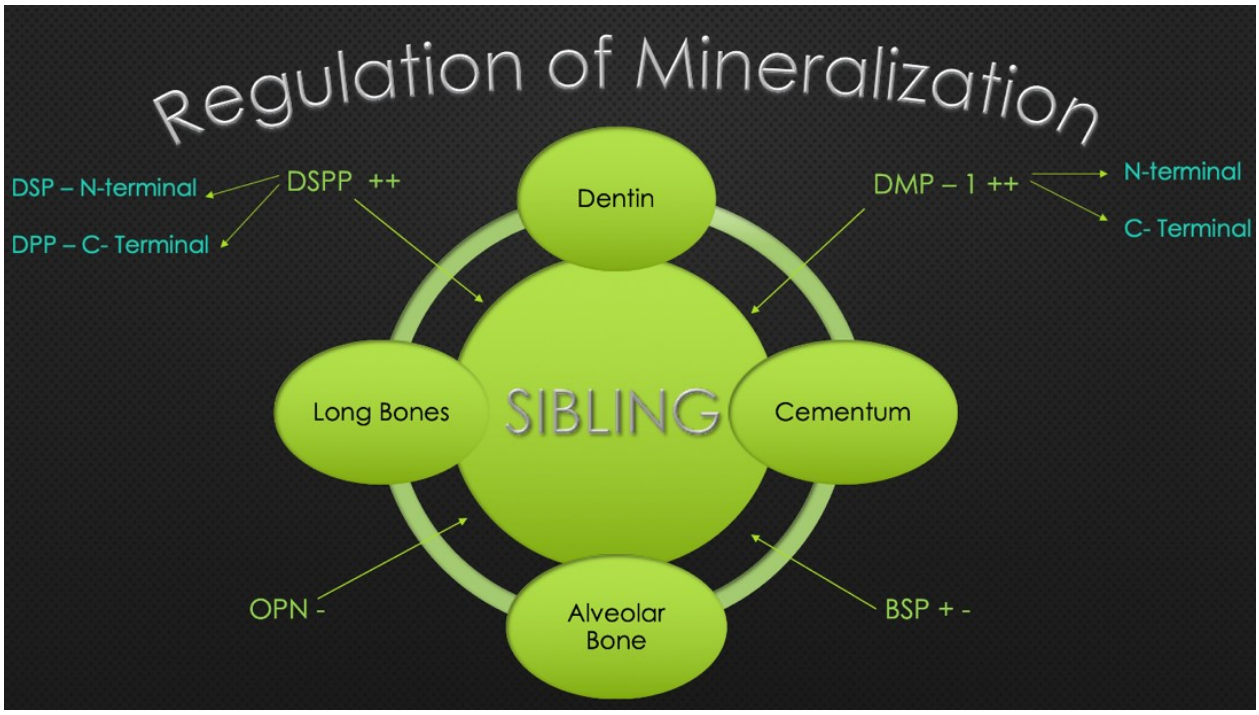

Figure 1.18. Interrelationship of the SIBLING proteins in biomineralization. (Source: Dab)

Previously known expression profile*			
DSP	Tooth	Dentin Cementum PDL	Differentiating odontoblasts Pericytes of dental pulp
	Bone		Present
	Non-mineralized tissues	Lung Kidney etc.	Yes
DMP1	Tooth	Dentin Cementum PDL	Odontoblasts Dental pulp cells Dentin Peritubular region Predentin Cementocytes Cementum matrix
	Bone		Osteocytes Osteoblasts
	Cartilage		Hypertrophic chondrocytes
	Non-mineralized tissues	Lung Kidney etc.	Yes
BSP	Tooth		Reactionary dentin Acellular cementum Cellular cementum Cementoblasts Alveolar bone
	Bone		Bone
	Cartilage		Mineralizing cartilage Growth plate
	Non-mineralized tissues	Lung Kidney etc.	No
OPN	Tooth		Predentin Tertiary dentin Mantle dentin Dentino-cemental junction Cellular Cementum
	Bone		Resting Lines/cement lines Below growth plate Osteocytes Sites of bone resorption
	Cartilage		Cartilage
	Non-mineralized tissues		No
**Profile of localized expressions is not based on skeletal growth and mineralization.*			

Table 1.1.Previously known expression profile of SIBLING proteins in various tissues.

(Source Dab)

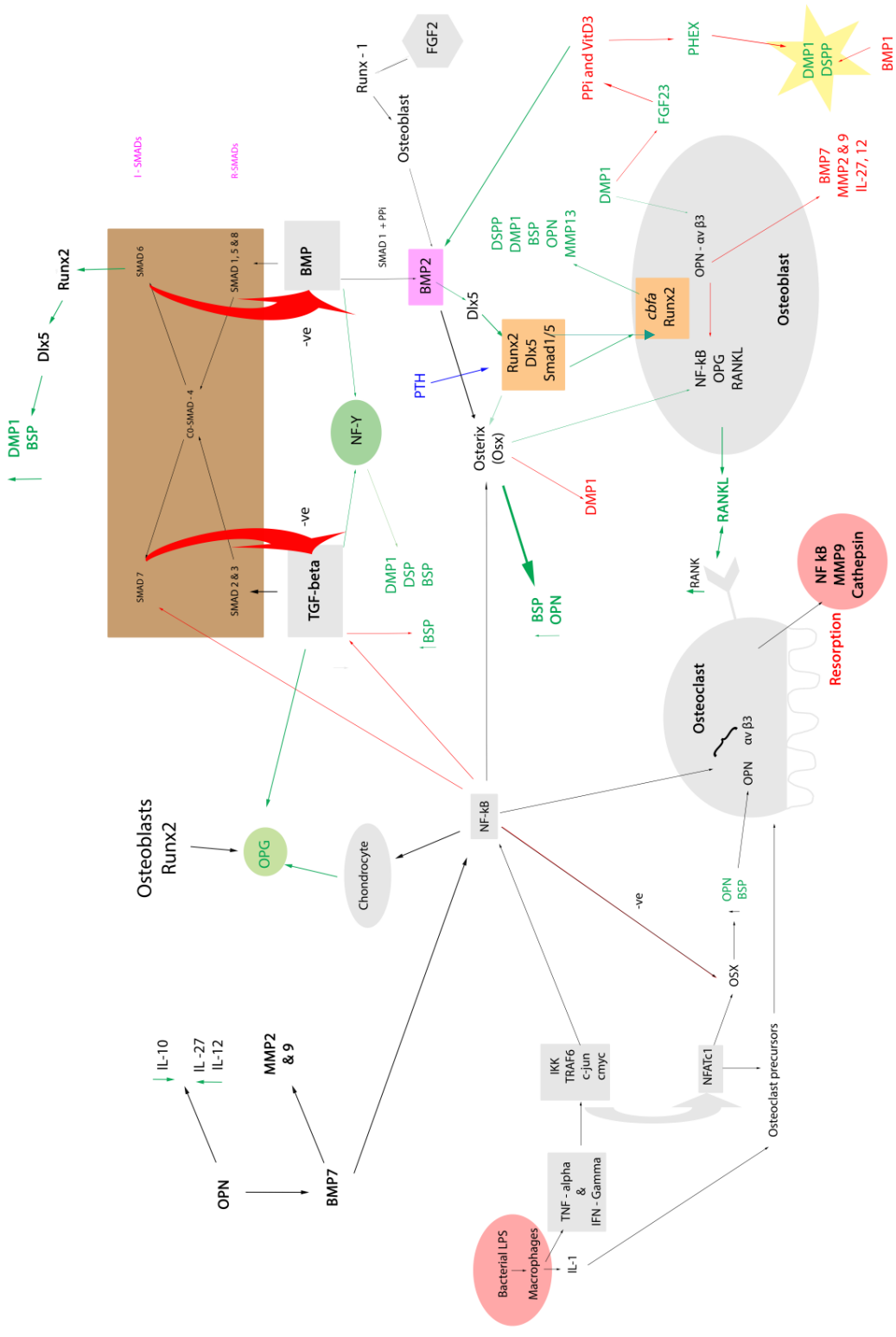

Figure 1.19. Interrelationship of the SIBLING proteins with various biological pathways.

(Source: Dab)

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Chapter 2. Material and Methods

Material and methods

A. *Sample Acquisition and Preparation*

15WT mice (C57BL/6J – The Jackson Laboratory; Bar Harbor, ME) were euthanized with CO_2 - at ages 1, 3 and 6 months. Five hemimandibles and 5 tibias each for 1, 3 and 6-month old mice were randomly selected from dissected samples for use in the experiments. Selection of the age group for tibia was based on the stages of skeletal maturity with high rate of longitudinal bone growth at 1 month in the tibia followed by slowing of growth at 3 months and finally no longitudinal growth but characteristic persistence of growth plate at 6 months. All procedures related to the animals were conducted in accordance with protocols approved by the Animal Welfare Committee at the University of Alberta Health Sciences (ACUC Committee approval #AUP0002086). Dissection was performed to retrieve the two hemi-mandibles and two hind limbs per mice. Sections of the long bone and whole hemi-mandibles were further fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline at 4°C overnight. The specimens for histology and immunohistochemistry were then decalcified (using protocol as previously described in the mentioned reference)in 15% EDTA (pH 7.4) at 4°C with continuous agitation using 300 ml of solution per long bone and mandible section. They were checked every 7 days and the total time of decalcification depended upon the age of the mice and the degree of calcification. (6) EDTA was changed to provide a fresh solution every 3 days (3d) until decalcification was complete, as confirmed by pin prick and deformation with hand.

B. *Hematoxylin and Eosin (H&E)*

Decalcified samples were sent to the University of Alberta histology core at for embedding in paraffin, and 5µm-thick serial sections were cut parallel to the long axis of the teeth and growth plate in a mesio–distal direction. Every 5th section was mounted and stained with hematoxylin and eosin (H&E) for morphological examination. Sections adjacent to those demonstrating teeth and alveolar bone were mounted and used for immunohistochemical staining and marker identification. H&E staining was performed in accordance with protocol developed by Sigma -Aldrich (7) and modified by our lab to examine the histological appearance and identification of the various structures in the mandible and tibia. The sections were observed under a simple American Optical One-fifty light microscope. (**Table2.1**)

H&E Staining Protocol		
	Repeat	
1. Xylene	X 2	10 mins
2. Blot excess Xylene from ends		
3. 100% EtOH		5 mins
4. 95% EtOH		5 mins
5. 95% EtOH		2 mins
6. 70% EtOH		2 mins
7. Distilled water		Dip
8. Stain with Harris Hematoxylin Solution		1-2 mins
9. Rinse slides with tap water***		5 mins
10. Differentiation solution		1-2 dips
11. Wash/Rinse with tap water		3 mins
12. Blue in Scott's tap water substitute		30 secs
13. 95% EtOH		2 mins
14. Eosin Y		1 min
15. 95% EtOH		3 mins
16. 100% EtOH	X 2	3 mins
17. Xylene	X 2	3 mins
18. Mounting with coverslip using permount (xylene based)		After drying

Table2.1.Hematoxylin and Eosin (H&E) protocol. *(Source- Dab)*

C. *Immunohistochemistry (IHC) Staining*

IHC staining was done to analyze the distribution and expression pattern of the four SIBLING family members DSPP, DMP1, BSP and OPN. MEPE was not part of our project since it is expressed at a very early stage and only expressed during embryonic development and postnatally day 5-9 in predentin and day 2 in osteoblasts. (8) (9) For IHC staining of DSPP/DSP, anti-DSP monoclonal antibody referred to as anti-DSP-2C12.3 (Baba et al. 2004a), was used at a dilution of 1:800. For IHC staining of DMP1, anti-DMP1-C-8G10.3 MAb recognizing the COOH- terminal region of DMP1 (Baba et al 2004b) was used at a dilution of 1:400. For BSP, polyclonal rabbit anti-mouse (Han 2014) (KLH conjugated synthetic peptide IgG derived from mouse bone sialoprotein) and for OPN, polyclonal (purified recombinant mouse OPN) (Miyazaki 1990, Lee 2016) goat anti-mouse were used as primary antibodies at a concentration of 1:200. (**Table 2.2**) Primary antibody concentrations were chosen to minimize background staining based on preliminary studies using various concentrations of the primary antibodies. Normal mouse immunoglobulin G (IgG) secondary antibody was used as a negative control for DSP and DMP-1, while normal rabbit anti-goat serum was employed as a negative control for BSP and OPN. (**Figure 2.1**) Biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was used at a concentration of 1:200 as the secondary antibody for DSP and DMP-1. Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used at a concentration of 1:200 as the secondary antibody for BSP and OPN. Rabbit IgG isotype control (bs-0295p-Bioss Inc.) was used as a control for staining.(1,2) In our experiments we followed IHC Protocol as previously developed in Chunlin Qin lab in Texas, USA by Dr. Gibson. (5)

Antibody	Antibody type – Catalog No.	Manufacturer
Anti-DSP-2C12.3	Monoclonal – MABT37	EMD Millipore
Anti-DMP1-C-8G10.3	Monoclonal - MABD19	EMD Millipore
Anti-BSP	Polyclonal–bs-4729R	Bioss
Anti-OPN	Polyclonal - O7635	Sigma-Aldrich

Table 2.2. Antibodies used in this study. *(Source - Dab)*

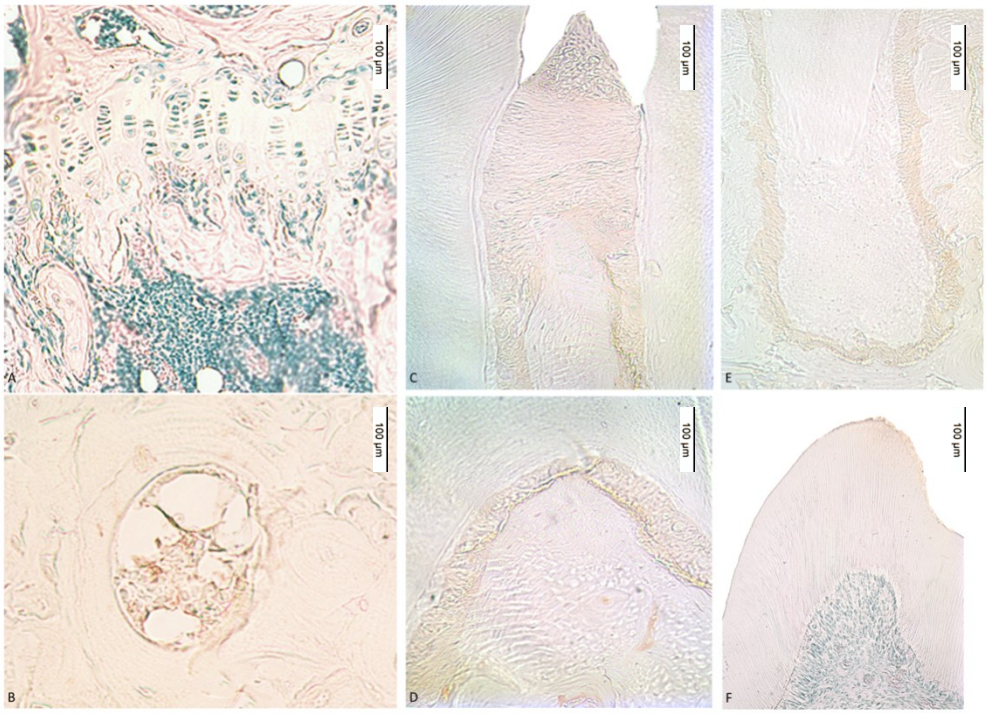

Figure 2.1.IHC control specimensusing mouse secondary antibody for DSP and DMP1 and normal rabbit anti-goat serum for BSP and OPN. (*A&B) IHC control of tibia: (A) POC (B) SOC), (C, D, E & F) IHC control of mandible: (C) interproximal alveolar bone, (D) furcation alveolar bone, (E) root (PDL & Cementum),(F) cusp dentin and predentin in mice. (IHC 20x) (Source – Dab)*

After de-paraffinizing, sections were blocked with 0.3% H_2O_2 in methanol for 30 minutes to inactivate endogenous peroxidase. Sections were treated with hyaluronidase to expose epitopes of target proteins. For monoclonal antibodies the blocking was achieved with the mouse IgG blocking reagent followed by incubation with primary antibody in protein concentrate for 1 h. For polyclonal antibodies the specimens were incubated for 24hours (24 h) at 4°C with blocking buffer (4% bovine serum albumin and 10% normal goat serum) to block nonspecific binding. Primary antibodies diluted in the blocking buffer were then applied for 1 h at room temperature. After washing in phosphate-buffered saline (PBS), secondary antibodies diluted in the same blocking buffer were placed on the samples for 1 h at room temperature. All IHC experiments were carried out using the Vectastain ABCkit and 3,3' Diaminobenzidine DAB kit (Vector Laboratories; Burlingame, CA) following the manufacturer's instructions to develop color for visualization of immunoreactivity. Finally, sections were counterstained with methyl green and observed under a simple light microscope. (**Table 2.3**)

IHC Monoclonal Antibody	IHC Polyclonal Antibody
Day 1	
1. Xylene x 3mins;	Xylene x 3mins;
2. 100% ethanol x 5mins;	100% ethanol x 5mins;
3. 100% methanol x 2 mins.	100% methanol x 2 mins.
4. 0.3% H_2O_2 in Methanol – 30 mins	0.3% H_2O_2 in Methanol – 30 mins
5. PBS wash	PBS wash
6. Hyaluronidase $1\mu g/\mu l$ at 37°C for 60 mins.	Hyaluronidase $1\mu g/\mu l$ at 37°C for 60 mins.
7. PBS	PBS
8. Overnight incubation at 4°C - M.O.M kit Mouse IgG Blocking reagent	Overnight incubation at 4°C – *Blocking buffer solution* (PBS + 2% Bovine Serum Albumin [BSA] + 10% Normal goat serum [NGS])
Day 2	
1. PBS	PBS
2. Incubate M.O.M protein concentrate	
3. Incubate with primary antibody in protein concentrate (DSP – 1:800; DMP1 – 1:400)	Incubate with primary antibody in *blocking buffer solution*. (BSP and OPN at 1:200)
4. PBS	
5. Incubate with secondary antibody Biotinylated Anti-mouse IgG Reagent of the M.O.M. Kit dilution 1:200 in protein concentrate.	Incubation with biotinylated goat anti-rabbit IgG at dilution 1:200 in *blocking buffer solution*.
6. Vectastain ABC kit reagent mixture	Vectastain ABC kit reagent mixture
7. PBS	PBS
8. Vectastain DAB solution	Vectastain DAB solution
9. H_2O for stopping color development	H_2O for stopping color development
10. Counterstain with Methyl green	Counterstain with Methyl green

Table 2.3.Immunohistochemistry protocol *(Source - Dab)*

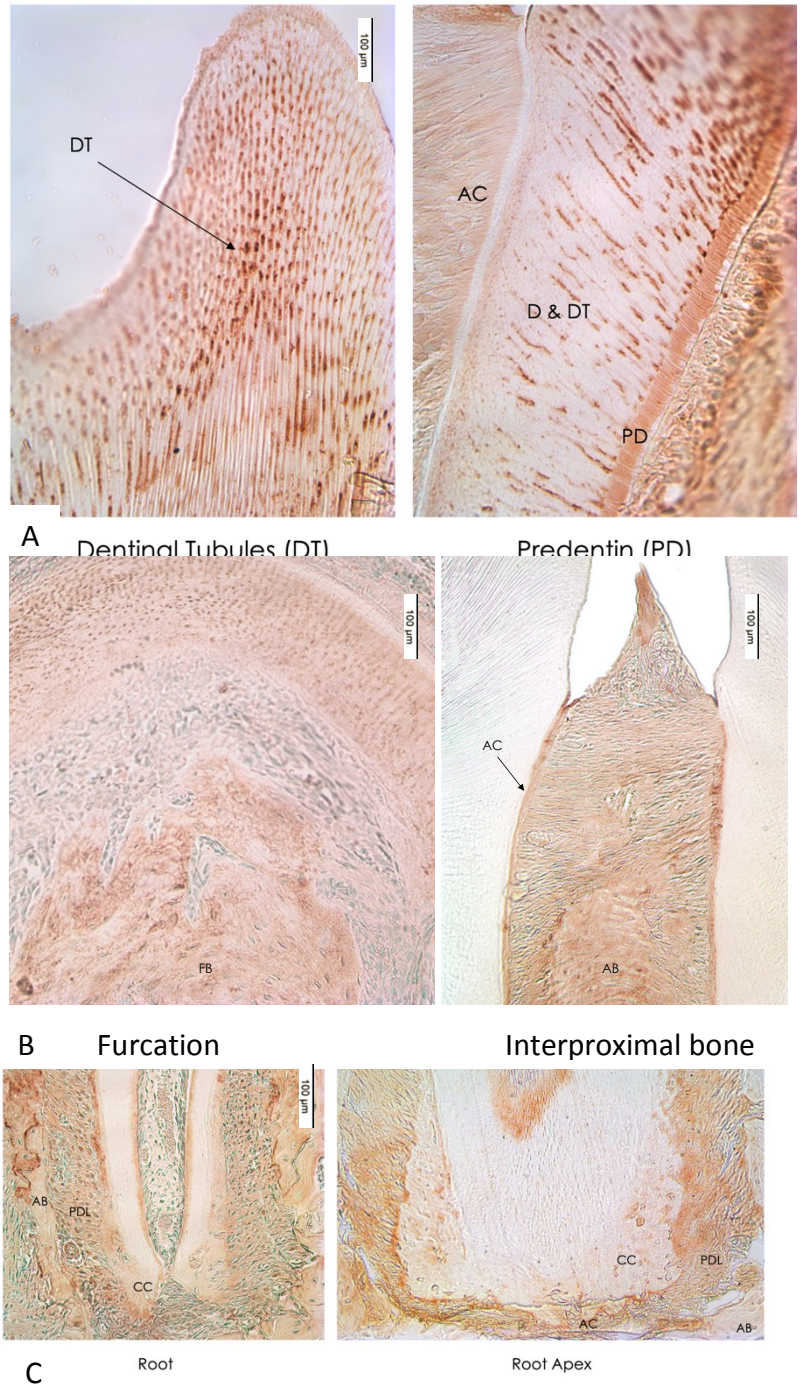

Figure 2.2.IHC specimens using antibodies for DSP, BSP and DMP1 showing the localization of expression in (A) cusp dentinal tubules (DT), predentin (PD), (B) acellular cementum (AC), interproximal & furcation alveolar bone (AB), (C) cellular cementum (CC), periodontal ligament (PDL). (*A-C) IHC 20x showing expression of various antibodies. DSP – cusp, DMP1 – furcation bone, BSP -interproximal bone and root apex. (Source –IHC Dab)*

D. Image Capture

The images were captured using a Leica MC170HD camera on upright microscope Leica DMRE (DM2000) set at brightfield. Light and camera settings were controlled by Leica application suite LASv4.9 software (Leica microsystems). Overall images were captured at 10x, 20x and 40x objective lenses. We chose 20x as a standard magnification to capture images. All photomicrographs were white balanced to achieve image standardization. The camera settings used for 20x magnification were an exposure of 70ms, gain of 1.1x, saturation at 125 and gamma set at 0.80 using LAS software resulting in average background values for RGB channels. (**Figure 2.2**)

E. Semi - quantification of IHC expression

The standardized images were visualized microscopically at 20x using the same parameters for illumination. These images were further analyzed using ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA] with IHC profiler plugin and FIJI plugin. The software follows a basic principle that the staining density is proportional to the concentration of the stain, which in turn transmits RGB vectors of certain optical density with wavelengths specific to the stain as per the Lambert-Beer law. The ImageJ IHC profiler plugin deconvolutes (separates) the image into two images- DAB, and counterstain images. (**Figure 2.3**) This plugin then analyzes the staining pattern by assigning a histogram profile for the deconvoluted DAB image. A histogram profile plot is between intensity values of pixels (X axis) vs. the number of pixels representing the intensity (Y axis). The histogram profile is then automatically divided in to 4 zones - high positive, positive, low positive and negative pixel color intensity bars. In digital image analysis, the pixel intensity values for any color range from 0 to 255, wherein, 0 represents the darkest shade of the color and 255 represent the lightest shade of the color as standard. The staining intensity was quantified between different age groups and proteins for expression. In the IHC method, visualization of the antibody-antigen reaction is accomplished using a secondary antibody conjugated to an enzyme, such as peroxidase, which catalyzes a brown color-producing reaction. (3)(4)

The pattern of the stain is broadly categorized based on the percentage of cells stained i.e. 75% – uniform; 25–75% – variable; and 0–25% – rare. The overall score of the staining intensity typically has four tiers ranging from 0 to 4. Keeping in view the standard grading procedure, the

histogram profile was further divided into 4 zones, viz. high positive, positive, low positive and negative.Wherein, the score of the zone is assigned as 4 for the high positive zone, 3 for the positive zone, 2 for the low positive zone and 1 for the negative zone. As a general procedure, images are opened in ImageJ,followed by deconvolution using the newly optimized color deconvolution plugin. With the selection of the 'H DAB' vector on the color deconvolution popup window, IHC profiler automatically plots a histogram profile of the DAB image and the corresponding scoring log is displayed on the screen.(3)(4)

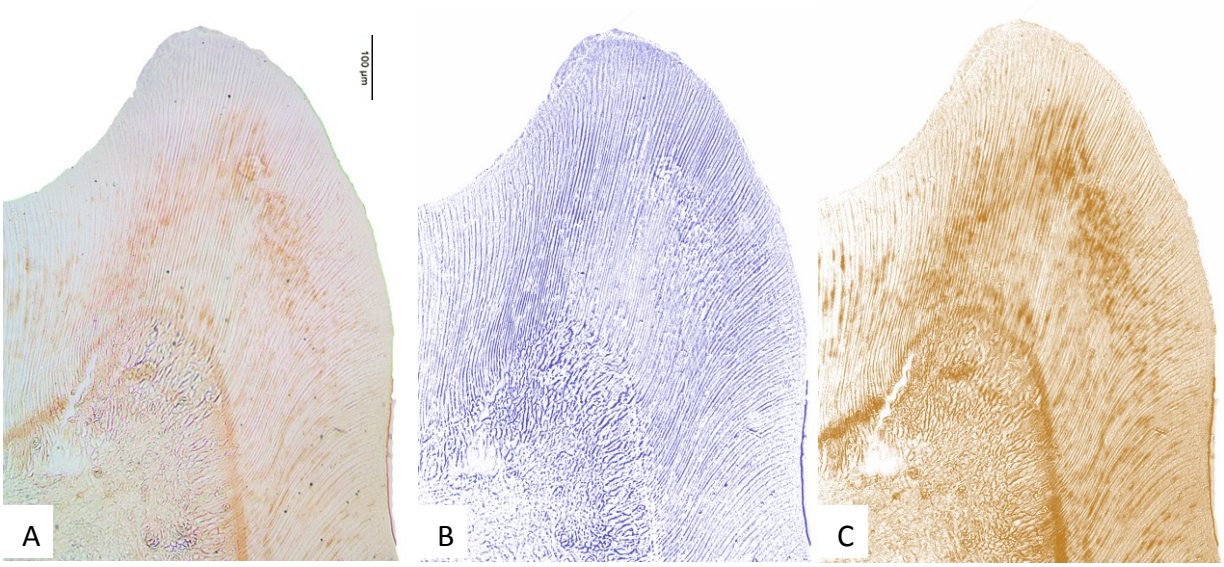

Figure 2.3. Image deconvolutionby ImageJ IHC profiler plugin *(A) Original image of tooth cusp before deconvolution by ImageJ. Separation following deconvolution into (B) counterstain (c) DAB image used by IHC profiler plugin for image intensity analysis.(Source – IHC Dab)*

F. Selection of field of view for image quantification:

During semi-quantification of tibia complete image at 20x without contrast enhancement was analyzed using ImageJ. For mandible sections in order to compensate for brightness each image was contrast enhanced by 0.5%. For the furcation bone, interproximal bone in order to isolate expression in bone being influenced by that from PDL, dentin and cementum a 2x2 area was selected randomly by each reviewer and assessed with ImageJ. Similarly, in the periapical region, a 2x2 area including the cellular cementum and PDL was selected for assessment. Since the software uses a DAB plugin which uses ratio between pixels the program automatically compensates for the magnification. (**Figure 2.4**)

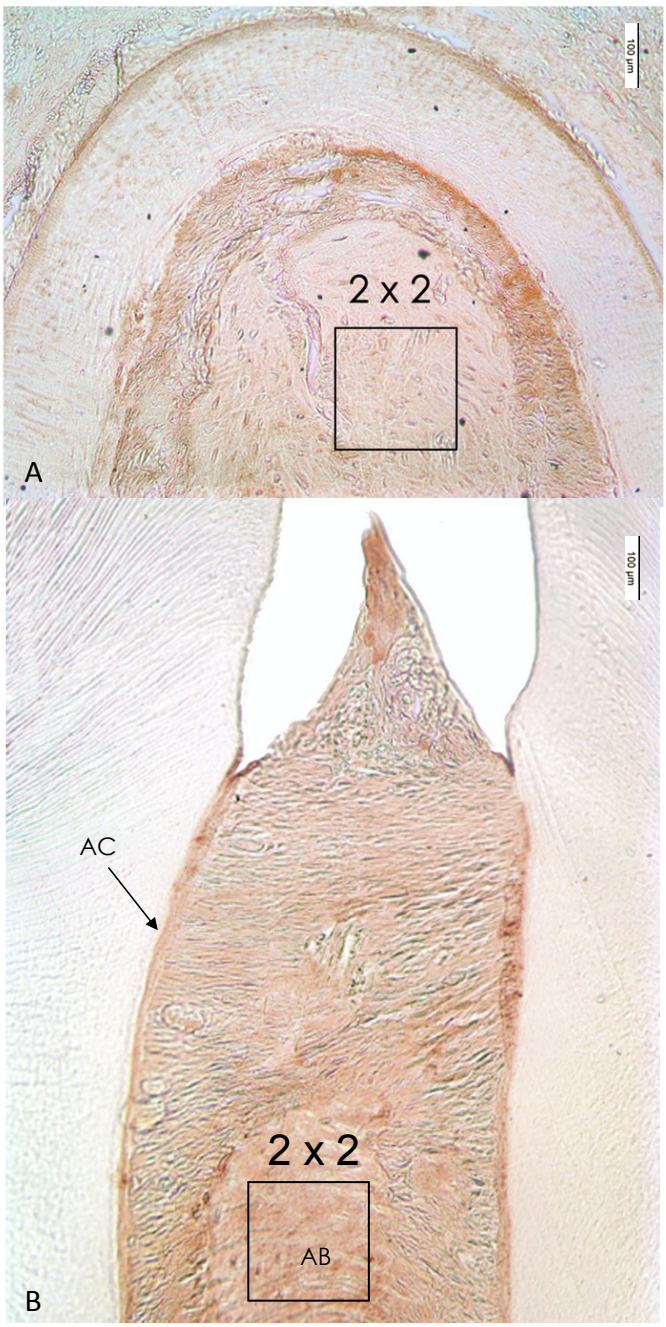

Figure 2.4.ROI (region of interest) selection in ImageJ IHC profiler plugin *(A&B) Original image of tooth furcation and interproximal bone before deconvolution by ImageJ. 2x2 area selection by 2 independent reviewers for image intensity analysis.* *(Source –IHC Dab)*

After the scoring was performed by 2 independent investigators, the expression between the different age groups and controls in long bone and mandible was compared. To achieve this, the values of each zone (high positive, positive, low positive and negative) were compared among

the controls and between the different ages. The high positive value was selected as a representative for graphs in each section analyzed. High positive was selected because it always gave a consistent reproducible result and avoided influence of background staining. (**Figure 2.5**) This process has advantages over the visual assignment of scores in the samples in that the computer allows high throughput of samples saving time and prevention of visual perception bias between independent investigators. We calculated the changes in the ratios between the different ages, proteins and interactions between the four proteins and the three age groups.

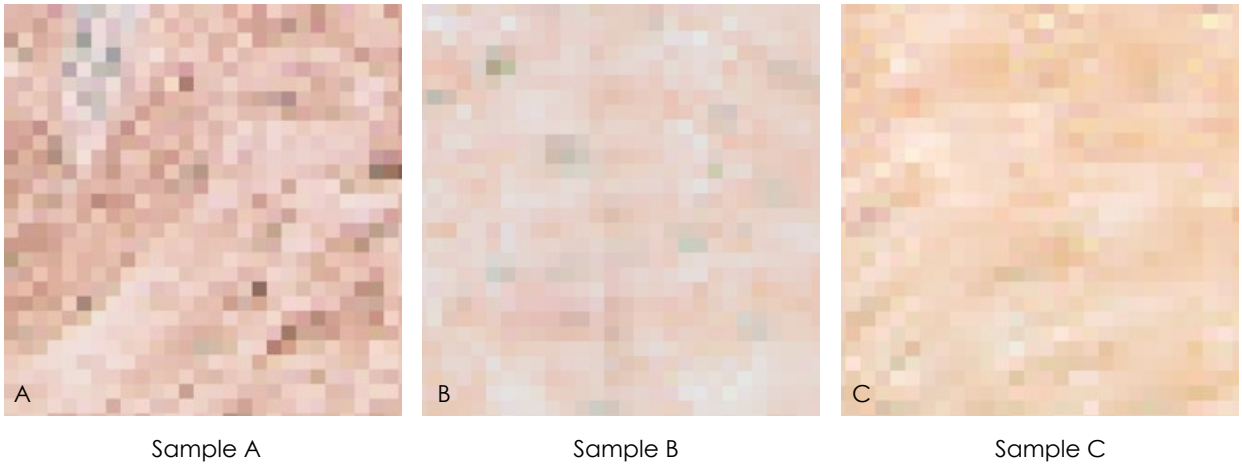

Figure 2.5. ImageJ automated sorting, segregation, and calculation of high positive, positive and low positive squares of DAB positive areas in three different samples (A, B, & C).

(Source –IHC Dab)

G. Statistical Analysis:

All data was processed using IBM SPSS statistics software 24 version for Mac, (11-2018) build 1.0.0.1347. Sample size analyzed for protein intensity (4 samples for each 4 proteins) (N=4) at each age (1, 3, and 6 months). Statistical analysis was done using repeated measures ANOVA to analyze changes in the expression intensity between and among 1month, 3month and 6-month immuno-stained samples. Descriptive statistics were run on SPSS to check the normal distribution. Mauchly's test for sphericity was conducted to check the significance of the variances of differences. When the condition of sphericity was not met i.e. differences between the variance of differences were significantly different the degrees of freedom were adjusted to have more conservative *F*-ratio. When the estimate of sphericity (ε) was <.75 Greenhouse-Geisser correction was used and when ε was >.75 Huynh-Feld was used to test within-subject

effects. Post hoc test using bonferroni corrections was used to compare and analyze significance of each protein by age group interactions. A separate univariate ANOVA was conducted for each age and plotted to analyze variations in *p*-values of SIBLING proteins from control at individual time points. Statistical significance was set at $P<0.05$. An interrater reliability analysis using the Kappa statistic was performed to determine consistency among raters. (10) Inter-rater reliability was tested on the same image in ImageJ software and running the IHC profiler to check variation among two observers. No difference in the measurements was noted. The interrater reliability for the raters was found to be Kappa = 0.88 ($p < .0.001$), 95% CI (0.848, 0.910).

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Chapter 3. Results

RESULTS

It was hypothesized that with the mineralization process during growth SIBLING proteins would show changes in the level at different ages. To test the expression pattern of SIBLING proteins in various age groups and in different mineralized tissues, we evaluated tibias and mandibles of 1-, 3-, and 6-month-old C57BL/6J mice for variation. For comparative study we histologically investigated the general morphology of tibias and mandibles of the variant age groups. For this purpose, H&E was the stain of choice. This was followed by immunohistochemical analysis to investigate the expression and distribution patterns of SIBLING proteins.

The following section will fully describe the major findings along with the data obtained from representative histologic sections at each observation age. (*Images for results were obtained from current thesis project experiments)

A. Haematoxylin and Eosin staining:

Tibia:

i. 1-month-old group:

For observation, the H&E-stained sections of *tibial head* (epiphysis) were divided into 4 distinct layers including; the articular cartilage, secondary ossification center (SOC), growth plate cartilage-bone interface and primary ossification center (POC).At this age in C57BL/6J mice, the articular cartilage and growth plate of the tibial heads were completely separated by a secondary ossification centre. (**Figure 3.1**) The articular cartilage was located immediately adjacent to the secondary ossification centre which abutted the growth plate. The tibias showed well organized growth plates with characteristic columnar organization. The proliferative zone was located adjacent to the plate's epiphyseal border, whereas the hypertrophic zone was close to the metaphysis. The growth plate had 5 distinct zones 1: Zone of proliferation (Resting zone - RZ); 2: Mature zone (proliferating zone - PZ); 3: Zone of hypertrophied chondrocytes (hypertrophic zone - HZ); 4: Zone of destruction (zone of chondrocyte destruction- DZ); 5: Zone of ossification (Calcification zone - CZ). At CZ there was a gradual change from calcified cartilage zone to ossification zone. At 1 month, the growth plate showed multiple layers of proliferating and hypertrophic chondrocytes.

ii. 3-month-old group:

At 3months, the total number of chondrocytes in the proliferating and hypertrophic zones of growth plate decreased significantly. The dimension of the hypertrophic zone was significantly smaller than the proliferative zone. The growth plate still revealed typical columnar organization, however, it showed a notably reduced size. Larger areas of hyalinization with degenerating chondrocytes were seen and stacked proliferative cells were surrounded by lacunae. The matrix had higher basophilic properties and stained intensely with hematoxylin. (**Figure 3.2**)

iii. 6-month-old group:

At 6 months, there were scattered hypertrophic and some proliferating chondrocytes. More organized calcified matrix was seen at the CZ. The trabeculae were reduced in number (Tb.N) but showed increased size (Tb.Th) and subsequent lower trabecular bone volume and lower bone volume fraction - BV/TV, %. (**Figure 3.3**)

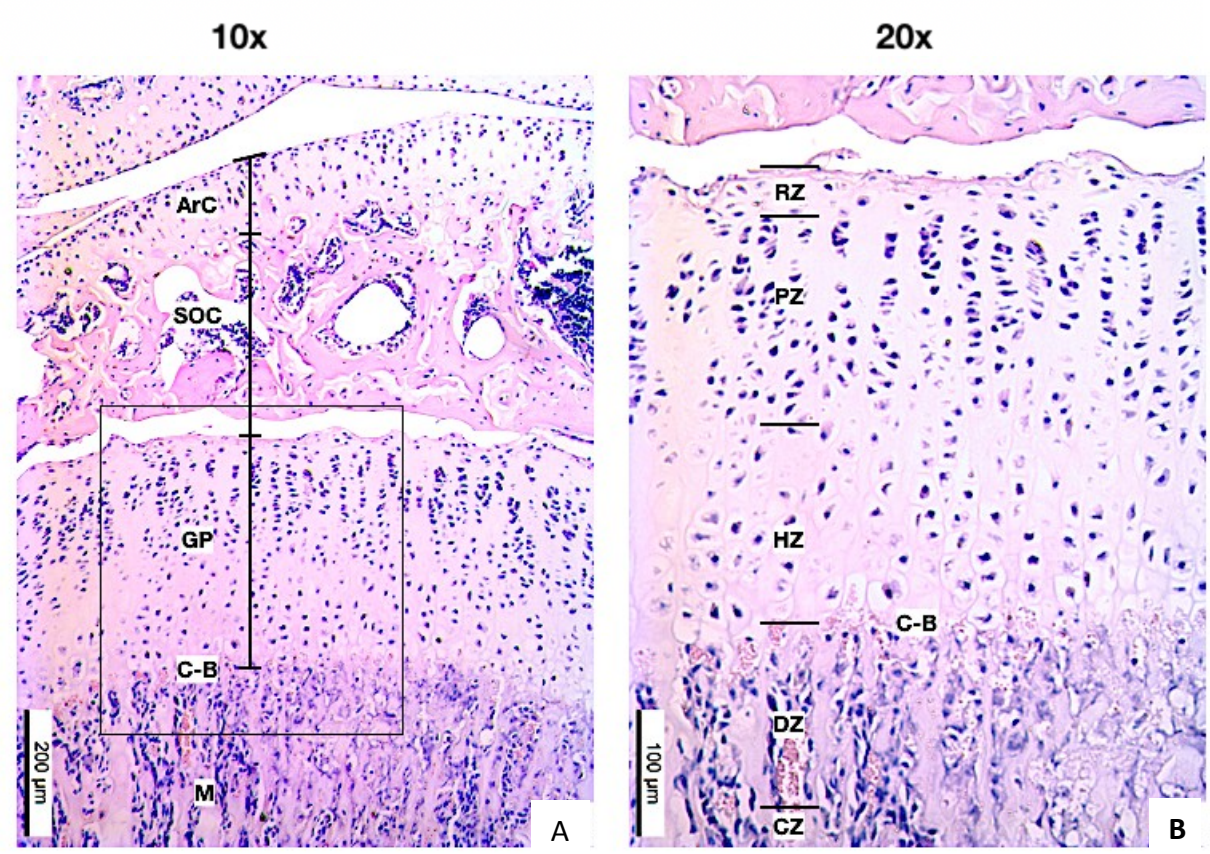


Figure 3.1. H&E Tibia showing growth plate, primary and secondary ossification center of 1-month-old mice.*A-B: H&E staining; images showed higher magnification of the growth plate (A) Hematoxylin-eosin stain (10X). (B) Higher magnification (20x) of picture A showing different zones of growth plate. The growth plate was divided into 5 distinct layers 1: Zone of proliferation (Resting zone - RZ); 2: Mature zone (proliferating zone - PZ); 3: Zone of hypertrophied chondrocytes (hypertrophic zone - HZ); 4: Zone of destruction (zone of chondrocyte destruction- DZ); 5: Zone of ossification (Calcification zone - CZ). ArC – Articular cartilage; SOC – Secondary ossification center; GP – Growth plate; C-B – Cartilage bone interface; M – Metaphysis. There was a gradual change from calcified cartilage zone to ossification zone. The growth plate showed multiple layers of proliferating and hypertrophic chondrocytes. The metaphysical region had multiple trabeculae in the marrow.* (Source: *H & E Dab)

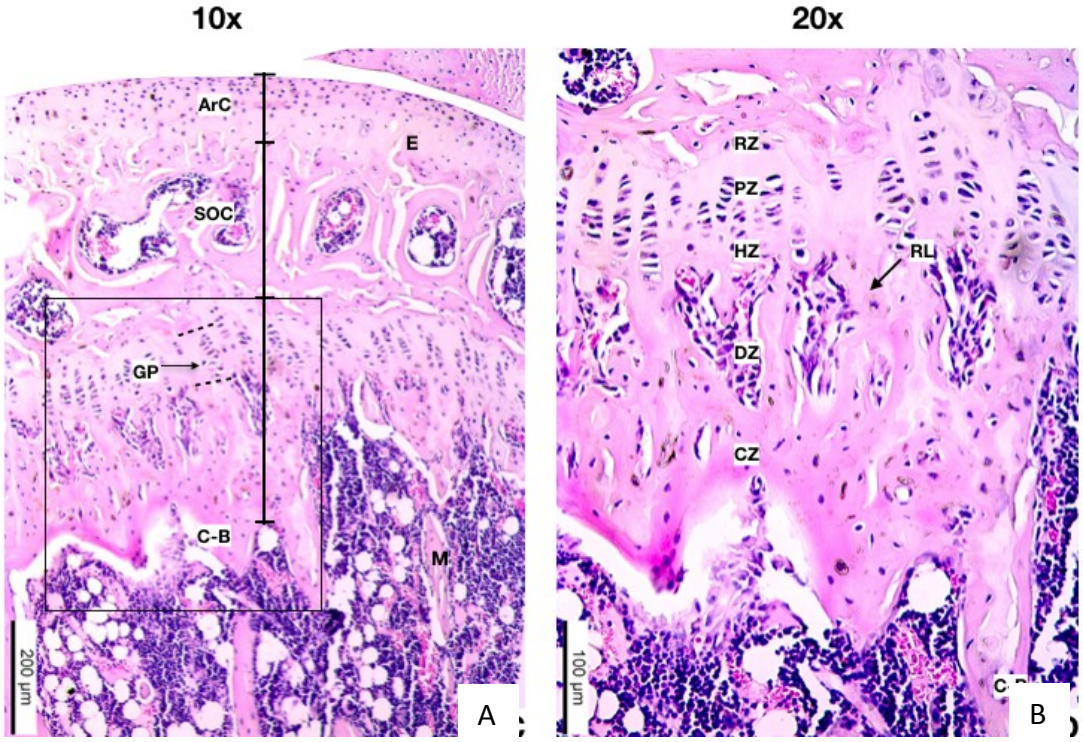


Figure 3.2.H&E Tibia showing growth plate, primary and secondary ossification center of 3-month-old mice.*A-B H&E staining; images showed higher magnification of the growth plate (A) Hematoxylin-eosin stain (10X). (B) Hematoxylin-eosin stain (20x)Higher magnification of boxed area in picture A showing different regions of growth plate. The growth plate was divided into 5 distinct layers 1: (Resting zone - RZ); 2: Mature zone (proliferating zone - PZ); 3: Hypertrophic zone - HZ); 4: Zone of chondrocyte destruction- DZ); 5: Zone of ossification (Calcification zone - CZ). ArC – Articular cartilage; SOC – Secondary ossification center; GP – Growth plate; C-B – Cartilage bone interface; ; E – Epiphysis M – Metaphysis; RL – Resting line. There was an abrupt change from calcified cartilage zone to ossification zone. 10x magnification was able to capture all zones and included a significant part of the metaphysis compared to 1-month-old mice where it was difficult to capture the initial segment of metaphysis.* (Source: *H & E Dab)

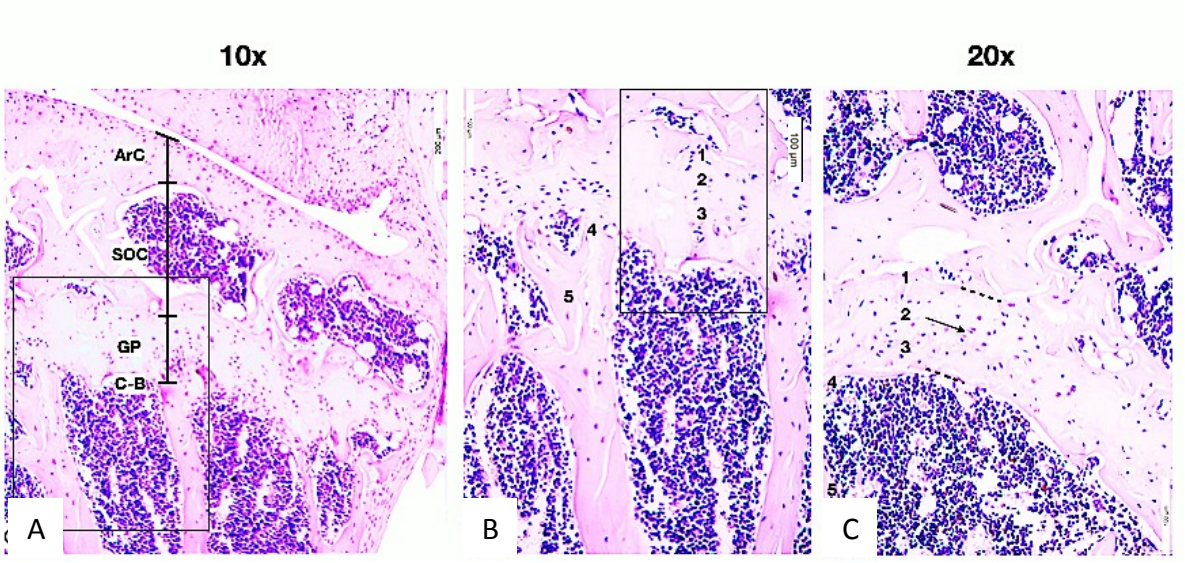


Figure 3.3. H&E Tibia showing growth plate, primary and secondary ossification center of 6-month-old mice. *A-C: H&E staining; images of the growth plate) A) Hematoxylin-eosin stain (10X). (B&C) Hematoxylin-eosin stain (20x). Higher magnification of boxed area in picture A showing different regions of growth plate. The growth plate was divided into 5 distinct layers 1: Zone of proliferation (Resting zone - RZ); 2: Mature zone (proliferating zone - PZ); 3: Zone of hypertrophied chondrocytes (hypertrophic zone - HZ); 4: Zone of destruction (zone of chondrocyte destruction- DZ); 5: Zone of ossification (Calcification zone - CZ). ArC – Articular cartilage; SOC – Secondary ossification center; GP – Growth plate; C-B – Cartilage bone interface; M – Metaphysis. There was a noticeable change from the calcified cartilage zone to the ossification zone. The 10x view was able to capture all zones and included a significant part of the metaphysis compared to 1-month-old mice where it was difficult to capture the initial segment of the metaphysis*. (Source: *H & E Dab)

Mandible:

In the H&E-sections of the *mandible*, the specimens showed erupted molars. The crowns of the teeth had fully developed dentin with normal thickness of predentin and a layer of odontoblasts lining the pulp chamber near the unmineralized dentin matrix. Acellular cementum lined theroot from the CEJ to the apical third of the root. A well-developed interdental papilla was seen with trans-septal fibers crossing the interdental alveolar bone inserting into the cementum on either side. The PDL and pulp were well developed and organized tissue structures.

At 1 month, the cusps had minimal attrition and no reactionary dentin was noted. There was a thin layer of cellular cementum at the apex. At higher magnification, a distinct layer of predentin was visible between the dentin and the odontoblastic layer. (**Figure 3.4**&**Figure 3.5**)

At 3, and 6months, the root structure was well developed. A thin layer of acellular cementum lined the cervical two thirds of the roots and reactionary dentin was visible at the working cusp. There was a distinct change in the direction of dentinal tubules with convergence toward the flattened cusp tip. This reactionary dentin zone stained intensely and there was an accentuated horizontal incremental line pattern. The predentin layer maintained a relatively constant width in all three age groups. (**Figure 3.6**, **Figure 3.7**, **Figure 3.8**&**Figure 3.9**)

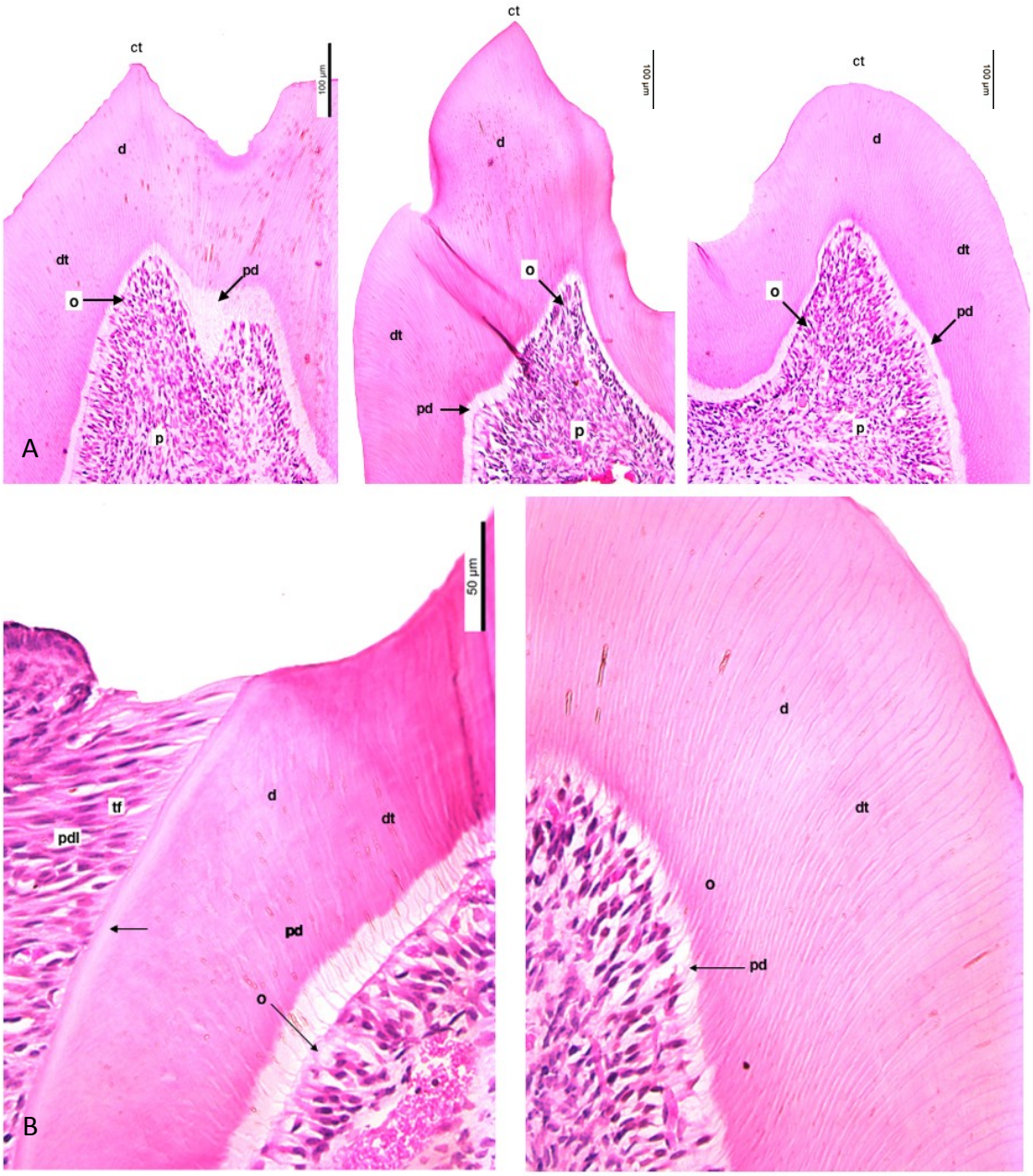


Figure 3.4. H&E Molar showing dentin, dentinal tubules and predentin

A) Cusp 20x B) Cusp 40xH&E staining: the photomicrograph shows mandibular molars, cusp tips (ct). (H&E 20x & 40x).; d - dentin; pd - predentin; o - odontoblastic layer; p - pulp; dt - dentinal tubules; ct - cusp tip; pdl – periodontal ligament; tf – transseptal fibers. Lower magnification shows a well-developed cusp with no attrition, a well-developed pale staining predentin. Higher magnification shows odontoblasts in the tooth pulp with their processes spanning through the predentin into dentin, s-shaped dentinal tubules. Acellular cementum is seen lining the outer side of the root dentin. (Source: *H & E Dab)

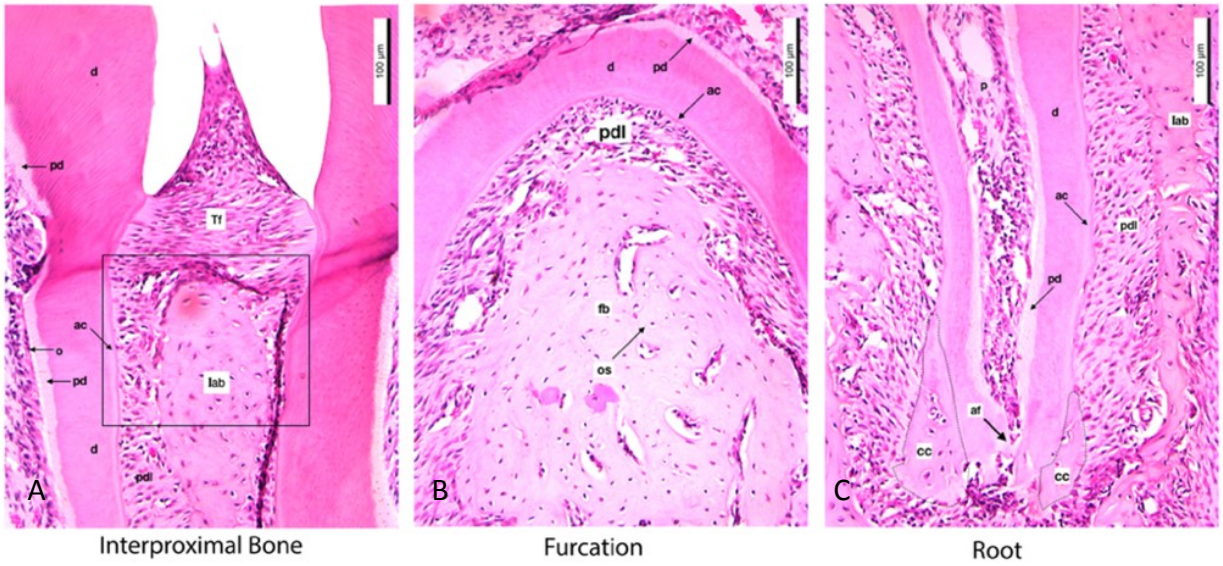


Figure 3.5. H&E Molar showing interdental, furcal and apical bone1-month-old mice.

H&E staining: the photomicrograph showing mandibular molar, A) interproximal alveolar bone (Iab), B) furcation alveolar bone (fb) and C) root. (H&E 20x).; The molars.; d - dentin; pd - predentin; o -odontoblastic layer; p - pulp; fb - furcal bone; pdl - periodontal ligament; tf - transeptal fibers; Iab - interproximal alveolar bone; cc - cellular cementum; ac - acellular cementum; af - apical foramen.The interproximal and furcation bone is well-developed and the root apex is still developing. (Source: *H & E Dab)

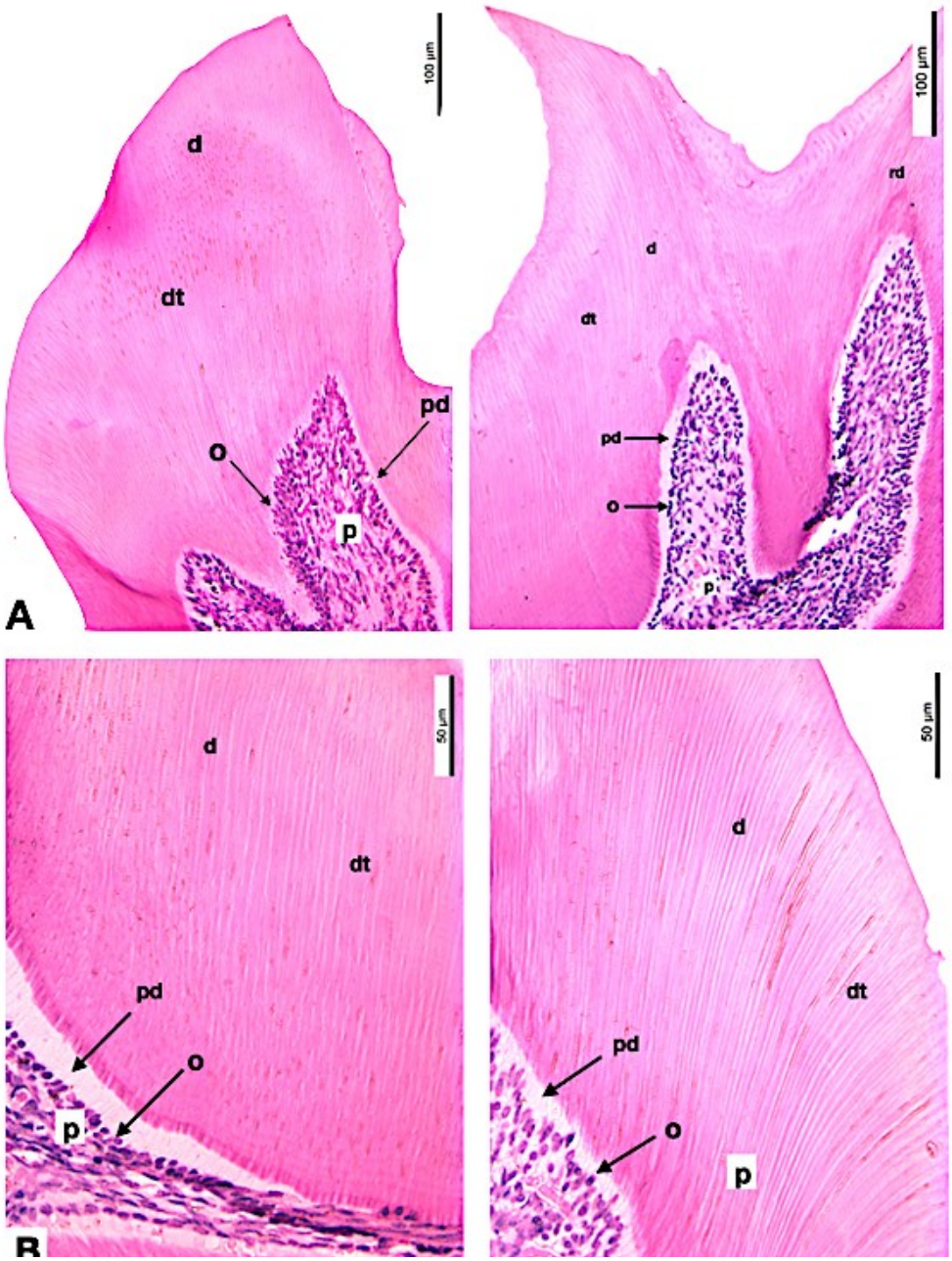


Figure 3.6. H&E Molar showing dentin, dentinal tubules and predentin3-month old mice.

A) Cusp 20x B) Cusp 40xH&E staining: the photomicrograph showing mandibular molar cusp tips (ct). (H&E 20x & 40x).; d - dentin; pd - predentin; o - odontoblastic layer; p - pulp; dt - dentinal tubules; ct - cusp tip; pdl – periodontal ligament; tf – transseptal fibers. Lower magnification shows a well-developed cusp with some attrition and reactionary dentin (rd), a well-developed pale staining predentin. Higher magnification shows odontoblasts in the tooth pulp with their processes spanning through the predentin into dentin, s-shaped dentinal tubules.(Source: *H & E Dab)

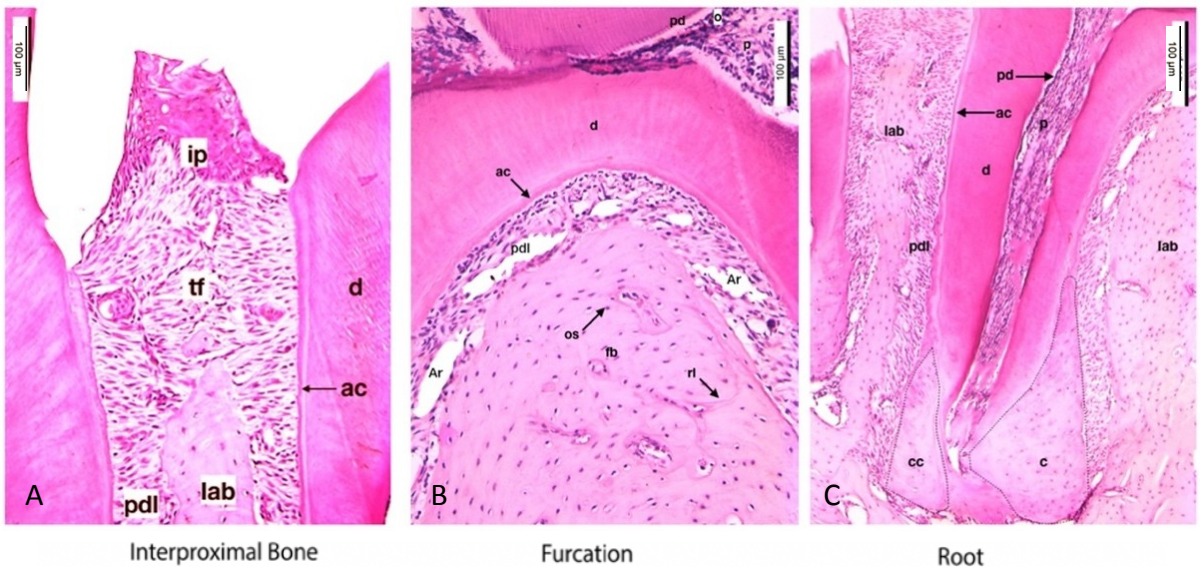


Figure 3.7. H&E Molar showing interdental, furcal and apical bone3-month-old mice.

H&E staining: the photomicrograph showing mandibular molar, A) interproximal alveolar bone (Iab), B) furcation alveolar bone (fb) and C) root. (H&E 20x).; The molars.; d - dentin; pd - predentin; o -odontoblastic layer; p - pulp; fb - furcal bone; pdl - periodontal ligament; tf - transeptal fibers; Iab - interproximal alveolar bone; cc - cellular cementum; ac - acellular cementum; af - apical foramen.The interproximal and furcation bone is well-developed and the root apex is continues developing with abundant cellular cementum. (Source: *H & E Dab)

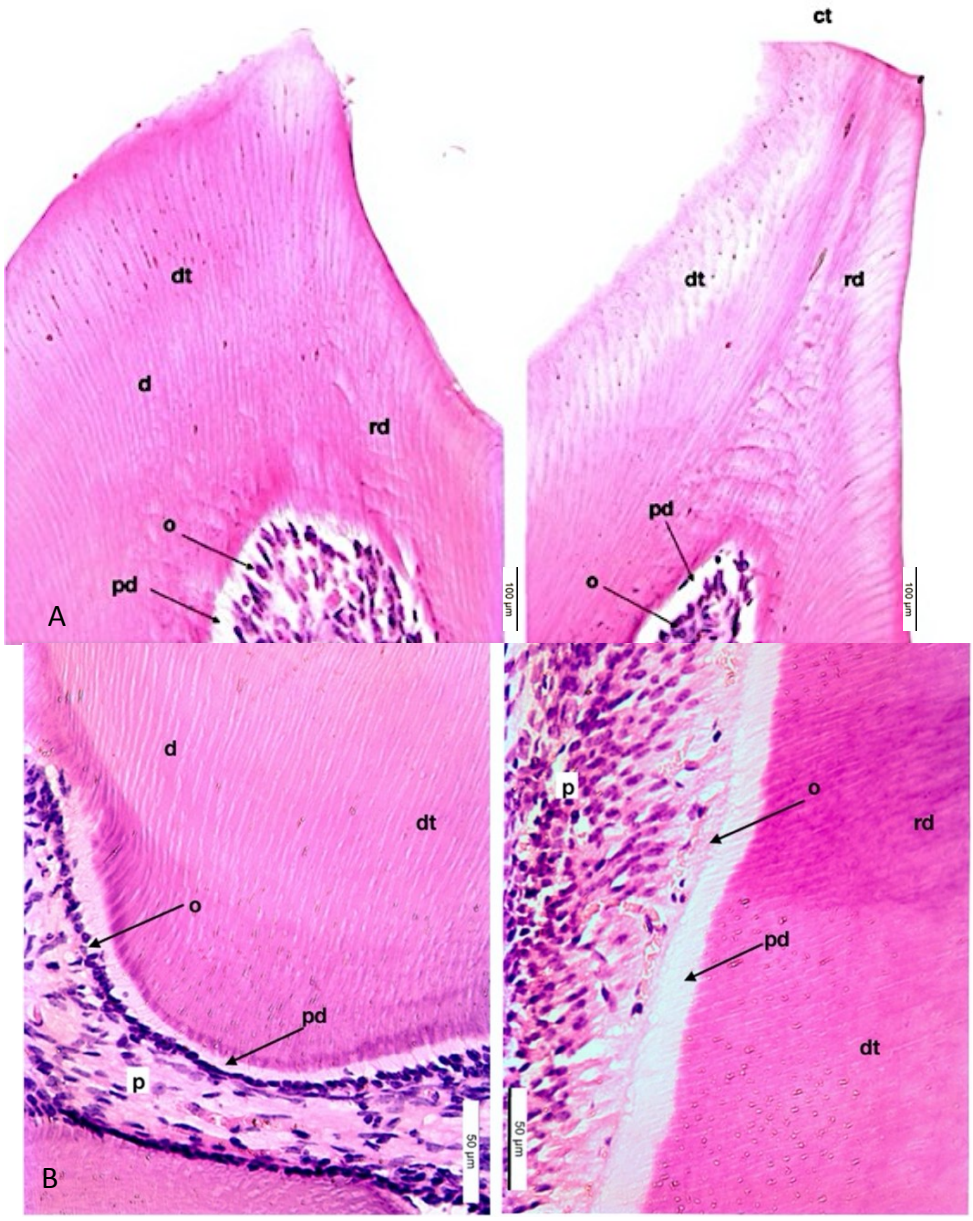


Figure 3.8. H&E Molar showing dentin, dentinal tubules and predentinin 6-month old mouse.*A) Cusp 20x (B) Cusp 40xH&E staining: the photomicrograph shows mandibular molars, cusp tips (ct). (H&E 20x & 40x).; d - dentin; pd - predentin; o - odontoblastic layer; p - pulp; dt - dentinal tubules; ct - cusp tip; pdl – periodontal ligament; tf – transseptal fibers. Lower magnification shows a well-developed cusp with prominent attrition and reactionary dentin (rd) formation following irregular pattern, a well-developed pale staining predentin. Higher magnification shows odontoblasts in the tooth pulp with their processes spanning through the predentin into dentin, s-shaped dentinal tubules.* (Source: *H & E Dab)

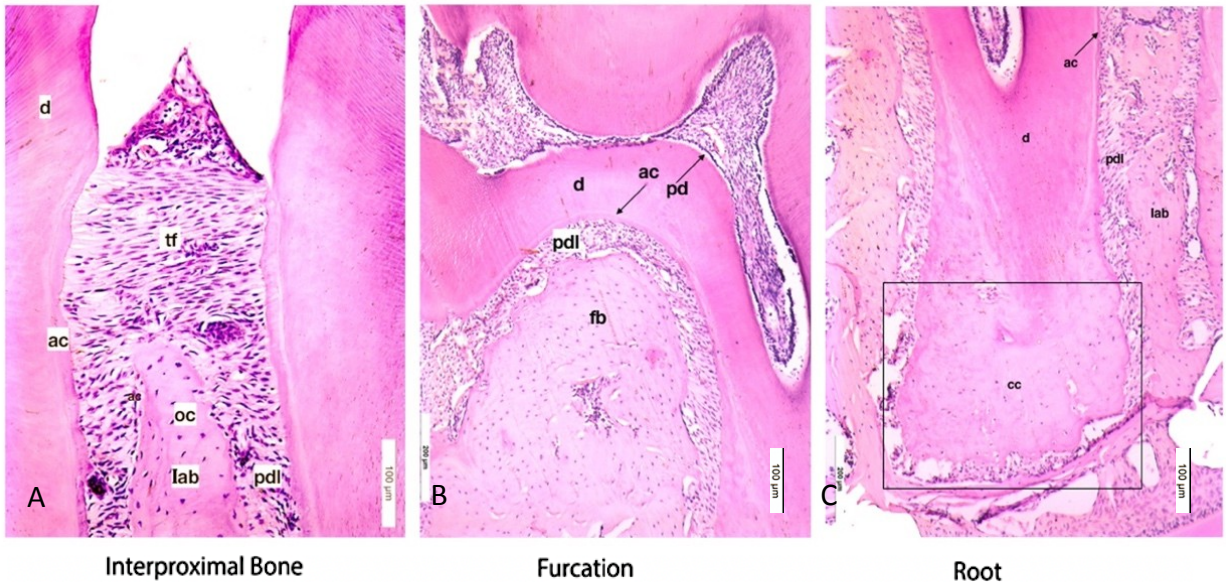


Figure 3.9. H&E Molar showing interdental, furcal and apical bone6-month-old mice.

H&E staining: the photomicrograph showing mandibular molar, A) interproximal alveolar bone (Iab), B) furcation alveolar bone (fb) and C) root. (H&E 20x).; The molars.; d - dentin; pd - predentin; o -odontoblastic layer; p - pulp; fb - furcal bone; pdl - periodontal ligament; tf - transeptal fibers; Iab - interproximal alveolar bone; cc - cellular cementum; ac - acellular cementum; af - apical foramen.The interproximal and furcation bone is well-developed and the root apex is continues to remodel with abundant cellular cementum (Source: *H & E Dab)

B. Immunohistochemistry:

Immunohistochemical staining of the tibia from 1-, 3-, and 6-month old mice showed that each of the four SIBLING proteins had its own distinct expression pattern in the different mineralized tissues. In addition, the distribution profile and intensity of these changed with age. The interrater reliability for the raters was found to be high Kappa = 0.88 ($p < .0.001$), 95% CI (0.848, 0.910). The detailed description and distribution of each SIBLING protein is outlined in the following section.

1. Anti-DSP antibody:

*Tibia:*The signal for DSP spread diffusely throughout the growth plate and POC at 1month. Lower levels at SOC and higher expression was found in areas of transformation into trabeculae. At 3months, the expression pattern of the DSP was found to be more pronounced and

localized at the calcifying front (CZ) of the growth plate. In addition, notable expression was seen in the degenerating chondroblasts at the cartilage-bone interface. This corresponded to the site where degeneration of chondrocytes, invasion by blood vessels, active formation of osteoid and conversion to bone via mineralization was taking place. The reversal lines in SOC also showed prominent DSP expression. At 6months, low intensity expression was seen at the SOC and the calcification front of the primary ossification center. Overall there was a 34-fold increase in the expression of DSP from 1month to 3months followed by an8-fold decrease in the expression of DSP from 3 to 6months. A similar pattern was observed at the secondary ossification center. (**Figure 3.10a&Figure 3.10b**)

Mandible: A strong signal for DSP was observed in the predentin and the peritubular regions. The signal was preferentially expressed around the dentinal tubules in the cusp and was higher a certain distance from the predentin following the outline of DEJ. Some areas showed an incremental signalling pattern perpendicular to the dentinal tubules. There was 22% increase in the expression from 1 to 3-months followed by a 38% increase between 3- to 6-months. At 6 months, the expression of DSP was stronger compared to expression at 1 month and localized to the peritubular region. With increasing age and as the development of reactionary dentin increased, the anti-DSP signals demonstrated stronger immunoreactivity. There were large areas with no signal at all age groups. There was no staining in the pulp or odontoblastic layer.

Interproximally, the interdental bone showed a 3-fold decrease in the expression of DSP from 1 to 3 months followed by a 2.4-fold reduction in the expression falling back to the initial levels. In the furcation region, there was a 68% decrease in the level of expression from 1 to 3 months followed by a further 17% decrease in the expression of DSP from 3 to 6 months. DSP was also expressed in the PDL around the root. At the root apex, there was a 4.4-fold decrease in the expression from 1 to 3 months whereas from 3 months to 6 months there was a 1.7-fold increase in the expression of DSP. The cellular cementum at the apex of the root showed DSP staining albeit with less intensity and no staining of the acellular cementum was observed in all age groups. (**Figure 3.11** a, **Figure 3.11** b, **Figure 3.11** c &**Figure 3.11** d)

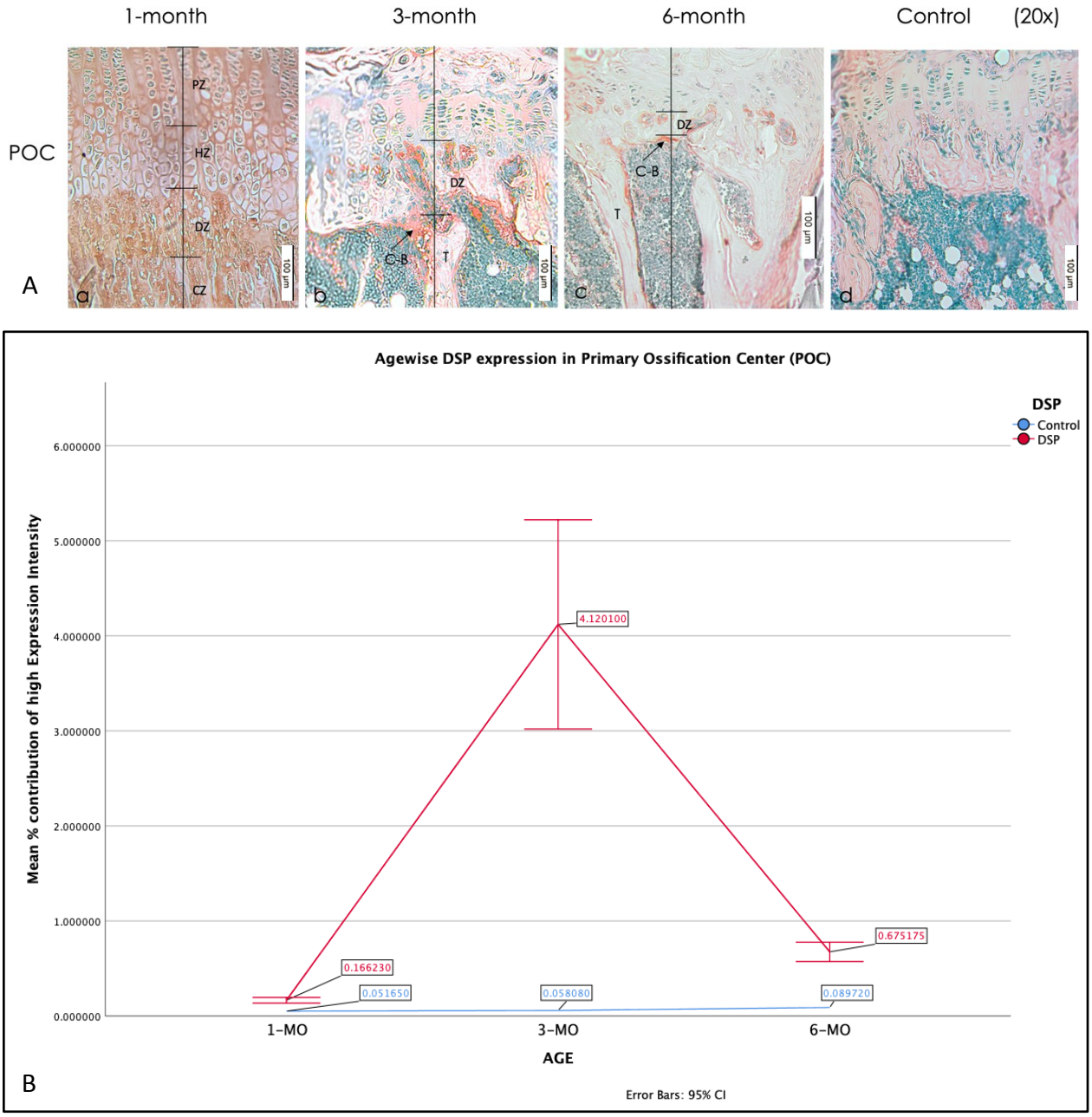


Figure 3.10a.Expression of staining intensity ofDSP at 1-, 3- and 6-months in primary ossification center (POC) *A) IHC of POC and growth plate at epiphysis in 1-, 3-, and 6-month old mice with anti-DSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly increased from 1- to 3-months followed by a decrease between 3- and 6-months.* *(Source- IHC Dab)*

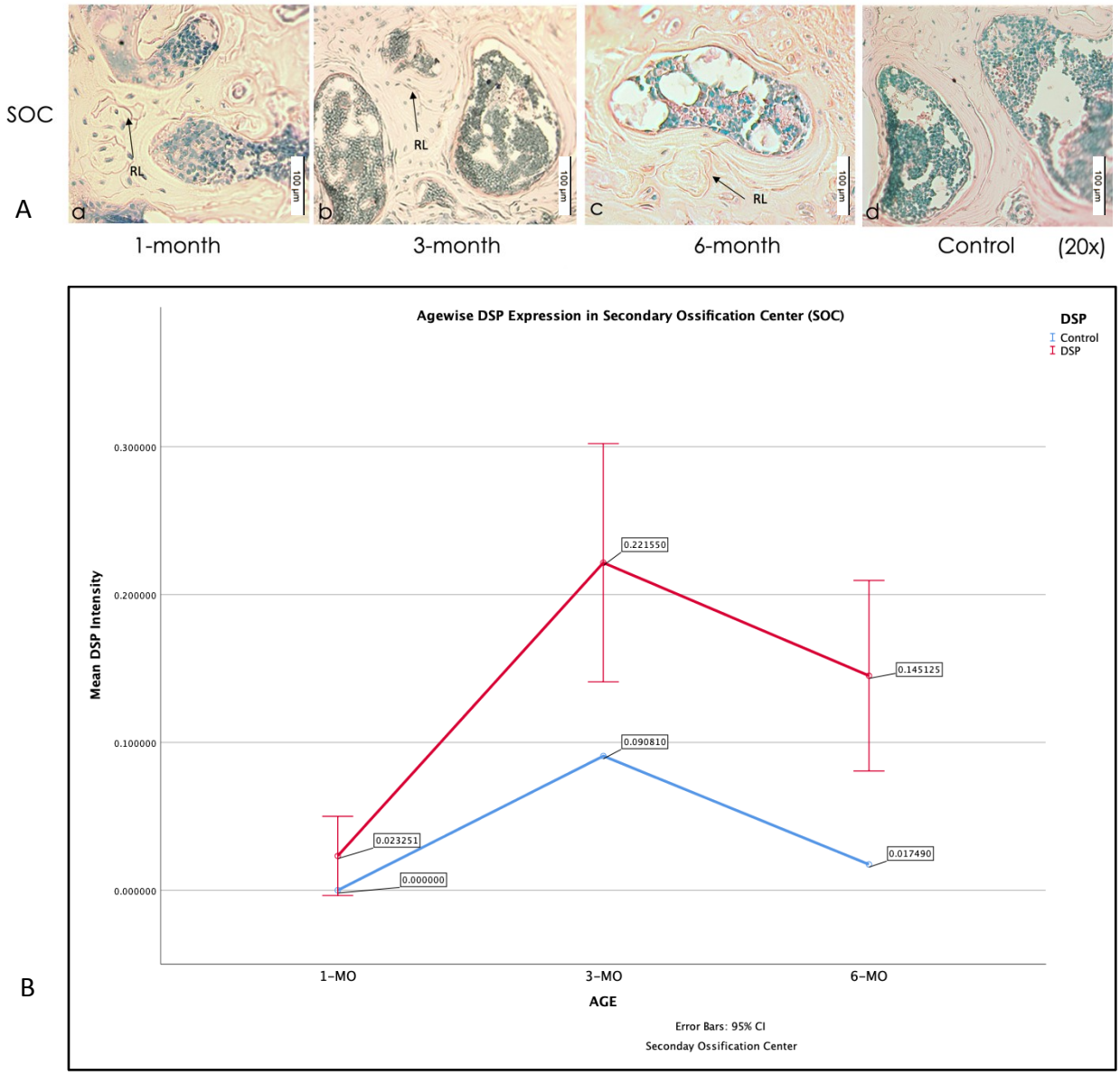


Figure 3.10b.Expressionof staining intensity ofDSP at 1-, 3- & 6-months in the secondary ossification center (SOC)*.A) IHC staining of SOC at epiphysis in 1-, 3-, and 6-month old mice with anti-DSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly increased from 1- to 3-months followed by a decrease between 3- and 6-months. (Source- IHC Dab)*

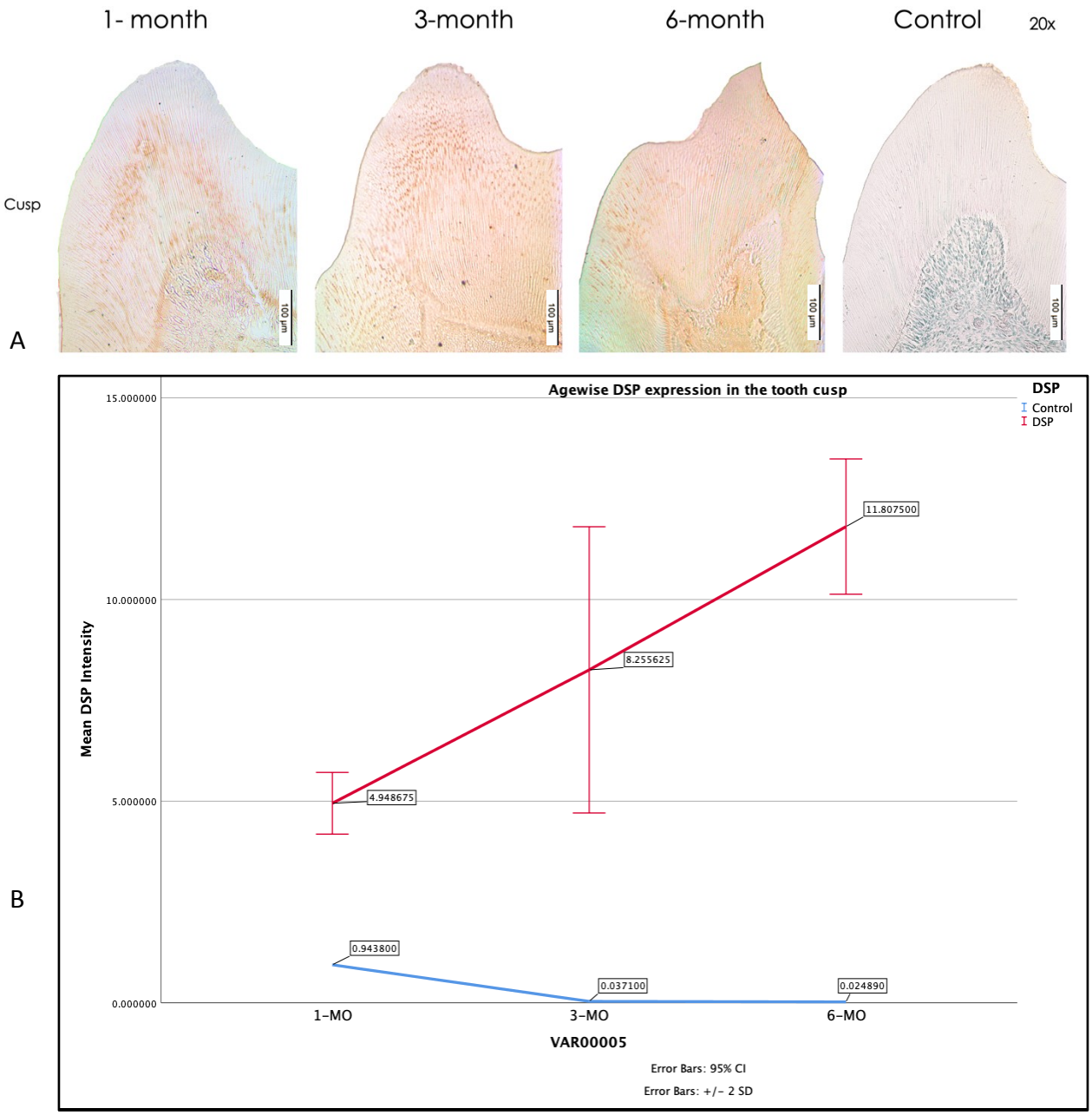


Figure 3.11a.Expression of staining intensity ofDSP at 1-, 3- and 6-months in dentin and predentin in the tooth cusp. *A) IHC of dentin and predentin at tooth cusp in 1-, 3-, and 6-month old mice with anti-DSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity increased from 1- to 3-months followed by a further increase between 3- and 6-months. (Source- IHC Dab)*

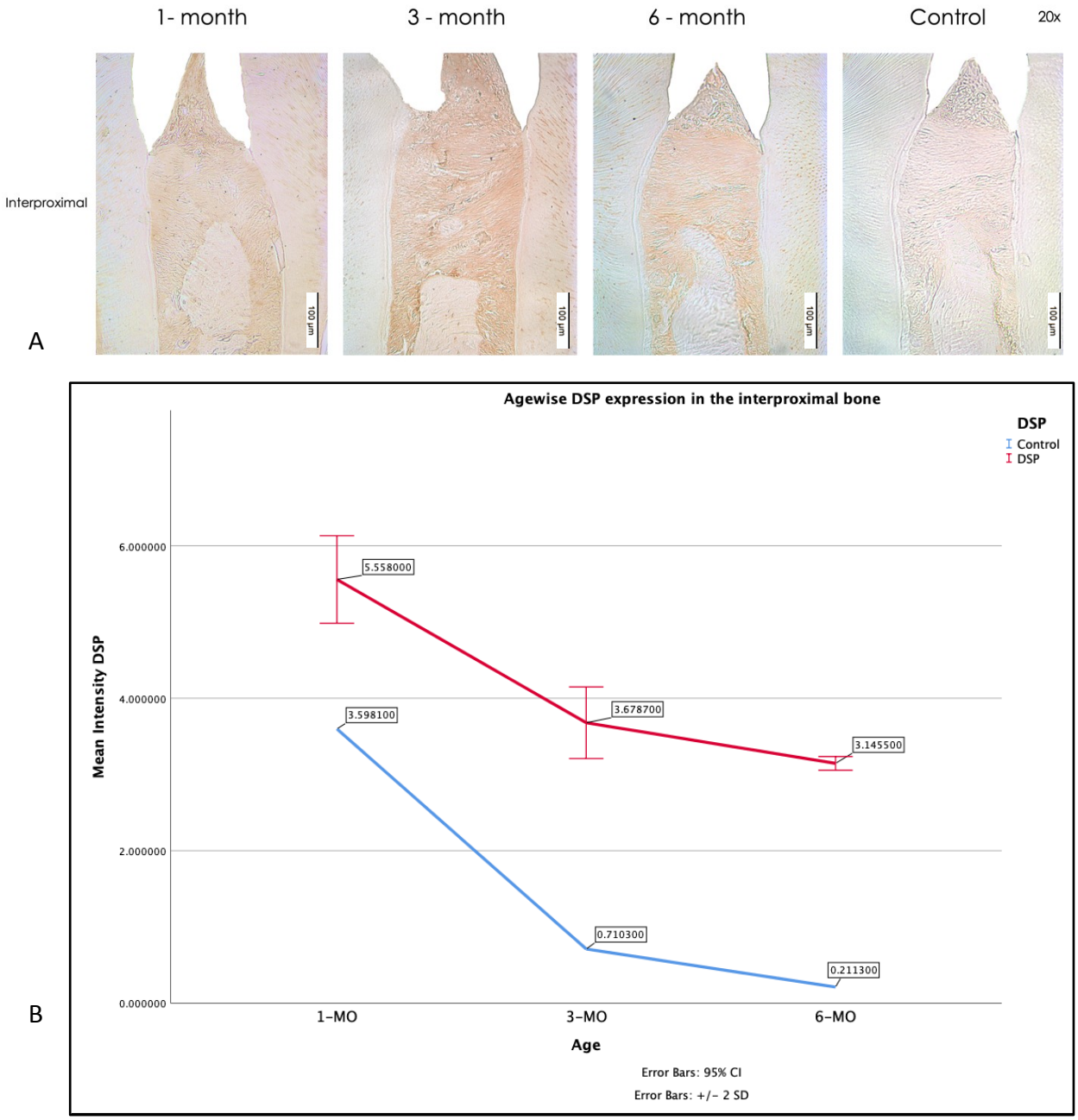


Figure 3.11b.Expression of staining intensity ofDSP at 1-, 3- and 6-months in Interproximal bone.*A) IHC of interproximal alveolar bone in 1-, 3-, and 6-month old mice with anti-DSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly decreased from 1- to 3-months followed by a slight decrease between 3- and 6-months. (Source-IHC Dab)*

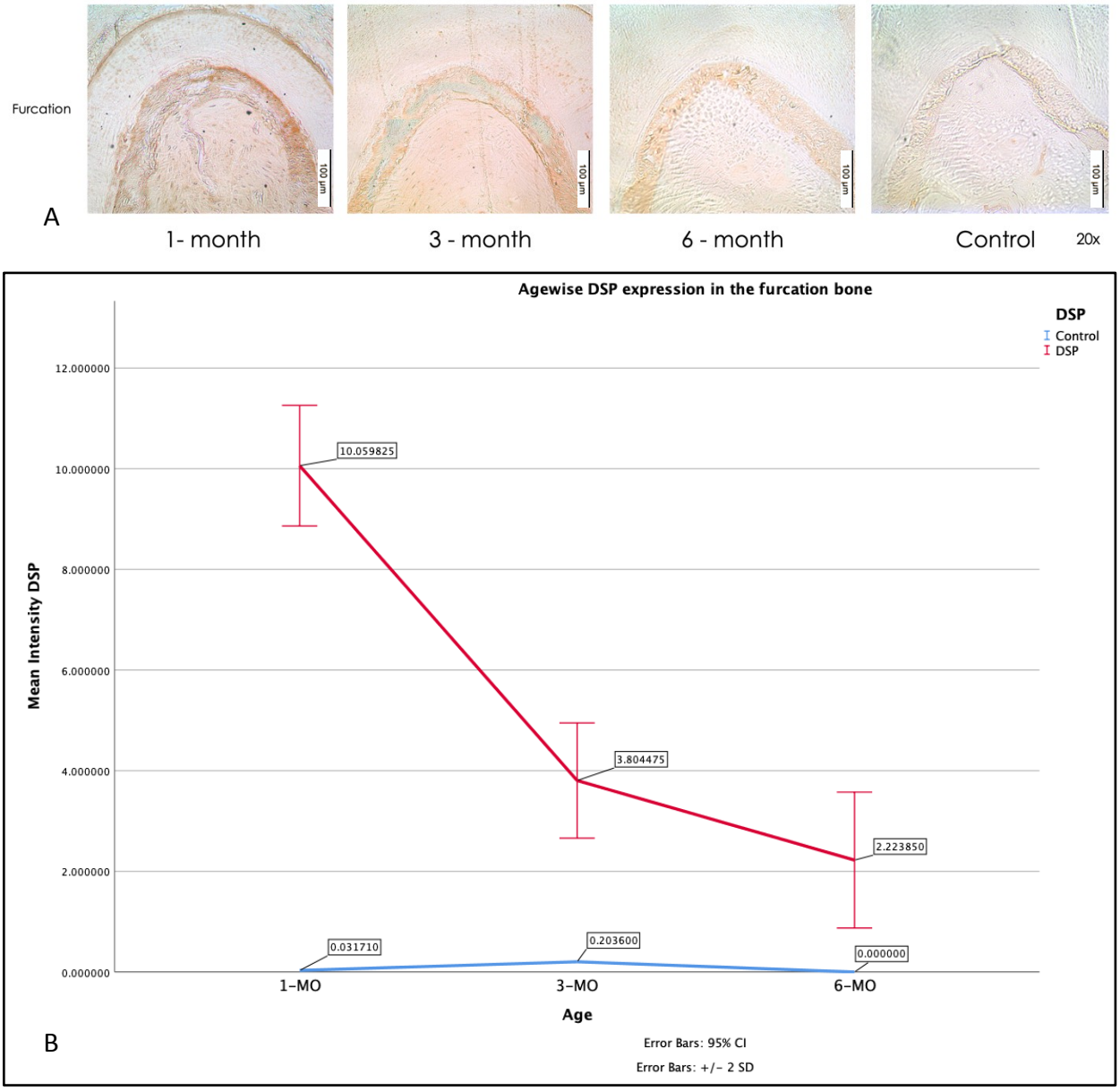


Figure 3.11c.Expression of staining intensity ofDSP at 1-, 3- and 6-months in Furcation bone.*A) IHC of furcation alveolar bone in 1-, 3-, and 6-month old mice with anti-DSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly decreased from 1- to 3-months followed by a slight decrease between 3- and 6-months. (Source- IHC Dab)*

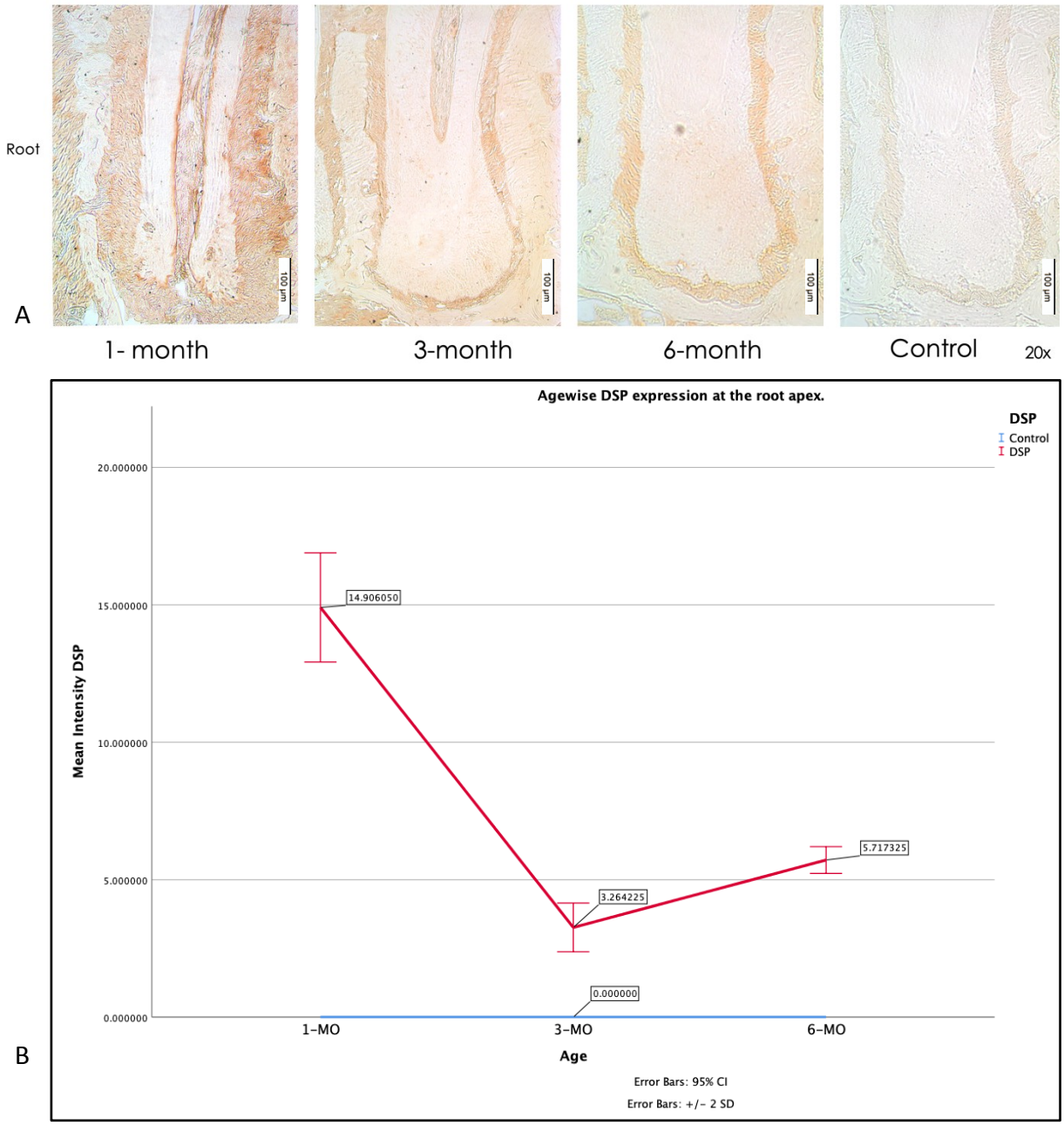


Figure 3.11d.Expression of staining intensity of DSP at 1-, 3- and 6-months in cellular cementum and PDL of tooth root. *A) IHC staining of cellular cementum and PDL in tooth root in 1-, 3-, and 6-month old mice with anti-DSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly decreased from 1- to 3-months followed by a slight increase at 6-months.* *(Source- IHC Dab)*

2. *Anti-DMP1 antibody:*

Tibia: There was diffuse expression of DMP1 throughout the growth plate of 1-month-old mice and particularly strong expression was noted at the interface of the degenerating chondroblasts and newly forming bony trabeculae. This expression was pronounced at the CZ interface of 3-month-old mice and there was a significant increase in expression from 1 to 3 months followed by a 200-fold decrease in DMP1 expression by 6months of age. Although the expression was reduced, it still was concentrated along the CZ. A particularly strong signal for DMP1 was visualized in the bone surrounding marrow spaces in the secondary ossification center. This expression declined from 1to 3 months by 49% but still was localized around the same region. The expression pattern for DMP1 showed a further decline at 6 months. (**Figure 3.12** a &**Figure 3.12**b)

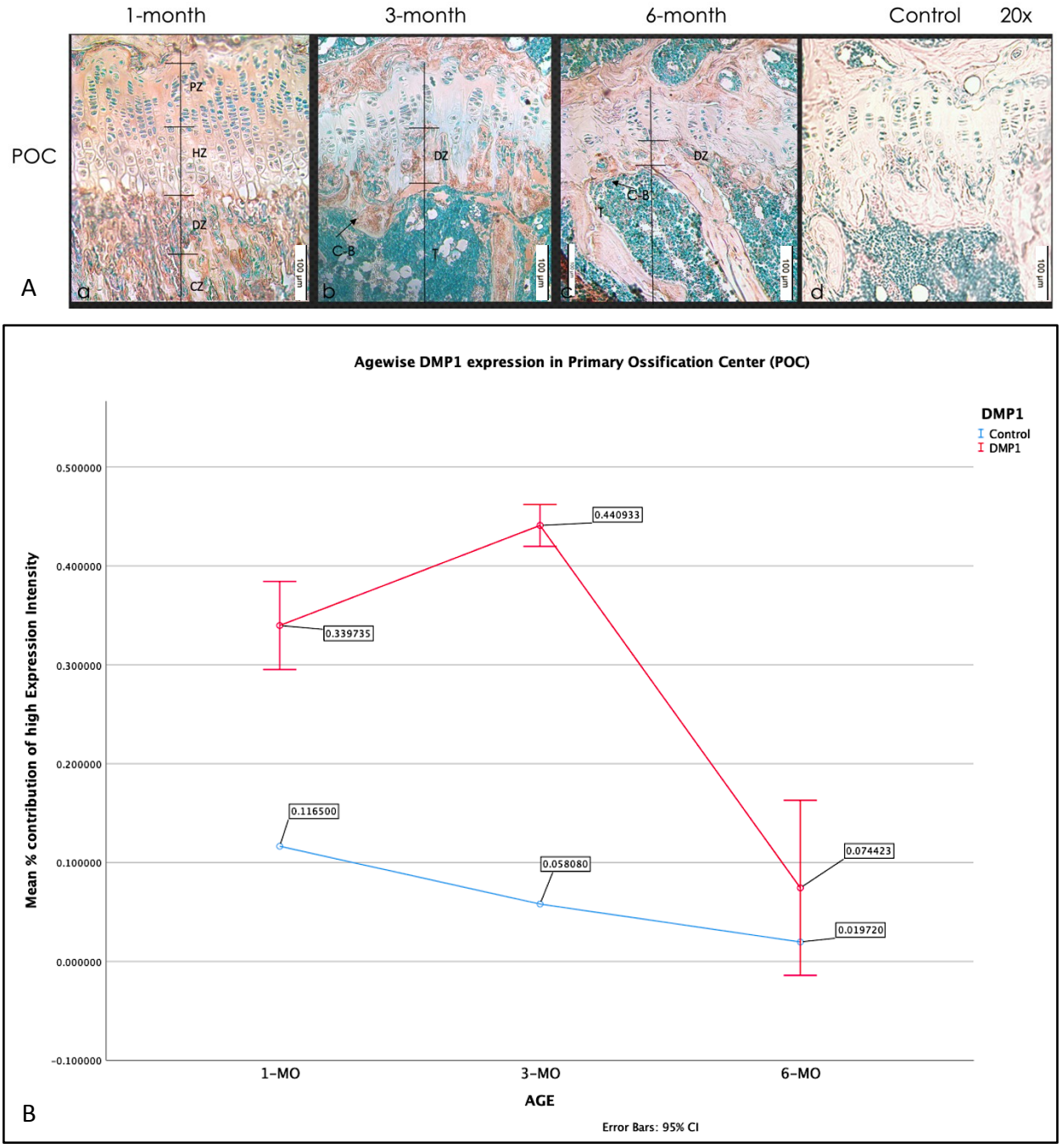


Figure 3.12a. Expression of staining intensity ofDMP1at 1-, 3- and 6-months in primary ossification center (POC). *A) IHC staining of POC and growth plate at epiphysis in 1-, 3-, and 6-month old mice with anti-DMP1 (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Highest expression was seen at 3 months. Intensity increased from 1- to 3-months and then decreased between 3- and 6-months.* *(Source- IHC Dab)*

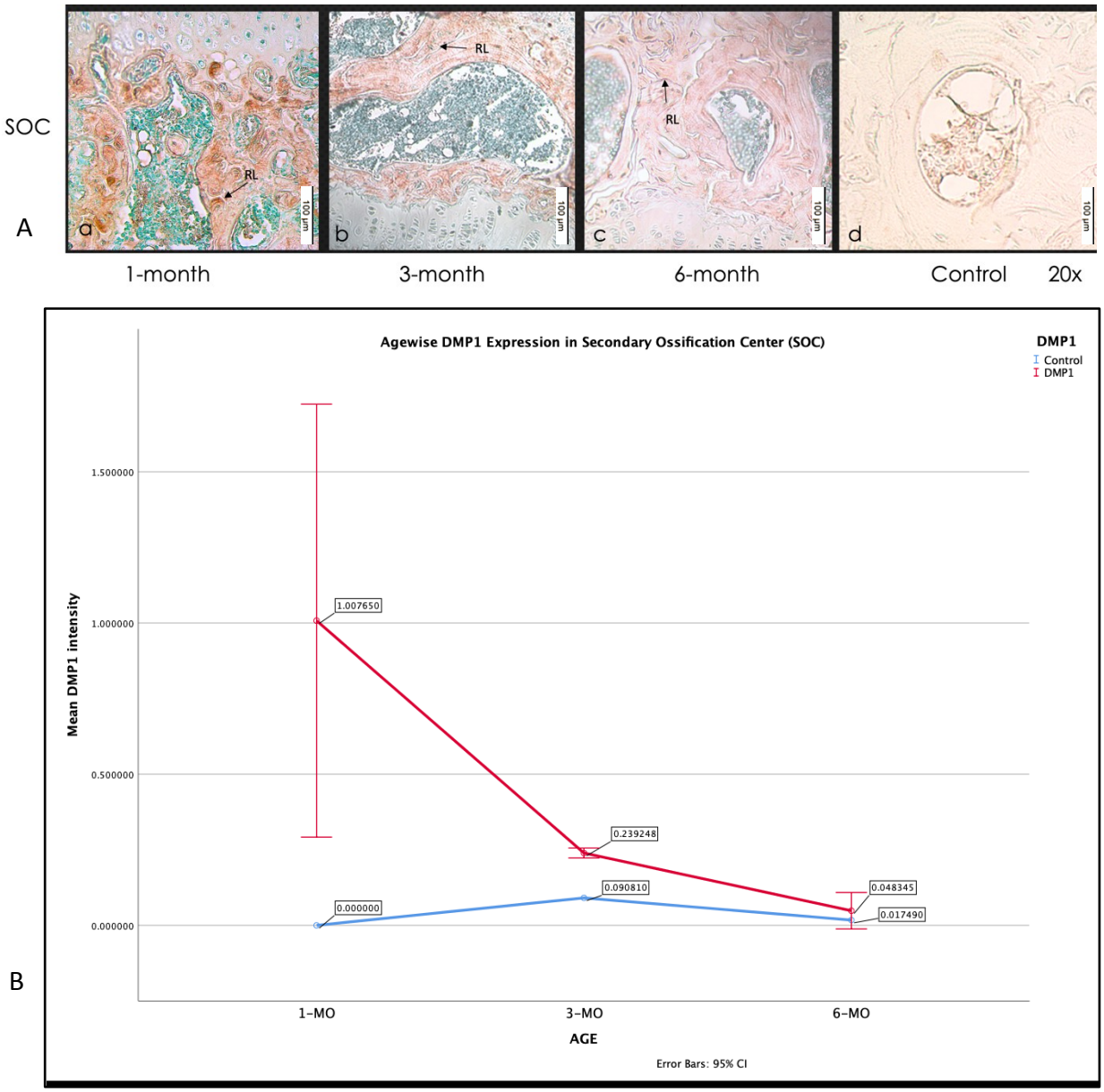


Figure 3.12b. Expression of staining intensity ofDMP1at 1-, 3-& 6-months in secondary ossification center (SOC). *A) IHC staining of SOC at epiphysis in 1-, 3-, and 6-month old mice with anti-DMP1 (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity decreased from 1- to 3-months followed by a further decrease between 3- and 6months. Highest expression was seen at 1 month.* *(Source- IHC Dab)*

Mandible: In the *mandibular molar* region, a strong signal was observed around predentin and along the peritubular region. The expression followed the dentinal tubules. The expression pattern of DMP1 showed significantly stronger expression with a 62% increase from 1 to 3 months followed by a slight but insignificant decline between 3 and 6 months. In the interproximal bone, DMP1 was expressed at 1month followed by a 200-fold increase in the expression at 3months. This was followed by a significant decrease in expression from 3 to 6months falling below 1month levels. The expression of DMP1 in the furcation was high at 1month followed by a slight decrease at 3months. This expression showed a negative slope from 3 to 6months showing a 47% decrease from 3-month expression levels. The roots showed a significant strong signal for DMP1 within the dentin, periapical PDL and cellular cementum. There was a significant increase in expression from 1 to 3 and 3 to 6months with an overall 72% increase at 3 months from 1-month levels. (**Figure 3.13** a, **Figure 3.13**b, **Figure 3.13**c &**Figure 3.13**d)

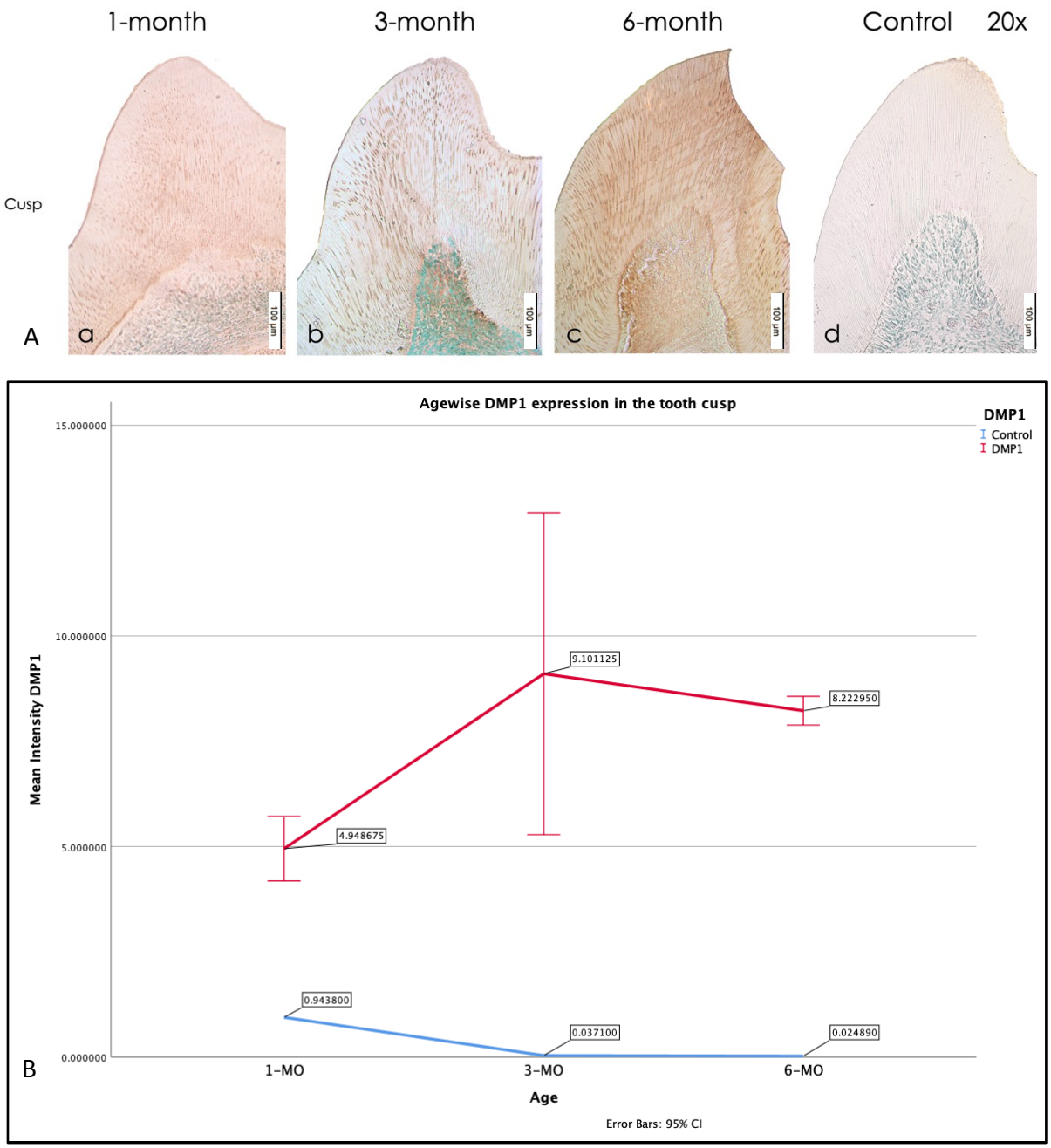


Figure 3.13a.Expression of staining intensity ofDMP1 at 1-, 3- and 6-months in dentin and predentin of tooth cusp. *A) IHC staining of dentin and predentin at tooth cusp in 1-, 3-, and 6-month old mice with anti-DMP1 (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly increased from 1- to 3-months followed by a slight decline at 6-months. (Source- IHC Dab)*

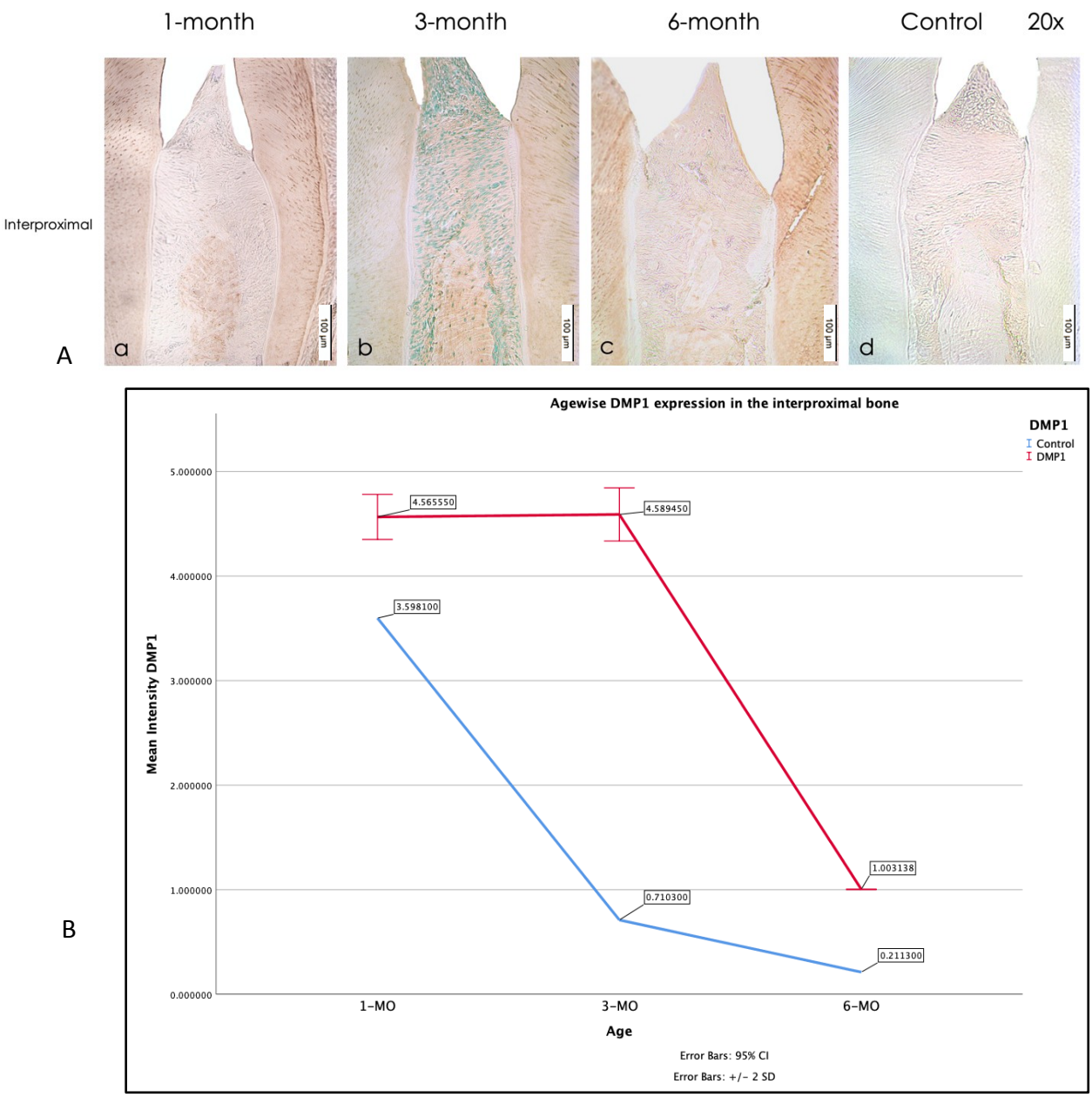


Figure 3.13b.Expression of staining intensity ofDMP1 at 1-, 3- and 6-months in interproximal bone. *A) IHC staining of interproximal alveolar bone in 1-, 3-, and 6-month old mice with anti-DMP1 (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. There was a negligible intensity change from 1- to 3-months followed by a decrease between 3- and 6-months.* *(Source- IHC Dab)*

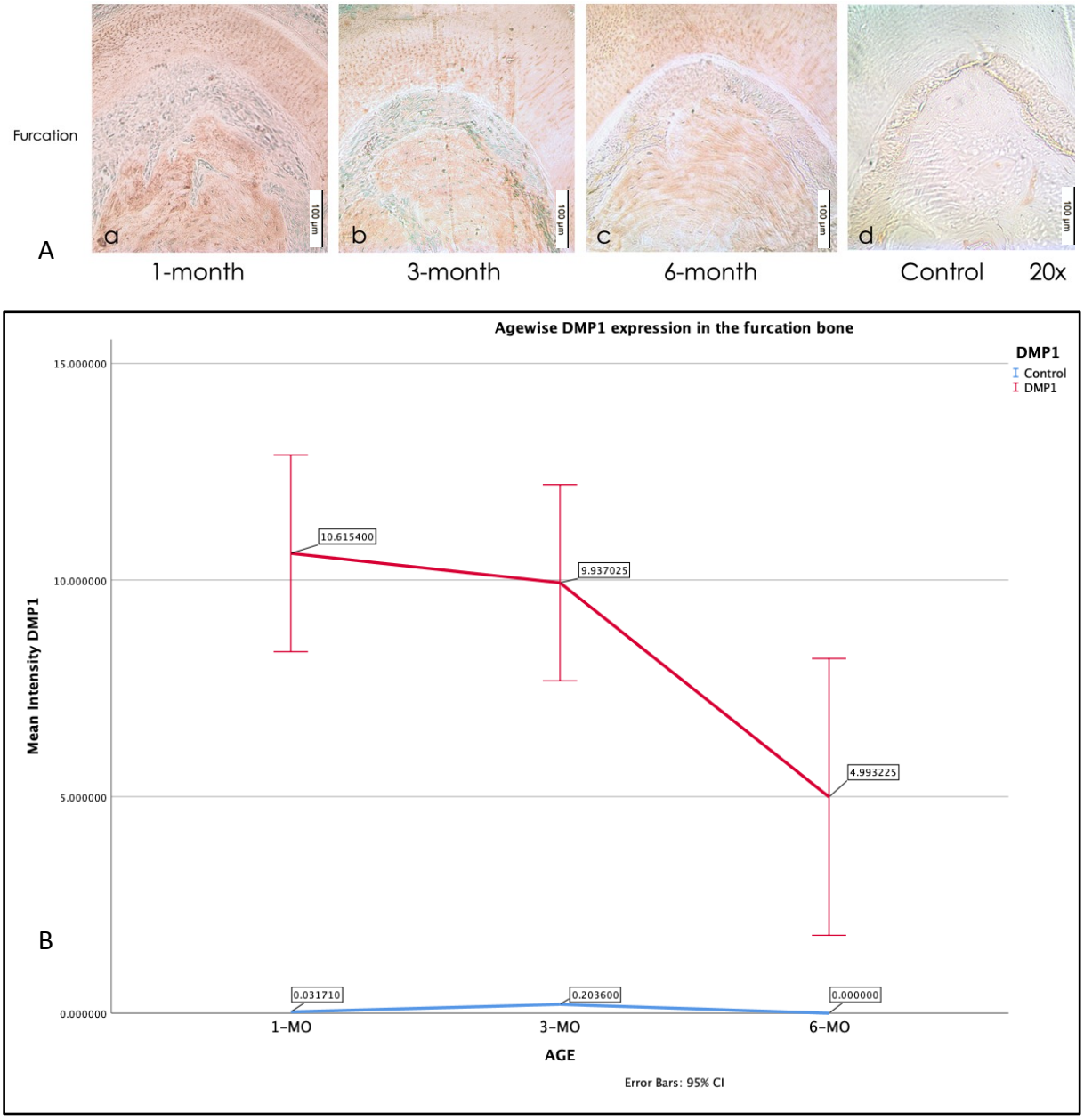
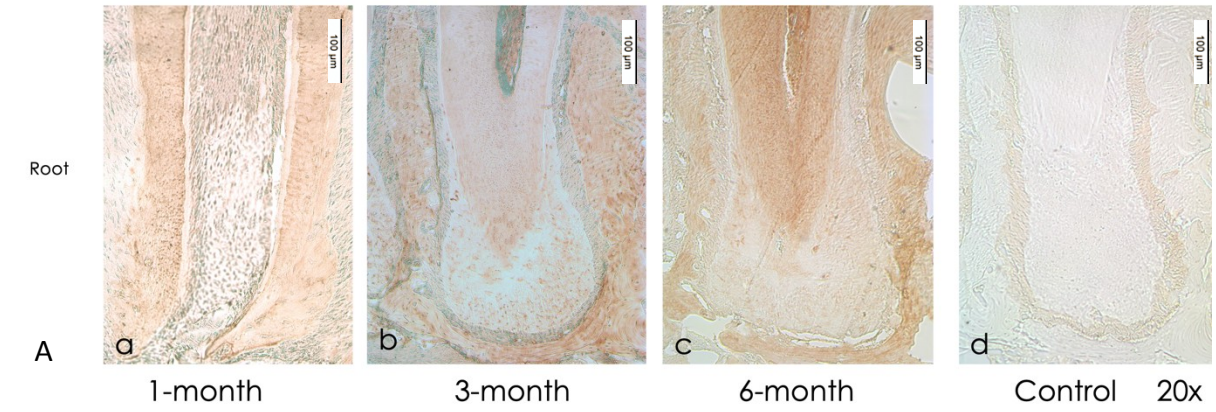


Figure 3.13c.Expression of staining intensity ofDMP1 at 1-, 3- and 6-months in furcation bone. *A) IHCstaining of furcation alveolar bone in 1-, 3-, and 6-month old mice with anti-DMP1 (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity decreased from 1- to 3-months and decreased furtherbetween3- and 6-months. (Source- IHC Dab)*



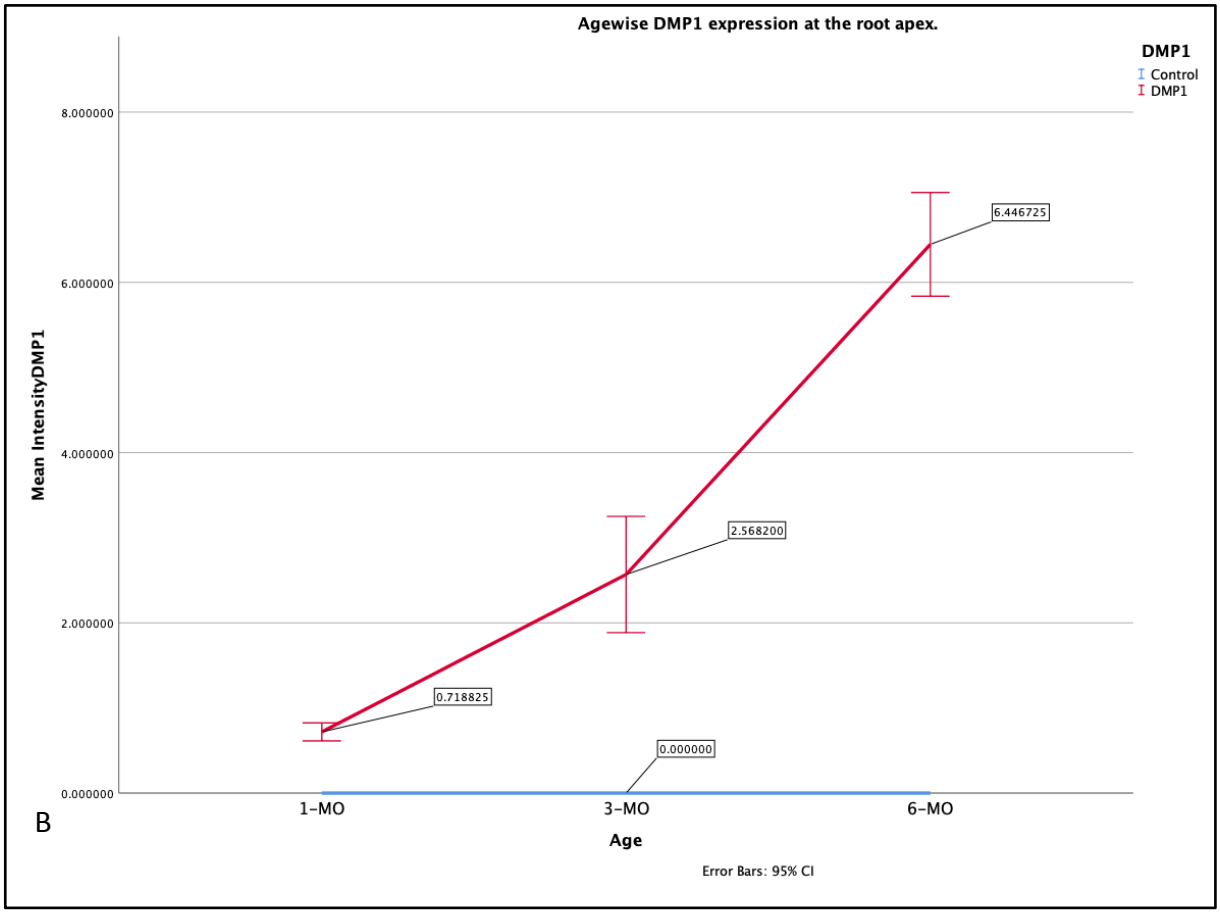


Figure 3.13d.Expression of staining intensity ofDMP1 at 1-, 3- and 6-months in tooth root. *A) IHC staining of cellular cementum and PDL in tooth root in 1-, 3-, and 6-month old mice with anti-DMP1 (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity increased from 1- to 3-months followed by an increase between 3- and 6-months. (Source- IHC Dab)*

3. *Anti-BSP antibody:*

Tibia: At 1month, the tibial growth plate and primary ossification center showed diffuse BSP expression. This was followed by a 300% increase in expression at 3months. The expression of BSP in the growth plate was especially concentrated in the resting zone (RZ), proliferating and hypertrophic layers of growth plate. This expression was different from other proteins which were concentrated along the calcification border at the chondrocyte-bone interface. There was a 48% decrease in BSP expression at 6-months. The expression was mainly localized in the hypertrophic and degenerating chondrocytes as well as the calcification zone. In the SOC, a pattern of expression mainly localized in resting lines was observed. Similar to POC, BSP expression in the SOC showed statistically significant increases from 1 to 3 months followed by a decline in its expression between 3- and 6- months.(**Figure 3.14** a &**Figure 3.14** b)

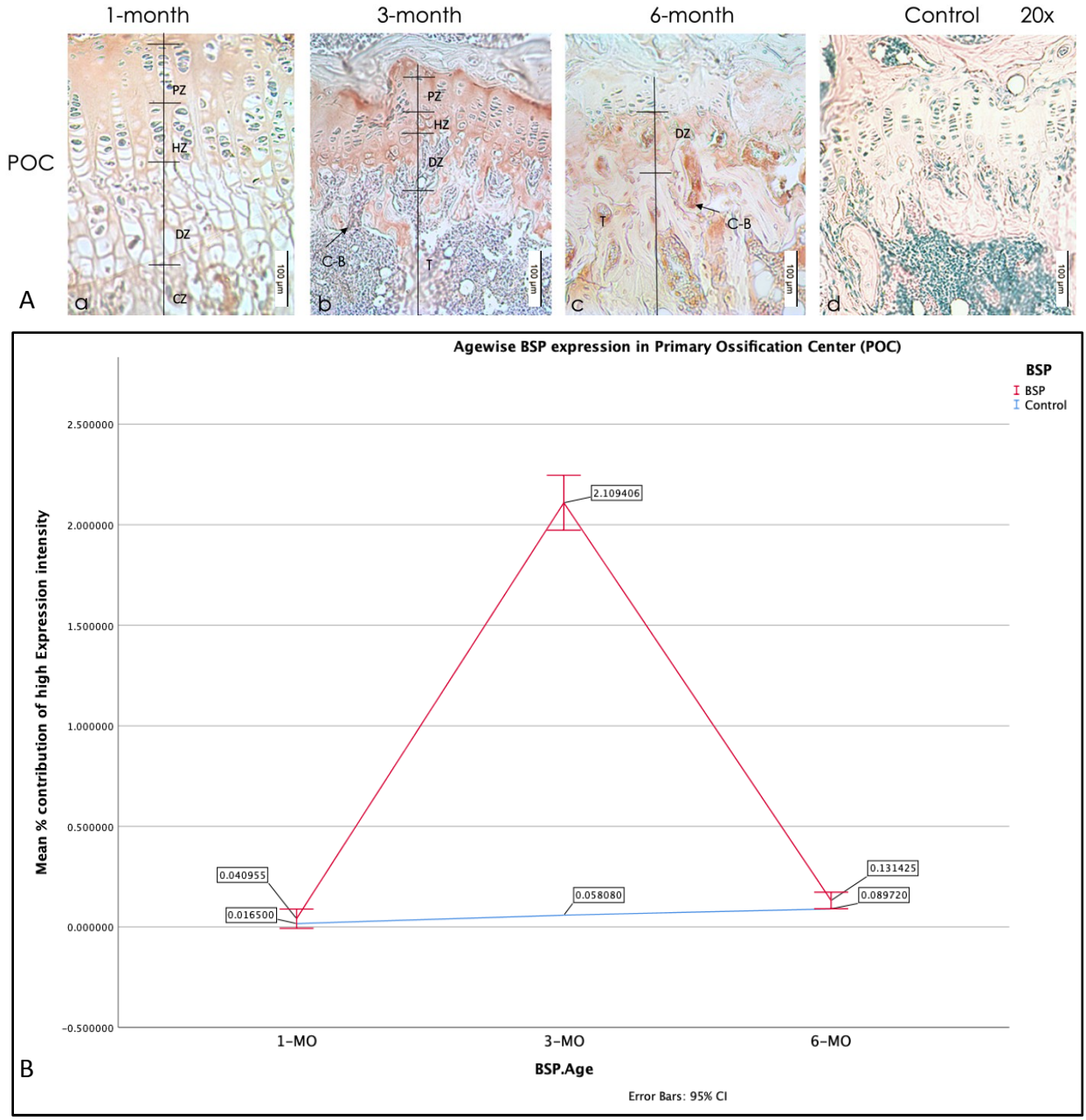


Figure 3.14a. Expression of staining intensity ofBSP at 1-, 3- and 6-months inprimary ossification center (POC). *A) IHC of POC and growth plate at epiphysis in 1-, 3-, and 6-month-old mice with anti-BSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity increased from 1- to 3-months followed by a decrease to 6-months. Highest expression was found at 3 months. (Source- IHC Dab)*

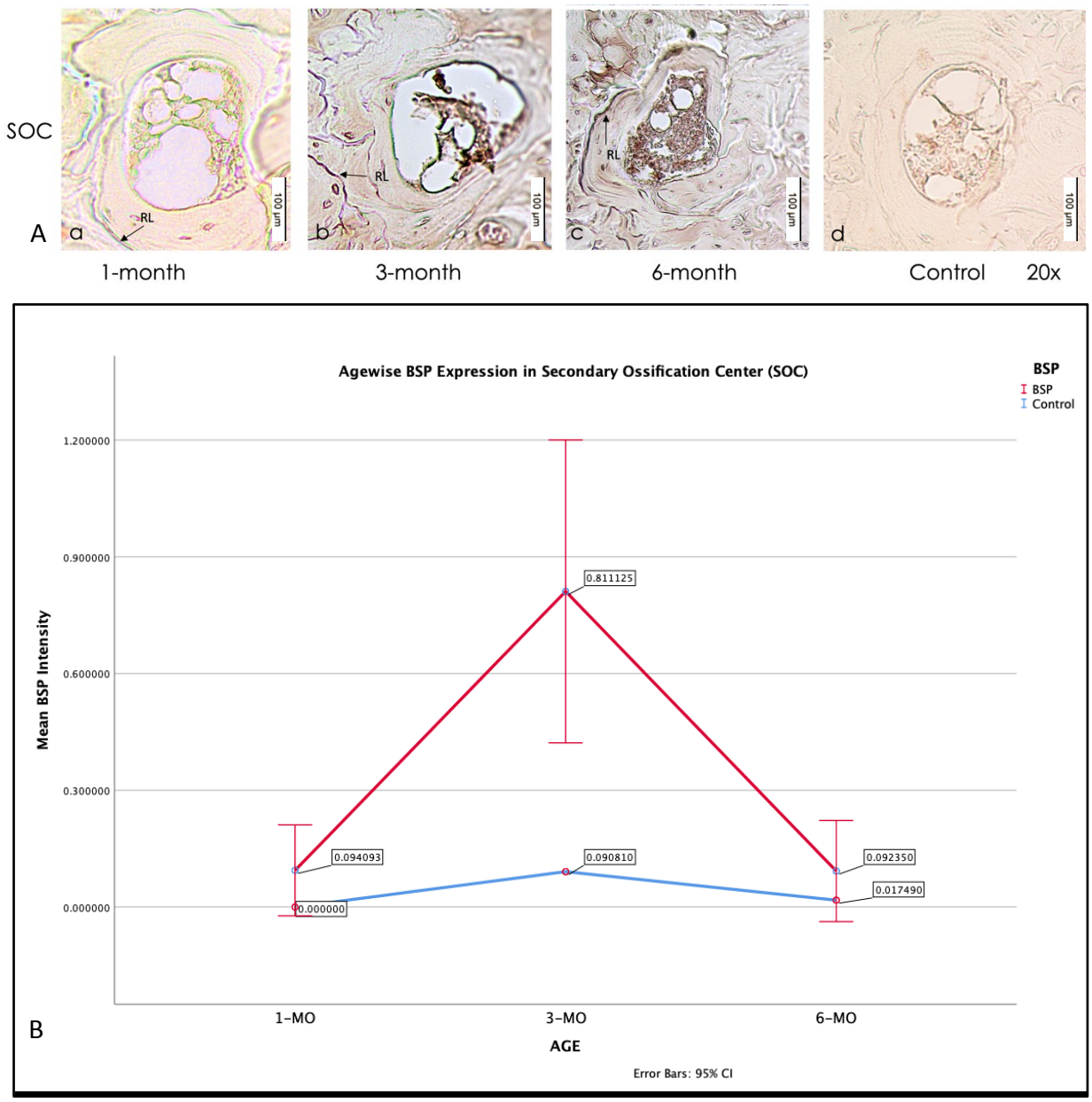


Figure 3.14b. Expression of staining intensity ofBSP at 1-, 3- and 6-months in secondary ossification center (SOC). *A) IHC of SOC at epiphysis in 1-, 3-, and 6-month-old mice with anti-BSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly increased from 1- to 3-months and decreased between 3- and 6-months. (Source- IHC Dab)*

Mandible: Intense BSP signals were observed with polyclonal anti-BSP antibody. The anti-BSP signals were observed predominantly in the dentin matrix and predentin. The BSP signal in predentin was especially strong at 3months. In the cusp, there was a 78% increase in the BSP expression from 1 to 3 months followed by a 16% decrease in expression at 6months. Strong BSP signals (brown staining) were observed in the interdental alveolar bone, furcation bone, and acellular cementum. Signals for cellular cementum were localized to the cervical and apical portions of the root. IHC analysis revealed expression of BSP in the alveolar bone. In the interproximal region, there was slight increase in its expression from 1- to 3- months followed by a 166% increase from 3- to 6-months. Within the furcal bone, there was a slight decrease from 1- to 3--month group followed by a363% increase in expression at 6months. In the apical third of the root, BSP was expressed in the dentin, cellular cementum and the PDL. Within the cellular cementum and PDL there was a 3% increase in expression from 1- to 3-months followed by a 9% increase in the expression of BSP from 3- to 6-months in the root. The expression level at 6months was 3-times that noted at 1 month. (**Figure 3.15** a, **Figure 3.15**b, **Figure 3.15**c &**Figure 3.15**d)

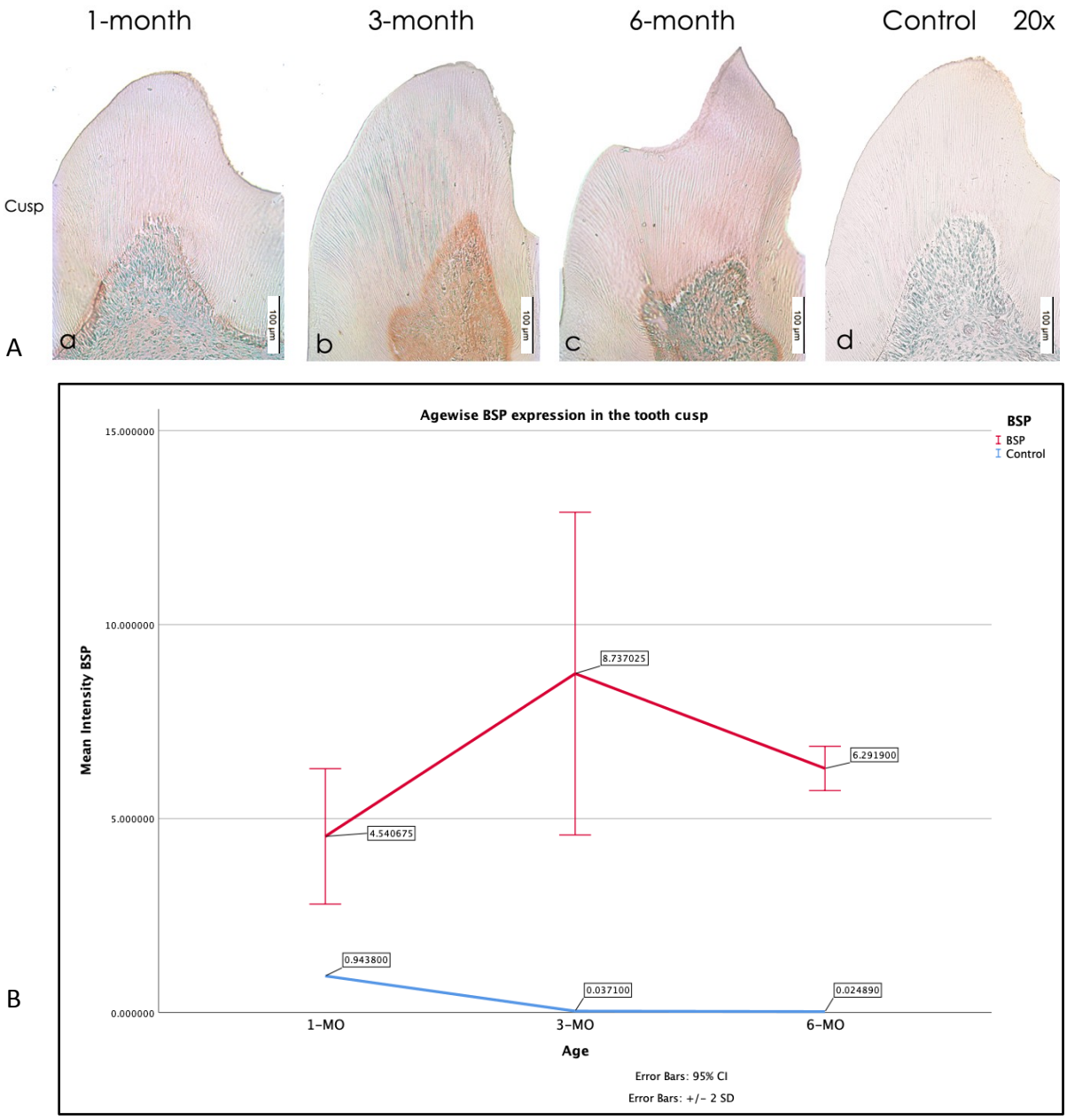


Figure 3.15a. Expression of staining intensity ofBSP at 1-, 3- and 6-months in dentin and predentin of tooth cusp. *A) IHC of dentin and predentin at tooth cusp in 1-, 3-, and 6-month-old mice with anti-BSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity increased from 1- to 3-months and decreased between 3- and 6-months.(Source- IHC Dab)*

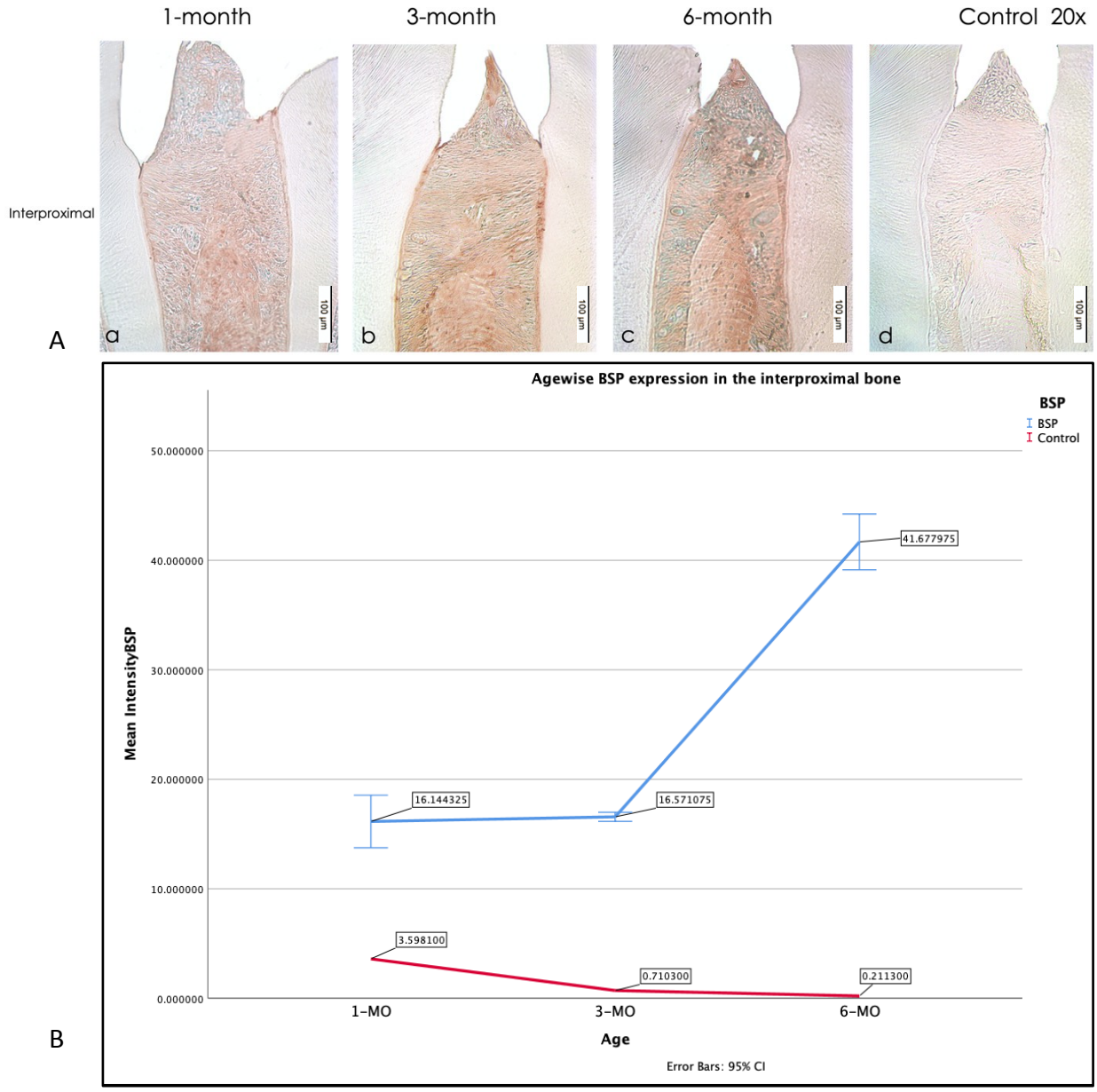


Figure 3.15b.Expression of staining intensity ofBSP at 1-, 3- and 6-months in interproximal bone. *A) IHC staining of interproximal alveolar bone in 1-, 3-, and 6-month-old mice with anti-BSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. There was minimal change in intensity from 1 to 3months followed by an increase between 3 and 6months. (Source-IHC Dab)*

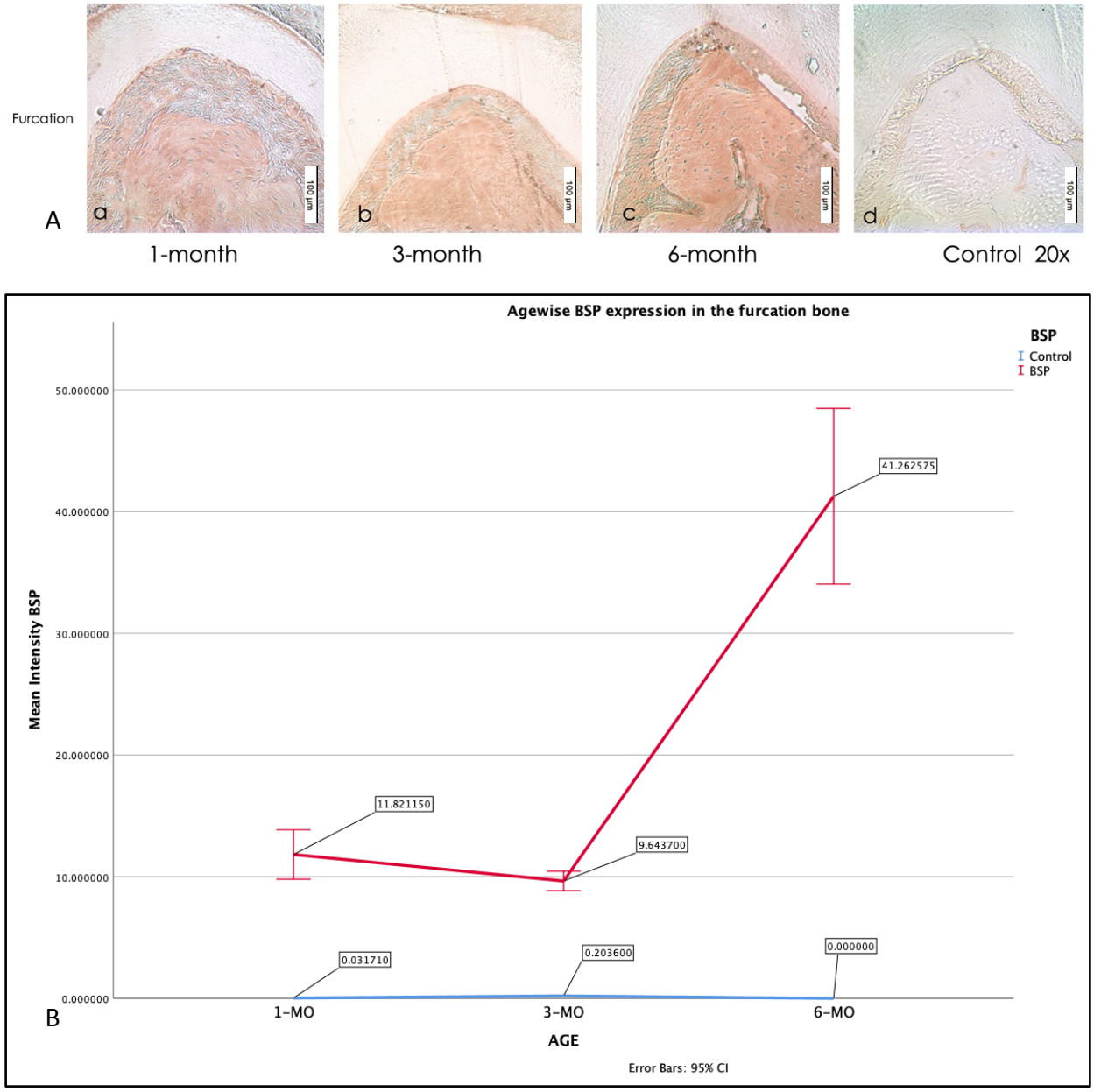


Figure 3.15c. Expression of staining intensity ofBSP at 1-, 3- and 6-months in furcation bone. *A) IHC staining of furcation alveolar bone in 1-, 3-, and 6-month-old mice with anti-BSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity decreased slightly from 1- to 3-months followed by a significant increase between 3- and 6-months. (Source-IHC Dab)*

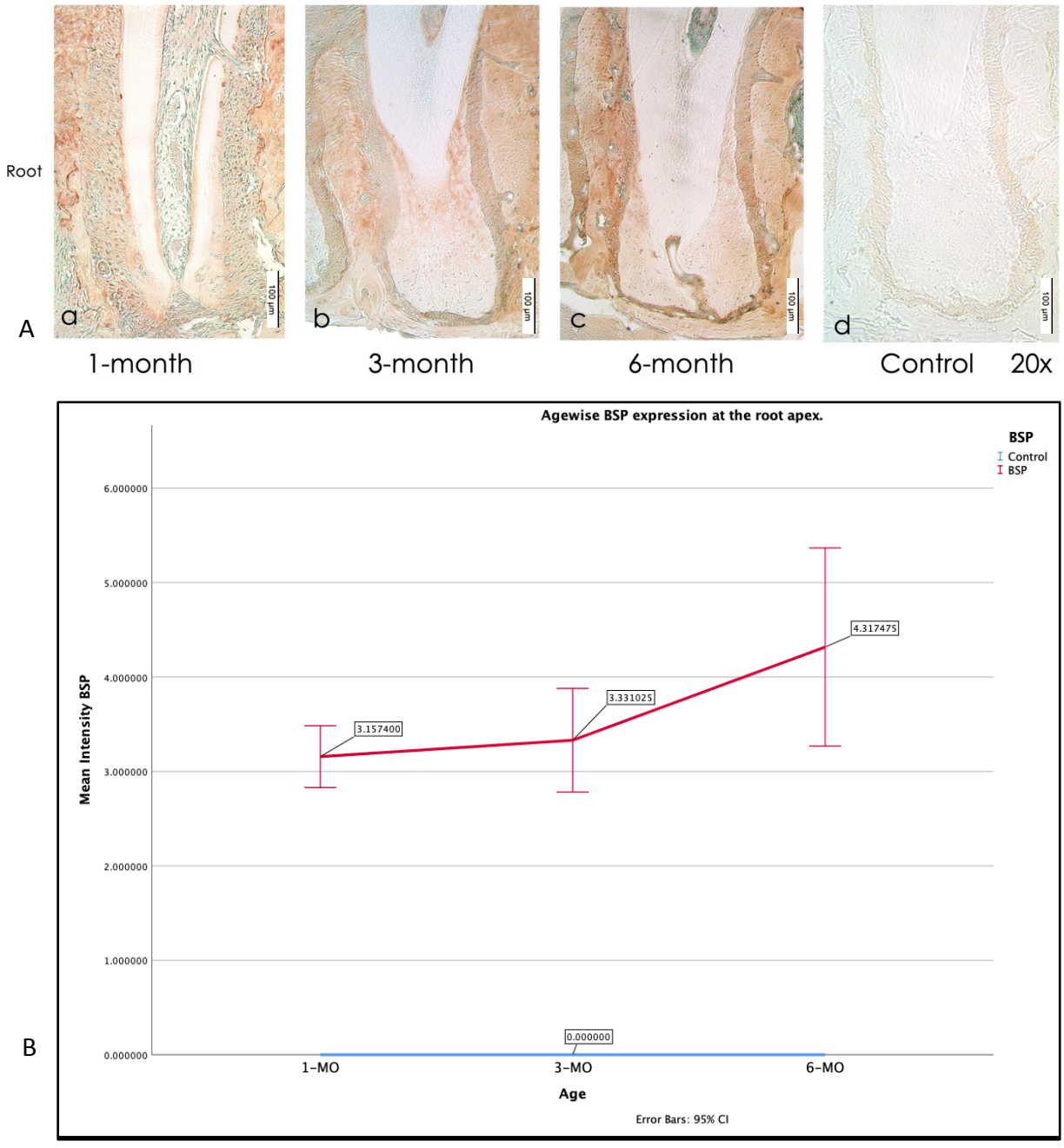


Figure 3.15d. Expression of staining intensity ofBSP at 1-, 3- and 6-months in cellular cementum and PDL of tooth root.*A) IHC staining of cellular cementum and PDL in tooth root in 1-, 3-, and 6-month-old mice with anti-BSP (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity increased slightly from 1- to 3-months followed by a significant increase between 3- and 6-months. (Source- IHC Dab)*

4. *Anti-OPN antibody:*

Tibia: OPN was expressed in the calcification zone of the growth plate and POC of tibia at all ages. There was a slight increase in the expression of OPN from 1- to 3 months followed by a 68% decrease in the expression from 3- to 6-months. A similar expression pattern was observed in the secondary ossification center within the same age groups. (**Figure 3.16**a &**Figure 3.16** b)

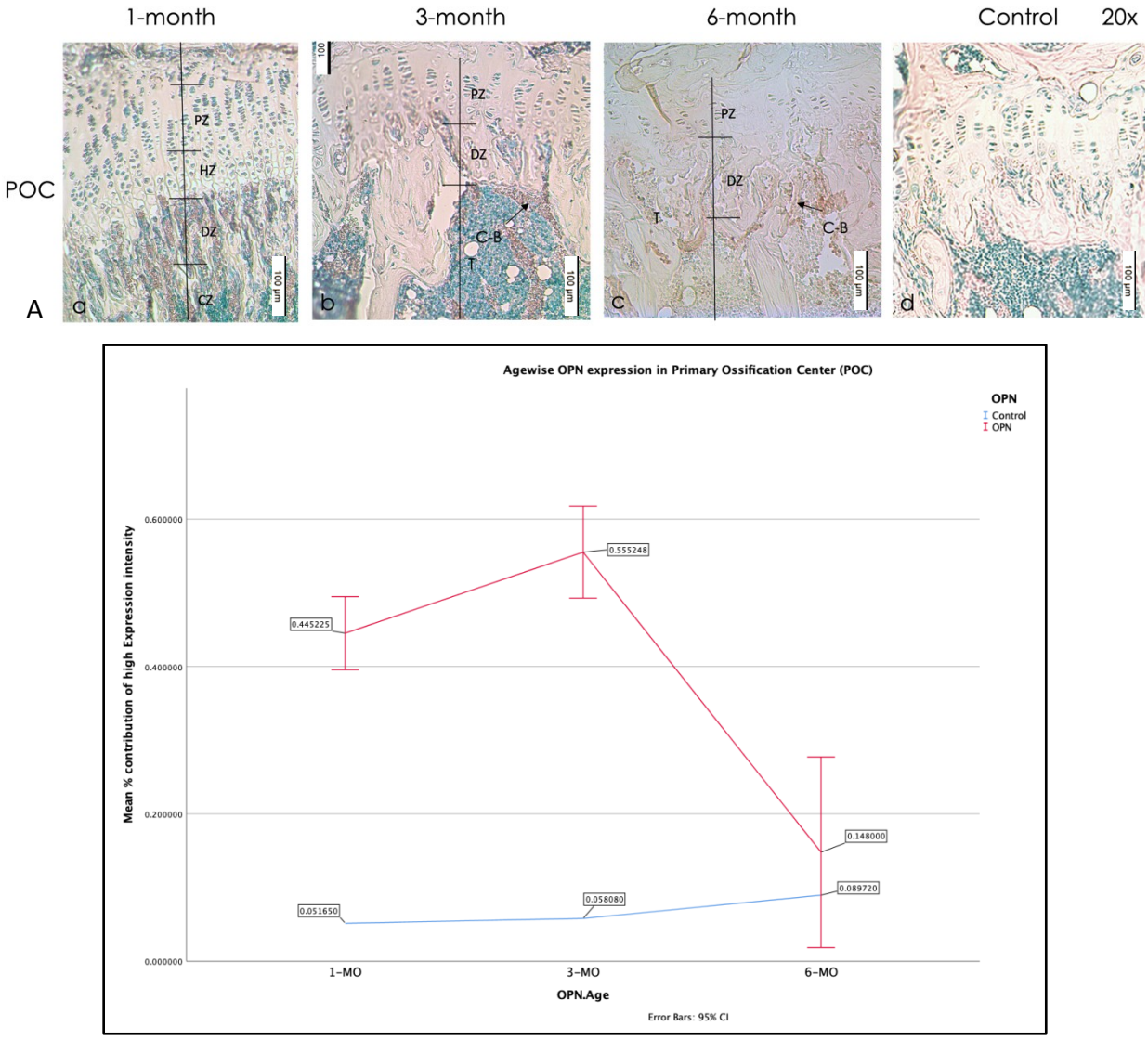


Figure 3.16a. Expression of staining intensity of OPN at 1-, 3- and 6-months in primary ossification center (POC). *A) IHC staining of POC and growth plate at epiphysis in 1-, 3-, and 6-month-old mice with anti-OPN (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity increased from 1- to 3-months followed by a significant decrease between 3- and 6-months.* *(Source- IHC Dab)*

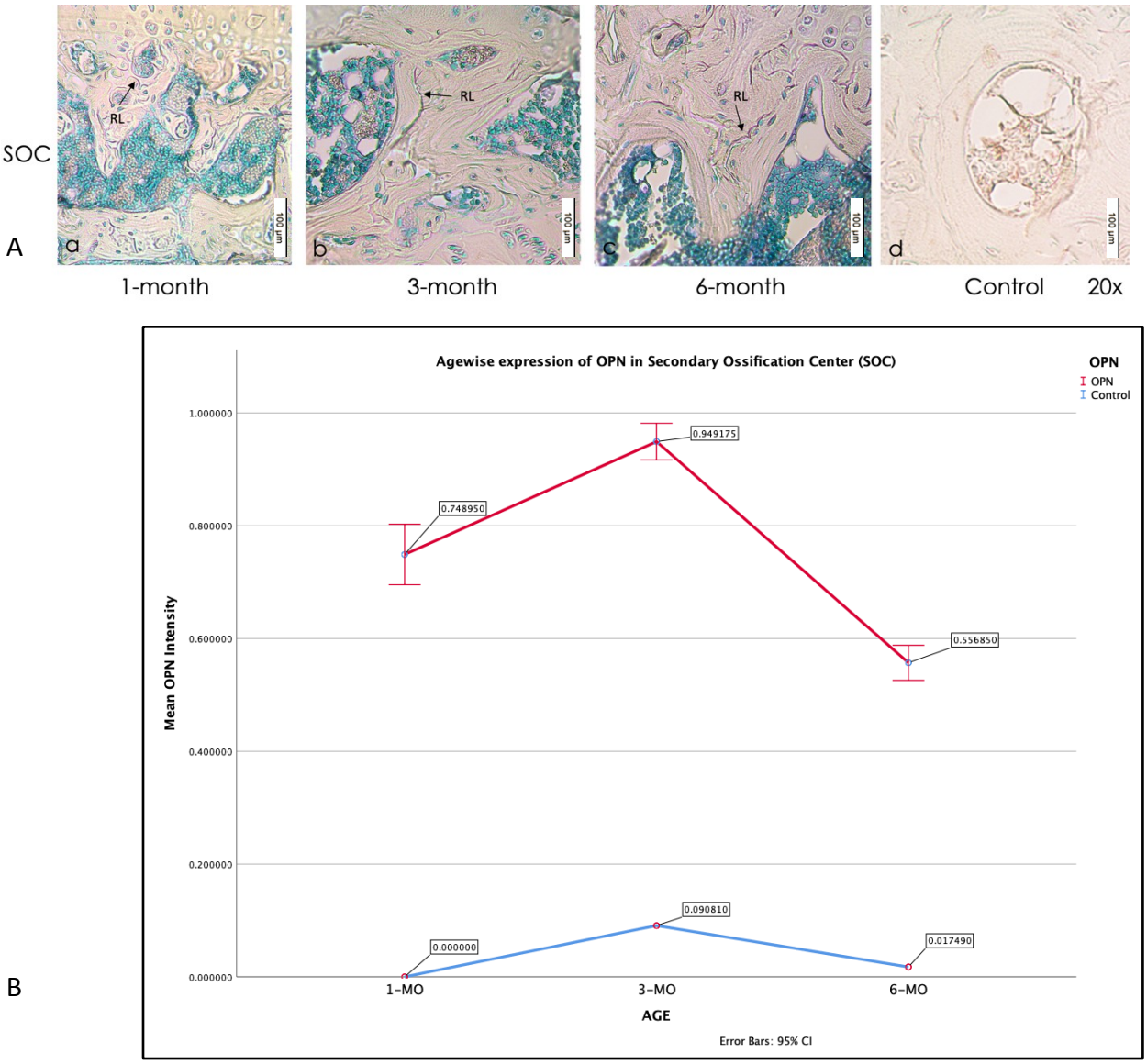


Figure 3.16b. Expression of staining intensity ofOPN at 1-, 3- & 6-months in secondary ossification center (SOC). *A) IHC staining of SOC at epiphysis in 1-, 3-, and 6-month-old mice with anti-OPN (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity increased from 1- to 3-months and decreased between 3- and 6-months.* *(Source- IHC Dab)*

Mandible: In the cuspal region, OPN was expressed along the dentinal tubules and reactionary dentin. There was a fall in the expression of OPN with an 91% decrease from 1- to 3-months followed by a 5% increase in expression from 3 to 6months. The interproximal bone showed a similar expression pattern with a 75% decrease from 1 to 3months followed by a 37% increase from 3- to 6- months. There was no expression of OPN in the acellular cementum in any age group. OPN expression in the furcal bone remained consistent with a 6% increase from 1 to 3 months followed by a 21% decrease from 3 to 6months. A similar pattern of expression was observed in the root with a 6% increase from 1 to 3months followed by a 11% decrease from 3 to 6months. All changes were statistically significant. (**Figure 3.17** a, **Figure 3.17**b,**Figure 3.17** c &**Figure 3.17** d)

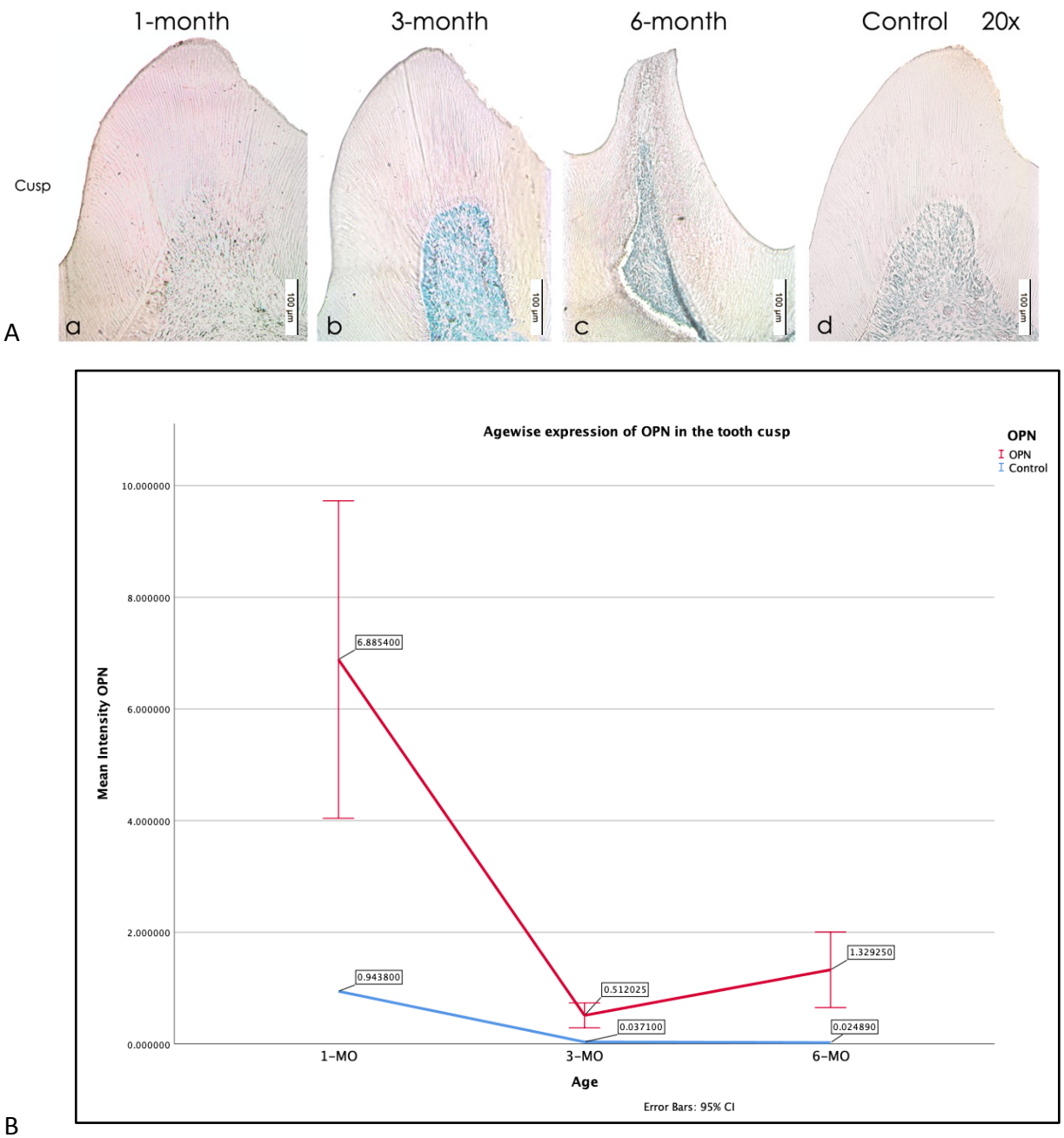


Figure 3.17a.Expression of staining intensity ofOPN at 1-, 3- and 6-months in dentin and predentin of tooth cusp. *A) IHC staining of dentin and predentin at tooth cusp in 1-, 3-, and 6-month-old mice with anti-OPN (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity significantly decreased from 1 to 3months followed by a slight increase between 3 to 6months. (Source- IHC Dab)*

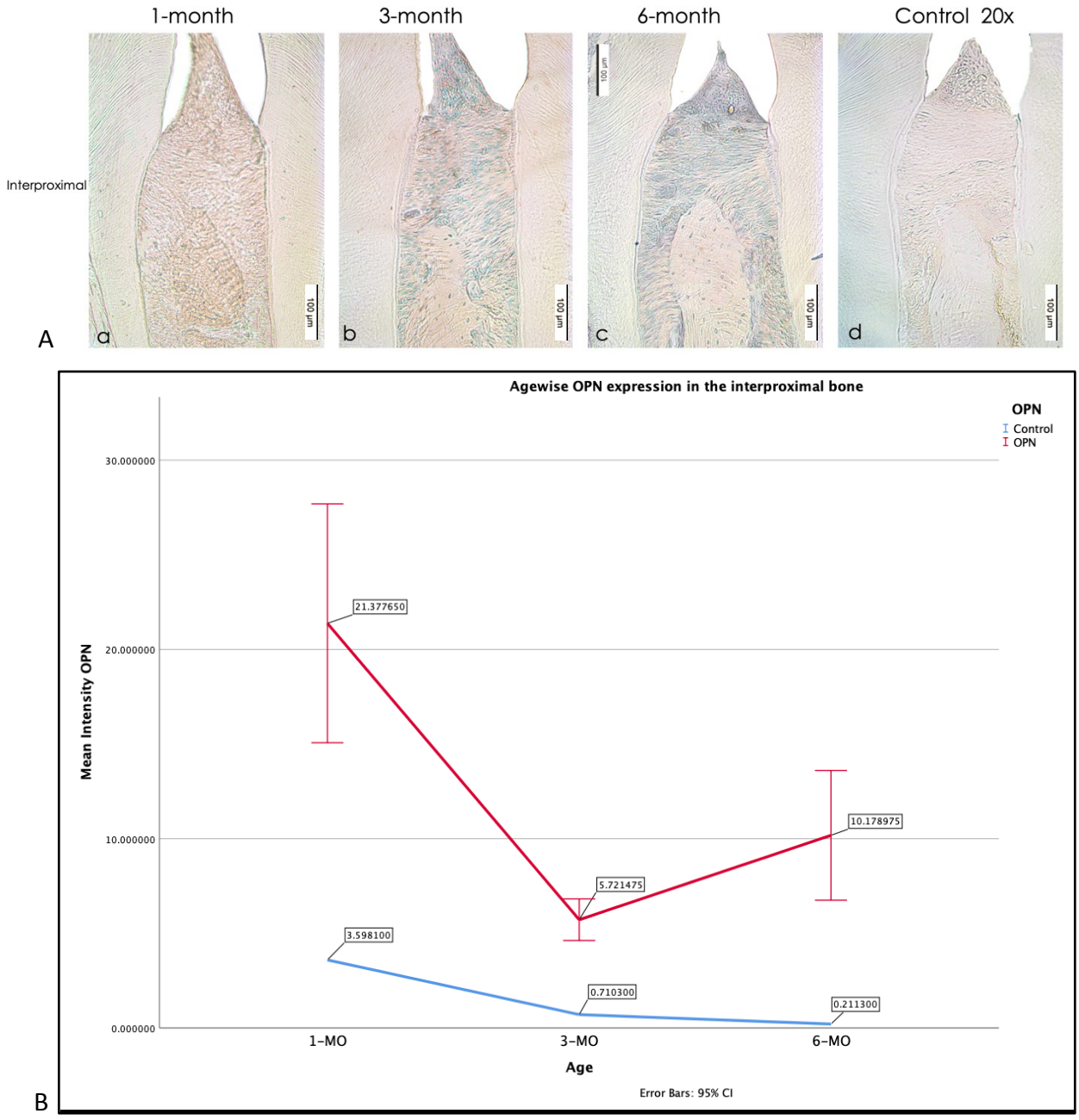


Figure 3.17b. Expression of staining intensity ofOPN at 1-, 3- and 6-months in interproximal bone. *A) IHC staining of interproximal alveolar bone in 1-, 3-, and 6-month-old mice with anti-OPN (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity decreased significantly from 1- to 3-monthsandslightly increased between 3- and 6-months.(Source- IHC Dab)*

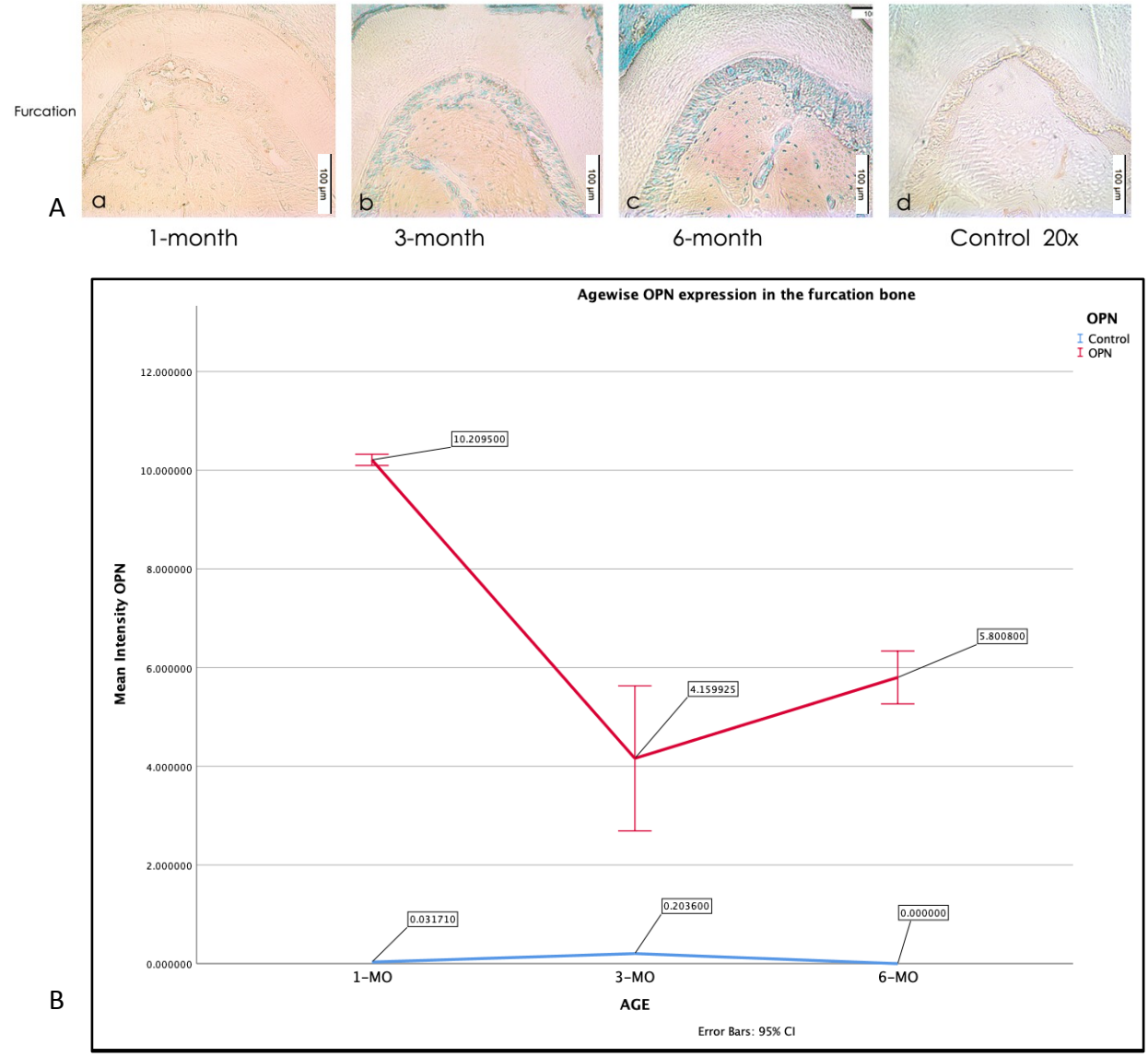


Figure 3.17c. Expression of staining intensity ofOPN at 1-, 3- and 6-months in furcation bone. *A) IHC staining of furcation alveolar bone in 1-, 3-, and 6-month-old mice with anti-OPN (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity decreased significantly from 1- to 3-months followed by an increase between 3- and 6-months. (Source- IHC Dab)*

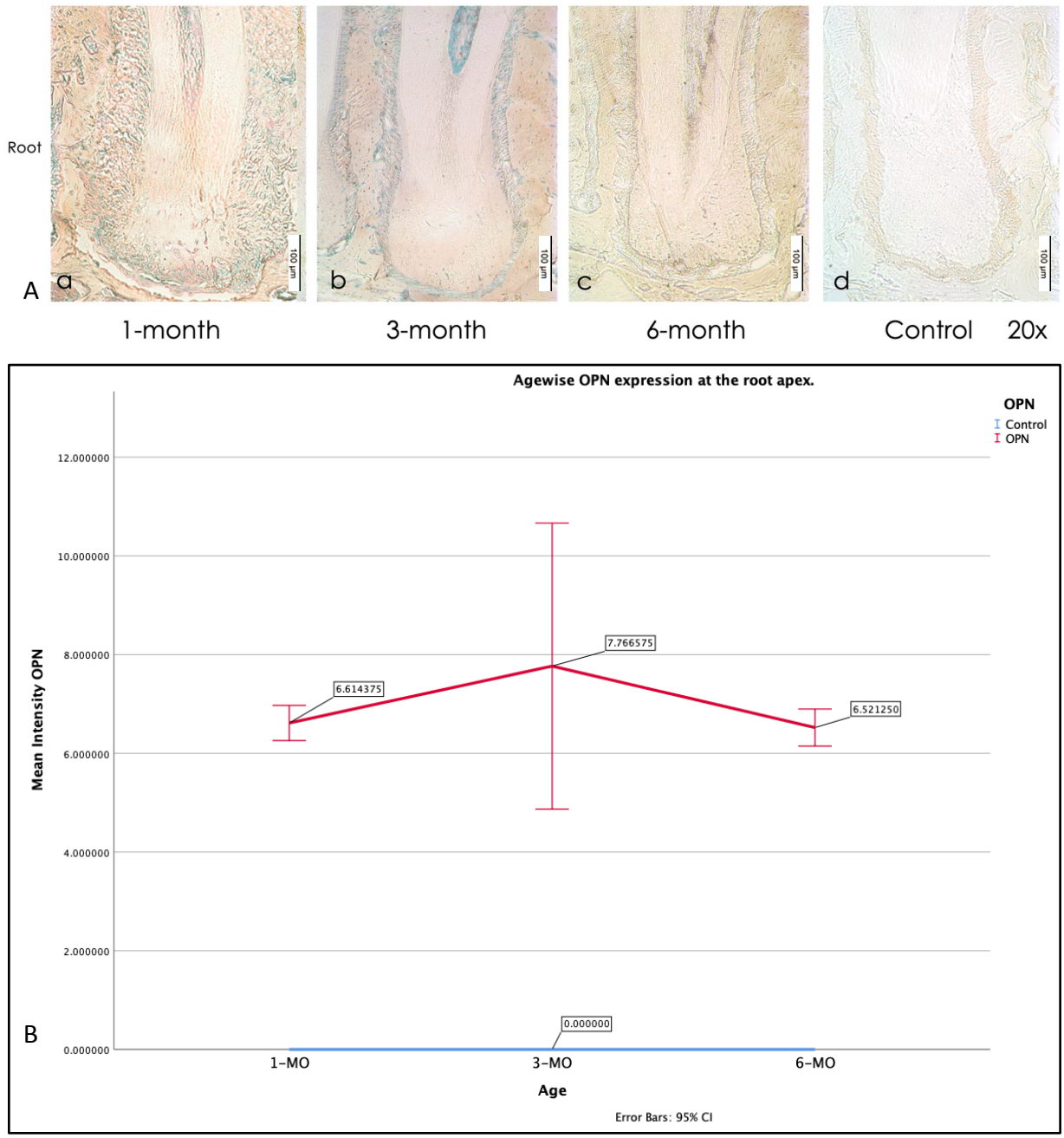


Figure 3.17d.Expression of staining intensity of OPN at 1-, 3- and 6-months in cellular cementum and PDL of tooth root. *A) IHC staining of cellular cementum and PDL in tooth root in 1-, 3-, and 6-month-old mice with anti-OPN (IHC 20x) B) Line graph for staining intensity at 1-, 3-, and 6-months. Intensity show insignificant changes between 1 and 3, and 3 and 6months respectively.* *(Source- IHC Dab)*

Expression Region	Protein / Age	DSP	DMP1	BSP	OPN
Dentinal Tubules (Peritubular)	1-month	+	+	+	+
	3-month	+	+	+	+
	6-month	+	+	+	+
Predentin	1-month	+	+	_	_
	3-month	_	+	+	_
	6-month	_	+	_	_
Interproximal Bone	1-month	+	+	+	+
	3-month	+	+	+	+
	6-month	+	+	+	+
Furcation Bone	1-month	+	+	+	+
	3-month	+	+	+	+
	6-month	+	+	+	+
Acellular Cementum	1-month	_	_	+	_
	3-month	_	_	+	_
	6-month	_	_	+	_
Cellular Cementum	1-month	+	+	+	+
	3-month	+	+	+	+

	6-month	+	+	+	+
Periodontal Ligament	1-month	+	+	+	_
	3-month	+	+	+	_
	6-month	+	+	+	_
Primary Ossification center	1-month	+	+	+	+
	3-month	+	+	+	+
	6-month	+	+	+	+
Secondary Ossification center	1-month	+	+	+	+
	3-month	+	+	+	+
	6-month	+	+	+	+
Growth Plate Degenerating Hypertrophic Chondrocytes	1-month	+	+	+	+
	3-month	+	+	+	+
	6-month	+	+	+	+
Growth Plate Resting and proliferating chondrocytes	1-month	_	_	_	_
	3-month	_	_	+	_
	6-month	–	–	–	–

Table 3.1. Regions of expression of four SIBLING proteins at 1-, 3- and 6-months. *Highlighted green boxes represent spatial expression of a protein in a region not localized by other SIBLING proteins at that specific age of mice.* *(Source- Dab)*

5. *Regional comparison of SIBLING's expression:*

Primary ossification center (POC): Repeated measures ANOVA of IHC expression intensity was conducted on the tibia in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody and analyzed using ImageJ. A line graph and box plot of mean % contribution of high staining intensity showing expression of these proteins versus control was explored for all age groups. At 1 month, post hoc test using bonferroni correction showed the intensity of DMP1, DSP and OPN versus control was significantly higher with $p<.001$, $p=0.002$,and $p<.001$, respectively. The intensity of BSP was insignificant versus control at 1 month $(p>0.05)$. At 3 months, the intensity of DSP, DMP1, and BSP versus control was significant with p-values $<.001$, $p<.001$, and $p<.001$, respectively. OPN had statistically insignificant but higher intensity of expression versus control at 3 months $p>0.05$. At 6 months, the intensity of only DSP was statistically significant versus control $p\text{-value}<.001$. Although the antibody expression was localized at the trabecular margins the intensity of DMP1, BSP, and OPN was statistically insignificant versus control at 6 months $p>0.05$. DSP's mean expression difference was significant at 3 months from 1 month and 6 months however, the mean difference was insignificant between 1 and 6 months. DMP1's mean intensity expression difference was significant between 1, 3 and 6 months. BSP's mean expression difference was significant at 3 months from both 1 month and 6 months however, the mean difference was insignificant between 1 and 6 months. For OPN, there was no change in the intensity of expression between and 3 months, but the mean expression difference was significant between 3 months compared to 6 months and 6 months compared with 1 month. A repeated analysis of variance showed that the effect of age on the expression of proteins was significant. (*Repeated measures ANOVA results: ANOVA POC*) (**Figure 3.18b**)

Secondary ossification center (SOC): Repeated measures ANOVA of IHC expression intensity was conducted on the tibia in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody and analyzed using ImageJ. A line graph and box plot of mean % contribution of high staining intensity showing expression of these proteins versus control was explored for all age groups. At 1 month, post hoc test using bonferroni correction showed the intensity of DMP1, and OPN versus control was significant with $p\text{-value}<.001$ and $p=.001$, respectively. The intensity of BSP and DSP was insignificant versus control at 1 month

(p>0.05). At 3 months, the intensity of BSP and OPN versus control was significant with *p-value<.001*. DMP1 and DSP had statistically insignificant but higher intensity of expression versus control at 3 months *(p>0.05)*. At 6 months, post hoc test using bonferroni correction showed that the intensity of BSP *(p<.001)* and DSP *(p=.011)* were statistically significant versus control. Although the antibody expression was localized at the trabecular margins and resting lines the intensity of DMP1, and OPN was statistically insignificant versus control at 6 months *(p>0.05)*. DSP's mean expression difference was significant between 1, 3 and 6 months *(p<.05)*. DMP1 mean intensity expression difference had insignificant change between 3 and 6 months but was significant between 1 to 3 and 1 to 6 months. There was no difference in BSP's mean expression between 1 and 6 months, but it was significant between 1 to 3, 3 to 6 months, and vice versa. OPN's mean intensity expression difference was significant between all ages. A repeated measures ANOVA showed that the effect of age on the expression of proteins was significant. (*ANOVA SOC*) **(Figure 3.18c)**

Tooth cusp: Repeated measures ANOVA of IHC expression intensity was used to analyze the images. At 1 month, post hoc test using bonferroni correction showed the intensity of all four SIBLING proteins was significant *(p<0.001)*. At 3 months, the intensity of DSP, DMP1 and BSP was significant versus control with *p-value<.001*. OPN had statistically insignificant but higher intensity of expression versus control at 3 months *(p>0.05)*. At 6 months, post hoc test using bonferroni correction showed that the intensity of DSP, DMP1 and BSP was statistically significant versus control *(p<.001)*. The mean intensity of expression of OPN at 6 months was also significant *p=.009 (p<.05)*. DSP's mean expression difference was significant between 1, 3 and 6 months *(p<.05)*. DMP1 mean intensity expression difference had insignificant change between 3 and 6 months but was significant between 1 to 3 and 1 to 6 months. BSP's mean expression difference was insignificant between 1 and 6 months and 3 and 6 months, but it was significant between 1 to 3 months and vice versa. OPN's mean intensity expression difference was insignificant between 3 and 6 months however, it was significant between 1 to 3, 1 to 6 and vice versa.An analysis of variance showed that the effect of age on the expression of proteins was significant. (*ANOVA Cusp*) **(Figure 3.19b)**

Root apex: At 1 month, post hoc test using bonferroni correction showed the intensity of DSP, BSP and OPN was significant *(p<0.001)*. However, DMP1 expression was insignificant but higher than control. At 3 months, the intensity of DSP, DMP1, BSP and OPN was significantly higher than control with *p-value<.001*. At 6 months, post hoc test using bonferroni correction showed that the intensity of DSP, DMP1, BSP, and OPN was statistically significant versus control *(p<.001)*. DSP's mean expression difference was significant between 1, 3 and 6 months *(p<.05)*. DMP1 mean intensity expression difference was significant between all ages. BSP's mean expression difference was insignificant between 1 and 3 months but was statistically significant between all other ages. OPN's mean intensity was higher but the expression difference was insignificant between all ages. An ANOVA showed that the effect of age on the expression of proteins was significant. (*ANOVA Root*) (**Figure 3.19c**)

Interproximal bone: Repeated measures ANOVA of IHC expression intensity was conducted in the interproximal bone to assess significance at and between each age. At 1 month, post hoc test using bonferroni correction showed the intensity of BSP and OPN was significant *(p<0.001)*. However, the expression intensity of DMP1 and DSP versus control was insignificant. At 3 months, the intensity of DSP, DMP1, BSP and OPN was significantly higher than control with *p-value<.001*. At 6 months, post hoc test using bonferroni correction showed that the intensity of BSP, and OPN was statistically significant versus control (*p<.001 and p=.05,* respectively). The expression intensity was insignificant at 3 months for DMP1 and DSP. DSP's mean expression difference was significant between 1, 3 and 6 months *(p<.05)*. DMP1 mean intensity expression difference was significant between all ages. BSP's mean expression difference was insignificant between 1 and 3 months but was statistically significant between all other ages. OPN's mean intensity was insignificant between 3 and 6 months. However, the mean expression difference between all other ages (1 to 3 and 1 to 6 months) was significant. An analysis of variance showed that the effect of age on the expression of proteins was significant. (*ANOVA Interproximal bone*) **(Figure 3.20b)**

Furcation bone: At 1 month, post hoc test using bonferroni correction showed the intensity of all four SIBLING proteins in the furcation bone versus control was significant *(p<0.001)*. At 3 months, post hoc test showed the intensity of DSP, DMP1, BSP and OPN was

significantly higher than control with *p-value<.001*. At 6 months, the intensity of DMP1, BSP, and OPN was statistically significant versus control (*p=.013, p<.001 and p=.004*, respectively). The expression intensity was insignificant at 3 months for DSP *(p>.05)*. DSP's mean expression difference was significant between 1, 3 and 6 months *(p<.05)*. DMP1 mean intensity expression difference was significant between all ages except between 1 and 3 months *(p>.05)*. BSP's mean expression difference was insignificant between 1 and 3 months but was statistically significant between all other ages. OPN's mean expression difference was significant between all ages (p<.05).A repeated measures ANOVA was conducted to compare the age wise expression intensity of SIBLING proteins from 1 to 3 to 6 months and their variation among each other. An analysis of variance showed that the effect of age on the expression of proteins was significant. (*ANOVA Furcation*) **(Figure 3.20c)**

Temporal pattern: *In the tibial secondary ossification center* (which completes ossification prior to the primary ossification center), DMP1 appears earlier than DSP, BSP and OPN. DMP1's levels fall at 3 months, a time when the levels of DSP and BSP rise. *In the alveolar bone*, the levels of DSP are higher at 1 month. At 3 months, the levels of DSP fall and DMP1 levels peak. Concomitantly, the levels of OPN fall during this time. At 6 months, the levels of DSP and DMP1 fall however, the levels of BSP rise. There is also a slight rise in the level of OPN. Therefore, in the alveolar bone DSP comes first followed by DMP1 and then BSP a pattern that is novel to this research. *In the cusp*, there is an increased expression from 1 to 3 to 6 months for DSP, DMP1. The levels for BSP fall from 3 to 6 months with levels of OPN rising. BSP and DMP1 follow similar pattern. *In the root*, DMP1 and BSP follow similar pattern of consistent rise while OPN expression remains high throughout. DSP on the other hand decreases from 1 to 3 months followed by increase at 6 months. As the levels of DSP fall the levels of DMP1 and BSP increase.

6. *Repeated measures ANOVA results:*

ANOVA POC:

Data for primary ossification center were analyzed using a mixed-design ANOVA with a within-subject factor subscale of age (1 month, 3 months and 6 months), an interaction of age and proteins and a between subject factor of proteins (DSP, DMP1, BSP and OPN). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, ($\chi^2(2) = 31.9$, $p<.000$). Greenhouse-Geisser was used since the epsilon was <0.75($\varepsilon = 0.527$). Therefore, Greenhouse-Geisser correction was applied to correct the degrees of freedom and reduce the Type I error. Main effects of subscale time * protein interaction, F (4.2, 15.8) = 83.8, $p<.0001$, $\eta_p^2 = .957$, time F (1.05, 15.8) = 374.5, $p<.0001$, $\eta_p^2 = .961$, proteins F (4, 15) = 155, $p<.0001$, $\eta_p^2 = .976$.

ANOVA SOC:

Data for secondary ossification center were analyzed using a mixed-design ANOVA with a within-subject factor subscale of age (1 month, 3 months and 6 months), an interaction of age and proteins and a between subject factor of proteins (DSP, DMP1, BSP and OPN). Mauchly's Test of Sphericity indicated that the assumption of sphericity had not been violated, ($\chi^2(2) = 5.5$, p=.064) and therefore, the variances of differences were not statistically different. Assuming sphericity all the main results and interactions were significant. Main effects of subscale time * protein interaction, F (8, 30) = 25, p<.0001, $\eta_p^2 = .870$, time F (2, 30) = 22.5, p<.0001, $\eta_p^2 = .600$, proteins F (4, 15) = 40.8, p<.0001, $\eta_p^2 = .916$.

ANOVA Cusp:

Data for tooth cusp were analyzed using a mixed-design ANOVA with a within-subject factor subscale of age (1 month, 3 months and 6 months), an interaction of age and proteins and a between subject factor of proteins (DSP, DMP1, BSP and OPN). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, ($\chi^2(2) = 8.9$, $p<.011$). Greenhouse-Geisser was used since the epsilon was <0.75($\varepsilon = 0.668$). Therefore, degrees of freedom were corrected using Greenhouse-Geisser correction and reduce the Type I error. Main effects of subscale time * protein interaction, F (5.34, 18.7) = 29.8, $p<.0001$, $\eta_p^2 = .895$, time F (1.3, 18.7) = 6.3, $p=.015$, $\eta_p^2 = .309$, proteins F (4, 14) = 118, $p<.0001$, $\eta_p^2 = .971$.

ANOVA Furcation:

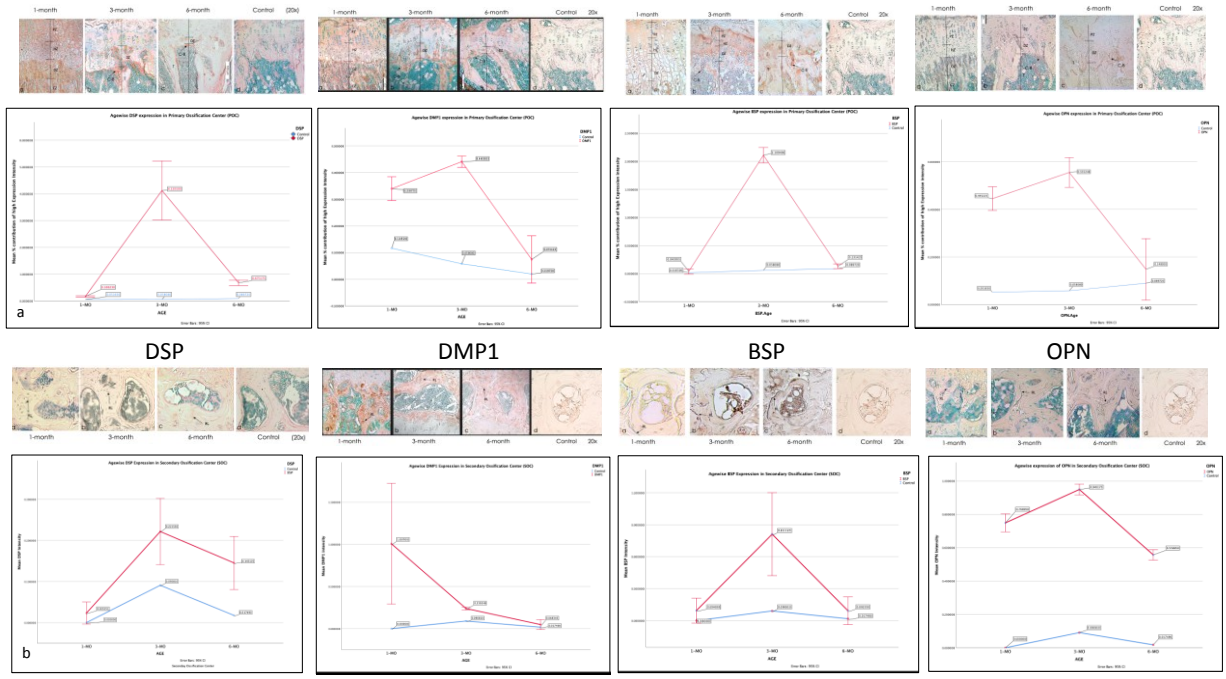
Data for furcation bone were analyzed using a mixed-design ANOVA with a within-subject factor subscale of age (1 month, 3 months and 6 months), an interaction of age and proteins and a between subject factor of proteins (DSP, DMP1, BSP and OPN). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, ($\chi^2(2) = 6.05$, p=.049). Greenhouse-Geisser was used since the epsilon was <0.75($\varepsilon = 0.74$). Therefore, degrees of freedom were corrected using Greenhouse-Geisser correction and reduce the Type I error. Main effects of subscale time * protein interaction, F (5.9, 22.2) = 234, p<.0001, η_p^2= .984, time F (1.5, 22.2) = 105.9, p<.0001, η_p^2 = .876, proteins F (4, 15) = 461, p<.0001, η_p^2 = .992.

ANOVA Interproximal bone:

Data for interproximal bone were analyzed using a mixed-design ANOVA with a within-subject factor subscale of age (1 month, 3 months and 6 months), an interaction of age and proteins and a between subject factor of proteins (DSP, DMP1, BSP and OPN). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, ($\chi^2(2) = 14.96$, p=.001). Greenhouse-Geisser was used since the epsilon was <0.75($\varepsilon = 0.60$). Therefore, degrees of freedom were corrected using Greenhouse-Geisser correction and reduce the Type I error. Main effects of subscale time * protein interaction, F (4.8, 18.1) = 193.3, p<.0001, η_p^2= .981, time F (1.2, 18.1) = 106.4, p<.0001, η_p^2 = .876, proteins F (4, 15) = 1665, p<.0001, η_p^2 = .998.

ANOVA Root:

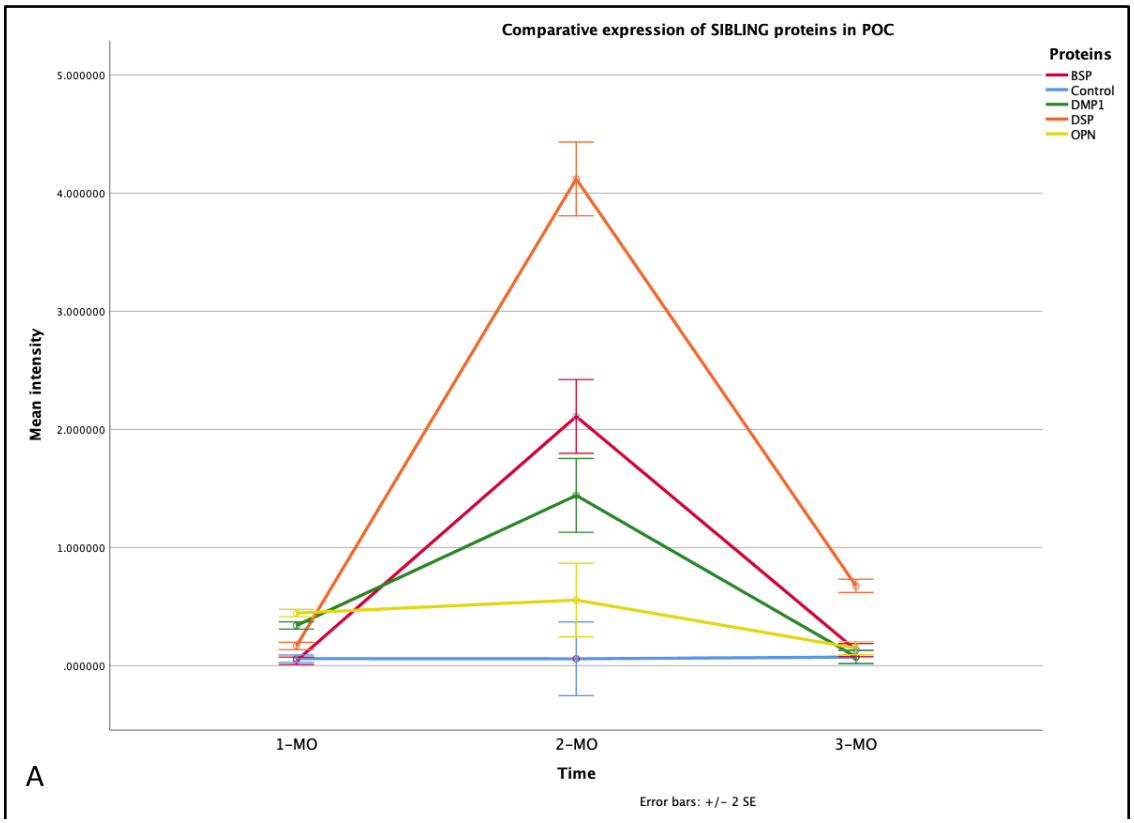
Data for root apex were analyzed using a mixed-design ANOVA with a within-subject factor subscale of age (1 month, 3 months and 6 months), an interaction of age and proteins and a between subject factor of proteins (DSP, DMP1, BSP and OPN). Mauchly's Test of Sphericity indicated that the assumption of sphericity had not been violated, ($\chi^2(2) = 1.56$, p=.458) and therefore, the variances of differences were not statistically different. Assuming sphericity all the main results and interactions were significant. Main effects of subscale time * protein interaction, F (8, 30) = 165.6, p<.0001, η_p^2= .978, time F (2, 30) = 58.3, p<.0001, η_p^2 = .795, proteins F (4, 15) = 440.2, p<.0001, η_p^2 = .992.



SIBLING expression in Tibia

Figure 3.18a.Comparative expression of staining intensity of SIBLING proteins at 1-, 3- and 6-months in (a) POC and (b) SOC. *IHC of tibia in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN (IHC 20x) with Line graphs for staining intensity at 1-, 3-, and 6-months. Intensity showed significant higher expression at 3 months versus 1-, and 6-months DMP1 showed higher expression at 1 month in SOC. (Source- Dab)*

Primary Ossification Center ANOVA and post hoc analysis:



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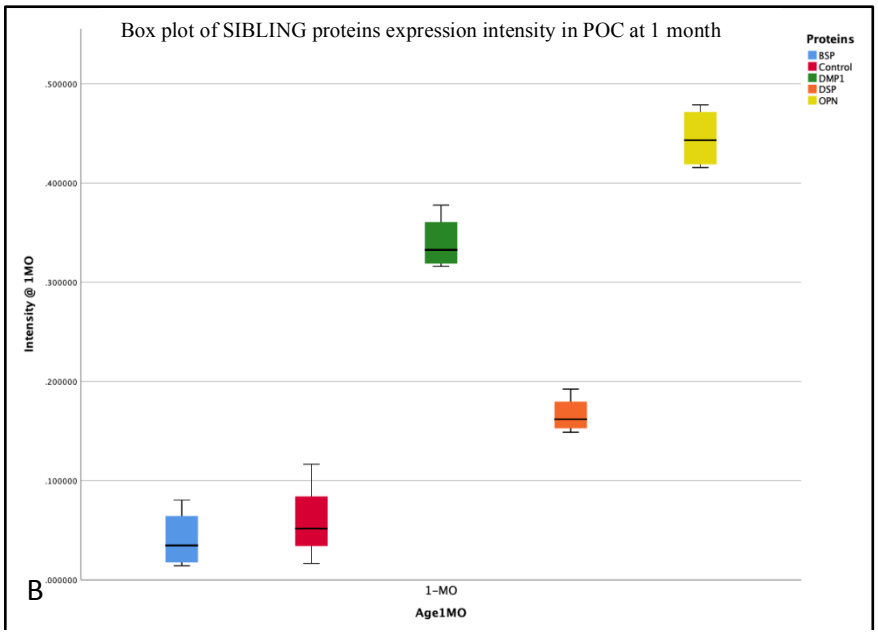
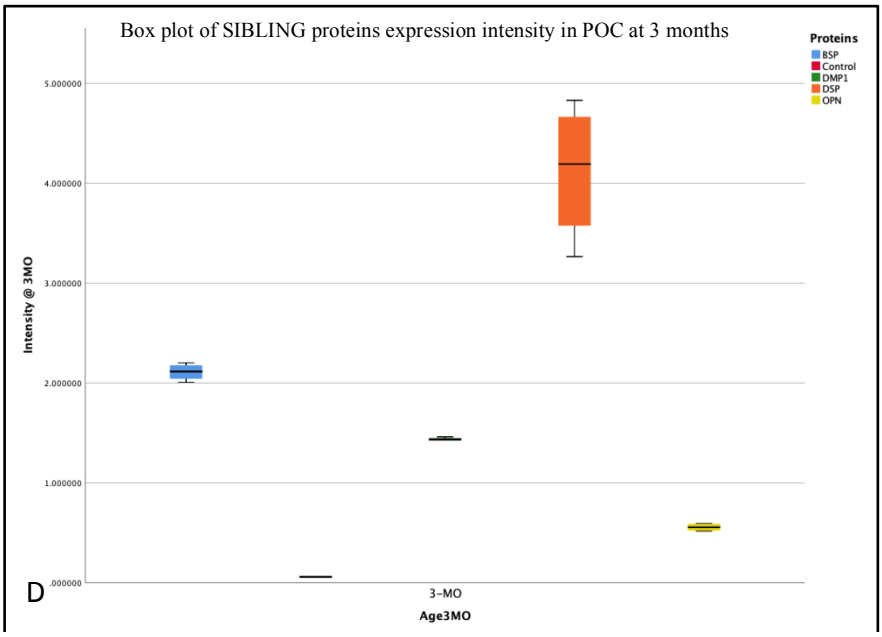


B

Univariate analysis for significance of SIBLING proteins versus control at 1 month

C

Control	BSP	.01812000	.021820585	1.000	-.05358328	.08982328
	DMP1	-.2806600*	.021820585	.000	-.35236328	-.20895672
	DSP	-.1071550*	.021820585	.002	-.17885828	-.03545172
	OPN	-.3861500*	.021820585	.000	-.45785328	-.31444672

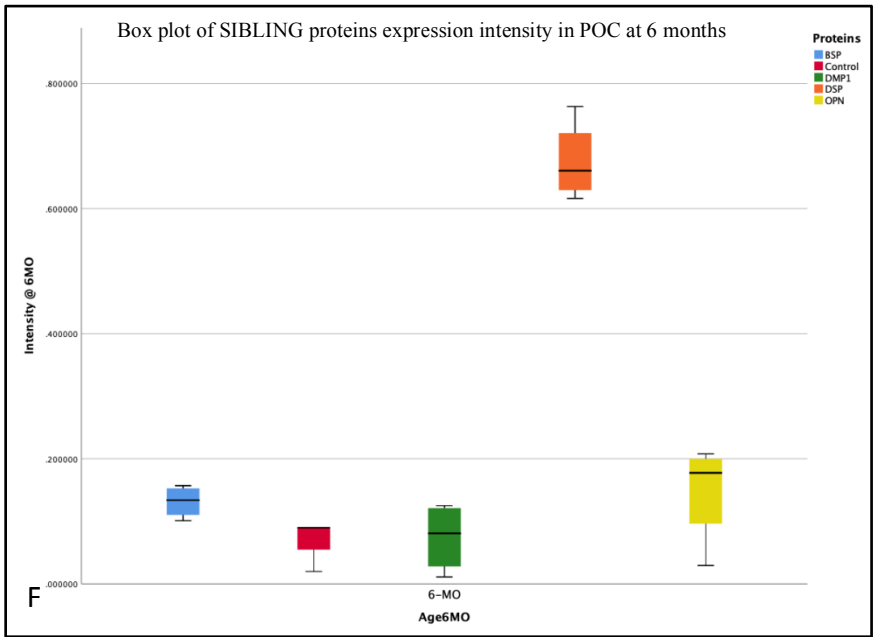


D

Univariate analysis for significance of SIBLING proteins versus control at 3 months

Control	BSP	-2.051326*	.220773077	.000	-2.7767943	-1.3258567
	DMP1	-1.382853*	.220773077	.000	-2.1083213	-.65738365
	DSP	-4.062020*	.220773077	.000	-4.7874888	-3.3365512
	OPN	-.49716750	.220773077	.397	-1.2226363	.22830135

E



F

Univariate analysis for significance of SIBLING proteins versus control at 6 months

Control	BSP	-.05920500	.039600253	1.000	-.18933296	.07092296
	DMP1	-.00220250	.039600253	1.000	-.13233046	.12792546
	DSP	-.6029550*	.039600253	.000	-.73308296	-.47282704
	OPN	-.07578000	.039600253	.749	-.20590796	.05434796

G

Post hoc tests

Dependent VariableDSP Intensity

Test: Bonferroni

(I) AGE	(J) AGE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-3.164382*	.253716365	.000	-3.9086143	-2.4201497
	6-MO	-.41477000	.253716365	.410	-1.1590023	.32946232
3-MO	1-MO	3.1643820*	.253716365	.000	2.42014968	3.90861432
	6-MO	2.7496120*	.253716365	.000	2.00537968	3.49384432
6-MO	1-MO	.41477000	.253716365	.410	-.32946232	1.15900232
	3-MO	-2.749612*	.253716365	.000	-3.4938443	-2.0053797

Based on observed means.
The error term is Mean Square(Error) = .161.

H *. The mean difference is significant at the 0.05 level.

Dependent VariableDMP1 Intensity

Test: Bonferroni

(I) AGE	(J) AGE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-.8692740*	.023251945	.000	-.93747949	-.80106851
	6-MO	.23160600*	.023251945	.000	.16340051	.29981149
3-MO	1-MO	.86927400*	.023251945	.000	.80106851	.93747949
	6-MO	1.1008800*	.023251945	.000	1.03267451	1.16908549
6-MO	1-MO	-.2316060*	.023251945	.000	-.29981149	-.16340051
	3-MO	-1.100880*	.023251945	.000	-1.1690855	-1.0326745

Based on observed means.
The error term is Mean Square(Error) = .001.

I *. The mean difference is significant at the 0.05 level.

Dependent VariableBSP Intensity

Test: Bonferroni

(I) BSP.Age	(J) BSP.Age	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-1.663076*	.034507179	.000	-1.7642971	-1.5618557
	6-MO	-.08702000	.034507179	.098	-.18824074	.01420074
3-MO	1-MO	1.6630764*	.034507179	.000	1.56185566	1.76429714
	6-MO	1.5760564*	.034507179	.000	1.47483566	1.67727714
6-MO	1-MO	.08702000	.034507179	.098	-.01420074	.18824074
	3-MO	-1.576056*	.034507179	.000	-1.6772771	-1.4748357

Based on observed means.
The error term is Mean Square(Error) = .003.

J *. The mean difference is significant at the 0.05 level.

Dependent VariableOPN intensity

Test Bonferroni

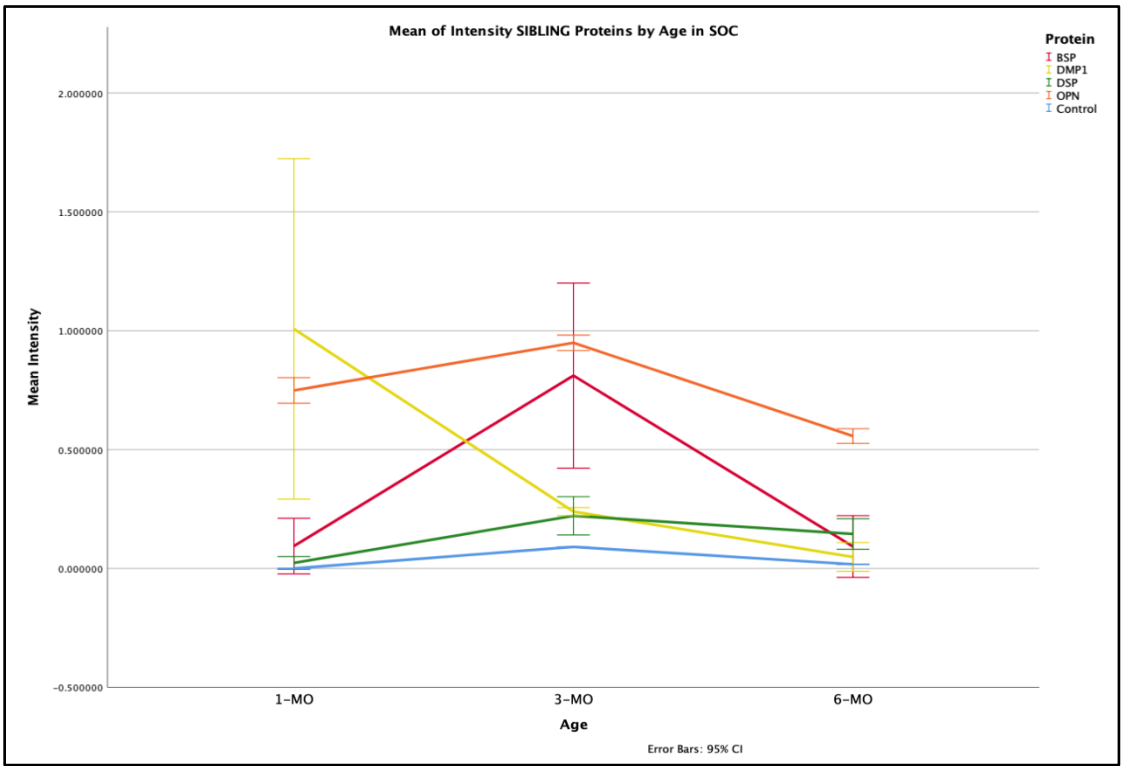
(I) OPN.Age	(J) OPN.Age	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-.08930400	.034852218	.092	-.19153685	.01292885
	6-MO	.23016600*	.034852218	.000	.12793315	.33239885
3-MO	1-MO	.08930400	.034852218	.092	-.01292885	.19153685
	6-MO	.31947000*	.034852218	.000	.21723715	.42170285
6-MO	1-MO	-.2301660*	.034852218	.000	-.33239885	-.12793315
	3-MO	-.3194700*	.034852218	.000	-.42170285	-.21723715

Based on observed means.
The error term is Mean Square(Error) = .003.

*. The mean difference is significant at the 0.05 level.

Figure 3.18b.Comparative expression of staining intensity of SIBLING proteins at 1-, 3- and 6-months in POC. *IHC of tibia in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody (A) Line graph of mean % contribution of high staining intensity as analyzed by ImageJ. (B) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 1 month. (C) At 1 month, post hoc test using bonferroni correction showed the intensity of DMP1, DSP and OPN versus control was significant with p-value<.001, p=0.002, p<.001, respectively. The intensity of BSP was insignificant versus control at 1 month (p>0.05). (D) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 3 months. (E) At 3 months, post hoc analysis using bonferroni correction showed the intensity of DSP, DMP1, and BSP versus control was significant with p-value=<.001, p<.001, p<.001, respectively. OPN had statistically insignificant but higher intensity of expression versus control at 3 months p>0.05. (F) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 6 months. (G) At 6 months, post hoc test using bonferroni correction showed that the intensity of only DSP was statistically significant versus control p-value=<.001. Although the antibody expression was localized at the trabecular margins the intensity of DMP1, BSP, and OPN was statistically insignificant versus control at 6 months p>0.05. (H) DSP's mean expression difference was significant at 3 months from 1 month and 6 months however, the mean difference was insignificant between 1 and 6 months. (I) DMP1 mean intensity expression difference was significant between 1, 3 and 6 months. (J) BSP's mean expression difference was significant at 3 months from both 1 month and 6 months however, the mean difference was insignificant between 1 and 6 months. (K) For OPN, there was no change in the intensity of expression between and 3 months, but the mean expression difference was significant between 3 months compared to 6 months and 6 months compared with 1 month.*

Secondary Ossification Center ANOVA and post hoc analysis:



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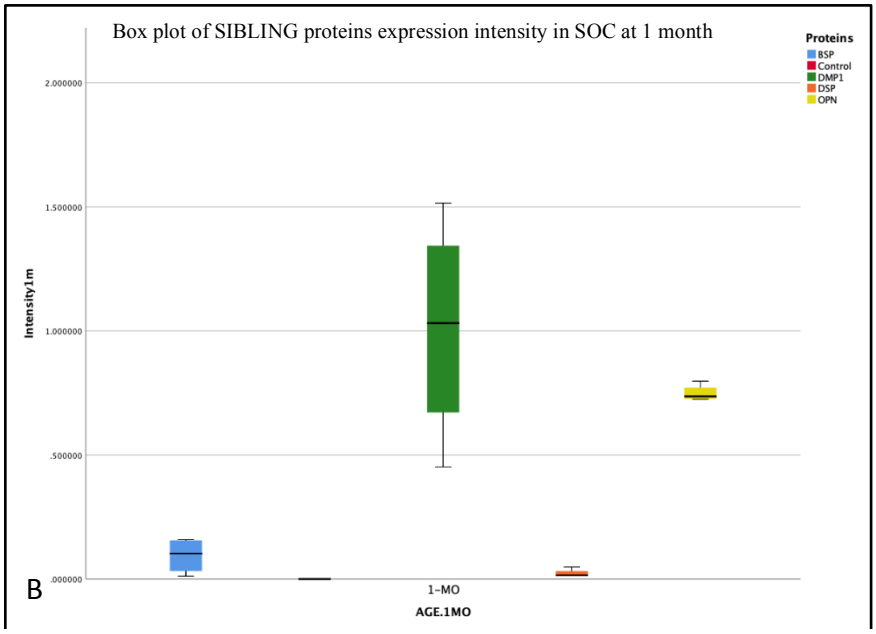
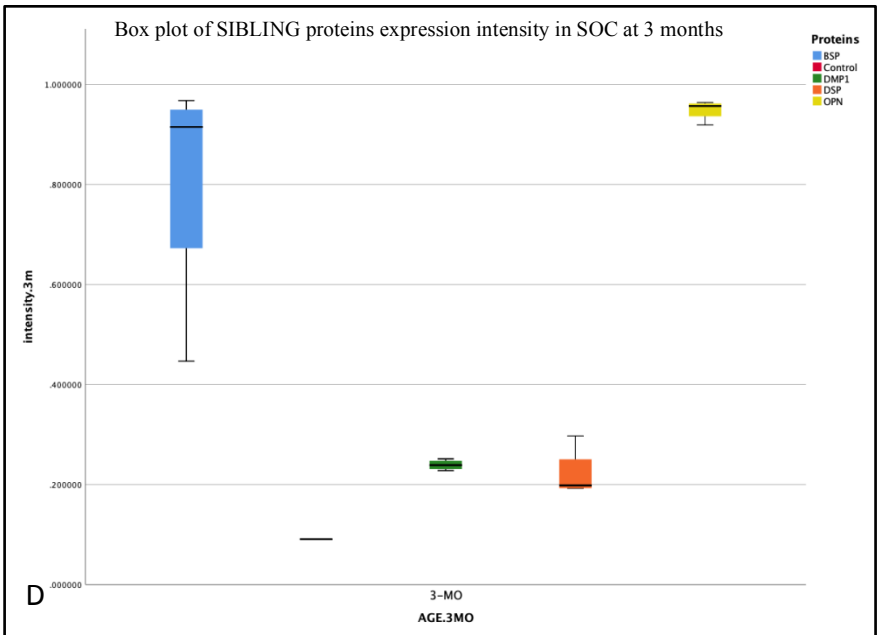


B

Univariate analysis for significance of SIBLING proteins versus control at 1 month

Control	BSP	-.09409250	.144640231	1.000	-.56938588	.38120088
	DMP1	-1.007650*	.144640231	.000	-1.4829434	-.53235662
	DSP	-.02325050	.144640231	1.000	-.49854388	.45204288
	OPN	-.7489500*	.144640231	.001	-1.2242434	-.27365662

C

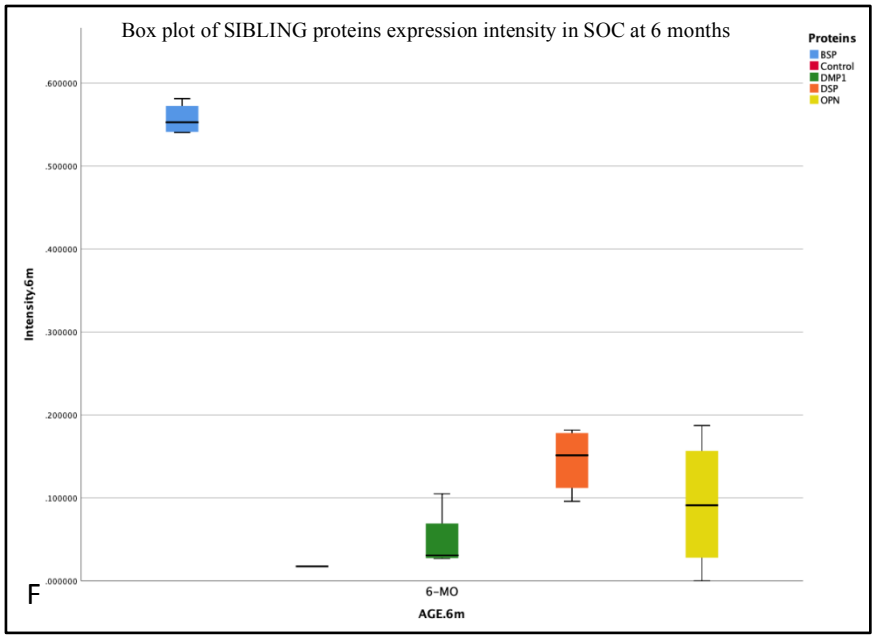


D

Univariate analysis for significance of SIBLING proteins versus control at 3 months

Control	BSP	-.7203150*	.079316314	.000	-.98095147	-.45967853
	DMP1	-.14843750	.079316314	.809	-.40907397	.11219897
	DSP	-.13074000	.079316314	1.000	-.39137647	.12989647
	OPN	-.8583650*	.079316314	.000	-1.1190015	-.59772853

E



F

Univariate analysis for significance of SIBLING proteins versus control at 6 months

Control	BSP	-.5393600*	.031811501	.000	-.64389382	-.43482618
	DMP1	-.03085500	.031811501	1.000	-.13538882	.07367882
	DSP	-.1276350*	.031811501	.011	-.23216882	-.02310118
	OPN	-.07486000	.031811501	.327	-.17939382	.02967382

G

Dependent Variable DSP

Test

Post hoc tests

(I) AGES	(J) AGES	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1-MO	3-MO	-.1768016*	.024461027	.000	-.24855372	-.10504948
	6-MO	-.1009976*	.024461027	.008	-.17274972	-.02924548
3-MO	1-MO	.17680160*	.024461027	.000	.10504948	.24855372
	6-MO	.07580400*	.024461027	.038	.00405188	.14755612
6-MO	1-MO	.10099760*	.024461027	.008	.02924548	.17274972
	3-MO	-.0758040*	.024461027	.038	-.14755612	-.00405188

Based on observed means.
The error term is Mean Square(Error) = .001.

H *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityDMP1

Test Bonferroni

(I) AGES	(J) AGES	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1-MO	3-MO	.59656000*	.164897705	.017	.11286159	1.08025841
	6-MO	.76394600*	.164897705	.004	.28024759	1.24764441
3-MO	1-MO	-.5965600*	.164897705	.017	-1.0802584	-.11286159
	6-MO	.16738600	.164897705	1.000	-.31631241	.65108441
6-MO	1-MO	-.7639460*	.164897705	.004	-1.2476444	-.28024759
	3-MO	-.16738600	.164897705	1.000	-.65108441	.31631241

Based on observed means.
The error term is Mean Square(Error) = .068.

I *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityBSP

Test Bonferroni

(I) AGES	(J) AGES	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1-MO	3-MO	-.5917880*	.097895903	.001	-.87894841	-.30462759
	6-MO	-.00210400	.097895903	1.000	-.28926441	.28505641
3-MO	1-MO	.59178800*	.097895903	.001	.30462759	.87894841
	6-MO	.58968400*	.097895903	.001	.30252359	.87684441
6-MO	1-MO	.00210400	.097895903	1.000	-.28505641	.28926441
	3-MO	-.5896840*	.097895903	.001	-.87684441	-.30252359

Based on observed means.
The error term is Mean Square(Error) = .024.

J *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityOPN

Test Bonferroni

(I) AGES	(J) AGES	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-.1783420*	.016080779	.000	-.22551214	-.13117186
	6-MO	.15018200*	.016080779	.000	.10301186	.19735214
3-MO	1-MO	.17834200*	.016080779	.000	.13117186	.22551214
	6-MO	.32852400*	.016080779	.000	.28135386	.37569414
6-MO	1-MO	-.1501820*	.016080779	.000	-.19735214	-.10301186
	3-MO	-.3285240*	.016080779	.000	-.37569414	-.28135386

Based on observed means.
The error term is Mean Square(Error) = .001.

K *. The mean difference is significant at the 0.05 level.

Figure 3.18c.Comparative expression of staining intensity ofSIBLING proteins at 1-, 3- and 6-months in SOC. *IHC of tibia in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody(A) Line graph of mean % contribution of high staining intensity as analyzed by ImageJ. (B) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 1 month. (C) At 1 month, post hoc test using bonferroni correction showed the intensity of DMP1, and OPN versus control was significant with p-value<.001 and p=.001, respectively. The intensity of BSP and DSP was insignificant versus control at 1 month (p>0.05). (D) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 3 months. (E) At 3 months, post hoc test using bonferroni correction showed the intensity of BSP and OPN versus control was significant with p-value<.001. DMP1 and DSP had statistically insignificant but higher intensity of expression versus control at 3 months (p>0.05). (F) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 6 months. (G) At 6 months, post hoc test using bonferroni correction showed that the intensity of BSP (p-value<.001) and DSP (p=.011) were statistically significant versus control. Although the antibody expression was localized at the trabecular margins and resting lines the intensity of DMP1, and OPN was statistically insignificant versus control at 6 months (p>0.05). (H) DSP's mean expression difference was significant between 1, 3 and 6 months (p<.05). (I) DMP1 mean intensity expression difference had insignificant change between 3 and 6 months but was significant between 1 to 3 and 1 to 6 months. (J) There was no difference in BSP's mean expression between 1 and 6 months, but it was significant between 1 to 3, 3 to 6 months, and vice versa. (K) OPN'smean intensity expression difference was significant between all ages.*

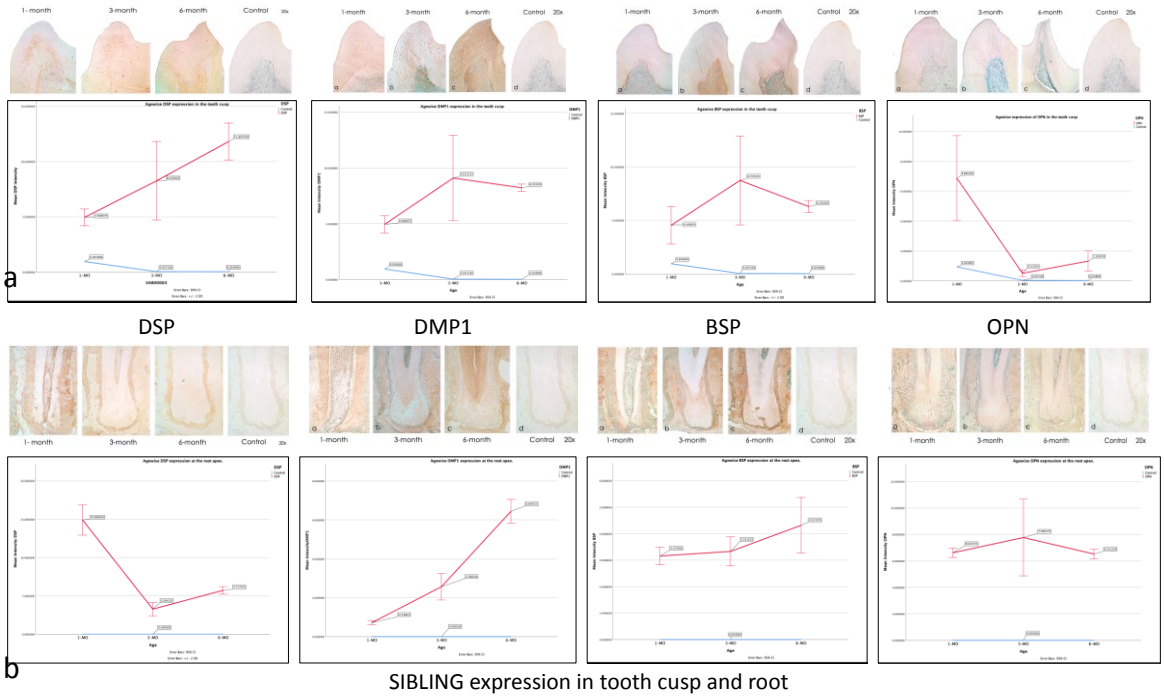
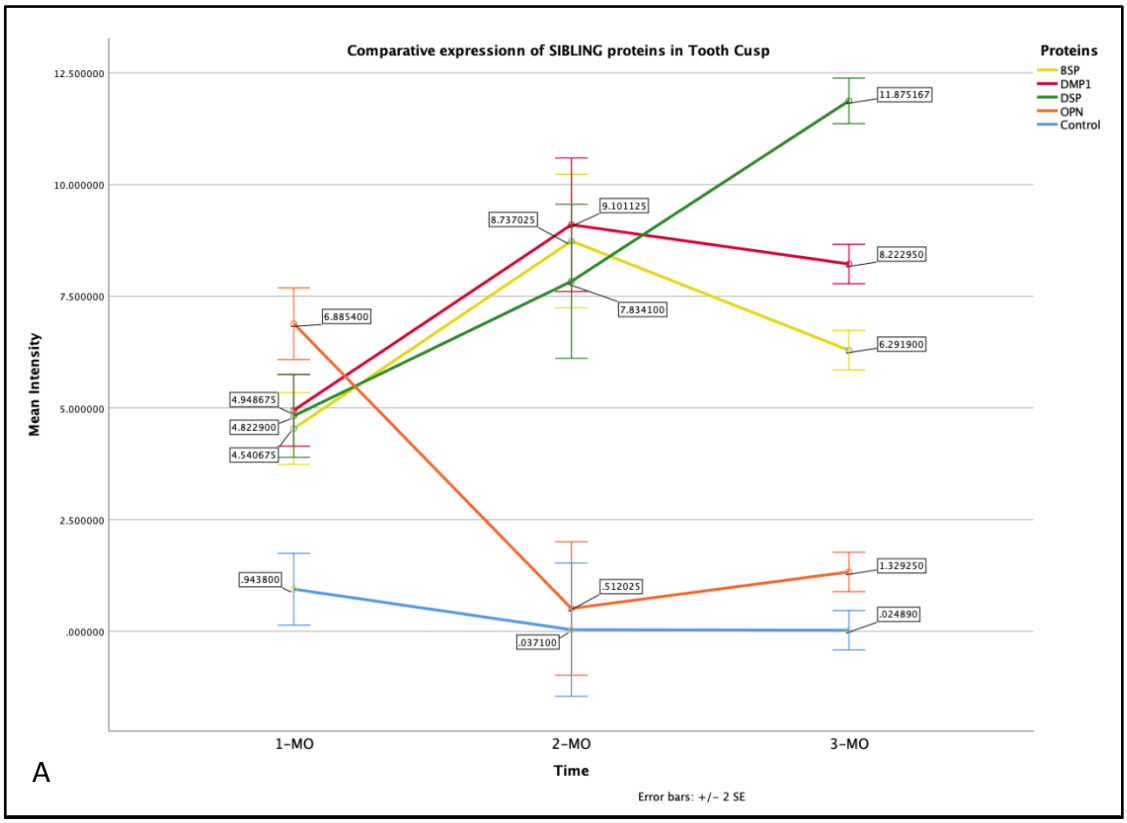
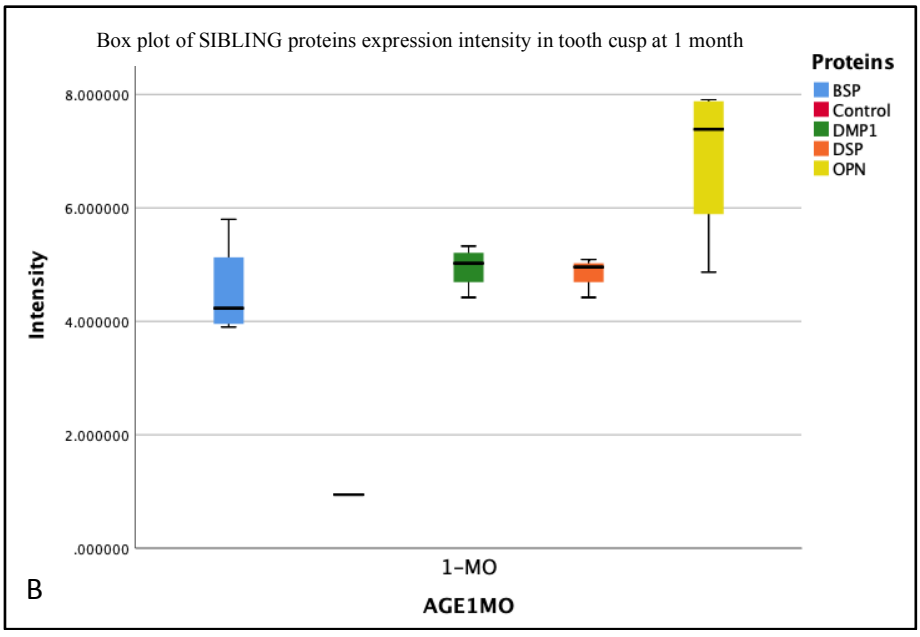


Figure 3.19a.Comparative expression of staining intensity ofSIBLING proteins at 1-, 3- and 6-months in (a) cusp and (b) root apex. *IHC of tibia in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN (IHC 20x) with line graphs for staining intensity at 1-, 3-, and 6-months. DSP and OPN Intensity pattern showed significant decreased expression at 3 months versus 1-, and 6-months. DMP1 and BSP in cusp showed similar expression with an increase at 3 months followed by minimal change at 6 months. On the other hand, DMP1 and BSP in the root showed continued increases from 1-, 3- to 6- months BSP was exclusively expressed in the predentin at 3 months.*

Tooth Cusp ANOVA and post hoc analysis:



A

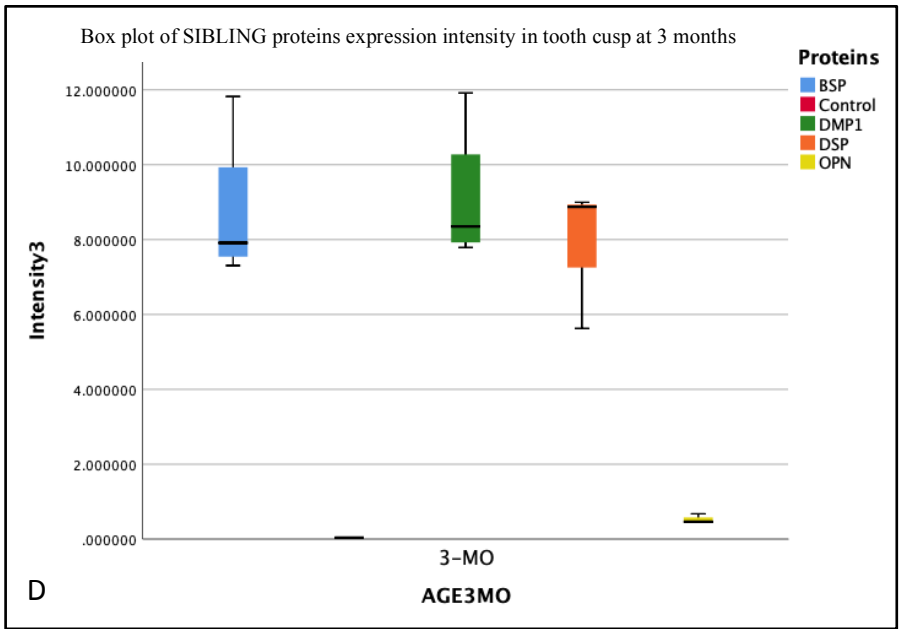


B

Univariate analysis for significance of SIBLING proteins versus control at 1 month

Control	BSP	-3.596875*	.567834295	.000	-5.4853191	-1.7084309
	DMP1	-4.004875*	.567834295	.000	-5.8933191	-2.1164309
	DSP	-3.879100*	.613331138	.000	-5.9188528	-1.8393472
	OPN	-5.941600*	.567834295	.000	-7.8300441	-4.0531559

C

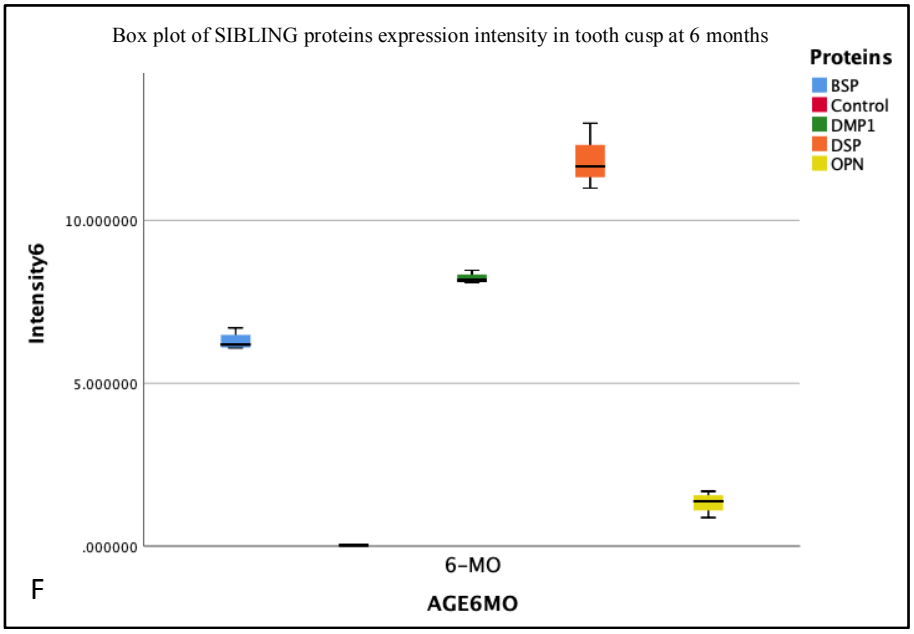


D

Univariate analysis for significance of SIBLING proteins versus control at 3 months

Control	BSP	-8.699925*	1.05655923	.000	-12.213720	-5.1861304
	DMP1	-9.064025*	1.05655923	.000	-12.577820	-5.5502304
	DSP	-7.797000*	1.14121440	.000	-11.592332	-4.0016680
	OPN	-.47492500	1.05655923	1.000	-3.9887196	3.03886962

E



F

Univariate analysis for significance of SIBLING proteins versus control at 6 months

Control	BSP	-6.267010*	.312057536	.000	-7.3048184	-5.2292016
	DMP1	-8.198060*	.312057536	.000	-9.2358684	-7.1602516
	DSP	-11.85028*	.337060663	.000	-12.971238	-10.729315
	OPN	-1.304360*	.312057536	.009	-2.3421684	-.26655156

G

Post hoc tests

Dependent VariableDSP Intensity

Test: Bonferroni

(I) AgeDSP	(J) AgeDSP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-2.464220*	.729898295	.025	-4.6052483	-.32319175
	6-MO	-5.303278*	.729898295	.000	-7.4443063	-3.1622497
3-MO	1-MO	2.4642200*	.729898295	.025	.32319175	4.60524825
	6-MO	-2.839058*	.729898295	.011	-4.9800863	-.69802975
6-MO	1-MO	5.3032780*	.729898295	.000	3.16224975	7.44430625
	3-MO	2.8390580*	.729898295	.011	.69802975	4.98008625

Based on observed means.
The error term is Mean Square(Error) = 1.332.

H *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensity DMP1

Test: Bonferroni

(I) AgeDMP1	(J) AgeDMP1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-3.140620*	.713978652	.005	-5.2349508	-1.0462892
	6-MO	-2.435638*	.713978652	.023	-4.5299688	-.34130722
3-MO	1-MO	3.1406200*	.713978652	.005	1.04628922	5.23495078
	6-MO	.70498200	.713978652	1.000	-1.3893488	2.79931278
6-MO	1-MO	2.4356380*	.713978652	.023	.34130722	4.52996878
	3-MO	-.70498200	.713978652	1.000	-2.7993128	1.38934878

Based on observed means.
The error term is Mean Square(Error) = 1.274.

I *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensity BSP

Test: Bonferroni

(I) AgeBSP	(J) AgeBSP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-3.175740*	.829788456	.012	-5.6097785	-.74170153
	6-MO	-1.2171980	.829788456	.529	-3.6512365	1.21684047
3-MO	1-MO	3.1757400*	.829788456	.012	.74170153	5.60977847
	6-MO	1.95854200	.829788456	.128	-.47549647	4.39258047
6-MO	1-MO	1.21719800	.829788456	.529	-1.2168405	3.65123647
	3-MO	-1.9585420	.829788456	.128	-4.3925805	.47549647

Based on observed means.
The error term is Mean Square(Error) = 1.721.

J *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensity OPN

Test Bonferroni

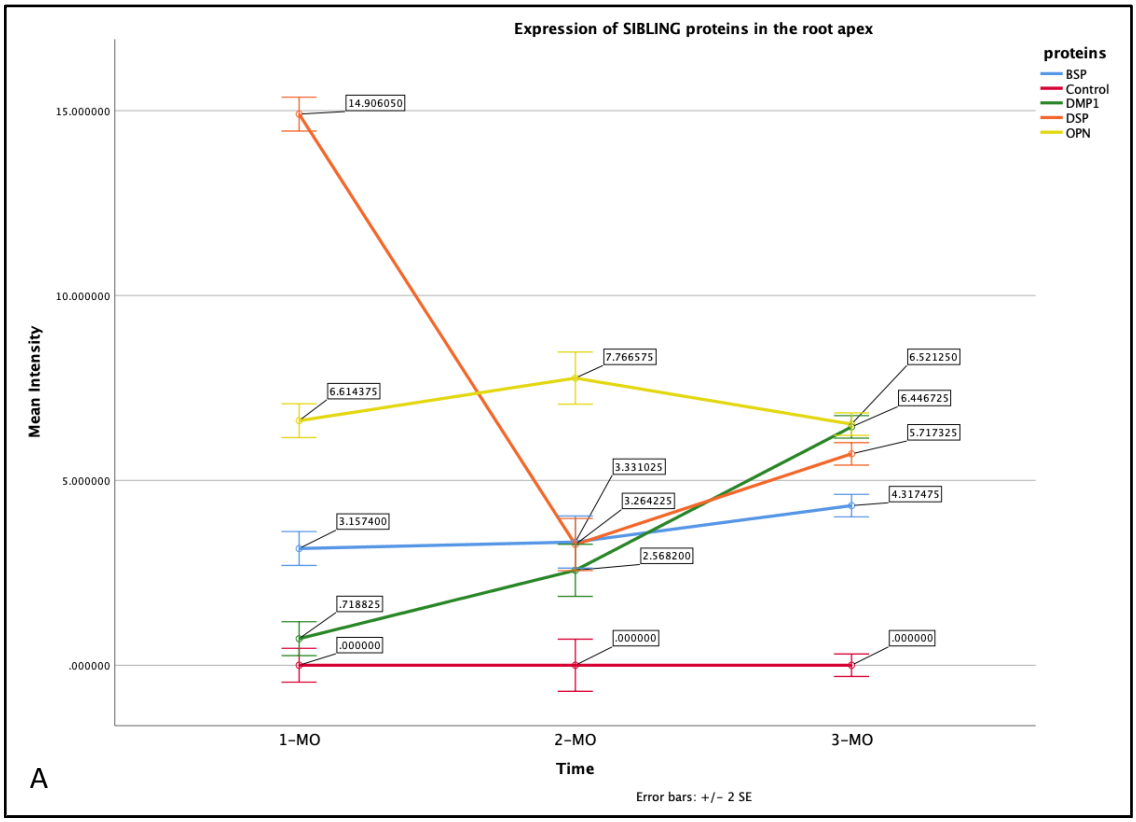
(I) AgeOPN	(J) AgeOPN	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	5.2800400*	.534985820	.000	3.71075321	6.84932679
	6-MO	4.6287020*	.534985820	.000	3.05941521	6.19798879
3-MO	1-MO	-5.280040*	.534985820	.000	-6.8493268	-3.7107532
	6-MO	-.65133800	.534985820	.763	-2.2206248	.91794879
6-MO	1-MO	-4.628702*	.534985820	.000	-6.1979888	-3.0594152
	3-MO	.65133800	.534985820	.763	-.91794879	2.22062479

Based on observed means.
The error term is Mean Square(Error) = .716.

K *. The mean difference is significant at the 0.05 level.

Figure 3.19b.Comparative expression of staining intensity ofSIBLING proteins at 1-, 3- and 6-months in the tooth cusp. *IHC of mandible in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody(A) Line graph of mean % contribution of high staining intensity as analyzed by ImageJ. (B) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 1 month. (C) At 1 month, post hoc test using bonferroni correction showed the intensity of all four SIBLING proteins was significant (p<0.001). (D) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 3 months. (E) At 3 months, post hoc test using bonferroni correction showed the intensity of DSP, DMP1 and BSP was significant versus control with p-value<.001. OPN had statistically insignificant but higher intensity of expression versus control at 3 months (p>0.05). (F) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 6 months. (G) At 6 months, post hoc test using bonferroni correction showed that the intensity of DSP, DMP1 and BSP was statistically significant versus control (p<.001). The mean intensity of expression of OPN at 6 months was also significant p=.009 (p<.05). (H) DSP's mean expression difference was significant between 1, 3 and 6 months (p<.05). (I) DMP1 mean intensity expression difference had insignificant change between 3 and 6 months but was significant between 1 to 3 and 1 to 6 months. (J) BSP's mean expression difference was insignificant between 1 and 6 months and 3 and 6 months, but it was significant between 1 to 3 months and vice versa. (K) OPN'smean intensity expression difference was insignificant between 3 and 6 months however, it was significant between 1 to 3, 1 to 6 and vice versa.*

Root Apex ANOVA and post hoc analysis:



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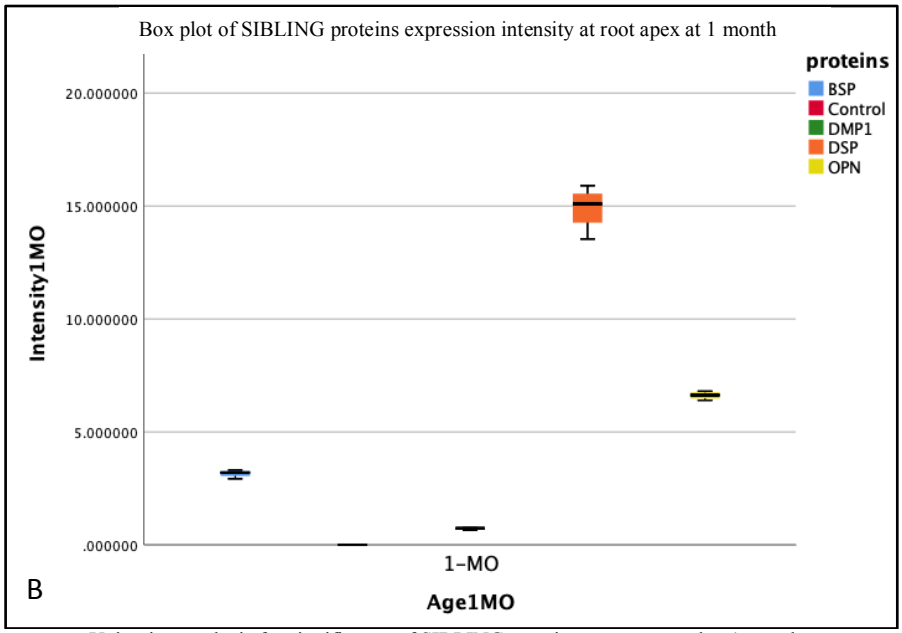
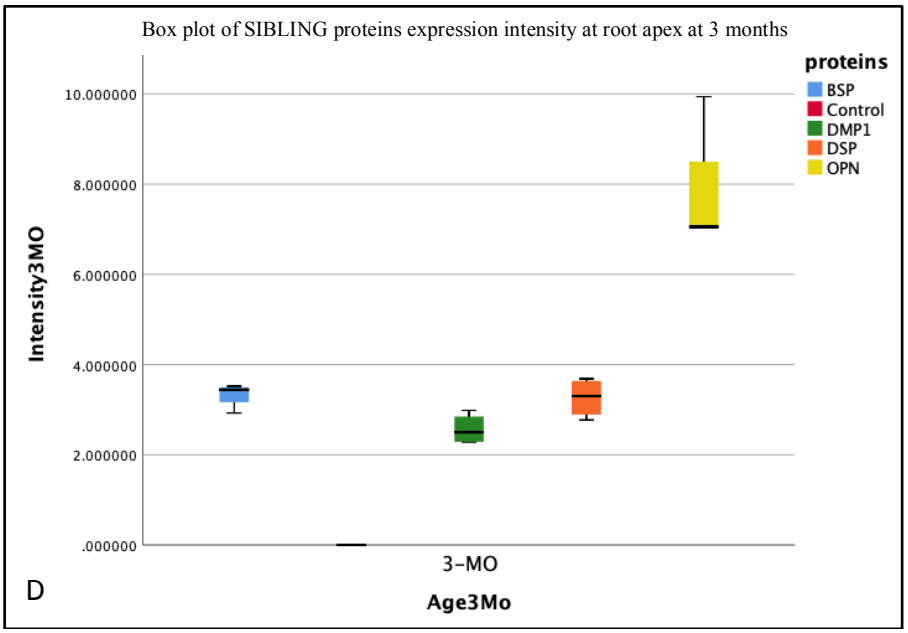


B

Univariate analysis for significance of SIBLING proteins versus control at 1 month

Control	BSP	-3.157400*	.323699361	.000	-4.2210886	-2.0937114
	DMP1	-.71882500	.323699361	.422	-1.7825136	.34486358
	DSP	-14.90605*	.323699361	.000	-15.969739	-13.842361
	OPN	-6.614375*	.323699361	.000	-7.6780636	-5.5506864

C

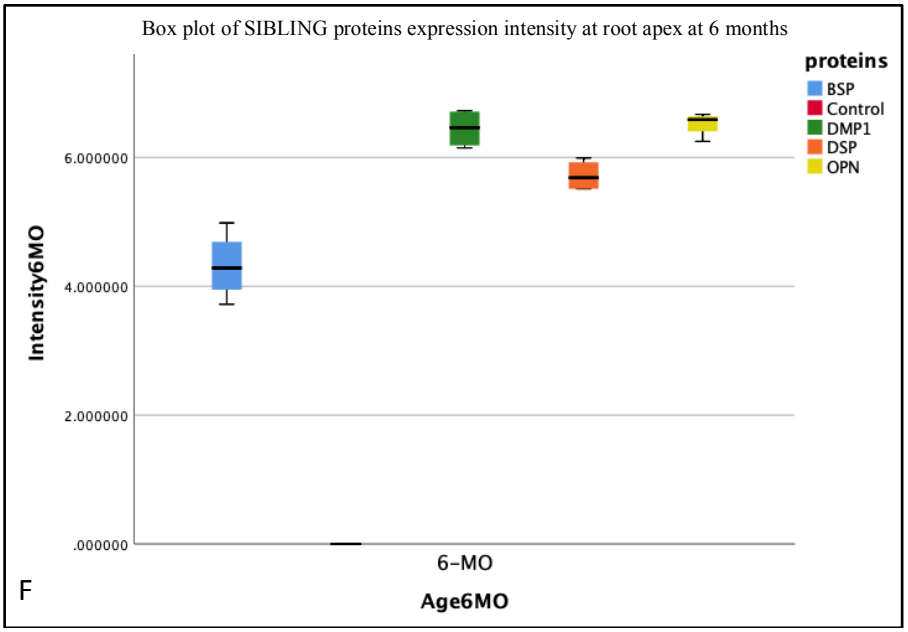


D

Univariate analysis for significance of SIBLING proteins versus control at 3 months

Control	BSP	-3.331025*	.498649182	.000	-4.9696054	-1.6924446
	DMP1	-2.568200*	.498649182	.001	-4.2067804	-.92961955
	DSP	-3.264225*	.498649182	.000	-4.9028054	-1.6256446
	OPN	-7.766575*	.498649182	.000	-9.4051554	-6.1279946

E



F

Univariate analysis for significance of SIBLING proteins versus control at 6 months

Control	BSP	-4.317*	.215	.000	-5.024	-3.611
	DMP1	-6.447*	.215	.000	-7.154	-5.740
	DSP	-5.717*	.215	.000	-6.424	-5.010
	OPN	-6.521*	.215	.000	-7.228	-5.814

G

Dependent VariableIntensityDSP

Test: Bonferroni

Post hoc tests

(I) AgeDSP	(J) AgeDSP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	9.3134600*	.406902098	.000	8.11988427	10.5070357
	6-MO	7.3509800*	.406902098	.000	6.15740427	8.54455573
3-MO	1-MO	-9.313460*	.406902098	.000	-10.507036	-8.1198843
	6-MO	-1.962480*	.406902098	.003	-3.1560557	-.76890427
6-MO	1-MO	-7.350980*	.406902098	.000	-8.5445557	-6.1574043
	3-MO	1.9624800*	.406902098	.003	.76890427	3.15605573

Based on observed means.
The error term is Mean Square(Error) = .414.

H *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityDMP1

Test: Bonferroni

(I) AgeDMP1	(J) AgeDMP1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-1.479500*	.168144109	.000	-1.9727212	-.98627883
	6-MO	-4.582320*	.168144109	.000	-5.0755412	-4.0890988
3-MO	1-MO	1.4795000*	.168144109	.000	.98627883	1.97272117
	6-MO	-3.102820*	.168144109	.000	-3.5960412	-2.6095988
6-MO	1-MO	4.5823200*	.168144109	.000	4.08909883	5.07554117
	3-MO	3.1028200*	.168144109	.000	2.60959883	3.59604117

Based on observed means.
The error term is Mean Square(Error) = .071.

I *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityBSP

Test: Bonferroni

(I) AgeBSP	(J) AgeBSP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-.13890000	.224327284	1.000	-.79692463	.51912463
	6-MO	-.9280600*	.224327284	.008	-1.5860846	-.27003537
3-MO	1-MO	.13890000	.224327284	1.000	-.51912463	.79692463
	6-MO	-.7891600*	.224327284	.020	-1.4471846	-.13113537
6-MO	1-MO	.92806000*	.224327284	.008	.27003537	1.58608463
	3-MO	.78916000*	.224327284	.020	.13113537	1.44718463

Based on observed means.
The error term is Mean Square(Error) = .126.

J *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityOPN

Test Bonferroni

(I) AgeOPN	(J) AgeOPN	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	-.92176000	.537392471	.361	-2.4981063	.65458628
	6-MO	.07450000	.537392471	1.000	-1.5018463	1.65084628
3-MO	1-MO	.92176000	.537392471	.361	-.65458628	2.49810628
	6-MO	.99626000	.537392471	.290	-.58008628	2.57260628
6-MO	1-MO	-.07450000	.537392471	1.000	-1.6508463	1.50184628
	3-MO	-.99626000	.537392471	.290	-2.5726063	.58008628

Based on observed means.

KThe error term is Mean Square(Error) = .722.

Figure 3.19c.Comparative expression of staining intensity ofSIBLING proteins at 1-, 3- and 6-months at root apex. *IHC of mandible in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody(A) Line graph of mean % contribution of high staining intensity as analyzed by ImageJ. (B) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 1 month. (C) At 1 month, post hoc test using bonferroni correction showed the intensity of DSP, BSP and OPN was significant (p<0.001). However, DMP1 expression was insignificant but higher than control. (D) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 3 months. (E) At 3 months, post hoc test using bonferroni correction showed the intensity of DSP, DMP1, BSP and OPN was significantly higher than control with p-value<.001. (F) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 6 months. (G) At 6 months, post hoc test using bonferroni correction showed that the intensity of DSP, DMP1, BSP, and OPN was statistically significant versus control (p<.001). (H) DSP's mean expression difference was significant between 1, 3 and 6 months (p<.05). (I) DMP1 mean intensity expression difference was significant between all ages. (J) BSP's mean expression difference was insignificant between 1 and 3 months but was statistically significant between all other ages. (K) OPN'smean intensity was higher but the expression difference was insignificant between all ages.*

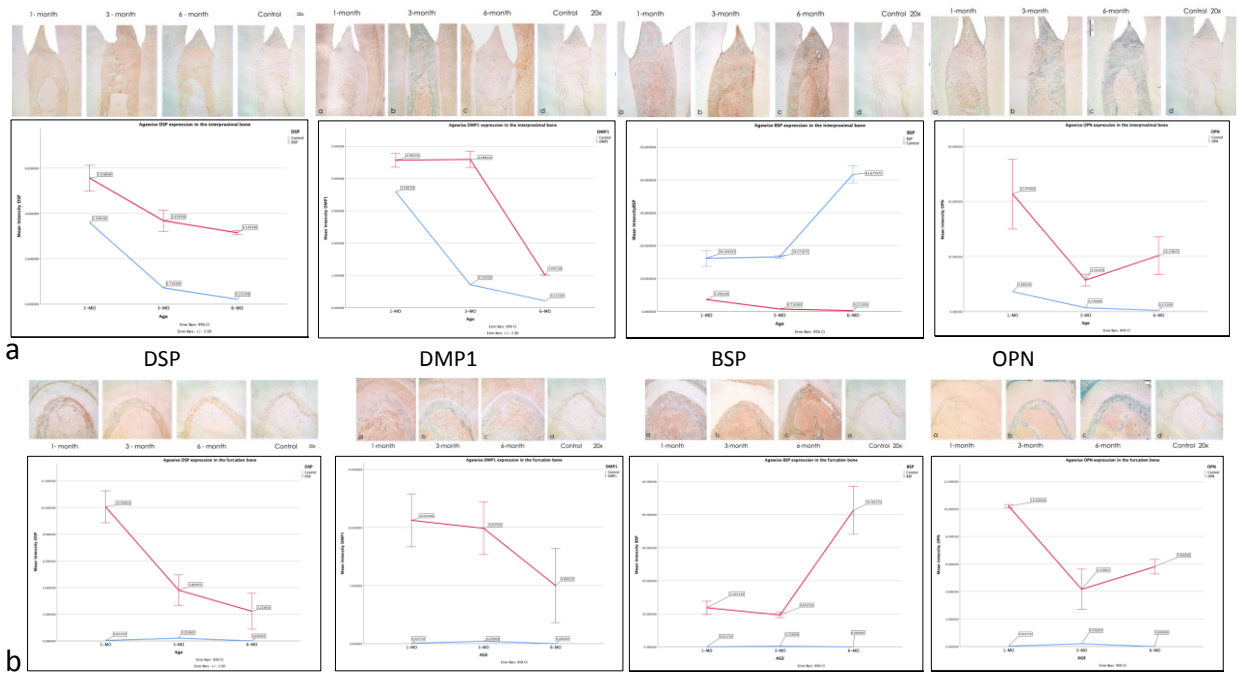
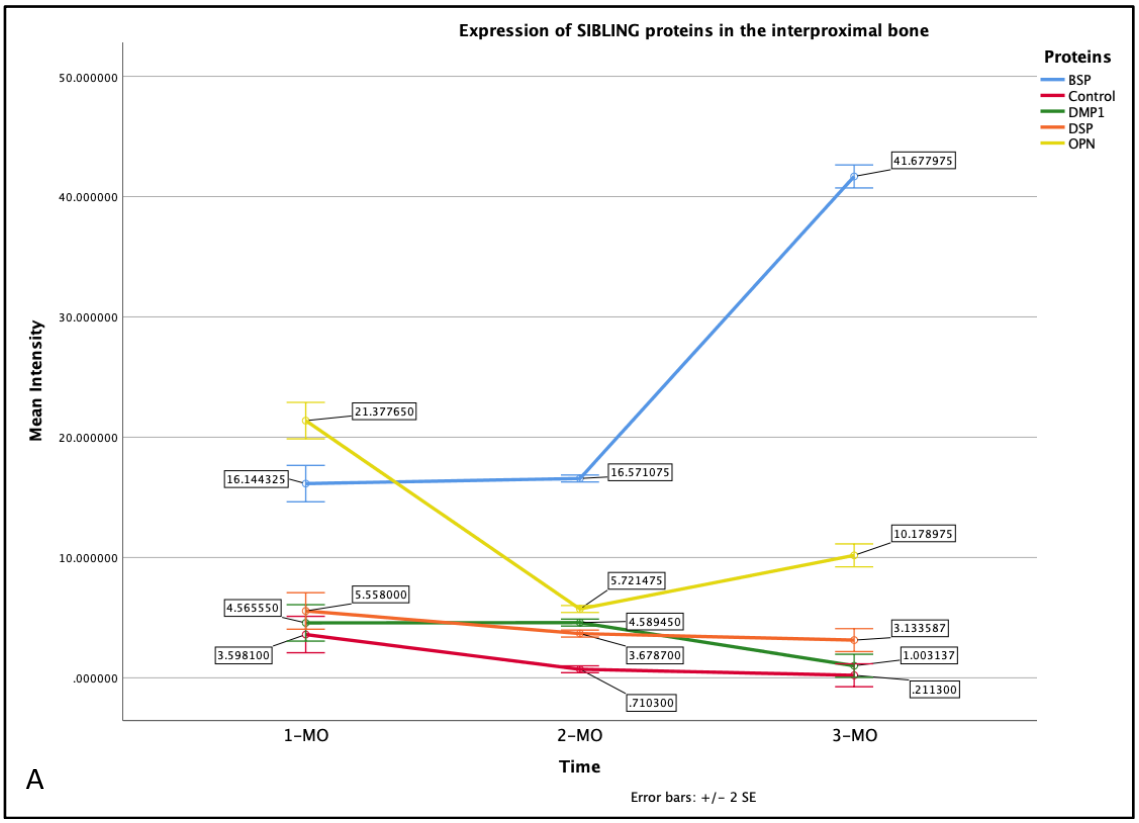
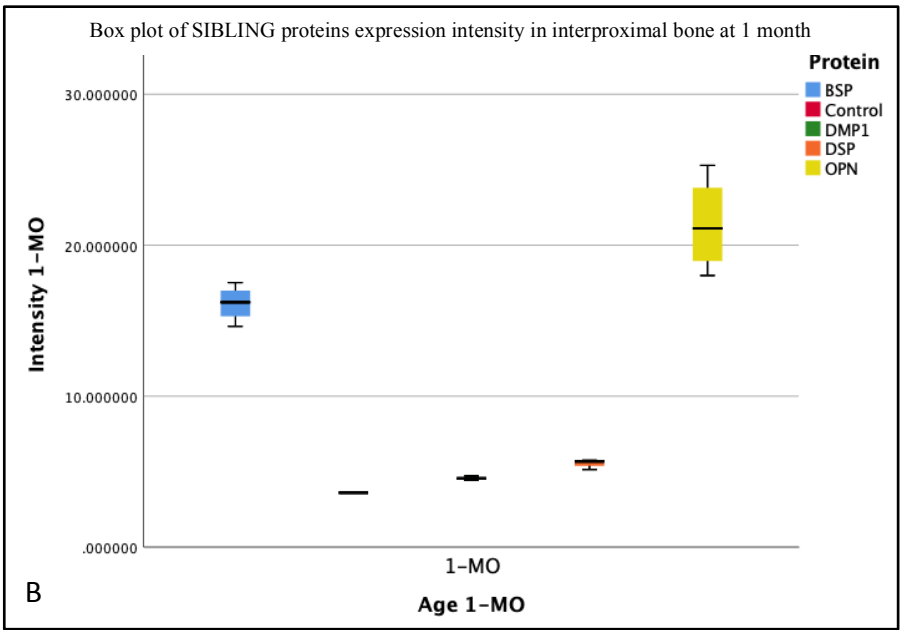


Figure 3.20a.Comparative expression of staining intensity ofSIBLING proteins at 1-, 3- and 6-months in (a) interproximal and (b) furcation bone. *IHC of mandible in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN (IHC 20x) with line graphs for staining intensity at 1-, 3-, and 6-months. Intensity showed significant decreased expression at 3 months versus 1-, and 6-months, DMP1 showed early and increased staining intensity in furcation. BSP on the other hand increased from 3- to 6- months temporally being the last to express in alveolar bone. BSP was exclusively expressed in the acellular cementum.*

Interproximal Bone ANOVA and post hoc analysis:

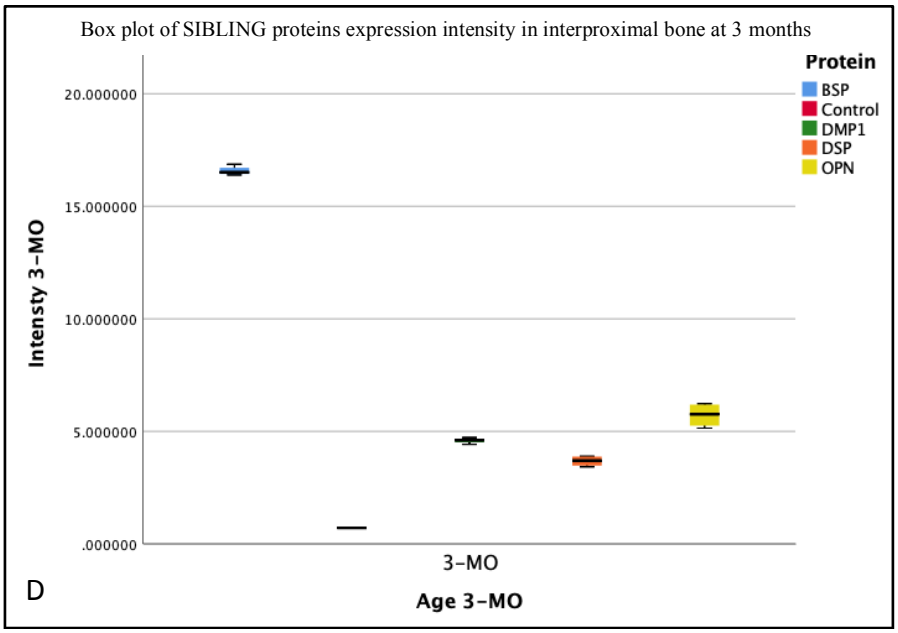




Univariate analysis for significance of SIBLING proteins versus control at 1 month

Control	BSP	-12.54623*	1.07148973	.000	-16.067182	-9.0252684
	DMP1	-.96745000	1.07148973	1.000	-4.4884066	2.55350658
	DSP	-1.9599000	1.07148973	.873	-5.4808566	1.56105658
	OPN	-17.77955*	1.07148973	.000	-21.300507	-14.258593

C

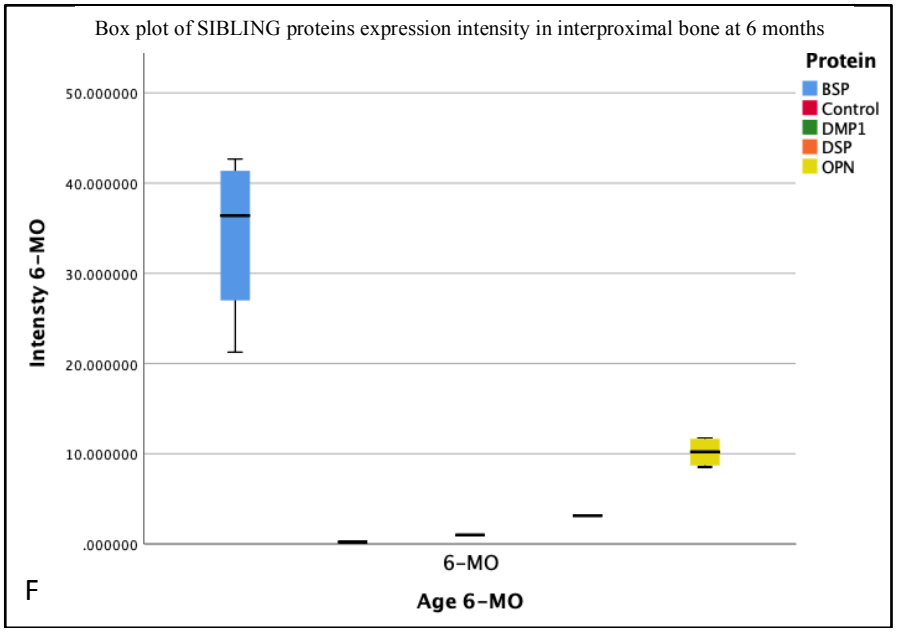


D

Univariate analysis for significance of SIBLING proteins versus control at 3 months

Control	BSP	-15.86078*	.204026335	.000	-16.531213	-15.190337
	DMP1	-3.879150*	.204026335	.000	-4.5495884	-3.2087116
	DSP	-2.968400*	.204026335	.000	-3.6388384	-2.2979616
	OPN	-5.011175*	.204026335	.000	-5.6816134	-4.3407366

E



F

Univariate analysis for significance of SIBLING proteins versus control at 6 months

Control	BSP	-33.96668*	3.07866946	.000	-44.083302	-23.850048
	DMP1	-.79183750	3.07866946	1.000	-10.908464	9.32478908
	DSP	-2.9342000	3.07866946	1.000	-13.050827	7.18242658
	OPN	-9.9676750	3.07866946	.055	-20.084302	.14895158

G

Post hoc tests

Dependent VariableIntensity DSP
Test Bonferroni

(I) Age	(J) Age	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	2.0810000*	.135630048	.000	1.68315311	2.47884689
	6-MO	2.6168900*	.135630048	.000	2.21904311	3.01473689
3-MO	1-MO	-2.081000*	.135630048	.000	-2.4788469	-1.6831531
	6-MO	.53589000*	.135630048	.010	.13804311	.93373689
6-MO	1-MO	-2.616890*	.135630048	.000	-3.0147369	-2.2190431
	3-MO	-.5358900*	.135630048	.010	-.93373689	-.13804311

Based on observed means.
The error term is Mean Square(Error) = .046.

H *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensity DMP1
Test Bonferroni

(I) Age DMP1	(J) Age DMP1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	.55844000*	.060901707	.000	.37979556	.73708444
	6-MO	3.5272900*	.060901707	.000	3.34864556	3.70593444
3-MO	1-MO	-.5584400*	.060901707	.000	-.73708444	-.37979556
	6-MO	2.9688500*	.060901707	.000	2.79020556	3.14749444
6-MO	1-MO	-3.527290*	.060901707	.000	-3.7059344	-3.3486456
	3-MO	-2.968850*	.060901707	.000	-3.1474944	-2.7902056

Based on observed means.
The error term is Mean Square(Error) = .009.

I *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityBSP
Test Bonferroni

(I) AgeBSP	(J) AgeBSP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	.23616000	.643998961	1.000	-1.6528977	2.12521767
	6-MO	-19.74956*	.643998961	.000	-21.638618	-17.860502
3-MO	1-MO	-.23616000	.643998961	1.000	-2.1252177	1.65289767
	6-MO	-19.98572*	.643998961	.000	-21.874778	-18.096662
6-MO	1-MO	19.749560*	.643998961	.000	17.8605023	21.6386177
	3-MO	19.985720*	.643998961	.000	18.0966623	21.8747777

Based on observed means.
The error term is Mean Square(Error) = 1.037.

J *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensity OPN

Test Bonferroni

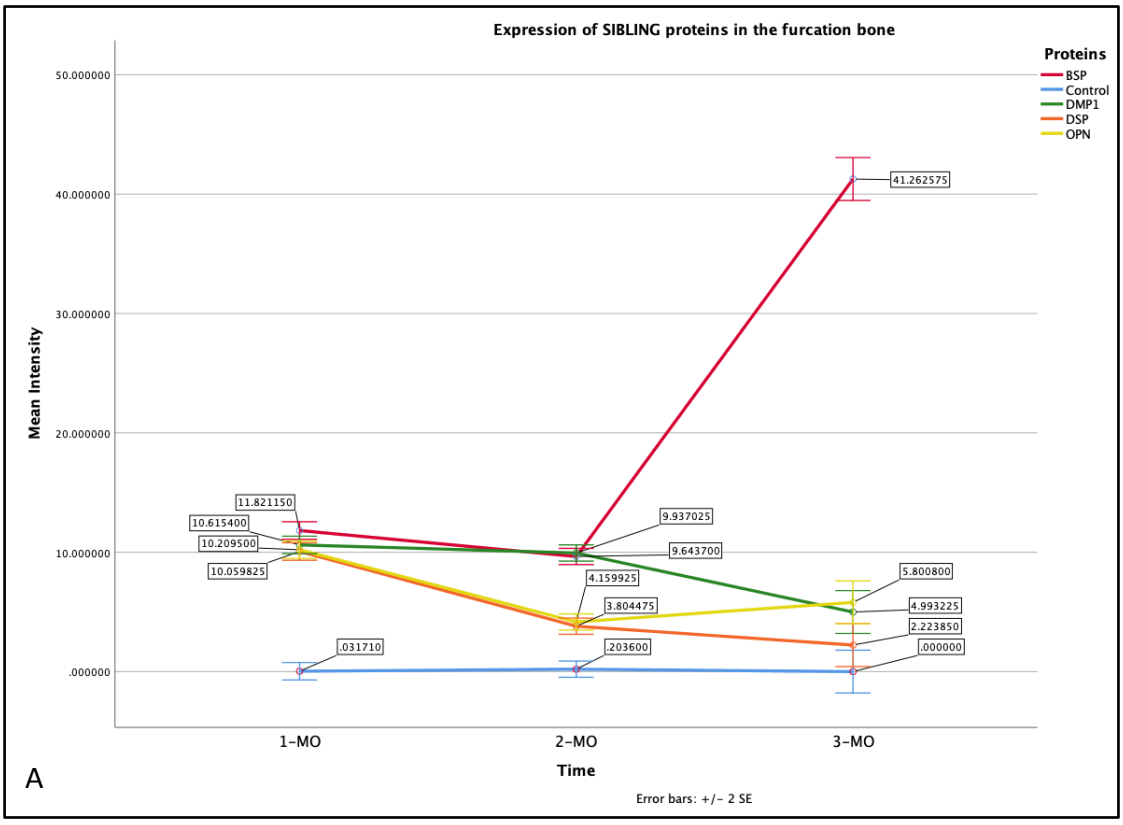
(I) Age OPN	(J) Age OPN	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	13.102500*	1.32564442	.000	9.21395529	16.9910447
	6-MO	9.6363000*	1.32564442	.000	5.74775529	13.5248447
3-MO	1-MO	-13.10250*	1.32564442	.000	-16.991045	-9.2139553
	6-MO	-3.4662000	1.32564442	.084	-7.3547447	.42234471
6-MO	1-MO	-9.636300*	1.32564442	.000	-13.524845	-5.7477553
	3-MO	3.46620000	1.32564442	.084	-.42234471	7.35474471

Based on observed means.
The error term is Mean Square(Error) = 4.393.

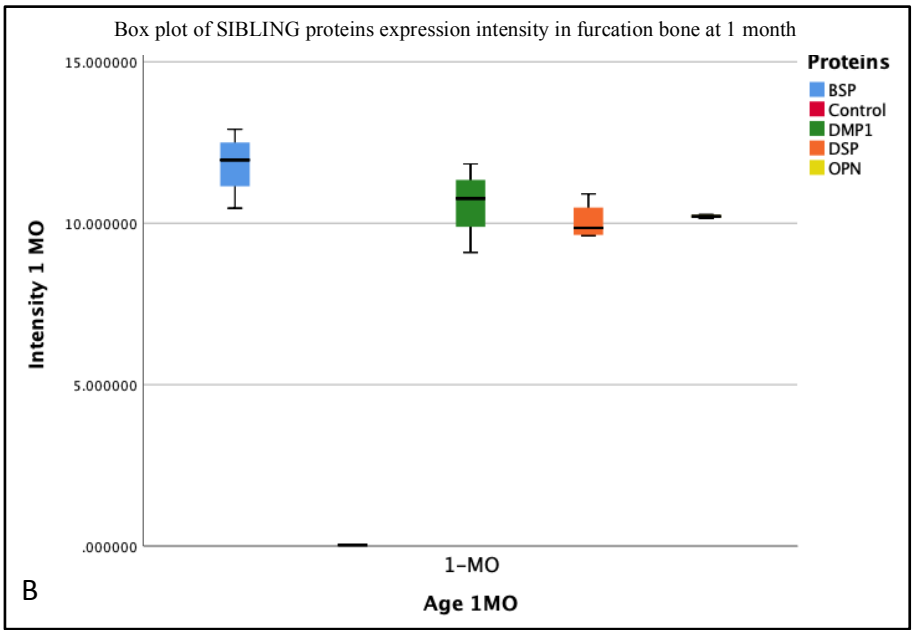
K *. The mean difference is significant at the 0.05 level.

Figure 3.20b.Comparative expression of staining intensity of SIBLING proteins at 1-, 3- and 6-months in the interproximal bone. *IHC of mandible in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody(A) Line graph of mean % contribution of high staining intensity as analyzed by ImageJ. (B) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 1 month. (C) At 1 month, post hoc test using bonferroni correction showed the intensity of BSP and OPN was significant (p<0.001). However, the expression intensity of DMP1 and DSP versus control was insignificant. (D) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 3 months. (E) At 3 months, post hoc test using bonferroni correction showed the intensity of DSP, DMP1, BSP and OPN was significantly higher than control with p-value<.001. (F) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 6 months. (G) At 6 months, post hoc test using bonferroni correction showed that the intensity of BSP, and OPN was statistically significant versus control (p<.001 and p=.05, respectively). The expression intensity was insignificant at 3 months for DMP1 and DSP. (H) DSP's mean expression difference was significant between 1, 3 and 6 months (p<.05). (I) DMP1 mean intensity expression difference was significant between all ages. (J) BSP's mean expression difference was insignificant between 1 and 3 months but was statistically significant between all other ages. (K) OPN'smean intensity was insignificant between 3 and 6 months. However, the mean expression difference between all other ages (1 to 3 and 1 to 6 months) was significant.*

Furcation Bone ANOVA and post hoc analysis:



A

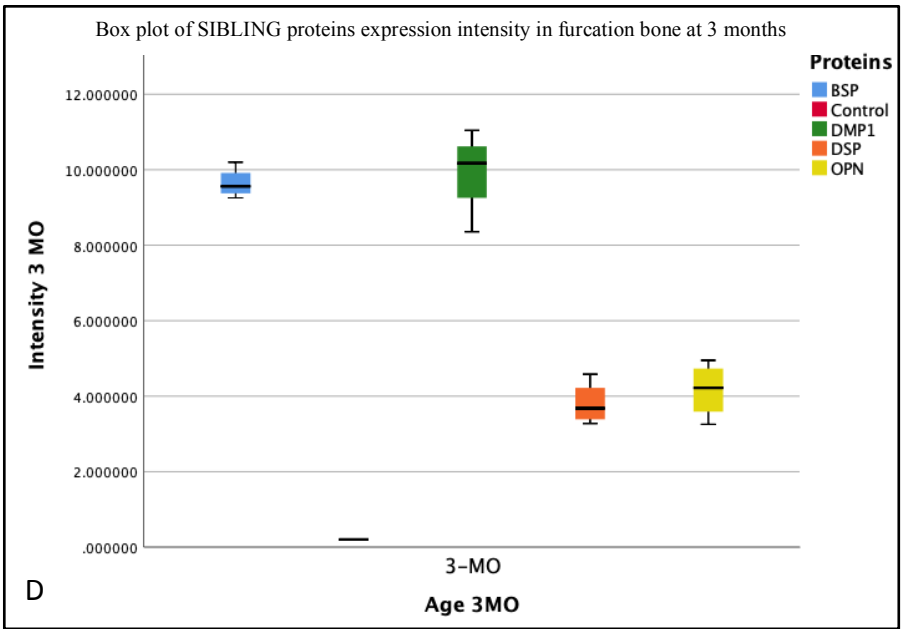


B

Univariate analysis for significance of SIBLING proteins versus control at 1 month

Control	BSP	-11.78944*	.517904987	.000	-13.491296	-10.087584
	DMP1	-10.58369*	.517904987	.000	-12.285546	-8.8818342
	DSP	-10.02812*	.517904987	.000	-11.729971	-8.3262592
	OPN	-10.17779*	.517904987	.000	-11.879646	-8.4759342

C

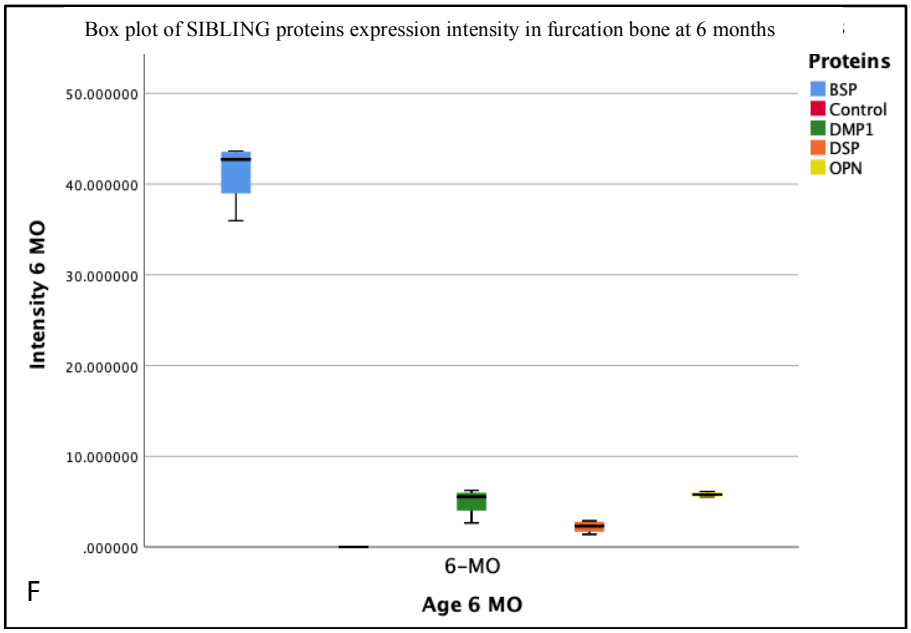


D

Univariate analysis for significance of SIBLING proteins versus control at 3 months

Control	BSP	-9.440100*	.480424041	.000	-11.018792	-7.8614081
	DMP1	-9.733425*	.480424041	.000	-11.312117	-8.1547331
	DSP	-3.600875*	.480424041	.000	-5.1795669	-2.0221831
	OPN	-3.956325*	.480424041	.000	-5.5350169	-2.3776331

E



F

Univariate analysis for significance of SIBLING proteins versus control at 6 months

Control	BSP	-41.26258*	1.26940564	.000	-45.433891	-37.091259
	DMP1	-4.993225*	1.26940564	.013	-9.1645409	-.82190912
	DSP	-2.2238500	1.26940564	1.000	-6.3951659	1.94746588
	OPN	-5.800800*	1.26940564	.004	-9.9721159	-1.6294841

G

Post hoc tests

Dependent VariableIntensityDSP

Test: Bonferroni

(I) MO1Age	(J) MO1Age	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	4.9699020*	.390028134	.000	3.82582308	6.11398092
	6-MO	6.2751220*	.390028134	.000	5.13104308	7.41920092
3-MO	1-MO	-4.969902*	.390028134	.000	-6.1139809	-3.8258231
	6-MO	1.3052200*	.390028134	.026	.16114108	2.44929892
6-MO	1-MO	-6.275122*	.390028134	.000	-7.4192009	-5.1310431
	3-MO	-1.305220*	.390028134	.026	-2.4492989	-.16114108

Based on observed means.
The error term is Mean Square(Error) = .380.

H*. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityDMP1

Test: Bonferroni

(I) AGE	(J) AGE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	.50832200	.826346507	1.000	-1.9156201	2.93226411
	6-MO	4.5040820*	.826346507	.001	2.08013989	6.92802411
3-MO	1-MO	-.50832200	.826346507	1.000	-2.9322641	1.91562011
	6-MO	3.9957600*	.826346507	.003	1.57181789	6.41970211
6-MO	1-MO	-4.504082*	.826346507	.001	-6.9280241	-2.0801399
	3-MO	-3.995760*	.826346507	.003	-6.4197021	-1.5718179

Based on observed means.
The error term is Mean Square(Error) = 1.707.

I *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityBSP

Test: Bonferroni

(I) AGE	(J) AGE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	1.70758200	1.37727721	.739	-2.3324184	5.74758242
	6-MO	-23.54680*	1.37727721	.000	-27.586798	-19.506798
3-MO	1-MO	-1.7075820	1.37727721	.739	-5.7475824	2.33241842
	6-MO	-25.25438*	1.37727721	.000	-29.294380	-21.214380
6-MO	1-MO	23.546798*	1.37727721	.000	19.5067976	27.5867984
	3-MO	25.254380*	1.37727721	.000	21.2143796	29.2943804

Based on observed means.
The error term is Mean Square(Error) = 4.742.

J *. The mean difference is significant at the 0.05 level.

Dependent VariableIntensityOPN

Test Bonferroni

(I) AGE	(J) AGE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1-MO	3-MO	4.8052820*	.286417525	.000	3.96512657	5.64543743
	6-MO	3.5333020*	.286417525	.000	2.69314657	4.37345743
3-MO	1-MO	-4.805282*	.286417525	.000	-5.6454374	-3.9651266
	6-MO	-1.271980*	.286417525	.005	-2.1121354	-.43182457
6-MO	1-MO	-3.533302*	.286417525	.000	-4.3734574	-2.6931466
	3-MO	1.2719800*	.286417525	.005	.43182457	2.11213543

Based on observed means.
The error term is Mean Square(Error) = .205.

K *. The mean difference is significant at the 0.05 level.

Figure 3.20c.Comparative expression of staining intensity ofSIBLING proteins at 1-, 3- and 6-months in the interproximal bone. *IHC of mandible in 1-, 3-, and 6-month-old mice with anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibody(A) Line graph of mean % contribution of high staining intensity as analyzed by ImageJ. (B) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 1 month. (C) At 1 month, post hoc test using bonferroni correction showed the intensity of all four SIBLING proteins versus control was significant (p<0.001). (D) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 3 months. (E) At 3 months, post hoc test using bonferroni correction showed the intensity of DSP, DMP1, BSP and OPN was significantly higher than control with p-value<.001. (F) Box plot showing expression of anti-DSP, anti-DMP1, anti-BSP and anti-OPN antibodies versus control at 6 months. (G) At 6 months, post hoc test using bonferroni correction showed that the intensity of DMP1, BSP, and OPN was statistically significant versus control (p=.013, p<.001 and p=.004, respectively). The expression intensity was insignificant at 3 months for DSP (p>.05). (H) DSP's mean expression difference was significant between 1, 3 and 6 months (p<.05). (I) DMP1 mean intensity expression difference was significant between all ages except between 1 and 3 months (p>.05). (J) BSP's mean expression difference was insignificant between 1 and 3 months but was statistically significant between all other ages. (K) OPN'smean expression difference was significant between all ages (p<.05).*

Chapter 4. Discussion

DISCUSSION

Unmineralized predentin and osteoid are seen lying sandwiched between their precursor cells and the mineralized front. This unmineralized zone is maintained uniformly throughout. **(Figure 3.1 &Figure 3.4)**The dynamic process of conversion of unmineralized predentin/osteoid into mineralized dentin/bone occurs at the most distal end is precisely controlled. The rate of formation equalling the rate of mineralization. Thus, maintenance of a uniform layer indicates tight regulation of complex crosstalk among cells and several components of ECM including type I collagen, NCPs (like SIBLING proteins), and proteoglycans. (1)

SIBLING family members have been investigated in the mineralized tissues and their role in biomineralization indicated. (2)(3)Few have reported their relative expression in the teeth and tibia at defined age groups until they reach skeletal maturity. Therefore, the distribution pattern of SIBLINGs (DSP, DMP1, BSP, and OPN) is ill-defined and data-scarce in long bone and mandible across different age groups. In this study, we observed differences in the distribution and level of expression of these proteins in the bone and mandible at the three different age groups.The four SIBLING proteins and their expression patterns in tibia and mandible are discussed separately below.

A. Staining pattern with Hematoxylin and Eosin:

At 1 month, the growth plate showed multiple layers of proliferating, hypertrophic chondrocytes and degenerating chondrocytes. The first few layers (the resting zone) with flattened cells are quiescent chondrocytes that provide constant source of dividing cells. In the PZ,(a zone of proliferation)rapid mitosis of the stacked chondrocytes takes place. The HZ is the zone where the chondrocytes stop mitosis and begin storing glycogen, lipids and alkaline phosphatase making them hypertrophic. The DZ is the zone where the chondrocytes undergo apoptosis and the cartilaginous matrix begins to undergo changes for future mineralization. The CZ is a zone where chondrocytes have degenerated, and the matrix is being calcified. In this zone the chondroclasts breakdown the calcified cartilage and replace it with mineralized bone tissue via osteoclasts and osteoblasts. A well-defined trabecular pattern was seen in the metaphysis following transition from cartilage to bone at CZ. At 1 month, the newly forming

trabeculae and growth plate are responsible for a rapid increase in the length of the bone.At 3-months, the total number of chondrocytes in the growth plate decrease significantly while the width of calcification zone increases. At 6-months, the cell population consists of scattered hypertrophic and some proliferating cells with reduced number of trabeculae possibly undergoing slow remodeling.

Taken together, increased osteoid is present at 1 month. With further skeletal development and increase in compressive function of tibia there is increase in the rate of mineralization from 1 to 3 months. On attaining the skeletal maturity at 6 months (represented by reduced thickness of growth plate of tibia) there is concomitant decrease in the rate of mineralization.

B. Immunohistochemistry:

*Anti-Dentin Sialophosphoprotein (DSP) antibody (N-terminal):*In the current study, DSP was localized at the bone-forming front i.e. the degenerating chondrocyte-bone interface and trabeculae. This is consistent with previous studies that showed stronger signals for DSPP mRNA adjacent to the growth plate. (10) Also, in our study, we showed the expression of anti-DSP antibody in the tibia was higher at 3months compared to 1and 6months of age. The expression pattern followed the rate of bone mineralization. At 1month, C57BL/6 mice tibia were still developing with a high rate of osteoid formation, and the mineral content had not peaked. (11)During the rapid phase of development (1 to 3 months), the total amount of mineralized bone would not be expected to vary greatly. At the same time, relatively there is a continual increase in the amount of osteoid until 3months of age. Once the cartilaginous primordium has been formed and the mice reach 3months, the tibia is gradually placed under compression. At the same time there is a concomitantshift in the rate of conversion of osteoid into the mineralized matrix at the cartilage-bone interface. The decreased osteoid volume is followed by an increase in trabecular thickness, and mineral density. (12)(13)During HA formation and growth, *in vitro* studiesfor DSP have shown slight capacity to nucleate or inhibit. (8,9) Our results show highest expression of DSP at 3 months. Thus, it is conceivable that the increased expression of DSP at 3months compared to its expression at 1month follows the pattern of conversion of osteoid into mineralized matrix. At 6months, the C57BL/6 mice reach their peak bone mass and scant new bone is formed and reduced remodeling occurs compared to

3months. (12) (13) The reduction in expression of DSP in the tibia at 6months happened at the same time as the reduction in the size of the growth plate and skeletal maturity. It is possible that this reduced expression follows the reduced rate of mineralization and remodeling of the trabeculae with age. The results from the current study were coherent with previous studies showing localization and *in vivo* effects of DSP on bone mineralization (14) and the expression followed a pattern consistent with skeletal development.

In the molars, the current study showed the expression of anti-DSP antibody in the predentin, mantle dentin, peritubular region, alveolar bone, cellular cementum, and periodontal ligament. Previous immunohistochemical analysis study with anti-DSP monoclonal antibody confirmed the NH_2 terminal was secreted in dentin ECM. Expression in the peritubular region, mantle dentin, and predentin is consistent with the previous study by Huang showing DSP to be primarily present in the ECM and organic extract. (15)(16)Some studies show DSP's role in regulating matrix formation (17),its presence might possibly facilitate nucleation of HA along the collagen fibril converting the predentin to dentin. To our best understanding, one *in vivo* study on the function of DSP indicated its possible role in the initiation of dentin mineralization but not maturation. (18) It has also been shown that the NH_2 terminal (DSP) may play an inhibitory role by preventing premature mineralization of predentin by serving as an antagonist to DPP's mineralizing-accelerating action. Thus, DSP may participate in inhibiting or controlling the activity of DPP and perhaps transport or diffusion to the predentin-dentin junction. (1) Further, $DSP^{-/-}$ mice showed structures resembling thinner unmineralized predentin layer instead of mineralized dentin and more severely disrupted dentinal tubules. Together, these findings suggested DSP's role in contributing to dentin volume and its inhibitory role preventing early or too rapid mineralization of predentin during dentinogenesis. (19)(17) The expression of DSP in these regions may indicate DSP's potential role as initiator and regulator of dentin mineralization.

The intensity of expression was seen at 1month followed by higher expression of anti-DSP in 3-and 6-month-old mice as observed in the peritubular region along dentinal tubules and predentin. The intensity of expression in these structures increased from 1 to 3 to 6 months and then showing slight increase at 6months. At 1month, the molars are still undergoing development while at 3months, molars are fully grown, their eruption is complete, and the dentin matrix

mineralized. At 6months the molars undergo accelerated attrition following masticatory function resulting in reactionary dentin formation and upregulated mineralization. This decline in DSP expression from 1 to 3months and slight increase at 6months followed a pattern similar to the slowing down of mineralization process at 3 months with slight increase from 3to 6months. (17) (20)Visually the decline was more prominent in the predentin. (17)(21)

Signals for DSP were present at all ages in the alveolar bone present interproximally, in the furcation and that surrounding the apical half of root. It showed a decline in expression from 1 to 3 to 6months. The cells adjacent to the bone matrix stained intensely positive as did the forming cellular cementum and periodontal ligament following an expression pattern similar to dentin. The matrix of acellular cementum showed an immunonegative reaction. DSPP is secreted by a variety of cells and is associated with the initial formation of the periodontal ligament.(19)(22)DSPP then undergoes proteolysis into DSP and DPP. Detection of DSP expression in PDL, cellular cementum, dentin and alveolar bone is consistent with previous findings of DSP confirmed by *in situ* hybridization and IHC. (22) Root cellular cementum and periodontal ligament showed a higher level of expression at 1month followed by a decrease at 3 months and then by a slight increase at 6months of age. A similar expression pattern was observed for the alveolar bone, a pattern trailing active root formation and remodeling consistent with the eruption pattern. Lack of expression in acellular cementum is suggestive of this materials novel formation/homeostasis pattern different from cellular cementum. (22)For maintaining the periodontal tissue health (PDL, bone and cementum) NH_2 terminal of DSPP (DSP) has been shown to play a crucial role. (19) (48) It has also been shown it may play a collaborative role with other proteins like BSP to maintain PDL space. The results of our IHC-based study showed that the expression of DSP varies at different age groups in various mineralized tissues depending on the degree of mineralization. Higher expression at 1month indicate its possible involvement in conversion of unmineralized precursors to mineralized tissue. The dip in expression at 3 months followed a slowing of the mineralization process at this age. The continued expression with slight increase of DSP at 6months, although at a lower level than 1 month, suggests a possible pattern following remodeling. Together, these observations suggest that DSP expression pattern in the tooth structures, bone, and periodontium may possibly follow their mineralization dynamics.

Anti-Dentin matrix protein 1 antibody (C-terminal): Both full length *Dentin Matrix protein 1 (DMP1)* and DSPP undergo glycosylation and phosphorylation following proteolytic processing. They also have similar expression localization. Like DSPP, DMP1 is also present in the amino and carboxy fragments in the ECM of bone and dentin. (16)(23) In our immunohistochemistry experiment, we used the anti-DMP1 C-terminal which demonstrated that the majority of DMP1 was present in the ECM of bone and dentin. (3) This terminal, due to its high negative charge and phosphorylation, has been implicated as an initiator in mineral deposition by acting as a nucleator for hydroxyapatite. (3)(23)In the presence of type I collagen phosphorylated DMP1 C-terminal fragments isolated from rat bone have been shown by Tartaix et. al. to accelerate nucleation of hydroxyapatite.(24) Another DMP1 KO study done on mice showed a decrease in the mineral-to-matrix ratio and normal collagen crosslinking ratio suggesting normal matrix formation but deficient mineralization. This study also showed the crystal size of the mineral that was formed in DMP1 KO was larger in size that WT indicating its involvement in regulating crystal growth. (25) In the present study, the immunostaining of predentin and CZ of growth plate clearly showed that the COOH-terminal fragment of DMP1 was found in zones of the long bones and dentin cited as areas of mineralization. Presence at these sites the sites where unmineralized precursors (osteoid/predentin) are actively converted to mineralized tissues, suggests a potential role as an initiator of mineralization.

In the epiphyseal growth plates of the tibia, the DMP1-C terminal was found to have intense staining at the CZ (zone with degenerating chondrocytes) and mineralization front in our experiment. This result aligns with previous studies that show its presence in the mineralized phase at CZ tightly bound to the apatite crystals. (27)(28)Localization of expression in the degenerating hypertrophic chondrocytes indicates it may possibly have some regulatory function. The ECM of degenerating chondrocytes undergoes vascular invasion, resorption by chondroclasts, and deposition of osteoid. This is followed by osteoblastic/osteoclastic remodeling of osteoid into mineralized bone via nucleation of hydroxyapatite under the influence of some molecules. DMP1 may have some regulatory role in the process. (26)(27) In our study, the expression of DMP1 increased from 1 to 3months of age followed by a decline at 6months.Yang showed a significant increased osteocytic expression of DMP1 when subjected to mechanical loading. (29)(30) The process of bone growth showed rapid formation from 1 to

3months, followed by increasing mineralization at 3 months in response to increasing mechanical loading. As the tibia attain skeletal maturity at 6 months, slow remodeling of bone ensues. The expression pattern of DMP1 showed a similar pattern with increase in expression at CZ from 1 to 3months which then decreased at 6months. For the secondary ossification center, however, the expression of DMP1 was highest at 1month. Previous studies suggested SOC develops early between 5-14 days in C57BL/6 mice and hinted the importance of SOC in protecting the growth plate. It has also been previously shown that DMP1 expression increases with compressive mechanical loading *in vitro* and *in vivo*. (29) The location of the growth plate in the metaphysis is such that epiphyseal SOC mitigates the compressive and tangential forces. SOC therefore acts as a cushion to absorb the compressive forces to which the proliferative and hypertrophic chondrocytes are highly sensitive. Additionally, it has been shown that removal of SOC causes apoptosis of the proliferating and hypertrophic chondrocytes in growth plate. (49) This suggests that the mineralization of the bone in SOC starts early contributing to an increase in the width of bone at the epiphyseal end and continues to decline thereafter. Therefore, it is possible the higher expression of DMP1 at 1 month portrays its pattern following earlier SOC development.

The differentiation of mesenchymal cells into odontoblasts under the influence of DMP1 has been shown by several studies indicating its role as a signaling molecule during physiological dentinogenesis. It is also thought to be a key regulator of odontoblast differentiation, dentin tubular system formation, and mineralization. (35) It has been previously shown to be expressed in predentin and dentin and is predominantly expressed in the peritubular region and cementum. In cementum, it is primarily present in cementocytes and the surrounding matrix. (35) (21) (37) In line with previous studies, we observed DMP1 expression in the peritubular region, predentin, alveolar bone, and cellular cementum. Moreover, our observations are consistent with DMP1 C-terminal accumulation in mineralized dentin. The C-terminal may get entrapped in the collagenous ECM framework during the process of biomineralization contributing therefore to its distribution pattern in the mineralized dentin. The levels of DMP1 increased from 1 to 3 months and remained high at 6 months. Due to attrition of teeth more reactionary dentin is being laid down and therefore, it is possible that the level of DMP1 at 3 and 6 months follows this pattern. Within alveolar bone, a product of intramembranous ossification,

there was an increase in expression of DMP1 from 1 to 3months. This was followed by a decline in its expression. It is likely that DMP1 shows a pattern similar to the maturation of alveolar process and minimal remodeling at 6months. Taken together, DMP1 may somehow play an important role in regulating mineralization of dentin and tibia.

There have been significant efforts to evaluate the relationship between DSPP and DMP1 in dentinogenesis since dentin demineralization defects have been shown to be present in KO mice of both. (31)(32)(33)(34)Based on the previous studies showing lower level of DSPP in the dentin of DMP1 deficient mice indicating DMP1 may potentially have role in regulating the transcription of DSPP. (33)An experimental DSPP transgenic expression in DMP1 KO mice salvaged the defects in the alveolar bone and tooth. (31) Another study showed that DMP1 binding to the DSPP promotor region activated the differentiation of odontoblasts. (35) These findings suggest DMP1 and DSP may have a synergistic role and may help in the mineralization process. The pattern observed in the primary ossification center of tibia and teeth for both DMP1 and DSP appears similar. The findings of the present study ,in part, agree with the studies showing the complementary effects of DMP1 and DSP.

Anti-Bone sialoprotein (BSP) antibody: has been shown to have broader interaction with various components of ECM including (36) mineralized and organic parts of dentin and bone. The levels of BSP have been shown to vary in various extracts. BSP is least abundant in the unmineralized matrix, moderately present in decalcified exposed bone extract and most abundant in the mineralized phase tightly bound to apatite crystals. (28) Its presence in all extracts shows BSP molecules may bind to type 1 collagen (predentin and osteoid), hydroxyapatite crystals or both. In our study BSP initially showed a broad distribution in the tibia at 1month of age. (28) At 3months the distribution was confined to the growth plate in the matrix of the cartilage, osteoid and the calcification front. While at 6months it was confined to the degenerating chondrocytes and calcification border. A similar age-wise intensity of expression was observed with accentuated signals at the resting lines (an area of temporary cessation of bone growth) of SOC. These observations signify its broader expression profile and differential distribution pattern. At 1 month there is rapid formation of osteoid with insufficient time for its conversion to mineralized matrix. Since BSP was shown to have binding affinity for type I collagen, its expression was diffuse at this age. At 3months there is active conversion of osteoid into

mineralized bone. BSP has been shown to have bonding to both collagen and hydroxyapatite.(28) Its expression in all the layers of growth plate and at CZ shows it may have affinity for matrix high in collagen as well as hydroxyapatite. Its expression in all layers of growth plate may also indicate that BSP may have a role to play in controlling the rate of mitosis of chondrocytes in the resting and proliferating zones. Its presence in unmineralized predentin, growth plate and acellular cementum shows BSP may have higher binding affinity for GAG's like chondroitin sulphate and proteoglycans. (41) These observations further suggest BSP may have broader interaction with different components of these tissues.

In the mandible, analogous to bone, BSP has been shown to be present in all the extracts of dentin with affinity to type I collagen, hydroxyapatite or both. (28) BSP concentration has been reported to be one-tenth in dentin compared to bone. (37) BSP was expressed in the predentin and peritubular dentin as observed in previous studies. (21) The expression increased from 1month to 3months and dropped slightly at 6months. This change in immunostaining intensity may be explained by an increase in attrition following eruption and establishment of functional contacts. It also suggests BSP in addition to DSP and DMP1 may play a protective role in the dentin. The expression in the alveolar bone at the interproximal and furcation region increased from 3 to 6months. The pattern of expression in alveolar bone is different from long bones. A possible explanation is that the alveolar bone forms by intramembranous ossification, a process which is different form endochondral ossification. Moreover, at 3months, the molars are fully erupted and undertake increased masticatory function from 3 to 6months. This calls for increased remodeling of the alveolar bone compensating for increased attrition of the tooth cusps. If BSP has some role in mineralization this increased remodeling could possibly explain the increase in expression of BSP from 3 to 6months. Previous experiments have also shown strong signals of BSP in the osteoid surrounding the osteocytes. (38) The expression of BSP in the root and periodontal ligament region showed a consistent increase from 1 to 6months. In our experiment, BSP was expressed at particularly higher levels in the acellular cementum. (**Figure 4.1**) Acellular cementum forms by mineralization of collagen fibers inserting into the root surface. BSP has been shown to have a high affinity for collagen and additionally mediates hydroxyapatite formation. (40) BSP in the presence of collagen increases the nucleation potency of hydroxyapatite 10-fold. (39-41) The expression corroborates with the previous studies

showing a particular concentration of BSP in the acellular cementum lining the tooth root surface. (31) Also, a lack of BSP has been shown to cause periodontal defects in BSP null mice due to a lack of acellular cementum formation and subsequent attachment. (42) BSP therefore seems to play an important role in the formation and maintenance of the periodontal apparatus.

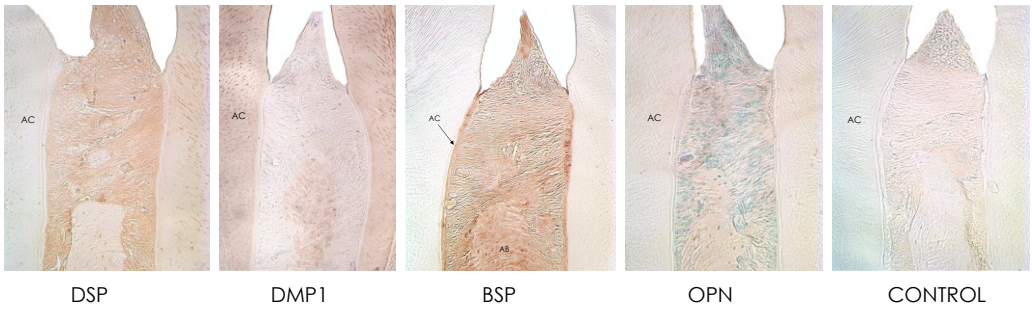


Figure 4.1. Expression of SIBLING proteins at acellular cementum. Anti-BSP antibody shows strong immunopositive staining in the acellular cementum. *(Source- IHC Dab)*

Anti-Osteopontin (OPN) antibody: has been shown to regulate mineralization *in vitro* and *in vivo* and is a constituent of both cementum and alveolar bone. (43)(36)It's role as a potent inhibitor of mineral nucleation, crystal growth, and proliferation has been clearly documented. (44) Detecting within both dentin and bone, OPN has been previously shown to be present.In our experiment in the tibial epiphyseal growth plate and metaphysis, OPN was mainly localized in the unmineralized osteoid and the remodeling region at the calcification front (CZ) border. This expression is consistent with previous studies showing its signal in the mineralization and remodeling regions of the bone. (38) In the secondary ossification center, the immunostaining was more prominent at the cement lines (corresponding to the bone remodeling reversal lines). These observations are coherent with previous studies with OPN detection in the unmineralized matrix, cellular compartments and in proteins bound to the mineral phase following immunoblotting of protein extractions. (28)(45) The increase in the expression of OPN from 1 month to 3-month-old group follows an increase in conversion of osteoid to mineralized bone. Since previous studies have demonstrated its role in inhibiting mineralization (46) and that the expression of other SIBLING proteins in our experiment have shown similar increased expression from 1 to 3 months, it is possible that OPN plays a role in regulating the precise rate of crystal nucleation and growth by controlled inhibition. Similarly, the decline in expression at

6months follows a pattern that is in line with its reduced need with slowing of the mineralization process corresponding to age and skeletal maturity.

In the mandible, OPN was detected in the reactionary dentin but was low in the primary dentin of tooth consistent with existing evidence. (28)Previous studies have shown a relatively lower level of OPN expression in dentin than in bone. (6)(37)This observation may indicate a different functional and developmental role for OPN in dentin compared to bone. This tissue specific difference could be attributed to continual active remodeling of bone matrix versus minimal to no remodeling in dentin. Thus, one of the potential roles of OPN (regulation of remodeling of bone matrix) may not be fully required in dentin. In the interproximal and furcal bone, the expression pattern showed a slowing of the OPN expression from 1 to 6months. This pattern may indicate controlled remodeling of the alveolar process following the initial rapid growth of the jaws. It is likely that the jaw has already completed its initial mineralization since mandibular bone develops via intramembranous ossification.IHC staining by Zhang in 5-week old mice showed OPN expression was mainly observed in the areas containing newly formed bone in the alveolar process. (38)At the root apex, the positive signals in the cellular cementum remained relatively consistent in our study, showing minor variations in the three age groups. This consistently elevated signal indicates the need for OPN's continued inhibition during remodeling for precise control of mineralization. Moses obtained similar signal patterns for OPN in bone, dentin, cellular cementum and periodontal ligament. (21) A previous study showed that the absence of OPN leads to increased volume and mineral density of dentin, cellular cementum and alveolar bone indicating OPN's inhibitory role in their development. Interestingly, acellular cementum apposition was not altered showing it may have a different mineralization mechanism. (47) Our results in addition to showing the localization of OPN, also showed that its expression varied considerably for the three age groups. The dentin and interproximal bone showed higher expression at 1month with reduction of expression at 3 months that was consistent with reduced mineralization and slightly increased levels at 6months consistent with an increase in the rate of cusp attrition and concomitant reactionary dentin formation. The increased expression pattern in the furcation and root at 3months followed increased remodeling in these regions. In total, OPN's expression pattern suggests a possible role of in regulating the pace and extent of mineralization of bone and dentin.

C. Summary of interprotein comparison:

IHC staining intensity of all SIBLING proteins in the tibia of 1-, 3-, and 6-month old mice showed significant higher intensity of expression at 3 months versus 1-, and 6-months, consistent with increased conversion of osteoid to bone in this age group and was reduced at 6months, consistent with slow remodeling at skeletal maturity. However, DMP1 showed higher expression at 1 month, showing its early involvement in the mineralization process in SOC. SOC forms between 5 and 14 days postnatally in C57BL6 mice and has been shown to function as a protector of the growth plate by compressive and shearing forces. Previous researchers have shown increased apoptosis of chondrocytes in the proliferative and hypertrophic layers in the absence of SOC. Our results were consistent with the need for early mineralization of SOC prior to POC which, therefore, may need early expression of DMP1, which further has been shown to regulate the expression of DSP. (**Figure 3.18a**)

Alveolar bone development occurs by intramembranous ossification. Staining intensity of SIBLING proteins showed a significant decrease at 3 months versus 1-, and 6-months, consistent with early formation and slowing of the mineralization process in alveolar bone followed by an increase at 6 months, indicating pattern following increased function, attrition, and remodelling. There was early and increased staining intensity of DMP1 in the furcation, consistent with increasing compression-induced expression as opposed to that of interproximal bone which is under tangential forces. BSP expression, on the other hand, increased from 3- to 6- months temporally being the last protein expressed in alveolar bone. BSP was exclusively expressed in the acellular cementum indicating its specific role.(**Figure 3.19a**)

The intensity pattern in the teeth showed notably decreased expression at 3 months versus 1 and 6months, consistent with slowing of the mineralization process followed by an increase at 6 months, again indicating it may have a regulatory role during increased function, attrition, and remodeling. DMP1 and BSP expression in the cusp showed similar expression with an increase at 3 months followed by minimal change at 6 months. On the other hand, DMP1 and BSP expression in the root showed a continued increase from 1, 3 to 6months. This pattern difference between the cusp and root may possibly be due to continued remodeling in the cellular cementum and PDL versus minimal remodeling in highly mineralized cusp. BSP was exclusively

expressed in the predentin at 3 months indicating diverse distribution and its specific role in the mineralization process. (**Figure 3.20a**)

Overall, in the primary ossification center and growth plate, all proteins showed the highest expression at 3 months. This could be correlated with the 3months stage of development in these mice having the highest conversion of osteoid to mineralized bone. All four proteins could be coordinating with each other to allow accurate temporospatial orientation of the mineralized product. In the teeth, the presence of DSP and OPN at early stages suggests their role in the well-controlled regulation of nucleation of apatite onto newly formed collagenous dentin matrix.

The expression profile of DMP1, DSP, BSP, and OPN showed clear variations at different stages of skeletal maturity. The expression patterns between the alveolar bone and tibia varied, indicating that the expression of these molecules is different in intramembranous bones versus those formed by endochondral ossification. The expression also changed over the time course in the various tissues of the teeth (dentin, predentin and cementum). The age-dependent changes in the expression of SIBLING proteins may be related to the changes in the need based functional contribution of progenitor cells. The shift in the expression pattern also indicates their possible role in development and function of these mineralized tissues. These proteins have been shown in the literature to function collectively to ensure proper formation. Thus, the changes in the bone parallel the expression pattern and vice versa.

D. Conclusion:

This analysis elaborated on the expression profile of four SIBLING proteins at three different ages of skeletal development in the mice. Their expression showed variations in staining intensity and temporospatial patterning, in the three age groups. In the tibia, the proteins showed strong immunohistochemical staining in the degenerating hypertrophic chondrocytes and calcification zone of the growth plate. The maximum intensity of expression was found at 3months of age which corresponds to the time of greatest conversion of osteoid into bone in the tibia. The staining intensity in the teeth corresponded to the relative time of greatest conversion of predentin into mineralized dentin. A rise in the expression of these proteins at 6 months

corresponded with increased rate of cuspal attrition. The IHC findings for DMP1 reinforce previous studies showing mechanical loading increased its expression and had similar localization pattern as DSP. BSP's staining in all zones of the growth plate, predentin and acellular cementum showed its broader expression pattern in an age dependent fashion. OPN's overall expression variation may indicate its role in controlled inhibition. Taken together, the SIBLING proteins were expressed in all age groups albeit at varying levels in different tissues.

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Limitations

1. Variations in temperature, pH and humidity of the laboratory on a daily basis likely influenced the processing conditions including degree and quality of staining and subsequent variation of the results.
2. All efforts were made to maintain consistency of the histologic sections including appropriate thickness and localization but the differences in sectioning plane of the tissues may have contributed to variations in the sections.
3. The background counterstaining could be controlled but needs repeated experiments to normalize equitable staining pattern.
4. Certain artifacts including tearing and folding of paraffin embedded sections, improper dehydration and hydration technique, mounting artifacts such as air bubbles and presence of water droplets were difficult to avoid at times and may have contributed to altered results.
5. The main limitation was that quantification of staining was semi-quantitative, and interpretation was done accordingly. In future, we plan to run Real Time PCR studies to determine the expression patterns of SIBLING proteins more accurately.

Future Directions

Examination of the localization and distribution pattern of the SIBLING proteins in our study showed the proteins to be present in PDL, acellular cementum, cellular cementum and alveolar bone. Furthermore, previous studies have shown the non-redundant role of BSP in the formation of acellular cementum and integrity of periodontal attachment apparatus. In addition, DMP1 and DSPP have been implicated to act synergistically and play an important role in dentinogenesis and osteogenesis. Therefore, a high-stringency antisense probe for all four SIBLING proteins should be utilized to analyze the expression of the transcripts in osteoblasts, odontoblasts, cementoblasts, cementocytes and fibroblasts. This will allow us to delineate the various cell lines expressing these proteins at defined time points during development and maturation.

In order to correlate the findings with associated conditions such as lack of acellular cementum formation in KO mice leading to periodontal defects, these experiments should be conducted in both WT and KO mice groups at 1-, 3-, and 6-months of age. Further, various stains such as alcian blue to check diminished acellular cementum, and Goldner's trichrome staining to check mineralized cementum matrix can be usedon WT vs *KO* mice. Picrosirius-red-stained sections viewed under polarized light could assess the degree of organization and directionality of periodontal ligament collagen fibrils between acellular cementum and bone. IHC staining with pankeratin could be used to check the integrity of the junctional epithelium. Tartrate-resistant acid phosphatase (TRAP) might be employed to check the activity of osteoclasts along the tooth and bone surfaces and the osteoblast marker osterix (OSX) – to localize osteoblasts.SEM could be utilized to confirm surface changes of the root topography. Combined, these experiments will allow us to analyze changes in the root surface e.g.

alteration/lack of acellular cementum and areas of resorption and correlate them with alterations in the PDL collagen fibers. Expression of *Spp1*for osteoblasts and *Ibsp* and *Alpl* for cementoblasts could be assessed to monitor their expression in the periodontium during root development.In addition, sections from 14 day- old mice could be used to look at early development e.g. root formation. Age-related changes in the cellular and acellular cementum could also be documented. Measurement of crystal size, mineral to matrix ratio, carbonate to phosphate ratio and collagen crosslinking by x-ray, FTIR etc. and comparison of these results to the expression of the four SIBLING proteins in corresponding wild type and knockout mice would add valuable information. In addition, experimental analysis of changes in the mineralized tissues, checking and correlating the expression of SIBLINGs in OPN/BSP null mice will help to understand their collaborative roles.

Since root resorption is a common phenomenon during tooth movement, expression of OPN during tooth movement could be analyzed since it has been shown to be important in force-induced bone and tooth resorption. In addition, TGF-beta1 and BMP2 are essential factors for tooth development. *In vitro* studies have shown that TGF-Beta 1 down-regulated the expression of DMP1 and DSPP and that BMP signalling regulated the expression of DSPP in odontoblasts. Reduced expression has been shown in cases of trauma to teeth caused by caries and/or subjected to calcium hydroxide pulp capping. The expression pattern of these proteins in injured or exposed dentin will allow further characterization of these proteins and help us further understand their role. The role of vitamin D and expression of SIBLING proteins in various mineralized tissue should also be investigated.

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