The Characterization of the Rat Bone Sialoprotein Knockout Phenotype

Benjamin Harvey
The University of Western Ontario

Supervisor
Goldberg, Harvey A.
The University of Western Ontario Co-Supervisor
Beier, Frank
The University of Western Ontario

Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
© Benjamin Harvey 2019

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Recommended Citation
https://ir.lib.uwo.ca/etd/6649

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.
ABSTRACT

Bone sialoprotein (BSP) is an extracellular phosphoprotein that has been associated with mineralizing tissues, such as the periodontium, the fibrocartilaginous enthesis, and long bone formation. In this study, we characterized the phenotype of BSP-deficient rats. Histomorphometric analysis discovered a thinning of the acellular cementum in both 20 and 50 week-old Bsp−/− rats with no related periodontal defects and an organized periodontal ligament. Analysis of the mature quadriceps tendon (QCT) enthesis determined that BSP and osteopontin are present in the calcified fibrocartilage of wild-type rats at 14 weeks. The developing QCT enthesis of Bsp−/− rats appears similar in mineral content, collagen organization, and morphology when compared to wild-type counterparts. Digital measurements of rat tibiae show that bone length does not differ between wild-type and Bsp−/− rats at day of birth. The results of this study suggest that BSP is present in the periodontal and enthesis tissues of the rat, but the rat displays less severe phenotypes than the Bsp−/− mouse.

KEYWORDS

Bone sialoprotein, mineralization, cementum, periodontal ligament, enthesis, calcified fibrocartilage, endochondral ossification
SUMMARY FOR LAY AUDIENCE

The formation of bone and related mineralized tissues is a complex and highly regulated process. Bone sialoprotein (BSP) is a protein that is expressed at the onset of mineralization in calcified tissues. Much can be learned about a protein by removing its corresponding gene from the genome of animal models and observing the effects. Research on BSP has mainly used the mouse as an animal model for understanding the normal physiological function of the protein, the role of the protein in relevant diseases, and the potential uses of the protein as a therapeutic. However, some experimental studies in the mouse model are limited due to its small size compared to the rat. This study aimed to characterize the effects of the absence of BSP in a novel rat model, and to lay the foundation of future studies on the role and function of BSP. Three regions of the rat were investigated: the dental tissue complex, the development of the tissue at the tendon-bone interface, and the early development of long bones. It was discovered that in the absence of BSP there is a slight defect in the mineralized dental tissues that did not lead to any tooth related defects, such as those observed in the mouse model. Secondly, the junction where the quadriceps femoris tendon inserts into the patella (kneecap) was investigated and although BSP is present in the tendon-bone interface in mature genetically unmodified rats (wild-type), there were no observed differences in the interface structure in the absence of BSP. Furthermore, it was discovered that the mineralization of the tendon-bone interface occurs after four weeks of age. Lastly, the tibia (long bone in lower hind leg) was examined to assess for any delays in its development. Rats lacking the BSP protein did not differ in the length of the tibia at day of birth, and initial analysis suggests that the overall mineral content is not significantly different than wild-type rats. This characterization provides a base of information for future investigations of BSP in the rat model.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my Mom, Mary-Alice, and my Dad, Jay, for making me the person I am today and for always providing the right guidance at the right time. Your continued support has been instrumental in my academic and personal success, I love you both very much. Additionally, I would like to thank my extended family for their encouragement and assistance throughout my education. I would also like to thank all of the close friends I made during my time at Western for making this experience unforgettable.

I would like to extend a thank you to my supervisor, Dr. Harvey Goldberg. You have helped make this experience a positive one. Although you did provide key research assistance, one of my favourite parts of having you as a supervisor was the numerous stories and words of wisdom you would provide any time we spoke. I wish you nothing but the best in the future.

I would like to thank Dr. Frank Beier, my co-supervisor, for his advice and direction over the last two years. Furthermore, I would like to thank the members of my advisory committee, Dr. Eric Ball and Dr. Peter Chidiac, for their guidance and valuable input. I would like to thank both Andrew Belling and Kevin Bartman for their assistance and for making our time together enjoyable.

I would like to acknowledge the assistance of Dale Fournier and Michael Chavez, for their microCT imaging and analysis, and Michael Pest, for his histology expertise. Last, but definitely not least, I would like to thank Wendy Dunn and all of the associated staff that cared for our animals and assisted me with management of the colony.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ ii
SUMMARY FOR LAY AUDIENCE .............................................................................................. iii
ACKNOWLEDGEMENTS ............................................................................................................. iv
TABLE OF CONTENTS .................................................................................................................. v
LIST OF FIGURES ....................................................................................................................... vii
LIST OF APPENDICES ................................................................................................................ ix
LIST OF ABBREVIATIONS ........................................................................................................ x

CHAPTER ONE: LITERATURE REVIEW .................................................................................. 1
1.1 Introduction ........................................................................................................................... 2
1.2 Skeletal Development .......................................................................................................... 2
1.3 Models of Biomineralization ............................................................................................... 7
1.4 SIBLING Family .................................................................................................................. 7
1.5 Bone Sialoprotein ............................................................................................................... 9
  1.5.1 Structure ...................................................................................................................... 10
  1.5.2 Functional Regions ................................................................................................. 11
  1.5.3 Tissue Expression .................................................................................................... 15
  1.5.4 In Vivo Function ....................................................................................................... 15
1.6 Murine Tooth Development ............................................................................................. 17
  1.6.1 Murine Tooth Anatomy .......................................................................................... 18
1.7 Enthesis Development ...................................................................................................... 25
  1.7.1 Enthesis Anatomy .................................................................................................. 27
1.8 Purpose of Thesis ............................................................................................................. 35
1.9 References ......................................................................................................................... 37

CHAPTER TWO: CHARACTERIZATION OF THE RAT BONE SIALOPROTEIN
KNOCKOUT PHENOTYPE ......................................................................................................... 49
2.1 Materials and Methods ...................................................................................................... 50
  2.1.1 Animals .................................................................................................................... 50
  2.1.2 Sanger DNA Sequencing ....................................................................................... 51
  2.1.3 Histology and Immunohistochemistry for Periodontal Tissues .................................. 51
  2.1.4 Histomorphometry ................................................................................................ 53
  2.1.5 Microcomputed Tomography ................................................................................ 54
  2.1.6 Histology and Immunohistochemistry for Enthesis Tissues .................................... 55
  2.1.7 Histology and Digital Analysis for Tibiae ................................................................. 58
LIST OF FIGURES

Figure 1.1 Endochondral ossification ................................................................. 4
Figure 1.2 Linear schematic of bone sialoprotein functional domains ......................... 12
Figure 1.3 Schematic of murine molar tooth cross section ......................................... 20
Figure 1.4 Bone sialoprotein localization in mouse periodontal tissues ....................... 23
Figure 1.5 Anatomical location of notable fibrocartilaginous entheses ......................... 30
Figure 1.6 Transitional zones of the fibrocartilaginous enthesis .................................. 32
Figure 2.1 Features of the Bsp\(^{−/−}\) rat ................................................................. 61
Figure 2.2 Body weight is unaffected in Bsp\(^{−/−}\) rats ............................................... 63
Figure 2.3 Bone sialoprotein is present in the mineralized tissues of the periodontium at 20 weeks ............................................................................................................. 66
Figure 2.4 Bone sialoprotein is present in the mineralized tissues of the periodontium at 50 weeks ............................................................................................................. 68
Figure 2.5 Potential underdevelopment of acellular cementum in the periodontal tissues of 20 week- and 50 week-old wild-type and Bsp\(^{−/−}\) rats ................................................................. 70
Figure 2.6 Apparent acellular cementum thinning observed in the periodontal tissues of 20 week- and 50 week-old wild-type and Bsp\(^{−/−}\) rats ................................................................. 72
Figure 2.7 Possible thinning of acellular cementum with no other differences in morphology in toluidine blue stained periodontal tissues of wild-type and Bsp\(^{−/−}\) rats ................................................................. 74
Figure 2.8 Graphical representation of histomorphometry of acellular cementum in 20 and 50 week-old wild-type and Bsp\(^{−/−}\) rats ................................................................. 77
Figure 2.9 No differences observed in the collagen organization of periodontal tissues at 20 weeks in wild-type and Bsp\(^{−/−}\) rats ................................................................. 79
Figure 2.10 No differences observed in the collagen organization of periodontal tissues at 50 weeks in wild-type and Bsp\(^{−/−}\) rats ................................................................. 81
Figure 2.11 No observable resorption of alveolar bone in Bsp\(^{−/−}\) rats ......................... 83
Figure 2.12 Bone sialoprotein is not present in the QCT enthesis of 14 or 28 day-old wild-type rats ............................................................................................................. 86
Figure 2.13 Osteopontin is not present in the QCT enthesis of 14 or 28 day-old wild-type or Bsp\(^{−/−}\) rats ............................................................................................................. 88
Figure 2.14 No mineralization is present in the entheses of wild-type and $Bsp^{-/-}$ rats at 14 or 28 days.

Figure 2.15 No morphological differences are apparent in the entheses of wild-type and $Bsp^{-/-}$ rats at 28 days.

Figure 2.16 No differences in cartilage morphology are apparent in the entheses of wild-type and $Bsp^{-/-}$ rats at 28 days.

Figure 2.17 No observable differences in collagen organization in the entheses of wild-type and $Bsp^{-/-}$ rats at 14 or 28 days.

Figure 2.18 Bone sialoprotein is present in the calcified fibrocartilage of 14 week-old wild-type rats.

Figure 2.19 Osteopontin is present in the calcified fibrocartilage of 14 week-old wild-type and $Bsp^{-/-}$ rats.

Figure 2.20 No morphological differences are apparent in the entheses of wild-type and $Bsp^{-/-}$ rats at 14 weeks.

Figure 2.21 Mineralization and development does not appear to be impaired in the developing tibiae of $Bsp^{-/-}$ rats.
LIST OF APPENDICES

APPENDIX A: Animal Use Statement.................................................................................. 123
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACLT</td>
<td>Anterior Cruciate Ligament Transection</td>
</tr>
<tr>
<td>AB-NFR</td>
<td>Alcian Blue - Nuclear Fast Red</td>
</tr>
<tr>
<td>ABP</td>
<td>Alveolar Bone Proper</td>
</tr>
<tr>
<td>AFS</td>
<td>Acid Flagellates Solution</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone Sialoprotein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFC</td>
<td>Calcified Fibrocartilage</td>
</tr>
<tr>
<td>CIFC</td>
<td>Cellular Intrinsic Fiber Cementum</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
</tr>
<tr>
<td>DMM</td>
<td>Destabilization of the Medial Meniscus</td>
</tr>
<tr>
<td>DMP1</td>
<td>Dentin Matrix Protein 1</td>
</tr>
<tr>
<td>Dpn</td>
<td>Days Post-Natal</td>
</tr>
<tr>
<td>DSPP</td>
<td>Dentin Sialophosphoprotein</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>HU</td>
<td>Hounsfield Units</td>
</tr>
<tr>
<td>IEE</td>
<td>Inner Enamel Epithelium</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IHH</td>
<td>Indian Hedgehog</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kVp</td>
<td>Peak Kilovoltage</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SIBLING</td>
<td>Small Integrin-Binding Ligand, N-Linked Glycoprotein</td>
</tr>
<tr>
<td>MEPE</td>
<td>Matrix Extracellular Phosphoglycoprotein</td>
</tr>
<tr>
<td>microCT</td>
<td>Micro Computed Tomography</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OEE</td>
<td>Outer Enamel Epithelium</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDL</td>
<td>Periodontal Ligament</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid Hormone-Related Protein</td>
</tr>
<tr>
<td>QCT</td>
<td>Quadriceps Femoris Tendon</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic Acid</td>
</tr>
<tr>
<td>Sex</td>
<td>Scleraxis</td>
</tr>
<tr>
<td>Sox</td>
<td>Sex Determining Region on Y-box</td>
</tr>
<tr>
<td>SST</td>
<td>Supraspinatus Tendon</td>
</tr>
<tr>
<td>TB</td>
<td>Toluidine Blue</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffered Saline - Tween 20</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TGFβr</td>
<td>Transforming Growth Factor β Receptor</td>
</tr>
<tr>
<td>UFC</td>
<td>Uncalcified Fibrocartilage</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
CHAPTER ONE

LITERATURE REVIEW
1.1 Introduction

Bone formation, development, and remodeling are complicated processes involving several different cell types and proteins. Biomineralization is a critical component involved in bone and other mineralized tissue and is the formation of minerals by living organisms (Addadi and Weiner, 2014; Weiner and Dove, 2003). Biomineralization occurs in all kingdoms of eukaryotes, with over 60 biologically produced minerals identified (Lowenstam and Weiner, 1989). Prokaryotes were the first to develop this mineral forming ability 3500 million years ago, but it was not until the dawn of the Cambrian period 540 million years ago when complex biomineralization developed (Addadi and Weiner, 2014). Biominerals differ from standard minerals because of their amalgamation of inorganic and organic materials. The inorganic mineral found in bones and teeth of vertebrates is called hydroxyapatite, which is the most abundantly produced phosphate mineral (Lowenstam and Weiner, 1989). Biomineralized tissues have developed various roles throughout evolution, such as their involvement in body support, protection, and mineral homeostasis. These roles have been well characterized in humans and other chordates.

1.2 Skeletal Development

The development of bone in humans is a tightly regulated and critical process that relies on signaling and communication. This hard connective tissue is composed of an inorganic mineral component, calcium phosphate crystals in the form of hydroxyapatite (HA; Ca_{10}(PO_4)_6(OH_2)) that provides rigidity, and an organic matrix component of primarily type I collagen which provides tensile strength and elasticity (reviewed in Ralston, 2017). The organic phase also contains non-collagenous proteins, which make up roughly 10% of the organic matrix (reviewed in Ralston, 2017) and play key roles in mineralization regulation, cell signaling and adhesion, and bone remodeling (Gordon et al., 2007).
The two mechanisms of skeletal development are intramembranous and endochondral ossification (Olsen et al., 2000; Provot and Schipani, 2005; Long, 2012). Both processes make bone, but the end product is achieved in a different manner. Briefly, bones formed through intramembranous ossification (flat craniofacial bones and the clavicle) do not have a cartilage anlage stage prior to mineralization, unlike endochondral ossification which forms the rest of the skeleton (Streeter, 1949; Ortega et al., 2012). Endochondral ossification (reviewed in Olsen et al., 2000; Provot and Schipani, 2005; Mackie et al., 2008; Long, 2012) begins with the condensation of mesenchymal progenitor cells which undergo chondrogenesis where they differentiate into chondrocytes. These chondrocytes secrete a cartilage matrix that is rich in type II collagen as they proliferate and develop the long axis of the bone anlage (Figure 1.1). As the anlage is elongating, chondrocytes at the center undergo hypertrophy and begin to form an extracellular matrix (ECM) that is different than that of regular chondrocytes as it contains type X collagen. The cartilage around the hypertrophic chondrocytes calcifies, and the cells begin to secrete angiogenic factors (e.g. vascular endothelial growth factor) which induce the ingrowth of blood vessels into the tissue allowing the arrival of osteoblast and osteoclast precursors. Once differentiated, the osteoclasts degrade the calcified cartilage and the osteoblasts replace it with trabecular bone, thus creating the primary ossification center at the center of the bone (diaphysis). This process continues as the bone elongates. Secondary ossification centers form at the ends of the bone (epiphyses) and are separated from the diaphysis by the epiphyseal growth plate. This cartilage plate contains resting, proliferating, and hypertrophic chondrocytes and is responsible for the longitudinal elongation of the bone until it mineralizes and fuses with the surrounding bone following puberty. Bone development and maintenance is dependent on the function of three main cell types: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are mononucleated cells that are derived from
Figure 1.1 Endochondral ossification. (A) Mesenchymal progenitor cells have undergone chondrogenesis and have formed the cartilage anlage comprised of resting, proliferating, and hypertrophic chondrocytes. (B) The cartilage surrounding the hypertrophic chondrocytes calcifies and the cells release angiogenic factors that prompts the vascular invasion of the anlage (red). Blood vessels allow for the arrival of osteoblast and osteoclast precursors, which eventually differentiate and function in tandem to replace the cartilage anlage with trabecular bone. (C) Mineralization of the tissue begins at the primary ossification center in the diaphysis of the bone anlage (orange). Towards the ends of the developing bone are the epiphyseal growth plates, which contain resting, proliferating, and hypertrophic chondrocytes that function to longitudinally elongate the bone. After the primary ossification center and growth plate is developed, vascular invasion occurs at the epiphyses followed by cartilage calcification and eventual replacement with trabecular bone (secondary ossification; orange). Figure obtained from (Solomon et al., 2008).
mesenchymal stem cells and function to deposit and subsequently mineralize the bone matrix, or osteoid (Olsen et al., 2000; reviewed in Ralston, 2017). As the bone continues to develop, osteoblasts mature and either undergo apoptosis, become bone lining cells, or get entrapped within the mineralized matrix where they become osteocytes (Crockett et al., 2011). Osteocytes, the most abundant cell found in bone (Crockett et al., 2011; reviewed in Ralston, 2017), are connected via processes called canaliculi that allow for nutrient transport and signaling between cells for proper homeostasis, and they play an important role in mechanosensation (Bonewald, 2011; Klein-Nulend et al., 2013; reviewed in Ralston, 2017). Osteoclasts are multinucleated cells derived from the fusion and differentiation of monocyte/macrophage precursor cells and are responsible for the breakdown of both the inorganic mineral and organic matrix components of bone during bone turnover (Wagner and Karsenty, 2001; Boyle et al., 2003).

Sex steroid hormones have been found to play a role in bone development, contributing to sexual size dimorphism observed in many gonochoristic animals. The two main groups of hormones responsible for the differences are androgens, which are expressed more in males, and estrogens, which are expressed more in females. Despite the different expression levels, males and females both require androgens and estrogens for proper development (Saggese et al., 2002). The expression of sex hormones increases dramatically at sexual maturation, or puberty (Saggese et al., 2002). Androgens and estrogens have both been seen to play a role in bone mass accumulation, partially due to their ability to inhibit the apoptosis of osteoblasts (Kousteni et al., 2001) and promote osteoclast apoptosis (Hughes et al., 1996), but also have their own unique effects on bone. In males, androgens promote both the longitudinal and radial growth of long bone and contributes to a greater bone mass than females (reviewed in Vanderschueren et al., 2004). The increase in
bone mass is due to males having a higher rate of periosteal bone formation, as periosteal formation is stimulated by androgens but inhibited by estrogens (reviewed in Manolagas et al., 2013). In humans, a key contributor to sexual size dimorphism is attributable to the estrogen-induced closure of the growth plate (reviewed in Vanderschueren et al., 2004).

1.3 Models of Biomineralization

The method of bone mineralization is not completely understood due to the complex mechanisms involved, but theoretical models of mineral deposition have been described. Eidelman et al. showed that concentrations of calcium and phosphate in serum are supersaturated for the process of hydroxyapatite nucleation (Eidelman et al., 1987) but mineralization inhibitors, like albumin and fetuins, prevent the formation of ectopic calcifications by binding calcium (Schinke et al., 1996). One proposed mechanism of biomineralization involves the hole zones found every 67 nm within type 1 collagen fibrils in mineralized tissues due to the pattern of overlap (Katz and Li, 1973). Having been shown in mice and rats (Fratzl et al., 1991) as well as turkey tendon (Traub et al., 1992), the hole zones provide a site for HA crystal formation to initiate. Additionally, the hole zones have size exclusion characteristics (Toroian et al., 2007) which allow the exclusion of mineralization inhibitors larger than 40 kDa (Price et al., 2009). Nonetheless, other components must be involved as type 1 collagen alone is not adequate for the initiation of HA nucleation (Hunter et al., 1986). It has been postulated that phosphoproteins that bind to collagen at specific regions act as nucleating sites for HA crystal formation (Glimcher and Muir, 1984; Blumenthal et al., 1991).

1.4 SIBLING Family

The Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family are a group of non-collagenous phosphoproteins that are associated with calcified tissues. This five protein family
includes bone sialoprotein (BSP; \textit{IBSP}), osteopontin (OPN; \textit{SPP1}), dentin matrix protein (DMP1; \textit{DMP1}), dentin sialophosphoprotein (DSPP; \textit{DSPP}), and matrix extracellular phosphoglycoprotein (MEPE; \textit{MEPE}) (Fisher et al., 2001; Fisher and Fedarko, 2003). The SIBLING family share evolutionary history with calcium-binding enamel, milk, and salivary proteins due to a suggested gene duplication event (Kawasaki and Weiss, 2003; Kawasaki et al., 2004). Similarly, it is postulated that the SIBLING proteins are all descendants of a single gene that duplicated and diverged over history (Fisher et al., 2001) and that this divergence was a key event in the development of tissue mineralization in vertebrates (Kawasaki and Weiss, 2006). This postulation is due to similarities in their intron-exon boundaries, exon structure, biochemical properties, and key conserved regions. Interestingly, the overall primary protein sequences when compared do not show significant sequence similarity outside of conserved regions. In humans, the cluster of SIBLING genes are located on chromosome 4 within 375 kbp of each other (Kerr et al., 1993; Fisher and Fedarko, 2003; George and Veis, 2008) whereas in mice they are found on chromosome 5 (Young et al., 1994, Crosby et al., 1996). Despite being clustered on the same chromosome, the SIBLING proteins are responsible for different functions, although all seem to be involved with tissue mineralization.

SIBLING family members tend to function in the inhibition or promotion of mineralization. OPN and MEPE are involved in mineralization inhibition in both mineralized and non-mineralized tissues where they are suggested to prevent ectopic ossification (Schlieper et al., 2007; George and Veis, 2008). In contrast, BSP, DMP1, and DSPP are all promoters of mineralization (George and Veis, 2008). Animal models with null mutations may result in defects in tissue biomineralization, however no single knockout has fully inhibited mineralization from occurring (Fisher and Fedarko,
2003). This is likely due to the fact that biomineralization is a complex and well-regulated process with functional redundancy.

Members of the SIBLING family have similar biochemical features. All five proteins are intrinsically disordered (lack an ordered three-dimensional shape) and consequently are flexible in structure (George and Veis, 2008). Additionally, all members undergo extensive post-translational modifications such as phosphorylation, sulfation and glycosylation. MEPE is the only basic protein in the family (pI = 9.2) while the others are acidic with predicted isoelectric points spanning from 3.4 to 4.3 (Fisher and Fedarko, 2003). The acidic SIBLING proteins are all similar in that they tightly bind to hydroxyapatite (Fisher et al., 2001). Lastly, all members contain a highly conserved tripeptide Arg-Gly-Asp (RGD) integrin-binding domain found towards the C-terminus of the protein which allows for cell attachment and signaling (Fisher et al., 2001; George and Veis, 2008).

1.5 Bone Sialoprotein

BSP is a negatively charged extracellular phosphoprotein involved with mineralized tissues of vertebrates. The first isolation of BSP came from bovine cortical bone in 1963, where Herring and Kent extracted a 23 kDa glycoprotein (Herring and Kent, 1963) which was later shown to be a fragment of BSP (Fisher et al., 1983). Since then, BSP has been isolated from many sources such as frog (Shintani et al., 2008), human (Fisher et al., 1987), and rat (Oldberg et al., 1988). The mature form of the protein core without modifications has a molecular mass of 33-34 kDa (reviewed in Ganss et al., 1999), but due to extensive post-translational modifications the fully intact protein can be 70-80 kDa in size, based on gel electrophoresis (Fisher et al., 1983). The sequencing of the rat BSP cDNA by Oldberg et al. in 1988 allowed for the full characterization of the protein, noting key sequences and residues (Oldberg et al., 1988).
1.5.1 Structure

BSP in mammals has 327 amino acid residues on average, which includes a 16-residue signal sequence (reviewed in Ganss et al., 1999). Equilibrium centrifugation of bovine BSP in 1985 found the molecular weight to be 57.3 kDa (Franzen and Heinegard, 1985). Since then, mass spectrometry studies have calculated the molecular weight of human BSP to be 52.5 kDa (Zaia et al., 2001) and 49.0 kDa (Wuttke et al., 2001). The differences in mass are likely due to the heterogeneity of post-translational modifications the protein undergoes, specifically differences in glycosylation patterns, as was suggested from the broad mass spectrometry peaks (Zaia et al., 2001; Wuttke et al., 2001). Approximately 40% of the native protein mass is from post-translational phosphorylation, sulfation, and glycosylation (Stubbs et al., 1997). Analysis of bovine BSP by Salig and Flückiger found up to 11 phosphorylation sites in vivo, but the degree of phosphorylation varied (Salig and Flückiger, 2004). Additionally, mass spectroscopy analysis calculated an average of 5.8 phosphorylated serine residues per molecule of BSP (Salih, 2003). The majority of sulfation occurs towards the C-terminus of the protein, near the highly conserved RGD domain, where approximately 7-12 tyrosine residues are capable of undergoing sulfation (reviewed in Ganss et al., 1999).

Of the total amino acids, approximately a third of the protein is composed of glutamic acid and glycine residues (reviewed in Ganss et al., 1999). Both glutamic and aspartic acid are found in extended sequences and contribute to the pI of 3.9 and overall anionic nature of BSP (Stubbs et al., 1997; Zaia et al., 2001). Early predictions on the structure of BSP using hydrophilicity analysis suggested an intrinsically disordered, flexible protein with limited ability to form any secondary structure (Shapiro et al., 1993), and these predictions were later confirmed through one dimensional proton nuclear magnetic resonance (Fisher et al., 2001), circular dichroism (Wuttke
et al., 2001), and small-angle X-ray scattering (Tye et al., 2003). BSP contains three functionally conserved regions that contribute to its function: poly-glutamic acid sequences, an N-terminus collagen-binding domain, and a C-terminus integrin-binding domain (Goldberg and Hunter, 2012) (Figure 1.2).

1.5.2 Functional Regions

The main function of the negatively charged sequences in BSP is to allow for HA binding and nucleation. The ability for BSP to bind to HA is predominantly due to the two centrally located contiguous poly-glutamic acid sequences (Stubbs et al., 1997). If modified to a contiguous poly-alanine sequence, the binding of BSP to mineralized bone is reduced by 73% (Wazen et al., 2007) indicating the importance of the acidic poly-glutamic acid in BSP-HA binding. Since recombinant, unmodified forms of BSP cannot bind HA as effectively as native BSP, post-translational contribute directly or indirectly to the HA binding ability (Goldberg et al., 1996; Stubbs et al., 1997). In addition to binding HA, BSP is a potent nucleator as shown in vitro using a steady-state gel system (Hunter and Goldberg, 1993). This property has been linked to the presence of the glutamic acid-rich sequences, and even fragments containing the sequences were capable of nucleating HA (Goldberg et al., 1996; Baht et al., 2010). Using a steady-state agarose gel system, Harris et al. determined that the first poly-glutamic acid domain in porcine BSP cDNA is a more potent nucleator of HA (Harris et al., 2000) whereas Tye et al. determined, using peptides constructed from rat BSP cDNA, the second poly-glutamic acid domain to be more potent (Tye et al., 2003). The differences in nucleation potency among species may be due to slight differences in the poly-glutamic acid domains, specifically the number of contiguous glutamic acid residues. BSP binding to collagen has proven to enhance the HA nucleating ability (Baht et al., 2010). The collagen-binding domain of BSP is located towards the N-terminus covering residues 19-46 and
Figure 1.2 Linear schematic of bone sialoprotein functional domains. Mammalian BSP averages 327 amino acid residues in length, with three conserved functional regions: a collagen-binding domain (red) at the N-terminus, two poly-glutamic acid sequences (yellow) that bind and nucleate hydroxyapatite, and a C-terminus RGD integrin-binding sequence (blue) that promotes cell adhesion and signaling.
is highly conserved across mammalian species (Tye et al., 2005). This domain relies on hydrophobic interactions to bind collagen with high affinity, but also uses electrostatic interactions outside of the domain to form an initial low affinity complex (Fujisawa and Kuboki, 1992; Tye et al., 2005). Amino acid residues inside the collagen-binding domain do not undergo any post-translational phosphorylation or glycosylation (Wuttke et al., 2001; Zaia et al., 2001), and the collagen-binding ability of native versus recombinant BSP was equal suggesting that post-translational modifications do not play a role (Tye et al., 2005). In the collagen matrix, BSP tends to congregate in the hole zones of type I collagen fibrils that are associated with initial HA formation (as previously mentioned), but it is unclear whether this is due to sequence recognition or regional topography (Fujisawa et al., 1995).

The cell-binding ability of BSP is due to the RGD domain located at the C-terminus (Oldberg et al., 1988), with assistance from adjacent tyrosine-rich sequences (Stubbs et al., 1997). Cell attachment is mediated via RGD recognition by the αvβ3 integrin (Oldberg et al., 1988). This receptor is found on mature osteoblasts (Prince et al., 2001) as well as osteoblast precursors where BSP binding promotes differentiation (Zhou et al., 1995; Cooper et al., 1998). If the RGD sequence is mutated the integrin-binding ability to osteoblasts is significantly lessened, whereas BSP overexpression in primary osteoblasts in vitro resulted in a rise in osteoblast markers and mineral formation (Gordon et al., 2007). The αvβ3 integrin receptor has also been identified on osteoclasts (Miyuachi et al., 1991; Helfrich et al., 1992; Grano et al., 1994), and BSP binding is thought to induce bone resorption in vitro (Chenu et al., 1994; Raynal et al., 1996; Valverde et al., 2008). Additionally, RGD recognition of the αvβ3 integrin on osteoclast precursor cells promotes osteoclastogenesis (Valverde et al, 2005).
### 1.5.3 Tissue Expression

BSP expression occurs predominantly in mineralizing tissues (Oldberg et al., 1988). For instance, expression of BSP has been observed in osteoblasts (Bianco et al., 1991; Chen et al., 1991), osteoclasts (Bianco et al., 1991; Arai et al., 1995), osteocytes (Shapiro et al., 1993), hypertrophic chondrocytes (Bianco et al., 1991), cementoblasts (D’Errico et al., 1997; Foster et al., 2013), and odontoblasts (Chen et al., 1992). BSP expression in osteoblasts was detected in the Golgi apparatus using electron microscopy (Bianco et al., 1993), and Bianco et al. and Chen et al. determined that differentiation of osteoblasts induces expression as no mRNA was detected in osteoprogenitor cells (Bianco et al., 1991; Chen et al., 1991). The only non-mineralized sources observed to express BSP are trophoblasts in human placenta (Bianco et al., 1991), human platelets (Chenu and Delmas, 1992), and the salivary glands in humans and mice (Ogbureke and Fisher, 2004). Interestingly, BSP is highly expressed in certain cancers, such as breast and prostate, that metastasize to bone (Waltregny et al., 2000). Expression appears to be elevated in developing bone (Bianco et al., 1991, 1993; Chen et al., 1991, 1992), where mineralization ensnares the protein within the HA matrix giving it a homogeneous distribution (Bianco et al., 1993; Riminucci et al., 1995; Wuttke et al., 2001). During development, BSP is found at higher concentrations at sites of *de novo* bone formation (Chen et al., 1992; Shapiro et al., 1993; Riminucci et al., 1995) but mRNA expression markedly decreases once the tissue is fully developed (Chen et al., 1992).

### 1.5.4 In Vivo Function

Both *in vivo* and *in vitro* studies on BSP have tied its main function to HA formation in mineralized tissues. The HA nucleating ability of BSP is postulated to be due to the attraction of calcium ions (Ca$^{2+}$) to the acidic poly-glutamic acid regions, which have previously been associated with HA nucleation (Hunter and Goldberg 1993, 1994). As BSP interacts with positively charged calcium
ions, negatively charged phosphate ions are attracted and HA formation can begin. Interestingly, the phosphorylation of a serine residue (Ser-136) near the poly-glutamic acid sequences has been determined to enhance the HA nucleating ability of BSP (Baht et al., 2010).

The \textit{in vivo} function of BSP has been explored, with many studies completed on transgenic mice. Mice with an ablated $Bsp$ gene ($Bsp^{-/-}$) experienced a decrease in long bone length, thinner cortical bones, and an overall undermineralization of the skeleton when compared to their wild-type counterparts (Malaval et al., 2008). Additionally, $Bsp^{-/-}$ mice had greater trabecular bone volume, lower rate of bone formation, and decreased osteoclast activity, which suggests a decrease in osteoclast-mediated bone resorption (Malaval et al., 2008; Boudiffa et al., 2010). Malaval et al. also demonstrated a significant delay in the repair process for femur cortical defects in $Bsp^{-/-}$ mice compared to wild-type (Malaval et al., 2009). Investigating endochondral development during gestation, Holm et al. reported that $Bsp^{-/-}$ mice at embryonic day 15.5 exhibited a delay in ossification of tibiae and relative undermineralization, which was also displayed at birth (Holm et al., 2015).

BSP has also been associated with the development of periodontal tissues. Specifically, BSP has been shown to be present in alveolar bone, and in acellular cementum (Somerman et al., 1990). BSP is believed to play a crucial role in acellular cementum development, a tissue involved with the anchoring of teeth to alveolar bone. Mice lacking BSP experienced a defect in acellular cementum leading to a disruption of the periodontal ligament (PDL) attachment and corresponding increase in tooth and alveolar bone degradation (Foster et al., 2013) accompanied with incisor malocclusion (Soenjaya et al., 2015). The \textit{in vivo} function of BSP has also been investigated in the
enthesis tissue at the bone-tendon interface. In comparison to wild-type mice, the patellar tendon of 15 week-old $Bsp^{-/-}$ mice was determined to be weaker (Marinovich et al., 2016). The development and anatomy of periodontal and enthesis tissues will be further explained.

1.6 Murine Tooth Development

Murine tooth development has been comprehensively defined leading to four distinguishable stages; epithelial thickening, bud, cap, and bell (Caton and Tucker, 2009). In 1953, Lefkowitz et al. histologically examined and characterized embryonic odontogenesis in rats (Lefkowitz et al., 1953) as did Nagai et al. in 1976 (Nagai et al., 1976). The first indication of tooth development in rats occurs at embryonic day 12 (E12) with the formation of the oral groove. By E14 there is formation of the dental lamina, which is evident from the slight invagination accompanied with cell proliferation and epithelial tissue thickening. The invagination continues further into the mesenchyme on E15, and by E16 the development has entered the bud stage. At this point, there are proliferating fibroblasts derived from neural crest mesenchyme that condense around the lamina bud with higher concentration at the base. By E17, the development has progressed to the early cap stage, with the full cap forming by E18. A key feature of this stage is the presence of a BMP4-regulated transient structure of condensed epithelial cells in the center of the cap termed the enamel knot (Jernvall et al., 1998). The enamel knot cells are no longer proliferating and direct the movement of nearby proliferating cells to further develop the cap (Jernvall et al., 1994; Vahtokari et al., 1996). Once the cap is formed, the enamel knot cells undergo apoptosis. The condensed mesenchymal cells of the cap develop into the dental papilla, which eventually become the dentin and pulp in a mature tooth, and the epithelial cells above form the enamel organ. The enamel organ and dental papilla are enveloped by the dental follicle derived from surrounding mesenchymal cells. All together, they are termed the tooth germ.
From E17 to the bell stage at E21, the cells of the tooth germ differentiate. The enamel organ contains two epithelial layers – the outer enamel epithelium (OEE) and the inner enamel epithelium (IEE). The IEE cells develop into ameloblasts at E20 (Hoffman and Schour, 1940) that are responsible for the formation of enamel. The cells at the center of the dental papilla form the dental pulp, while the surrounding cells become odontoblasts which initiate dentinogenesis to produce dentin. The mesenchymal cells of the dental follicle differentiate to various lineages, such as cementoblasts, PDL fibroblasts, and osteoblasts. These cells give rise to the cementum, PDL, and alveolar bone respectively. Following birth, rat tooth development continues for roughly 42 days when all molars have fully erupted (Shaw et al., 1950).

### 1.6.1 Murine Tooth Anatomy

A mature tooth is composed of four dental tissues: dental pulp, enamel, dentin, and cementum (Figure 1.3). Dental pulp is the only non-mineralized tissue of the four but plays an important role in both development and tissue maintenance. This connective tissue enters at the apical portion of the tooth root and contains nerves and blood vessels. Odontoblasts are found on the surface layer of the dental pulp and are involved with the formation and maintenance of dentin (Yoshida and Ohshima, 1996). Dentin is a mineralized tissue that separates the pulp chamber from the cementum on the tooth root and enamel at the crown. In comparison, dentin is more mineralized than bone or cementum but less mineralized than enamel (Goldberg et al., 2011). The mineral phase is composed of hydroxyapatite and comprises 70% of the tissue by weight, compared to 20% from the organic matrix and remaining 10% from water (Tjaderhane et al., 2009). A unique characteristic of dentin is the presence of dentinal tubules, which are channels that extend from the pulp chamber to the dentinoenamel and dentinocemental junctions. The ECM of dentin is
comprised of collagen, accounting for 90% of the matrix, and non-collagenous proteins (Goldberg et al., 2011).

Cementum is an avascular mineralized tissue that covers the tooth root and functions to attach the tooth to the adjacent PDL, which in turn anchors to the alveolar bone. This ensures the tooth is adequately supported and the forces of mastication can be transferred from tooth to bone (Foster et al., 2013). Both the mineral and organic components of cementum are formed by cementoblasts. The mineral component, hydroxyapatite, comprises roughly 50% of the tissue while the organic matrix and water make up the other half (Goncalves et al., 2005). There are two forms of cementum found on the tooth – cellular and acellular cementum. Cellular intrinsic fiber cementum covers the apical portion of the root and functions to hold the tooth in the proper position and orientation, as well as aid in repair (Bosshardt and Selvig, 1997). The cellular cementum matrix contains embedded cementocytes, cementoblasts that got entrapped within the mineral. The organic matrix is primarily type I collagen, with small amounts of type III collagen and non-collagenous proteins.

Acellular extrinsic fiber cementum covers 40-70% of the cervical portion of the root (Goncalves et al., 2005) and increases in thickness with age. A major function of this cementum is to attach the tooth to the PDL. It accomplishes this through densely packed collagen fibers that are arranged perpendicular to the surface of the tooth (Yamamoto et al., 2015). These fibers are termed Sharpey’s fibers when they elongate and become continuous with the principal collagen fibers of the PDL. The organic matrix of acellular cementum is similar to cellular cementum, but with higher concentrations of the non-collagenous proteins bone sialoprotein (BSP) and osteopontin (OPN). Both proteins promote the differentiation of pre-cementoblasts to mature cementoblasts.
Figure 1.3 Schematic of murine molar tooth cross section. This schematic image is modeled after a coronal cross section of a murine first mandibular molar. The non-mineralized soft tissues of the periodontium are the gingiva, pulp chamber, and the periodontal ligament. The mineralized tissues of the periodontium are colour coded: enamel (blue), dentin (yellow), acellular and cellular cementum (green), and alveolar bone (orange). Figure obtained from (Foster, 2012).
(Saygin et al., 2000), while BSP is involved in cell binding and hydroxyapatite formation (Somerman et al., 1990; Foster et al., 2013)

BSP was first shown to be present in cementum tissue in 1990 (Somerman et al., 1990), and has since had its role investigated in vivo using a Bsp−/− mouse animal model. As previously mentioned, BSP localizes to the alveolar bone and cementum of wild-type animals (Figure 1.4). Foster et al. discovered using a scanning electron microscope that the acellular cementum of Bsp−/− mice was not mineralized, and this was supported with histological analysis with Goldner’s trichome staining, indicating that the acellular cementum does not mineralize in the absence of BSP (Foster et al., 2013). Bsp−/− mice fed a standard hard food diet experienced a significant underdevelopment as early as 14 days post-natal (dpn), as noted by reduction in the thickness of the acellular cementum analyzed via histomorphometry (Foster et al., 2013). Structural defects in the acellular cementum of Bsp−/− animals led to a disruption in the molar acellular cementum-PDL interface, where disorganization of the PDL collagen matrix was seen at 26 dpn, and by 60 dpn all Bsp−/− mice experienced detachment of the PDL from the acellular cementum, a phenotype not seen in wild-type counterparts (Foster et al., 2013).

The detachment and disorganization of the PDL resulted in other consequential defects. Improper attachment of the PDL led to the resorption of both tooth root and alveolar bone, and significant gingival epithelial downgrowth in Bsp−/− mice at 60 dpn (Foster et al., 2013). Resorption in Bsp−/− animals coincided with a significant increase in osteoclast-like cells (Foster et al., 2013). Soenjaya et al. determined that the periodontal defects experienced in the Bsp−/− mice were not due to the hard food diet, as 20 week-old Bsp−/− mice on soft food diet had similar phenotype severity as those
Figure 1.4 Bone sialoprotein localization in mouse periodontal tissues. BSP (brown staining) localizes to two mineralized tissues in the periodontium, the alveolar bone (AB) and the acellular cementum (AC), which are attached via the periodontal ligament (PDL). Image obtained from (Foster, 2012).
on hard diet, such as alveolar bone and molar tooth root resorption, PDL detachment and disorganization, and downgrowth of the gingival epithelium (Soenjaya et al., 2015). Regardless of diet, alveolar bone resorption resulted in an increase in exposure of the tooth root (Soenjaya et al., 2015). The absence of BSP also caused a reduction in the thickness of cementum found on the incisor of Bsp<sup>-/-</sup> mice, which resulted in an increased rate of malocclusion (Foster et al., 2013). Additionally, the incisors of Bsp<sup>-/-</sup> mice erupted significantly slower than wild-type (Soenjaya et al., 2015). Incisor malocclusion is more likely to be severe in Bsp<sup>-/-</sup> mice on hard diet, but those on soft diet still experienced malocclusion to a degree (Soenjaya et al., 2015).

1.7 Enthesis Development

The enthesis, also known as the osteotendinous junction, is an important tissue that serves to attach tendons and ligaments to bone (Benjamin et al., 2002). The development of the enthesis occurs independently from bone and tendon development, which allows for proper growth of each tissue type prior to their integration via the enthesis. Since the enthesis is a transition from soft to hard tissue, it would make sense that its development would incorporate factors associated with both tendon and bone development, and its independent development would ensure that there is no miscommunication between the two tissue types.

In tissue, tendons and ligaments tend to insert into prominences on the bone surface called bone eminences. The eminences form after the cartilage template of the long bone is constructed, where they develop from a pool of progenitors that attach to the anlage (Zelzer et al., 2014). This pool of progenitors expresses not only Sox9, a regulator of chondrocyte development (Akiyama et al., 2002), but also expresses scleraxis (Scx; Blitz et al., 2013; Sugimoto et al., 2013), a regulator of tenocyte differentiation (Cserjesi et al., 1995; Schweitzer et al., 2001). Zelzer <em>et al.</em> have proposed
a segregation model for enthesis formation involving the modularity of the tissue (Zelzer et al., 2014). This model suggests that the tissue develops from a common pool of progenitor cells which differentiate into either *Scx*-positive tenocytes or *Sox9*-positive chondrocytes.

In addition to the aforementioned signalling molecules, both bone morphogenic protein 4 (BMP4) and transforming growth factor β (TGFβ) play a role in bone eminence formation and enthesis development. Blitz et al. discovered that the absence of *Bmp4* in mesenchyme resulted in a lack of differentiation of eminence progenitors, but when under regulation of *Scx*, *Bmp4* promoted eminence formation at the enthesis (Blitz et al., 2013). The TGFβ signalling pathway has been connected to the differentiation of bone eminence progenitors, where the mesenchyme of *TgfβrII* conditional knockout mice lacked specified progenitors (Blitz et al., 2013). TGFβ is also responsible for tenocyte differentiation and tendon development (Pryce et al., 2009).

Attachment of tendon to bone involves the mineralization of the enthesis, which likely occurs in a process similar to the growth plate. Signaling factors found within the growth plate have also been linked to the developing enthesis, such as Indian hedgehog (IHH) and parathyroid hormone-related protein (PTHRP) (Fujioka et al., 1997; Zelzer et al., 2014). IHH is a signaling molecule involved with endochondral ossification regulation by stimulating chondrocyte proliferation and is postulated to direct enthesis mineralization. The IHH signaling pathway is a negatively regulated feedback loop where the end product, PTHrP, blocks expression of IHH in order to provide tight command of cartilage development. If PTHrP is removed from SCX-expressing cells, PTHrP-direction of osteoclasts is no longer achieved and proper enthesis attachment does not occur (Wang et al., 2013)
Skeletal development relies on mechanical loading and physical cues, including the enthesis. In the absence of muscle forces bone defects become apparent, including deformities in size, shape, and mineralization (Mikic et al. 2000; Gomez et al., 2007; Sharir et al., 2011). With regards to the enthesis, the bone eminences to which they insert also rely on muscle forces. Eminences still form with a lack of mechanical loading but they are undersized and underdeveloped, indicating the importance on the growth and proliferation of cells in the bone eminence (Blitz et al., 2009; Kahn et al., 2009). Muscle loading also plays a role in fibrocartilage formation and enthesis mineralization, as Thomopolous et al. found no fibrocartilage and a decrease in mineral in the supraspinatus tendon enthesis following eight weeks of muscle paralysis (Thomopoulous et al., 2007), which impairs the loading ability of the tissue.

1.7.1 Enthesis Anatomy

The main function of the enthesis is to transmit contractile forces from skeletal muscle to bone while also providing resistance to static and dynamic loads, which in turn allows for structured and controlled movements. Since the tissue can experience high mechanical stress, entheses injuries are fairly common and have been given terms such as tennis elbow and jumpers’ knee (Benjamin et al., 2006), but more serious injuries can result in the detachment of ligament or tendon at the bone interface. The structure and properties of the enthesis depends on its location, leading to the classification of two broad categories: the fibrous and fibrocartilaginous enthesis (Benjamin et al., 1995; Benjamin & McGonagle, 2001).

The fibrous enthesis, which has also been referred to as the diaphyseal-periosteal attachment, attaches to bone in two manners: directly or indirectly via the periosteum (Benjamin et al., 2002). Tendons that insert into bone through the periosteum tend to be much shorter and have a limited
ability to stretch. Fibrous entheses in the extremities insert into the diaphysis of long bones (Benjamin et al., 1986) but are also found in the vertebral column. Entheses of this nature are understudied as they do not undergo the same overuse injuries that are common with fibrocartilaginous entheses, and thus are not as clinically significant.

The fibrocartilaginous enthes, in comparison, is a more common and well-studied tissue. Unlike the fibrous enthes, the fibrocartilaginous enthes undergoes chondrogenesis during its development to produce a fibrocartilage tissue that provides a transition from tendon to bone. This thesis will focus on the fibrocartilaginous enthes that attaches the quadriceps femoris tendon (QCT) to the patella (Figure 1.5). Benjamin et al. identified four zones that are pivotal in the transition of tendinous to bony tissue: tendon, uncalcified fibrocartilage, calcified fibrocartilage, and bone (Figure 1.6; Benjamin et al., 1986).

The first zone is the tendon, a dense and fibrous connective tissue composed of parallel collagen fibers that run along the longitudinal axis. The primary cell type is tenocytes, which are spindle-shaped fibroblast-like cells that function to develop, maintain, and repair the tendon tissue (James et al., 2008). The extracellular matrix of tendons contains collagen, proteoglycans (e.g. decorin, biglycan, and versican), and elastin, among other less abundant non-collagenous components (Waggett et al., 1998; Screen et al., 2015). For collagen, type I is the predominant form found in tendons with lesser amounts of type III.

The second zone is an avascular region of unmineralized tissue referred to as the uncalcified fibrocartilage (UFC). Fibrocartilage cells of the UFC differ from tenocytes in that they increase in
size, become more spherical, and lose direct contact with neighbouring cells (Ralphs et al., 1998; Benjamin et al., 2006). Lack of direct communication ensures that osteocytes in the bone do not communicate with tenocytes of the tendon, postulated to prevent bone spur formation in the tendon or decrease the potential of infection spreading between tendon and bone (Ralphs et al., 1998; reviewed in Apostolakos et al., 2014). Unlike the tendon, the most abundant form of collagen in the UFC is type II, with lesser amounts of type I and III. The ECM of the UFC contains the proteoglycans decorin, biglycan, fibromodulin, and aggrecan (Waggett et al., 1998). Aggrecan is a crucial component of the ECM as it draws water into the tissue, allowing it to better resist compressive loading and dissipate stress, especially during bending away from bone. Entheses that have a larger range of insertion angle have a larger UFC zone to compensate for the added stress (Evans et al., 1990; Benjamin et al., 1991).

The second zone (UFC) is separated from the third zone, the calcified fibrocartilage (CFC), by a boundary referred to as the tidemark. This basophilic feature is representative of the divide between the mineralized and unmineralized regions of fibrocartilage, often called the mineralization or calcification front, and forms in a relatively straight manner (Benjamin et al., 2002). The transition to mineral occurs as a gradient, but the length of the gradient appears to vary depending on the organism and enthesis of study (Wopenka et al., 2008; Schwartz et al., 2012). Compared to the other tissues of the enthesis, the CFC has fewer cells. Type I collagen fibrils continue from the tendon through to the CFC but type II collagen is far more abundant with lesser amounts of type X collagen present (Fujioka et a., 1997). The CFC contains markedly less decorin than the tendon and UFC, but has strong expression of aggrecan (Waggett et al., 1998). As previously mentioned, this allows the tissue to resist compressive forces.
Figure 1.5 Anatomical location of notable fibrocartilaginous entheses. Anatomical schematics of the (A; adapted from Flandry and Hommel, 2011) enthesis at the insertion of the quadriceps femoris tendon into the back of the patella of the knee and the (B; adapted from Thomas et al., 2016) enthesis at the insertion of the supraspinatus tendon into the head of the humerus in the shoulder. Enthesis location in both are highlighted with a red circle.
Figure 1.6 Transitional zones of the fibrocartilaginous enthesis. The structure of the fibrocartilaginous enthesis represented (A) schematically and (B) histologically. The fibrocartilaginous enthesis plays an important role at the tendon-bone interface. As the tendon transitions into bone it passes through a region of fibrocartilage that is separated into uncalcified fibrocartilage (UFC) and calcified fibrocartilage (CFC). The tendon is primarily type I collagen, while the fibrocartilage is primarily type II. Separating the UFC from the CFC is the tidemark, which represents the boundary between unmineralized and mineralized tissue. Scale bar = 100 µm. Histological image is of the fibrocartilaginous enthesis of the mouse supraspinatus tendon of the shoulder, obtained from (Marinovich et al., 2016).
A

Tendon

Uncalcified Fibrocartilage (UFC)

Calcified Fibrocartilage (CFC)

Bone

B

Tendons

UFC

CFC

Tidemark

Bone
The fourth zone found in the fibrocartilaginous enthesis is subchondral bone. Conversely to the tidemark, the interface between the CFC and the underlying bone is uneven which promotes a secure and stable junction (Benjamin and Ralphs, 1998; Milz et al., 2002). Since BSP is involved in tissue mineralization, it is possibly involved with the calcification of the CFC.

As previously mentioned, the role of BSP in entheses has been investigated in the $Bsp^{-/-}$ mouse model. The following findings are from the work completed by Marinovich et al. in 2016 (Marinovich et al., 2016). BSP, along with OPN, were determined via immunohistochemistry (IHC) to be present in mineralized regions of the fibrocartilaginous entheses of the QCT in wild-type animals. A notable difference between $Bsp^{-/-}$ and wild-type animals was the elongation of the CFC zone of $Bsp^{-/-}$ mice, a 28% increase over their wild-type counterparts. Interestingly, both von Kossa staining and micro computed tomography (microCT) analysis suggested that there is no observable difference in mineral content of wild-type and $Bsp^{-/-}$ entheses. This finding was confirmed using Raman spectroscopy, a technique that analyzes the relative amount of chemical bonds present. The intensity of P-O bonds found in hydroxyapatite was compared to the intensity of C-H bonds in the organic matrix, and the data was consistent with the previous findings that mineral content of the $Bsp^{-/-}$ enthesis is normal.

BSP loss in the acellular cementum resulted in disruption of the PDL, but in contrast, the absence of BSP in the QCT enthesis did not affect the collagen organization of the tendon. As indicated previously, the patellar tendon, which serves to attach the patella to the tibial tuberosity, of $Bsp^{-/-}$ mice was significantly weaker than wild-type animals. The cross-sectional area of this tendon was found to be significantly larger in $Bsp^{-/-}$ mice by 7.5%, but both wild-type and $Bsp^{-/-}$ entheses
required the same load to induce tendon failure. The ultimate strength of the tendon, which is the load at failure per cross sectional area, was 16.5% lower in $Bsp^{-/-}$ mice, which indicates that in the absence of BSP the patellar tendon is weakened.

1.8 Purpose of Thesis

With ample investigations demonstrating defects of mineralized tissues in the $Bsp^{-/-}$ mouse animal model, we chose to explore the same tissues in the novel $Bsp^{-/-}$ rat. The rat provides worthwhile benefits over the mouse model that have the potential to advance the field of research on BSP, largely due to the considerable size difference between the two models. The first benefit regards the culturing of osteoblasts through calvarial extraction, where rats have a larger calvaria and provide higher cell counts (Orriss et al., 2012). Additionally, rat osteogenic cells in standard culture conditions thrive better than mouse and extracted osteoblasts are capable of producing mineralized nodules in 14 days compared to the 21 days in mice (Orriss et al., 2014). The second benefit is the use of the rat model for studies on osteoarthritis (OA). Current studies use the mouse model and tend to induce OA through surgical destabilization of the medial meniscus (DMM) or anterior cruciate ligament transection (ACLT; Glasson et al., 2007). ACLT surgery is more common amongst OA studies in other animal models but is difficult in mice due to their size and is not recommended as it produces a severe form of OA with possible subchondral bone erosion (Glasson et al., 2007). DMM surgery has produced more reliable results in mice but even with an adequate surgical technique, mice have a thinner articular cartilage than rats which makes it difficult to track the progression of OA (Teeple et al., 2013). A third benefit is the ease of studying the phenotypes of periodontal and enthesis tissues, as the larger size of the rat makes it easier to harvest and analyze.
Prior to reaping the benefits that the $Bsp^{-/-}$ rat model provides, it is considerably less studied than the mouse model in the context of BSP, and therefore the well-studied phenotypes of the $Bsp^{-/-}$ mouse must be defined in the rat. The objective of this thesis is to characterize the $Bsp^{-/-}$ rat phenotypes. Given the observed phenotypes in the $Bsp^{-/-}$ mouse, it is hypothesized that the $Bsp^{-/-}$ rat will experience defects in acellular cementum formation and subsequent disorganization of the periodontal tissues. Furthermore, this study will aim to understand and characterize the development and mineralization of the enthesis at the junction of the QCT and patella in the absence of $Bsp$. Lastly, it is hypothesized that there will be a delay in the ossification and development of the tibia in the $Bsp^{-/-}$ rat. Overall, it is hypothesized that BSP is required for the proper development of periodontal tissues, fibrocartilaginous entheses, and long bone. We hope that investigations on the phenotypes of the $Bsp^{-/-}$ rat will add to the previous knowledge of the $Bsp^{-/-}$ mouse, as well as determine whether the $Bsp^{-/-}$ rat is a viable animal model for future research.
1.9 References


CHAPTER TWO

CHARACTERIZATION OF THE RAT BONE SIALOPROTEIN KNOCKOUT PHENOTYPE
2.1 Materials and Methods

2.1.1 Animals

Animal care and all procedures followed guidelines of the Canadian Council on Animal Care and Animal Care and Veterinary Services at the University of Western Ontario (Approved protocol 2016-070; London, Canada). The Bsp\(^{-/-}\) rats were generated by SAGE Labs Inc using CRISPR/Cas9 genome editing technology. Rats used in the study were fed (Tekland Global 18% protein Harlan Laboratories, Indianapolis, IN, USA) either in the form of soft powder (soft diet) or hard pellets (hard diet) and were provided tap water ad libitum. All rats used in the study were bred from heterozygous parents and offspring were litter-matched for analysis.

Genotyping of Bsp\(^{-/-}\) and wild-type Sprague Dawley rats was performed on DNA isolated from ear tissue clippings using polymerase chain reaction (PCR) and restriction digest, followed by gel electrophoresis and visualized under ultraviolet light. Briefly, extracted DNA was combined with primers and mixed into FastMix Frenche PCR (i-StarTaq) tubes (iNtRON Biotechnology) before undergoing PCR. The resulting solution then was digested with BseRI (New England BioLabs) and run on a 1% agarose gel prior to visualization.

The experimental endpoints depend on the tissue being investigated but range from day 0.5 to 50 weeks and included both male and female animals. The use of both males and females was necessary in order to reach the appropriate sample sizes in all but the 50 week endpoint (all males). Timepoints for the periodontal tissues included 20 weeks (n = 9) and 50 weeks (n = 9). Enthesis tissue development was studied at 14 days (n = 8), 28 days (n = 9) and 14 weeks (n = 8). For endochondral ossification, the timepoint studied was 0.5 days post-natal (P0.5; n = 4 for mineral quantification, n = 7 for length measurements). Animals harvested at P0.5 were sacrificed with
decapitation, while all other endpoints were sacrificed by CO$_2$ asphyxiation as per the University of Western Ontario Animal Care Committee (ACC) protocol. Prior to tissue harvest, rats were weighed and the data was statistically analyzed in GraphPad Prism 6.0 using unpaired parametric t-tests (GraphPad Software, La Jolla, CA).

2.1.2 Sanger DNA Sequencing

The preparation of DNA for Sanger sequencing to confirm $Bsp$ gene mutation involved a combination of ethanol precipitation, PCR and subsequent product purification. Briefly, sample DNA was precipitated with an ethanol and salt solution, centrifuged, and resuspended in a Tris-EDTA (TE) buffer. Samples then underwent PCR to amplify DNA, which was confirmed by running the product on a 1.5% agarose gel. Using the reagents and protocol provided in the PureLink DNA Purification Kit (Thermo Fisher Scientific), samples were purified using a spin column and then diluted to the appropriate concentration needed for sequencing by the London Regional Genomics Center at the Robarts Research Institute in London, Ontario, Canada.

2.1.3 Histology and Immunohistochemistry for Periodontal Tissues

$Bsp^{-/-}$ and wild-type mandibles were harvested from 20 and 50 week-old rats and prepared for histological analysis as previously described with modifications (Foster, 2012). Briefly, mandibles were dissected and fixed in Bouin’s solution for 48 h and then decalcified in acid-formol saline solution (AFS; glacial acetic acid, neutral buffered formalin, sodium chloride) for 14-20 days and manually tested to ensure sufficient decalcification. Tissues were rinsed for 60 min in running tap water prior to standard histological processing by University Hospital Pathology Core, London, Ontario, Canada. Processed mandibles were embedded in paraffin and sections collected at 5 µm thickness with a rotary microtome and mounted on positively charged glass slides. Tissue sections
were deparaffinized in xylene and rehydrated with a graded ethanol series prior to histological analysis.

Immunohistochemical analysis of periodontal tissue was completed using a previously described protocol (Foster et al. 2013). Deparaffinized and rehydrated samples were incubated in 3% hydrogen peroxide before blocking nonspecific binding sites with 5% goat serum in TBS-T (Tris buffered saline with 0.1% Tween 20, Sigma-Aldrich) for 60 min. Samples were then incubated at 4 °C overnight with a rabbit anti-mouse BSP serum (1:200, Renny Franceschi, University of Michigan, Ann Arbor, USA) in 5% goat serum in TBS-T. The following day, slides were washed in TBS-T before being incubated with a horseradish peroxidise-conjugated goat anti-rabbit IgG antibody diluted in 5% goat serum in TBS-T for 2 h. After another wash with TBS-T, slides were stained with hydrogen peroxide and 3,3'-diaminobenzidine (DAB; Thermo Fisher Scientific) and then dehydrated, cleared and mounted in a toluene-based mounting medium.

Tissue morphology was assessed using a hematoxylin and eosin (H&E) stain described previously (Foster et al. 2013). Sections were submerged in Harris hematoxylin (Sigma-Aldrich) for 20 s and washed with running tap water for 10 min. Sections were then stained for 4 min in Eosin Y (Sigma-Aldrich) before being washed again with running tap water for 10 min. Sections were rinsed in deionized water, dehydrated, cleared and mounted in a toluene-based mounting medium.

Alcian blue with nuclear fast red (AB-NFR) staining and toluidine blue (TB) staining were also used to assess tissue morphology and acellular cementum structure. For AB-NFR staining, slides were stained with a 1% alcian blue (Sigma-Aldrich) in acetic acid solution for 30 min and then
rinsed with running water for 5 min. Slides were then counterstained with 0.1% nuclear fast red (Sigma-Aldrich) for 5 min before being dehydrated through a graded ethanol series, cleared in xylene and mounted with a toluene-based mounting medium. For TB staining, sections were stained with 0.1% toluidine blue (Sigma-Aldrich) for 3 min. Following a rinse in deionized water, sections were dehydrated by dipping in 90% and 100% ethanol, cleared in xylene and mounted in a toluene-based mounting medium.

Picrosirius red staining was used to evaluate collagen organization where sections were stained in an aqueous solution of 0.5% Sirius red (Direct Red 80, Sigma-Aldrich) in 1.3% 2,4,6-trinitrophenol (saturated picric acid, VWR) for 60 min. Following staining, sections were rinsed in acidified water before being dehydrated, cleared, and mounted in a toluene-based mounting medium. Due to the nature of the stain, plane and circular polarized light was used to image the samples and collagen organization was characterized based on appearance (Rich and Whittaker, 2005).

2.1.4 Histomorphometry
Acellular cementum thickness was quantified using ImageJ. Briefly, 20x magnification H&E images were used to digitally measure average acellular cementum thickness. Average thickness was calculated by dividing acellular cementum area by the length of the visible dentinocemental junction. Statistical analysis was completed on GraphPad Prism 6.0 using an unpaired parametric t-test (GraphPad Software, La Jolla, CA).
2.1.5 Microcomputed Tomography

Scanning of samples was completed by Dale Fournier of the Seguin Lab at the University of Western Ontario (London, Canada). The left hemi-mandible from wild-type and Bsp−/− rats was plotted in 1% agarose gel at opposite angles in order to fit in the scanner’s field of view. Embedded mandibles were stored at 4 °C overnight to ensure fixation of the agarose gel so the specimens will not shift during scanning. Prior to scanning, samples were equilibrated to room temperature and to radiation as the scanner warmed up. Hemi-mandibles were scanned using a cone-beam X-ray microCT imaging system (eXplore Locus SP/MS-8, GE Healthcare, London, Canada). The scanner was operated at a peak tube voltage of 80 kVp, tube current of 80 mA, integration time of 3000 ms, and with a 0.5 mm aluminum filtration. A total of 900 X-ray projections were captured at 0.4 degree increments as the sample rotated a full 360 degrees. Each projection angle was averaged over four frames to improve signal-to-noise characteristics and was brightfield and darkfield corrected in preparation for reconstruction.

The 900 X-ray acquisitions were reconstructed into a single 3D volume with voxel spacing of 11.442 µm using a Feldkamp filtered back-projection algorithm based on Reconstruction Toolkit library code (Feldkamp et al., 1984; Rit et al., 2014). The raw volumes were converted into VFFs (Sun TAAC Graphic File). Following reconstruction, image volumes were linearly rescaled into Hounsfield units (HU) based on internal calibration measures of air and water. A cortical bone substitute was also included in the scans (450-SB3, Gammex RMI, Middleton, WI, USA) with a physical density of 1.8247 g/mL and electron density of 1.696 relative to water. The full size of the field of view was 2300 x 2300 x 2820 voxels which was segmented into individual volumes per mandible. Image volume was spatially rebinned to isotropic voxel spacing of 22.884 µm to
enhance signal-to-noise characteristics and for file size reduction. Lastly, in order to be compatible with analysis software, the images were converted into DICOM files using MicroView (MicroView 2.5.0-4118, Parallax Innovations, Inc., Ilderton, Canada).

Analysis of microCT images was completed by Michael Chavez of the Foster Lab at The Ohio State University (Columbus, OH, USA). DICOM images calibrated to HU at 22.884 µm voxel size were loaded into AnalyzePro 1.0 (AnalyzeDirect, Overland Park, KS, USA). Using anatomical landmarks, mandibles were re-oriented to a standard position. For the frontal orientation of molar 1 (M1), the mesial root pulp chamber was aligned perpendicular to the transverse plane. The transverse plane view of M1 was re-oriented by adding a line through the center of the mesial and distal root pulp chamber and then the line was made perpendicular to the frontal plane. Sagittal orientation of the mandible was aligned to make the occlusion parallel to the transverse plane. To segment the various mineralized tissues (bone, dentin, cementum, and enamel), semiautomatic tracing was completed. For enamel density over 5500 HU was used, and for bone, dentin, and cementum tissues over 2500 HU was used with manual correction as necessary. Alveolar bone proper (ABP), also known as bundle bone or lamina dura, is the bone most closely associated with tooth attachment and periodontal ligament (PDL) fiber insertion. Bone over 2500 HU within 200 µm of the tooth surface was labeled as ABP and relevant measurements were made. Volume, density, and density variation was obtained from AnalyzePro and then graphed and analyzed in GraphPad Prism 6.0 with unpaired parametric t-tests (GraphPad Software, La Jolla, CA).

2.1.6 Histology and Immunohistochemistry for Enthesis Tissues

Bsp<sup>-/-</sup> and wild-type knee joints were harvested from 14 day, 28 day, and 14 week-old rats and prepared for histological analysis as previously described with modifications (Wang et al., 2007;
Marinovich et al., 2016). In brief, rats were euthanized and dissected knee joints were fixed in 4% paraformaldehyde for 48 h at room temperature prior to decalcification in Formical-2000 (StatLab). Manual testing was used to assess decalcification, but on average 14 and 28 day-old knee joints were decalcified for four days while 14 week-old knee joints required 10 days. Tissues were processed and prepared for histological analysis as previously described in Section 2.1.3.

Immunohistochemical analysis of enthesis tissue was completed using a previously described protocol with modifications (Foster et al. 2013). The protocol followed the one presented in Section 2.1.3 with the addition of staining for osteopontin (OPN) using the LF-175 rabbit anti-mouse OPN serum (1:200, Larry Fisher, National Institute of Dental and Craniofacial Research, Bethesda, Maryland, USA).

Whole-tissue von Kossa staining was performed to assess tissue mineralization as follows. Following tissue fixation, samples were incubated in a 2% silver nitrate solution for 48 h. Silver ions were precipitated by placing tissue samples in a reducing solution containing 0.6 M sodium hypophosphite for 48 h, and then another 24 h in 5% sodium hypophosphite, effectively leaving behind a black precipitate where calcium was present in the hydroxyapatite of mineralized tissue. Decalcification of the tissues was performed using Formical-2000 for 24 to 48 h and confirmed with manual testing. Samples were histologically processed, sectioned, and prepared for analysis as previously described. Sections were subsequently incubated in a 5% sodium thiosulphate solution for 5 min to remove unbound silver, rinsed with deionized water and then counterstained with 0.04% toluidine blue in 0.2 M acetate buffer solution for 30 min. After staining, samples were dried at 37 °C before being cleared in xylene and mounted in a toluene-based mounting medium.
Tissue morphology was assessed using an H&E stain. Sections were submerged in Harris Hematoxylin (Sigma-Aldrich) for 10 seconds, washed with running tap water for 10 min, rinsed with deionized water and stained for 1 min in Eosin Y (Sigma-Aldrich). Sections were rinsed again in deionized water, dehydrated, cleared and mounted in a toluene-based mounting medium. A second H&E protocol outlined in Section 2.1.3 was also performed. TB was used as an additional method to assess morphology and was performed as described in Section 2.1.3.

Cartilage structure was assessed using Safranin O with fast green staining. Sections were first stained with 0.02% fast green for 25 min, dipped in 1% glacial acetic acid and then stained in 0.1% Safranin O for 7 min. Sections were dehydrated, cleared and mounted in a toluene-based mounting medium.

Picosirius red staining of the enthesis tissue was used to assess the structure and organization of collagen in the QCT and was performed as described previously in Section 2.1.3.

Histomorphometric analysis of CFC length was performed as described previously with adaptations (Marinovich et al., 2016). Briefly, the CFC was measured by drawing three parallel lines along the orientation of the collagen fibers from the visible tidemark to the bone-CFC junction (identified by the irregular boundary) in a blinded manner. The triplicate length measurements were averaged prior to wild-type and Bsp<sup>-/-</sup> comparison using a parametric unpaired t-test.
2.1.7 Histology and Digital Analysis for Tibiae

Bsp\textsuperscript{−/−} and wild-type tibiae were harvested from 0.5 day-old rats (P0.5) and prepared for histological analysis as previously described with minor modifications (Wang et al., 2007; Usmani et al., 2012; Holm et al., 2015). In brief, rats were euthanized and dissected tibiae were fixed in neutral buffered 10\% formalin (Sigma-Aldrich) at 4 °C for 48 h. Tissues were processed and prepared for histological analysis as previously described in Section 2.1.3.

Von Kossa staining was performed to detect mineral content using a previously established protocol (Holm et al., 2015). Sections were incubated in 1\% silver nitrate under ultraviolet light for 30 min. Unbound silver was removed by incubating slides in 5\% sodium thiosulfate for 5 min. Finally, slides were stained with 1\% w/v alcian blue in 3\% acetic acid for 20 min before counterstaining with 0.1\% nuclear fast red for 5 min. Absolute mineral quantification was completed with pixel counting of von Kossa stain in trabecular and cortical bone using ImageJ. Additionally, ImageJ was used to digitally measure the length from images of tibiae. Statistical analysis was performed using an unpaired parametric t-test on GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

2.1.8 Histological analysis

Visual histological analysis was completed by comparing stained wild-type and Bsp\textsuperscript{−/−} rat tissue slides. A slide with 3-4 sections was selected from all samples at each time point for the purpose of staining. Following staining, images of each section on the slide were taken and the most representative image was used to compare wild-type to Bsp\textsuperscript{−/−} tissues. Staining of additional slides was completed if previously stained slides had a diminished quality. Tissue comparison was completed by aligning images and visually inspecting for noteworthy discrepancies or trends.
Images were further examined by Dr. Harvey Goldberg (University of Western Ontario) and if a histological difference was observed then additional investigation was completed using histomorphometry.

2.1.9 Statistical analysis

All quantitative data expressed as mean ± standard error and statistical analysis performed with an unpaired parametric t-test on GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).
2.2 Results

2.2.1 Confirmation of Bsp<sup>−/−</sup> rat knockout status

Prior studies on Bsp<sup>−/−</sup> mice have found significant phenotypes in the periodontal and enthesis tissues, as well as defects with long bone formation. Although these phenotypes are well described in the mouse model, a Bsp<sup>−/−</sup> rat was generated by SAGE Labs Inc using CRISPR/Cas9 genome editing to explore the phenotypes and to further our understanding of the role of BSP in mineralized tissues using the benefits of a rat model. DNA isolated from ear clippings of the Bsp<sup>−/−</sup> rat was sequenced by the London Regional Genomics Center (London, Ontario, Canada), which confirmed the knockout status of the model with an 8bp deletion 1 bp “A” insertion, which was absent in wild-type animals (n=8; Figure 2.1 A-B). The mutation occurred at location 3560-3567 in the genomic sequence and replaced a “TCTGAAAC” with a single “A” insertion, which matched the data provided by SAGE Labs Inc founders report. Additional confirmation of the knockout status was completed using quantitative PCR (qPCR) by Ashwin Baskaran, where no gene transcript was detected in calvarial extracts of Bsp<sup>−/−</sup> rats (Figure 2.1 C).

2.2.2 The absence of bone sialoprotein in Bsp<sup>−/−</sup> rats does not appear to affect body weight

Previous studies on the Bsp<sup>−/−</sup> mouse by the Malaval group have shown a significant difference between wild-type and Bsp<sup>−/−</sup> mice in body weight (Malaval et al., 2008; Bouleftour et al., 2014). However, other studies have shown the type of diet has an effect. Whereas Bsp<sup>−/−</sup> mice on hard diet weighed significantly less than their wild-type counterparts, mice fed only a soft diet showed no significant differences in weight (Soenjaya et al., 2015). Rat body weight was recorded prior to tissue harvest, and at all experimental timepoints, there were no significant differences observed in the weight of wild-type and Bsp<sup>−/−</sup> rat littermates (Figure 2.2). Rats at the 20-week timepoint were raised on a hard diet while all other timepoints were raised on soft diet.
Figure 2.1 Features of the $Bsp^{\text{+/--}}$ rat. A) BSP protein structure with locations of key regions: collagen-binding domain (red), two poly-glutamic acid sequences (yellow), RGD integrin-binding sequence (blue), and sgRNA target site (black). B) Section of wild-type mRNA showing the 8 bp deletion with 1 bp “A” insertion (blue; insertion not shown) at location 3560-3567 in the genomic sequence with sgRNA target site highlighted (teal). C) qPCR for $lbsp$ RNA isolated from cortical leg bones of wild-type and $Bsp^{\text{+/--}}$ rats at 28 days. D) Simulated agarose gel (1%) showing the results of the method used to determine the genotype of rats from ear clippings.
Wild Type mRNA showing the location of the 8bp deletion 1bp insertion "A" at location 3560-3567 in blue, sgRNA target site (actggaaaccgtttcagaggAGG) highlighted in teal. 1bp insertion "A" not shown.

A

Collagen-binding domain

Poly-glutamic acid sequences

Integrin-binding sequence (RGD)

NH₂

COOH

B

C

D

lbsp

Ladder

WT

Bsp⁻/⁻
Figure 2.2 Body weight is unaffected in Bsp^+/− rats. Body weight of wild-type and Bsp^+/− rats was recorded prior to dissection. No significant differences were found in body weight at any experimental timepoint: P0.5 (A, n=7), 14 days (B, n=8), 28 days (C, n=9), 14 weeks (D, n=8), 20 weeks (E, n=9), and 50 weeks (F, n=9).
2.2.3 Bone sialoprotein is present in the mineralized tissues of the periodontium

To determine the localization of BSP in the periodontal tissues, immunohistochemical analysis was performed. In wild-type rats, BSP was detected in cementum tissues and alveolar bone at 20 weeks (Figure 2.3) and 50 weeks (Figure 2.4). Conversely, immunohistochemistry confirmed the absence of BSP in $Bsp^-$ rats at both 20 weeks and 50 weeks.

2.2.4 Histomorphometry shows $Bsp^-$ rats exhibit an underdevelopment of acellular cementum

Various staining techniques were completed to characterize the periodontal tissues of $Bsp^-$ rats. At 20 weeks, general morphology of $Bsp^-$ tissues show no noteworthy differences from wild-type (Figure 2.5). Wild-type rats show a well-developed basophilic acellular cementum at this timepoint while $Bsp^-$ rats show a similar structured acellular cementum, although it appears to be thinner. The cells of the PDL appeared organized and structured with proper insertion into the acellular cementum with no detachment in both wild-type and $Bsp^-$ rats. In this study, rats harvested at 20 weeks were raised on a hard food diet which has been shown to induce a more severe phenotype of incisor malocclusion in $Bsp^-$ mice within the first nine weeks post-weaning (Soenjaya et al., 2015). During tissue harvesting, there was no evidence of incisor malocclusion in any $Bsp^-$ rats. By 50 weeks, there were no morphological differences, although acellular cementum of $Bsp^-$ rats still appeared thinner than wild-type (Figure 2.5). Intense pink staining observed in the $Bsp^-$ rat images represents eosin staining of erythrocytes and was not unique to $Bsp^-$ animals. In both wild-type and $Bsp^-$ tissues, the acellular cementum had grown thicker than at 20 weeks. Rats harvested at 50 weeks were raised on a soft food diet with no evidence of malocclusion in $Bsp^-$ rats. As was seen at 20 weeks, PDL morphology at 50 weeks appeared normal with no detachment from acellular cementum. Alcian blue with nuclear fast red (Figure 2.6) and toluidine blue (Figure 2.7) staining were also used to assess acellular cementum structure and both supported a structured
Figure 2.3 Bone sialoprotein is present in the mineralized tissues of the periodontium at 20 weeks. Immunohistochemistry was performed to detect the presence of BSP in the periodontal tissues visualized with diaminobenzidine staining (brown). BSP was detected in the alveolar bone and acellular cementum of wild-type tissues at 20 weeks. Suspected nonspecific staining was observed in the gingiva of both wild-type and $Bsp^{-/-}$ tissues. AB: alveolar bone, AC: acellular cementum, D: dentin, PDL: periodontal ligament, bar = 100 µm (n=9).
Figure 2.4 Bone sialoprotein is present in the mineralized tissues of the periodontium at 50 weeks. Immunohistochemistry was performed to detect the presence of BSP in the periodontal tissues visualized with diaminobenzidine staining (brown). BSP was detected in the alveolar bone and acellular cementum of wild-type tissues, while no BSP was detected in $Bsp^{-/-}$ rats at 50 weeks. Suspected nonspecific staining was observed in the gingiva of both wild-type and $Bsp^{-/-}$ tissues. AB: alveolar bone, AC: acellular cementum, D: dentin, PDL: periodontal ligament, bar = 100 µm (n=9).
Figure 2.5 Potential underdevelopment of acellular cementum in the periodontal tissues of 20 week- and 50 week-old wild-type and Bsp<sup>−/−</sup> rats. A standard regressive hematoxylin and eosin stain was used to assess the morphology of the periodontal tissues of wild-type and Bsp<sup>−/−</sup> rats at both 20 weeks and 50 weeks. Other than potential acellular cementum underdevelopment, no morphological differences were apparent. Bright pink stain seen in Bsp<sup>−/−</sup> rat represents eosin staining of erythrocytes and other acidophilic components of the tissue. AB: alveolar bone, AC: acellular cementum, D: dentin, PDL: periodontal ligament, bar = 100 µm (n=9 at both timepoints).
<table>
<thead>
<tr>
<th>Wild-type</th>
<th>$Bsp^{+/−}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Image of Wild-type at 20 wks" /></td>
<td><img src="image2" alt="Image of $Bsp^{+/−}$ at 20 wks" /></td>
</tr>
<tr>
<td><img src="image3" alt="Image of Wild-type at 50 wks" /></td>
<td><img src="image4" alt="Image of $Bsp^{+/−}$ at 50 wks" /></td>
</tr>
<tr>
<td>20 wks</td>
<td>50 wks</td>
</tr>
</tbody>
</table>

Legend:
- AB: Alveolar Bone
- PDL: Periodontal Ligament
- AC: Alveolar Crest
- D: Dentin
Figure 2.6 Apparent acellular cementum thinning observed in the periodontal tissues of 20 week- and 50 week-old wild-type and $Bsp^{-/-}$ rats. Alcian blue with nuclear fast red staining was used as an alternative method to assess the potential underdevelopment of the acellular cementum in wild-type and $Bsp^{-/-}$ rats at both 20 weeks and 50 weeks, as was observed with hematoxylin and eosin. Nuclei of the periodontal ligament stain pink, while the acellular cementum is highlighted in blue. The acellular cementum of $Bsp^{-/-}$ rats appeared to be thinner than wild-type, but no other structural differences were observed. AB: alveolar bone, AC: acellular cementum, D: dentin, PDL: periodontal ligament, bar = 100 µm (n=9 at both timepoints).
Figure 2.7 Possible thinning of acellular cementum with no other differences in morphology in toluidine blue stained periodontal tissues of wild-type and Bsp<sup>−/−</sup> rats. A standard toluidine blue stain was used as an alternative method to assess the structure and morphology of the periodontal tissues of wild-type and Bsp<sup>−/−</sup> rats at both 20 weeks and 50 weeks. Toluidine blue allows for increased contrast between the acellular cementum and adjacent periodontal ligament and mantle dentin. Additionally, toluidine blue provides a more detailed visualization of the cellular intrinsic fiber cementum. Other than a possible thinning of the acellular cementum in Bsp<sup>−/−</sup> rats, no structural differences were observed. AB: alveolar bone, AC: acellular cementum, CIFC: cellular intrinsic fiber cementum, MD: mantle dentin, PDL: periodontal ligament, bar = 100 µm (n=9 at both timepoints).
PDL and potentially thinner acellular cementum in Bsp\textsuperscript{−/−} rats, with no PDL detachment at both 20 and 50 weeks. Toluidine blue stain provides better contrast between the cellular intrinsic fiber cementum (CIFC) and surrounding dentin and PDL tissues. There was no apparent effect on the quantity or structure of the CIFC covering the apical portion of the tooth root in Bsp\textsuperscript{−/−} rats compared to wild-type (Figure 2.7). Histomorphometry performed on 20 and 50 week-old rats confirmed that Bsp\textsuperscript{−/−} rats had a significantly thinner acellular cementum on the first mandibular molar compared to wild-type animals (Figure 2.8).

Analysis of collagen organization using picrosirius red staining under circular polarized light found that both wild-type and Bsp\textsuperscript{−/−} rats exhibit a periodontal ligament that is highly organized with no striking differences at both 20 weeks (Figure 2.9) and 50 weeks (Figure 2.10). Collagen fibers remained organized as they extended from alveolar bone to their insertion in the acellular cementum as Sharpey’s fibers.

2.2.5 Alveolar bone structure appears unaffected by the absence of BSP in Bsp\textsuperscript{−/−} rats
Although the acellular cementum was underdeveloped in Bsp\textsuperscript{−/−} rats, no further related defects were observed. Studies on the Bsp\textsuperscript{−/−} mouse displayed alveolar bone resorption at 20 weeks due to defects in acellular cementum and loss of attachment of PDL fibers (Soenjaya et al., 2015). Preliminary microCT analysis (n=2) revealed no notable reduction in the height of alveolar bone in Bsp\textsuperscript{−/−} rats compared to wild-type (Figure 2.11 A-D). Furthermore, the analysis of microCT data found no apparent differences in the volume or density of alveolar bone proper, total alveolar bone, enamel, or dentin/cementum (Figure 2.11 E-L).
Figure 2.8 Graphical representation of histomorphometry of acellular cementum in 20 and 50 week-old wild-type and $Bsp^{\alpha}$ rats. Histomorphometry was performed on hematoxylin and eosin stained periodontal sections. At both 20 weeks (A) and 50 weeks (B), $Bsp^{\alpha}$ rats had a significantly thinner acellular cementum than wild-type counterparts. (n=9 at both timepoints, data represented as mean ± SEM, **** denotes p<0.0001)
Figure 2.9 No differences observed in the collagen organization of periodontal tissues at 20 weeks in wild-type and $Bsp^{+/-}$ rats. Picrosirius red was used to stain collagen to assess organization in the periodontal tissues, primarily the periodontal ligament, and viewed under circular polarized light. In both wild-type and $Bsp^{+/-}$ tissues a high degree of collagen organization and proper directionality was observed as indicated by the bright red/green staining. AB: alveolar bone, AC: acellular cementum, D: dentin, PDL: periodontal ligament, bar = 100 µm (n=9).
Figure 2.10 No differences observed in the collagen organization of periodontal tissues at 50 weeks in wild-type and $Bsp^{+/}$ rats. Picrosirius red was used to stain collagen to assess organization in the periodontal tissues, primarily the periodontal ligament, and viewed under circular polarized light. In both wild-type and $Bsp^{+/}$ tissues a high degree of collagen organization and proper directionality was observed as indicated by the bright red/green staining. AB: alveolar bone, AC: acellular cementum, D: dentin, PDL: periodontal ligament, bar = 100 μm (n=9).
Figure 2.11 No observable resorption of alveolar bone in $Bsp^{-/-}$ rats. MicroCT analysis showed no apparent differences in the alveolar bone structure of wild-type (A, B) or $Bsp^{-/-}$ (C, D) rats. The volume of alveolar bone proper (E), total bone (F), enamel (G), and dentin/cementum (H) was measured with no noted differences. Additionally, no apparent differences were observed in the density of alveolar bone proper (I), total bone (J), enamel (K), and dentin/cementum (L). HU: Hounsfield unit (n=2)
2.2.6 The developing enthesis: 14 and 28 days-old

A goal of this study was to determine whether the changes observed in the $Bsp^{-/-}$ mouse were also present in the $Bsp^{-/-}$ rat. Early timepoints in the rat enthesis were investigated first to determine when BSP expression occurred in order to characterize the development of the QCT fibrocartilaginous enthesis. Immunohistochemical (IHC) analysis was performed to investigate the presence of the two SIBLING proteins, BSP and OPN, in the enthesis tissues. At 14 days post-natal (dpn), there is no expression of BSP (Figure 2.12) or OPN (Figure 2.13) in the QCT enthesis or patella of wild-type rats. By 28 days post-natal, both BSP (Figure 2.12) and OPN (Figure 2.13) are expressed in the mineralized regions of the patella, yet neither protein is present in the QCT enthesis. IHC confirmed the absence of BSP in the patella of $Bsp^{-/-}$ rats at 28 days, while OPN expression in the patella was evident in both $Bsp^{-/-}$ and wild-type rats. Whole tissue von Kossa staining for mineral was completed, confirming that mineralization had not yet occurred in the patella or enthesis of 14 day-old rats (Figure 2.14). Coinciding with the expression of BSP and OPN, von Kossa staining indicates the presence of mineral in the patella of wild-type and $Bsp^{-/-}$ rats at 28 days with no mineralization occurring in the QCT enthesis at this time. Two protocols of hematoxylin and eosin staining were followed to assess the gross morphology of enthesis tissues at 28 days, given this was when mineralization had begun to occur in the patella (Figure 2.15). There were no apparent differences in morphology and no evidence of a tidemark in the QCT enthesis. Similarly, there were no apparent differences in the morphology of cartilage or proteoglycan content in the entheses of wild-type and $Bsp^{-/-}$ rats based on toluidine blue or Safranin-O with fast green staining (Figure 2.16). Lastly, picrosirius red staining for collagen organization demonstrated proper organization and fiber directionality at 14 and 28 days post-natal (Figure 2.17).
Figure 2.12 Bone sialoprotein is not present in the QCT enthesis of 14 or 28 day-old wild-type rats. Immunohistochemistry was performed to detect the presence of BSP in enthesis tissues visualized with diaminobenzidine staining (brown). BSP was not detected in the QCT enthesis or patella of wild-type tissues at 14 days. BSP was detected in the mineralized regions of the patella of wild-type tissues at 28 days, but mineralization of the QCT enthesis does not appear to be present. Suspected nonspecific staining was observed in the soft muscle tissue of both wild-type and $Bsp^{-/}$ tissues at both timepoints. QCT: quadriceps tendon, bar = 100 µm (n=8 for 14 dpn, n=9 for 28 dpn).
Figure 2.13 Osteopontin is not present in the QCT enthesis of 14 or 28 day-old wild-type or Bsp<sup>−/−</sup> rats. Immunohistochemistry was performed to detect the presence of OPN in enthesis tissues visualized with diaminobenzidine staining (brown). OPN was not detected in the QCT enthesis or patella of wild-type tissues at 14 days of age. OPN was detected in the mineralized regions of the patella of wild-type and Bsp<sup>−/−</sup> tissues at 28 days of age, but mineralization of the QCT enthesis does not appear to be present. Suspected nonspecific staining was observed in the soft muscle tissue of both wild-type and Bsp<sup>−/−</sup> tissues at both timepoints. QCT: quadriceps tendon, bar = 100 µm (n=8 for 14 dpn, n=9 for 28 dpn).
Figure 2.14 No mineralization is present in the entheses of wild-type and $Bsp^{-/-}$ rats at 14 or 28 days. Whole tissue von Kossa staining was performed on the QCT enthesis of 14 and 28 day-old wild-type and $Bsp^{-/-}$ rats. At 14 days, no mineral was observed in the patella or enthesis. At 28 days, mineral is present in the patella of both wild-type and $Bsp^{-/-}$ rats but remains absent in the enthesis. QCT: quadriceps tendon, bar = 100 µm (n=8 for both timepoints).
Figure 2.15 No morphological differences are apparent in the entheses of wild-type and $Bsp^{+/}$ rats at 28 days. A standard regressive hematoxylin and eosin stain, following two slightly different protocols, was used to assess the morphology of the enthesis tissues of 28 day-old wild-type and $Bsp^{+/}$ rats. QCT: quadriceps tendon, bar = 100 µm (n=8 for 14 dpn, n=9 for 28 dpn).
Figure 2.16 No differences in cartilage morphology are apparent in the entheses of wild-type and $Bsp^{+/}$ rats at 28 days. A standard toluidine blue and Safranin-O with fast green staining protocol was performed to assess cartilage morphology and proteoglycan content of the enthesis tissues of 28 day-old wild-type and $Bsp^{+/}$ rats, with no observable differences noted. QCT: quadriceps tendon, TB: toluidine blue, SOFG: Safranin-O with fast green, bar = 100 µm (n=9).
Figure 2.17 No observable differences in collagen organization in the entheses of wild-type and $Bsp^{−/−}$ rats at 14 or 28 days. Picrosirius red staining was performed to assess collagen organization in the QCT and enthesis of 14 and 28 day-old wild-type and $Bsp^{−/−}$ rats, and viewed under circular polarized light. In both wild-type and $Bsp^{−/−}$ tissues a high degree of collagen organization and directionality was observed as indicated by the bright red (thicker collagen, usually type I) and green (thinner collagen, usually type III) staining. QCT: quadriceps tendon, bar = 100 µm (n=8 for 14 dpn, n=9 for 28 dpn).
2.2.7 The developed enthesis: 14 weeks

By 14 weeks, both the patella and QCT enthesis have mineralized. IHC for BSP confirmed its presence in the mineralized regions of the patella and the calcified fibrocartilage of wild-type entheses, as well as the absence of BSP in Bsp<sup>-/-</sup> rat tissues (Figure 2.18). OPN was present in the patella bone and QCT enthesis of both wild-type and Bsp<sup>-/-</sup> rats, with no observable difference in localization (Figure 2.19). The morphology of 14 week-old enthesis tissues was investigated, but no apparent differences were observed (Figure 2.20). Of relevance, the length of the CFC in the QCT enthesis did not differ significantly between wild-type (196.3 µm ± 8.3 µm; n=5) and Bsp<sup>-/-</sup> rats (197.0 µm ± 4.3 µm; n=5). In both wild-type and Bsp<sup>-/-</sup> rats, evidence of a tidemark was present but was often not distinct.

2.2.8 Mineralization and growth of the tibiae in Bsp<sup>-/-</sup> rats is not impaired

Previous studies on the developing long bone in the Bsp<sup>-/-</sup> mouse demonstrated a delay in mineralization and mineral content at day of birth (P0.5). A combination of histological and digital analysis was used to assess the effects of the loss of Bsp on the development of tibiae in Bsp<sup>-/-</sup> rats. Using a von Kossa with alcian blue staining, there was no delay in the mineralization of tibiae at day P0.5 (Figure 2.21). Additionally, digital analysis of the length of tibiae at P0.5 revealed that there was no significant difference in the length of wild-type and Bsp<sup>-/-</sup> rats.
Figure 2.18 Bone sialoprotein is present in the calcified fibrocartilage of 14 week-old wild-type rats. Immunohistochemistry was performed to detect the presence of BSP in enthesis tissues visualized with diaminobenzidine staining (brown). BSP was detected in the calcified fibrocartilage of wild-type enthesis tissues at 14 weeks. Suspected nonspecific staining was observed in the soft muscle tissue of both wild-type and Bsp<sup>−/−</sup> tissues. Coverslipped images (bottom) shown to provide clear view of BSP localization. B: bone, CFC: calcified fibrocartilage, UFC: uncalcified fibrocartilage, QCT: quadriceps tendon, bar = 100 µm (n=8).
Figure 2.19 Osteopontin is present in the calcified fibrocartilage of 14 week-old wild-type and Bsp<sup>−/−</sup> rats. Immunohistochemistry was performed to detect the presence of osteopontin (OPN) in enthesis tissues visualized with diaminobenzidine staining (brown). OPN was detected in the calcified fibrocartilage of wild-type and Bsp<sup>−/−</sup> rats enthesis tissues at 14 weeks. Suspected nonspecific staining was observed in the soft muscle tissue of both wild-type and Bsp<sup>−/−</sup> tissues. Coverslipped images (bottom) shown to provide clear view of OPN localization. B: bone, CFC: calcified fibrocartilage, UFC: uncalcified fibrocartilage, QCT: quadriceps tendon, bar = 100 µm (n=8).
Figure 2.20 No morphological differences are apparent in the entheses of wild-type and \( Bsp^{\pm} \) rats at 14 weeks. A standard regressive hematoxylin and eosin stain was used to assess the morphology of the enthesis tissues of 14 week-old wild-type and \( Bsp^{\pm} \) rats, with no noteworthy morphological differences. B: bone, CFC: calcified fibrocartilage, UFC: uncalcified fibrocartilage, QCT: quadriceps tendon, bar = 100 µm (n=8).
Figure 2.21 Mineralization and development does not appear to be impaired in the developing tibiae of $Bsp^{/-}$ rats. Von Kossa (black) with alcian blue staining of tibiae at P0.5 highlights the mineral location in wild-type (A) and $Bsp^{/-}$ (B) rats. Mineral content (C) and overall tibia length (D) was digitally quantified, with no significant differences between wild-type and $Bsp^{/-}$ tissues. Bar = 100 µm (Mineral content n=4, length measurements n=7).
2.3 Discussion

2.3.1 The periodontium at 20 and 50 weeks

This study has demonstrated using histology and histomorphometry that the absence of BSP in the rat results in an underdevelopment of the acellular cementum that was observed at both 20 and 50 weeks. However, there was no evidence of detachment and degradation of the PDL from the acellular cementum and initial analysis of the alveolar bone with microCT showed no bone resorption. This is in contrast to what was observed by Foster et al. and Soenjaya et al. in the Bsp−/− mouse model (Foster et al., 2013; Soenjaya et al., 2015). In this study, the PDL of both wild-type and Bsp−/− rats was able to insert into the acellular cementum and remain inserted, allowing for the proper mechanotransduction of forces from tooth to alveolar bone via the PDL. Mechanical forces are key contributors to the structure of bone (Frost, 1987), and alveolar bone is a mechanosensitive tissue meaning it responds to mechanical forces in order to maintain tissue structure (Hansson and Halldin, 2012). Disruption in the PDL would interrupt the transmission of force, which is a major component in alveolar bone structure, resulting in resorption of the alveolar bone. Since the acellular cementum of Bsp−/− rats was intact, albeit narrowed, and the PDL remained organized, it is not a surprise that there is no resorption of alveolar bone. In mice, it is postulated that BSP is crucial in the formation and mineralization of acellular cementum, which contributed to the formation of the hypothesis that it would play a similar role in rats. The results of this study show that this is not the case and suggests that there may be a different developmental process or that the rat may have a protein present in the acellular cementum with functional redundancy to BSP. Cellular cementum appeared to be unaffected by the lack of BSP, which is consistent with studies in the Bsp−/− mouse and suggests that although the acellular and cellular cementum share a similar function, the developmental process must differ. Interestingly, although
both cementum structures are synthesized by cementoblasts, the origins of the cementoblasts differ (Zeichner-David et al., 2003) which may play a role in the specific development of each tissue.

Initially, rats to be harvested at 20 and 50 weeks were fed a powdered diet because it reduced rates of malocclusion significantly in the $Bsp^{-/-}$ mice, though defects in the periodontium were present whether soft or hard food was given (Soenjaya et al., 2015). Pilot investigations on 20 week-old rats raised on soft diet showed no readily observable phenotype, thus to provide a challenge to the $Bsp^{-/-}$ rat, a hard food diet was used to potentially induce a phenotype similar to the $Bsp^{-/-}$ mouse. Rats harvested at 50 weeks were already set up on a soft food diet, and due to time constraints, we could not investigate the effects of hard food for the timepoint. From morphological analysis, the acellular cementum appeared to be thinning at both 20 and 50 weeks. From this observation, histomorphometric analysis was used to further investigate and confirmed that rats raised on hard food diet (20 weeks) and soft food diet (50 weeks) both showed an underdevelopment of the acellular cementum. The acellular cementum at 50 weeks was notably thicker than at 20 weeks, but further investigations should be completed to determine whether 50 week-old rats on a hard food diet experience a similar thickening. During tissue harvesting, there was no visible evidence of incisor malocclusion in the $Bsp^{-/-}$ rat on either diet, in contrast to the observations of Soenjaya et al., which is consistent with the histological observations of an intact and ordered periodontium.

Of interesting note, there is a difference in the structure of the first mandibular molar of mice and rats. The first molar of the mouse has two large roots, one mesial and one distal (Li et al., 2017). The first molar of the rat has the same two larger roots, mesial and distal, but they have two additional smaller roots, one lingual and one buccal (Naveh et al., 2012). These roots add stability
to the periodontium of the first molar. Naveh et al. observed contact points between the buccal and lingual roots and the adjacent alveolar bone under moderate loading on the occlusal plane of the first mandibular rat molar, which was inferred to play a pivotal role in guiding tooth movement and transfer of mastication forces from tooth to bone (Naveh et al., 2012). The close contact of the roots with bone significantly restricts movement in the buccal-lingual direction. Soenjaya et al. observed alveolar bone resorption on all three mandibular molars in the Bsp\(^{-/-}\) mouse (Soenjaya et al., 2015). Mice and rats do not differ in the structure of the second molar, but the third molar of the mouse only has one root whereas the rat has two (Naveh et al., 2012; Yang et al., 2015). Whether these structural features play a role in the phenotype observed in the Bsp\(^{-/-}\) rat is unclear, but it represents a noteworthy difference in the architecture of the mandibular molars between mice and rats.

Future investigations will have to increase the sample size for microCT in order to draw any reliable conclusions, but initial examination suggests that there is no resorption of alveolar bone in Bsp\(^{-/-}\) rats at 20 weeks. Additional studies should also investigate if acellular cementum underdevelopment is caused by hypophosphatemia, hypocalcaemia, or a deficiency in tissue-nonspecific alkaline phosphatase, as was studied in the Bsp\(^{-/-}\) mouse. From the results, it can be concluded that BSP does not appear to be crucial in the development of the acellular cementum in rats, but its absence did lead to a thinner tissue that persisted at 50 weeks. Since the acellular cementum thickens with age, it is unlikely that Bsp\(^{-/-}\) rats older than 50 weeks will experience any unseen defects. BSP may play a role in the proper timing of acellular cementum mineralization, but itself is not required for mineralization or attachment of the PDL in the rat and it is possible that there is some functional redundancy among proteins in the tissue.
2.3.2 The developing and developed QCT enthesis

The role of BSP in the fibrocartilaginous enthesis was originally investigated by Marinovich because the development of the tissue bore similarities to the development of the growth plate (Marinovich, 2015). Marinovich investigated the role of BSP in the developing supraspinatus tendon (SST) and QCT entheses of mice at 14 and 21 days based on a previous study by Schwartz et al. which indicated mineralization occurring in the SST enthesis at 14 days in mice (Schwartz et al., 2012). Since Marinovich was unable to detect the presence of BSP or OPN in the QCT enthesis at 21 days, the timepoints of 14 and 28 days were chosen in order to characterize the developing QCT enthesis in the rat.

As was seen in the Bsp−/− mouse, there was no expression of either SIBLING protein, BSP or OPN, and no mineralization occurring in the patella or QCT enthesis of wild-type or Bsp−/− rats at 14 days. Not surprisingly, there were no observed differences in collagen organization and content at this time either. No differences were expected given the absence of BSP expression in wild-type tissues at this time. More in depth characterizations were completed on the QCT enthesis of 28 day-old rats, as mineralization had begun to occur at this time. Similar to what was seen in the mouse model at 21 days, mineralization in the rat was restricted to the patella and had not yet entered the enthesis at 28 days. This suggests that there is either a delay in enthesis mineralization following mineralization of the patella, or the development of the patella and QCT enthesis of the rat is delayed compared to the mouse. There were no observed differences in gross morphology, collagen organization, or cartilage structure between wild-type and Bsp−/− rats, which is explained by the enthesis still being unmineralized at 28 days.
The mature QCT enthesis was investigated at 14 weeks in order to characterize the expression of BSP in the developed enthesis, as well as assess if any gross morphological differences exist between wild-type and $Bsp^{-/-}$ tissues. By this point, the patella and QCT enthesis had fully mineralized and BSP was confirmed to be present in the CFC of wild-type tissues. OPN was observed in the CFC of both wild-type and $Bsp^{-/-}$ rats, consistent with the findings of Marinovich et al. (Marinovich et al., 2016). These two proteins have a close regulatory relationship, where BSP promotes hydroxyapatite (HA) nucleation (Hunter and Goldberg, 1993) while OPN acts as an inhibitor (Hunter et al., 1996), so it is not surprising to find them both in the mineralized CFC.

Marinovich et al. observed a morphological difference in the enthesis of $Bsp^{-/-}$ mice, and further investigated to find a lengthening of the CFC (Marinovich et al., 2016) which appeared to be absent in the $Bsp^{-/-}$ rat following visual and histomorphometric analysis. Future studies will also need to develop a better protocol for decalcification of tissues. Adult rats are roughly 10 times larger than adult mice, and thus have larger tissues and more mineral. EDTA decalcification is a common gentle method to remove the mineral component of bone, as was used in Marinovich et al., but larger tissues require multiple weeks and solution changes (Marinovich et al., 2016). Using physical endpoint testing, it was found that 28 days in a 0.65 M EDTA (pH 7.4) solution changed every four days was insufficient in fully decalcifying the knee tissues of 14 week-old rats. Due to this complication and time constraints, decalcifying of 14 week-old rat knees was completed using Formical-2000, a harsher but quicker method that uses formic acid to decalcify. Although this method sufficiently decalcified the tissue, it was difficult to maintain high tissue quality through processing and sectioning. If morphology of the enthesis in adult rats is investigated in the future, it is recommended that a better decalcifying protocol be used or developed.
A common issue among studies on BSP is the absence of a reliable primary antibody. Specifically, in our study there was significant nonspecific staining in both wild-type and $Bsp^{-/-}$ tissues. This issue was especially evident in muscle tissues where there should be no BSP expression, as was observed in the uncalcified tissues of the enthesis and periodontium. The earliest the periodontium was investigated was at 20 weeks, and by this point the tissues had mineralized and the only observed nonspecific staining was in the gingival epithelium. Analysis of the developing enthesis at timepoints prior to and including the initiation of mineralization also demonstrated nonspecific staining in the muscle tissues as well as throughout the QCT of both wild-type and $Bsp^{-/-}$ rats. This issue was minimized but not eliminated in 14 week-old enthesis tissues, despite following the same protocol. Multiple adaptations to the IHC staining protocol were made in an attempt to reduce or remove the nonspecific binding. Various antigen retrieval methods, such as heat-induced epitope retrieval with a sodium citrate buffer and protease-induced epitope retrieval with Proteinase K, were attempted but none proved to be beneficial. Different blocking buffer and primary antibody concentrations were attempted but these alterations only affected the overall staining intensity of the tissue, both specific and non-specific. Additionally, many of the commercially available antibodies claim to stain specifically but none that we tested (Abcam ab52128; BosterBio PA1887; BosterBio PA1505) were reliable (Abcam, Cambridge, United Kingdom; BosterBio, Pleasanton, CA, USA).

In the end, a protocol adapted from Brian Foster was used that incorporated the use of a hydrogen peroxide incubation to block endogenous peroxidases, as the chromogenic DAB staining uses horseradish peroxidase to produce a brown product (Foster, 2012), while using an antibody
provided by Dr. Renny Franceschi (University of Michigan, Ann Arbor, USA). This antibody is polyclonal and was produced using an unmodified mouse BSP sequence linked to glutathione S-transferase (GST), so it is possible that the antibody may also recognize GST present in tissue as one of the epitopes identified.

2.3.3 Endochondral ossification and development of the tibia

In the $Bsp^{-/-}$ mouse, a decrease in mineral content and overall bone length was present at both prenatal and neonatal timepoints (Holm et al., 2015), and at 4 months (Malaval et al., 2008). Holm et al. also observed a delay in mineralization of $Bsp^{-/-}$ mice during gestation (Home et al., 2015). In this study, there was no difference in the bone length of tibiae between wild-type and $Bsp^{-/-}$ rats at day of birth (P0.5). There were also no significant differences in mineral content, but the protocol for mineral staining requires optimization to produce more reliable results. The von Kossa mineral staining protocol followed the one presented in Holm et al. on $Bsp^{-/-}$ mice (Holm et al., 2015). In this protocol, tissues are not demineralized before sectioning which led to issues with tissue integrity. At P0.5, mice weigh 1-2 g while rats in this study weigh 5-7 g. The increase in size is accompanied by an increase in bone length and diameter and thus a relative increase in mineral content, which made tissue sectioning difficult. Whether it was during sectioning or staining, some sections experienced tissue loss. A modified von Kossa staining protocol that allows for decalcification was attempted, but this method resulted in overstaining of the tissue. Future studies will need to find an alternative method to mineral staining that retains the structure of the tissue.

The results presented suggest that an absence of BSP does not cause a relative under-mineralization or decrease in length of developing tibiae at P0.5. However, to be conclusive, additional samples and timepoints require analysis. There may be a compensatory method for
mineralization initiation in the $Bsp^{+/-}$ rat. In the $Bsp^{+/-}$ mouse, the mineral content of $Bsp^{+/-}$ mice caught up to wild-type by 52 weeks (Malaval et al., 2008). It is possible, although unlikely, that by P0.5 the $Bsp^{+/-}$ rat tibiae would catch up to their wild-type counterparts but future studies investigating the prenatal development are necessary to definitively conclude.

2.3.4 General discussion
As described, the $Bsp^{+/-}$ rat showed only a minor phenotype consisting of thinning of the acellular cementum, with an otherwise normal periodontium, enthesis and bone. This is in contrast to the significant phenotypes in these tissues demonstrated in the $Bsp^{+/-}$ mouse. Unfortunately, species and strain differences do lead to significant variability in the manifestations of genetic alterations.

A large area of concern within the scientific community when it comes to observed phenotypes is the effect of the specific background strain and how it may vary the results of a study. Within a single species, small genotypic differences can produce drastically different phenotypes. For example, even within a specific strain, C57BL/6, there are apparent differences that were noted between subspecies. Fontaine and Davis found that subspecies 6J and 6N, even without manipulation or challenge, already have significant differences in base blood glucose levels (Fontaine and Davis, 2016). Similarly, comparisons across different strains of mouse have shown significant variability in test results. Histomorphometric analysis showed that baseline analysis of the two mouse strains, C3H/HeJ and C57BL/6J, revealed a significant difference in cortical bone area in the tibia and femur combined with a drastic difference in peak bone density despite no genetic or environmental modifications (Sheng et al., 1999). These strain differences present issues when used as models for disease. For example, surgically induced osteoarthritis following destabilization of the medial meniscus is more severe in 129/SvEv mice than in DBA/1 mice
(reviewed in McCoy, 2015). Even the $Bsp^{-/-}$ mouse (129/CD1 strain) when backcrossed into BL6 background revealed a greatly diminished periodontal phenotype (personal communication with Harvey Goldberg, University of Western Ontario)

In this study, the $Bsp^{-/-}$ rat is a novel model for assessment of BSP’s role in biomineralization. Given that differences exist within strains of a single species, it is not surprising that noteworthy differences exist across species as well. Wu et al. investigated the differences between $MeCP2^{-/-}$ mice and rats and observed that rats experienced phenotypes that were nonexistent in mice, such as penile prolapse, while also not displaying classical phenotypes of the mouse model, such as hindlimb grasping (Wu et al., 2016). Even in cases where there is high conservation of gene sequence and expression, protein structure, and amino acid composition, Thomas et al. recognized opposite phenotypes in $Cntnap2$ knockout mice and rats, where mice showed hypoactivity and rats exhibited hyperactivity (Thomas et al., 2017).

Other studies have noted redundancy within genomes that affects the observation of phenotypes (as reviewed in Barbaric et al., 2007). Compensation effects occur in genes with genetic similarity, as in the case of $MyoD$ and $Myf5$ where a single knockout shows no phenotype with a consequential upregulation of the remaining gene. Or some protein families, such as the Caspase protease family, can experience severe and potentially lethal phenotypes when some members are removed while other knockouts exhibit minimal to no phenotype. Furthermore, redundancy exists among genes that are completely unrelated. In drosophila, a mutation to Hsp90 allowed for the appearance of phenotypes of other mutations that were formerly silent.
Some phenotypes are silent until the organism is challenged. In the *Mcr5* knockout mouse, there was no evident phenotype observed but a phenotype was unintentionally found when stress-induced analgesia with a swim test was performed and researchers noted a deficiency in water repulsion in the fur (Chen et al., 1997). Additionally, when the SIBLING protein OPN is knocked out of mice there is no overt phenotype, but when the symptoms of ulcerative colitis is induced using dextran sulfate sodium, *Opn*<sup>−/−</sup> mice experienced an increased severity of tissue destruction accompanied with a reduction in tissue repair (da Silva et al., 2009).

A limitation of this study was the use of both male and female animals. This was deemed necessary in order to reach the set sample size goals within the timeline of the study and was supported with information from literature. As was described previously (Section 1.2), sex hormones contribute to the sexual size dimorphism observed in gonochoristic animals with their increased expression during sexual maturation. No noteworthy differences are expected in the skeletal structure of the rat prior to sexual maturation, which occurs at 32-40 days for females (reviewed in Rivest, 1991; Lewis et al., 2002) and 42-48 days in males (Lewis et al., 2002; Campion et al., 2013), so both males and females were used when studying the young developing bone and entheses, as was done when studying these tissues in the *Bsp*<sup>−/−</sup> mouse (Holm et al., 2015; Marinovich et al., 2016). For the study of the QCT enthesis at 14 weeks there are differences in bone structure between males and females at this age, but no gender-based differences in enthesis structure have been reported.

With regards to periodontal tissues, there is limited characterization on the rat and mouse animal models describing any gender-based differences in periodontal structure. Studies in humans have found inconclusive evidence on alveolar bone thickness and density, where some studies reported that males had a thicker and more dense alveolar bone (Cassetta et al., 2013; Fadhil and Al-Khatib,
2015) while others reported no significant differences (Ono et al., 2008; Park et al., 2008; Choi et al., 2009; Farnsworth et al., 2011). Differences that may exist in alveolar bone density and thickness are postulated to be due to the larger mastication muscles and forces in males (Braun et al., 1996; Usui et al., 2007). A second limitation of this study was the lack of absolute proof that a truncated form of BSP was not being secreted from the cell in Bsp⁻/⁻ rats, however, the anti-serum used for BSP is polyclonal and presumably contains antibodies that would recognize sequences throughout the BSP molecule. Considering that bone sections of Bsp⁻/⁻ rats (alveolar, patellar, and femoral) were devoid of any immunoreaction, this would support the conclusion that BSP was not being expressed and released into the extracellular matrix.

In the realm of BSP and other SIBLING protein research, the majority of in vivo studies occur in the mouse animal model. However, there are some experimental procedures that are difficult to perform in the mouse because of its small size that are more easily done in the larger rat. The use of transgenic rats is novel and was justified based on the long-term goals of understanding the role of BSP in normal physiological conditions and pathological situations. For instance, BSP is believed to play a role in bone healing following the induction of a 1 mm cortical defect in wild-type and Bsp⁻/⁻ mice (Malaval et al., 2009). Cortical defects in the rat are not only easier due to the size increase, but also pose less risk of unintentional damage to other nearby tissues. BSP is also believed to play a role in osteoarthritis (OA; reviewed in Lis, 2008; Pesesse et al., 2012, 2014), which involves the degradation of articular cartilage in joints. The articular cartilage in the rat is thicker than in the mouse, which allows for the implementation of full and partial defects for studies on the role of BSP in OA (reviewed in McCoy, 2015). For the study of OA, the rat allows for the use of anterior cruciate ligament transection (ACLT) surgery, a common surgical induction
technique in OA studies that is not recommended in mice due to the difficulty of the surgery in small rodents and the severe phenotype it produces (Glasson et al., 2007). Even in the periodontal tissues, ligature-induced models of periodontal disease (Cai et al., 2008) are less difficult in rats due to the larger tooth sockets and tissue area. Furthermore, for studies involving cell culture of osteoblastic cells, isolation and culture of rat osteoprogenitor cells is far easier than that of cells isolated from mouse. The rat allows for higher cell counts during osteogenic cell extraction while also thriving better than mouse cells in standard culture conditions (Orriss et al., 2012, 2014).

2.3.5 Conclusions
This study aimed to characterize the phenotypes of the novel $Bsp^{−/−}$ rat. In the periodontal tissues, an underdevelopment of the acellular cementum that persisted into maturity was observed with no related periodontal defects. In the mature fibrocartilaginous enthesis, the presence of both BSP and OPN was identified in the CFC of wild-type animals. It was also determined that the QCT enthesis mineralizes at a later timepoint than 28 days. Lastly, there were no differences in tibial length of neonates while initial observations showed no difference in mineral content. Although our observations do not match the phenotypes demonstrated in the $Bsp^{−/−}$ mouse, it does provide the foundation for future studies utilizing the $Bsp^{−/−}$ rat.
2.4 References


APPENDIX A: Animal Use Statement

Statement of Permission for the Use of Animals for Experimental Research

All experimentation on animals was performed in compliance with the Animal Use Protocol 2015-015 held by principal investigator Dr. Harvey Goldberg of the Schulich School of Medicine and Dentistry and Department of Biochemistry at the University of Western Ontario (London, Ontario, Canada)

2015-015::1:

AUP Number: 2015-015
AUP Title: The role of bone sialoprotein in osteoarthritis and bone repair.
Yearly Renewal Date: 07/01/2016

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2015-015 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
CURRICULUM VITAE

Benjamin Harvey

Post-secondary Education and Degrees

Candidate for Master of Science – Biochemistry  
*The University of Western Ontario, London, ON*  
2017 – Present

**Bachelors of Medical Science**, Double Major in Physiology and Pharmacology  
*The University of Western Ontario, London, ON*  
2017

Honours and Scholarships

**Dean’s Honour List, The University of Western Ontario**  
2014, 2015, 2017  
- Awarded in recognition of academic success during the 2014, 2015, and 2017 academic terms

**The University of Western Ontario Entrance Scholarship of Excellence**  
2013  
- Received a $2,000 entrance scholarship for an entering average above 90%

Research Experience

**Masters in Biochemistry, Graduate Student**  
*Goldberg Lab, Schulich School of Medicine and Dentistry, London, ON*  
Sept 2017 – Present  
- Project title: The characterization of the rat bone sialoprotein knockout phenotype

**Laboratory Volunteer**  
*Stathopulos Lab, Schulich School of Medicine and Dentistry, London, ON*  
Sept 2016 – Apr 2017