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Bone Sialoprotein and Osteopontin Mediate Bone Development

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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BONE SIALOPROTEIN AND OSTEOPONTIN MEDIATE BONE DEVELOPMENT

(Thesis format: Integrated-Article)

by

Erik Martin Holm

Graduate Program in Biochemistry

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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ABSTRACT

The mechanism of biomineralization during endochondral ossification is an incompletely characterized process. The small integrin-binding ligand N-linked glycoprotein (SIBLING) group of proteins are anionic phosphoproteins found in the extracellular matrix of mineralized tissues and have been postulated to modulate mineral growth. Bone sialoprotein (BSP) has been shown to be a promoter of both osteoblastic differentiation and mineralization. In contrast, osteopontin (OPN) has been shown to be an inhibitor of mineralization. This thesis examined the phenotype of mineralization upon loss of either BSP or OPN. When the Bsp gene was ablated in mice (Bsp−/−) histological analyses revealed tibiae had delayed endochondral ossification, decreased mineralization, and shortened length. Additionally, the mice had altered growth plates with decreased chondrocyte proliferation and apoptosis, possibly contributing to the shortened bones. Studies on Bsp−/− osteogenic cultures agreed with the in vivo findings, demonstrating delayed osteogenic differentiation and mineralization. Adenoviral-mediated overexpression of BSP in Bsp−/− osteogenic cultures increased osteogenic gene expression and mineralization. Specific mutations of BSP that truncated the N-terminus, mutated poly-E to poly-A, and RGD integrin-binding to KAE, all resulted in increased mineralization and osteogenic gene expression. Loss of any one functional motif did not result in loss of functionality of BSP. Deletion of Opn (Opn−/−) in murine-derived osteogenic cultures resulted in increased mineralization with no physiological change in osteoblast gene expression. Supplementation with milk OPN and OPN-derived peptides reduced the enhanced mineralization of the Opn−/− osteoblasts without altering their
terminal differentiation characteristics. These studies demonstrate that the SIBLING proteins are potent mediators of mineralization.

KEYWORDS

Bone sialoprotein, osteopontin, hydroxyapatite, mineralization, endochondral ossification, osteoblast, chondrocyte, development
Chapter 1 entitled “Literature review” was written by E. Holm with suggestions from Drs. H. Goldberg, and F. Beier.

Chapter 2 entitled “Loss of bone sialoprotein leads to impaired endochondral bone development and mineralization” was adapted from Holm et al., 2014. (Submitted), and reproduced here. The manuscript was written by E. Holm with suggestions from Drs. J.E. Aubin, G.K. Hunter, and F. Beier, and H.A. Goldberg. All experiments were performed by E. Holm and were carried out in the laboratories of Drs. H.A. Goldberg and F. Beier.

Chapter 3 entitled “The role of bone sialoprotein in osteoblast differentiation and mineralization” was adapted from Holm et al., 2014 (in preparation). The manuscript was written by E. Holm, with suggestions from Drs. F. Beier and H.A. Goldberg. Under the supervision of E. Holm, fourth-year honours student K. Vincent expressed and purified the -28 recombinant adenovirus construct and post-doctoral fellow Dr. M. Siquiera expressed and purified the KAE recombinant adenovirus construct. All experiments were carried out in the laboratories of Drs. H.A. Goldberg and F. Beier.

Chapter 4 entitled “Osteopontin mediates mineralization and not osteogenic cell development in vitro” was adapted from Holm et al., 2014. (Submitted), and reproduced here. The manuscript was written by E. Holm, with suggestions from J.S. Gleberzon and Drs. E.S. Sørensen, F. Beier, G.K. Hunter, and H.A. Goldberg. Under the supervision of E. Holm, fourth-year honours student J.S. Gleberzon assisted with dissections and cell cultures. J.S. Gleberzon performed the experiments treating the osteogenic cultures with OPN-derived peptides, shown in Figure 4.5. All other experiments were performed by E.
Holm and were carried out in the laboratories of Drs. H.A. Goldberg, G.K. Hunter, and F. Beier.

Chapter 5 entitled “Discussion” was written by E. Holm with suggestions from Drs. H.A. Goldberg and F. Beier.
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<td>-28</td>
<td>28 residue N-terminal truncated BSP</td>
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<td>αMEM</td>
<td>alpha minimal essential medium</td>
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<td>Acan</td>
<td>aggrecan</td>
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<td>ACVS</td>
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dUTP  deoxyuridine triphosphate
E  embryonic day
EA  poly-glutamate regions of bone sialoprotein mutated to poly-alanine
ECM  extracellular matrix
EDTA  ethylenediaminetetraacetic acid
Enpp1  ectonucleotide pyrophosphate/phosphodiesterase 1
ERK  extracellular signal-regulated kinases
EV  empty vector
FAK  focal adhesion kinases
FBS  foetal bovine serum
FTIR  Fourier transform infrared imaging
FMOC  fluorenylmethyloxycarbonyl
g  gravitational force
G2P  glycerol-2-phosphate
HA  hydroxyapatite
HBSS  Hanks’ balanced saline solution
HEB  hypertrophic chondrocyte zone and endochondral bone
HPLC  high-performance liquid chromatography
Ibsp  integrin-binding sialoprotein
IF  immunofluorescence
IHC  immunohistochemistry
Ihh  indian hedgehog
IMDM  Iscove’s modified Dulbecco’s medium
KAE  lysine-alanine-glutamic acid
KO  gene ablated knockout
MAPK  mitogen activated protein kinase
MCSF  macrophage colony stimulating factor
MGP  matrix gla protein
MEPE  matrix extracellular phosphoglycoprotein
Micro-CT  micro-computed tomography
MMP  matrix metalloproteinase
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<td>bovine milk osteopontin</td>
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<td>MSCs</td>
<td>mesenchymal stromal (progenitor) cells</td>
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<td>NF-κB</td>
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<td>phosphate buffered saline</td>
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<td>shRNA</td>
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<td>small integrin-binding ligand N-linked glycoprotein</td>
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siRNA    small interfering ribonucleic acid
Sox      sex-determining region on Y box
Sp7      osterix
TNAP     tissue non-specific alkaline phosphatase
TRAP     tartrate-resistant acid phosphatase
TUNEL    terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VEGF     vascular endothelial growth factor
VSMC     vascular smooth muscle cell
WT       wild-type
CHAPTER ONE

LITERATURE REVIEW
1.1 General introduction

Bone is a complex tissue responsible for multiple aspects of mammalian life. Bone provides the anchor for muscles allowing them to attach and articulate movement [1-3]. Additionally, the skeleton acts as protective case for sensitive internal organs by shielding them from insult, and absorbing shock associated with locomotion [1, 2]. It also acts as a reservoir for phosphate and calcium ions, which is important for metabolism, replenishing the body when needed [1-3]. The marrow within the skeleton contains a large progenitor cell population, from which the cells important for tissue repair, maintenance, immune response, and homeostasis are found [1, 2]. Bone also acts as an endocrine organ, where it can interact with the pancreas thereby modifying mammalian metabolism [4].

Bone tissue development and mineralization is an incompletely characterized process. This is due, in part, to the complexity of bone and the involvement of many cell types from distinct origins that are each independently regulated yet must interact with one another. The dysregulation of different cell types within bone results in a variety of pathological conditions including, but not limited to: osteoporosis, osteopetrosis, osteogenesis imperfecta, and fibrodysplasia ossifican progressiva. The mechanism by which mineral deposition and growth is regulated is still unclear, but there are some proteins thought to be important in the mediation of this process. The body, and its fluids within, are supersaturated with respect to the concentration of ions required for hydroxyapatite, the main mineral component of bone, formation and growth, but biomineralization is restricted to discrete sites, such as bone and teeth, in normal development. The Small Integrin-Binding N-Linked Glycoprotein (SIBLING) group of
proteins, discussed below, presents an ideal target to understand mineralized tissue regulation. They are highly expressed in mineralizing tissues and have been shown to mediate mineral growth \textit{in vitro}.

1.2 Skeletogenesis

There are two mechanisms by which bone is formed during embryogenesis: endochondral ossification, and intramembranous ossification [5-7]. The mechanism that creates the long bones, vertebralae, and ribs, encompassing the majority of the mammalian skeleton, is endochondral ossification [5-7]. Briefly, this process involves the condensation of pluripotent mesenchymal progenitor cells that develop into one of two lineages: chondrocytes or osteoblasts. The collagen of endochondral bone is regimented in a linear pattern to provide the strength and rigidity necessary for tensile strength and shock absorption due to movement. The second mechanism by which bone is formed is intramembranous ossification [5-7]. This type of ossification results in the formation of the flat bones in our bodies, such as the calvariae. Intramembranous ossification initiates by the interaction between pluripotent mesenchymal cells and the epithelium, which directs the mesenchymal cells to differentiate into preosteoblasts [8-10]. These progenitors then condense, proliferate, and mature into osteoblasts that produce the intramembranous bone, without the cartilaginous intermediate [8, 11]. The collagen fibres of the intramembranous bone are often referred to as having a woven structure. The fibres are overlapping in a random array of directions and do not possess an organized pattern, such as that present in mature endochondral bone. The endochondral bones give rise to the structures that are responsible for providing strength and rigidity in order to
facilitate locomotion, articulation, and absorb shock. Intramembranous bone develops structures that are mainly responsible for protecting vital organs such as the brain.

1.2.1 Primary endochondral ossification

Endochondral bone development begins as a condensation of pluripotent mesenchymal progenitor cells, forming the primary template of bone (Figure 1.1) [12]. The condensation triggers the mesenchymal cells to differentiate into chondroprogenitors. This is controlled by the master chondrocyte transcription factors Sox5, 6, and 9 [13, 14]. The chondroprogenitors differentiate into chondrocytes, which begin to secrete extracellular matrix (ECM) forming the cartilaginous anlagen of the bone. The ECM secreted by these cells is composed of type II, IX, and XI collagen, proteoglycans, and other ECM proteins [11, 12]. The cells at the periphery of the cartilaginous anlagen begin to flatten and elongate, forming the perichondrium [11].

At the centre of the cartilaginous anlagen the chondrocytes terminally differentiate into hypertrophic chondrocytes [15]. As they become hypertrophic, the chondrocytes’ expression of transcription factors Sox 5, 6, 9, and type II collagen decrease [12, 16]. Concomitant with this reduction, there is an increase in expression of the transcription factor Runx2, type X collagen, other ECM proteins including bone sialoprotein (BSP), and angiogenic factors such as vascular endothelial growth factor (VEGF) [11, 17-19]. The ECM surrounding these cells then begins to mineralize, forming a calcified cartilaginous matrix [17, 18]. At this time, the cells in the perichondrium differentiate into preosteoblasts, expressing osteoblastic genes such as Runx2, alkaline phosphatase (Alp), type I collagen (ColI), among others. The ECM secreted by these cells then begins...
Figure 1.1 Endochondral ossification. (A) Condensation of pluripotent mesenchymal progenitor cells (pink) of the early anlage. (B) These cells differentiate into the chondroprogenitor cells (purple) and they begin to form the early cartilaginous anlage. (C) The chondrocytes terminally differentiate into the hypertrophic chondrocytes (blue) forming the early mineralized cartilaginous matrix (grey) at the centre of the anlage. The axial chondrocytes then begin to form the growth plates at either end of the cartilaginous anlage. The resting zone (purple) is localized at either end of the bone, and they differentiate to form the pancake-like cells of the proliferative chondrocyte zone (teal). Vascular invasion (red) begins in order to provide an avenue to recruit osteoclast precursors (yellow) and resorb the mineralized cartilage matrix. (D) Primary ossification begins as the osteoprogenitor cells (dark blue) are recruited into the resorbed areas, which differentiate and deposit osteoid in the diaphysis. The chondrocytes localize to the epiphysis of the bone separating the growth plates of the early bone.
A. Mesenchymal progenitor cell condensation

B. Chondrocyte progenitors and the early cartilaginous anlage

C. Mineralized cartilaginous anlage

D. Complete primary ossification
   - Resting zone
   - Proliferative zone
   - Hypertrophic zone
   - Primary ossification centre
to mineralize adjacent to the hypertrophic chondrocytes. This forms the bone collar of the developing bone [8, 17, 20].

Following the cartilaginous matrix calcifying around the hypertrophic chondrocytes, the majority of these cells begin to undergo apoptosis, leaving a porous cartilaginous matrix in which cells can migrate to begin the modeling of the bone [21]. VEGF is secreted by the hypertrophic chondrocytes prior to apoptosis to induce vascularisation of the cartilaginous anlage [11, 18, 22]. This provides an avenue for osteoclast recruitment from the peripheral blood supply to the anlage [5-7].

Following the vascular invasion and degradation of the cartilaginous matrix by chondrocyte secreted proteases and osteoclastic resorption, osteoblasts begin to secrete osteoid. Osteoid is an ECM composed of type I collagen and a variety of non-collagenous proteins, including BSP and osteopontin (OPN) [11]. This newly deposited matrix then becomes calcified, forming the primary ossification centre of endochondral bone [23]. The osteoblasts that were recruited to the bone then terminally differentiate and either become trapped in mature bone, becoming osteocytes, or undergo apoptosis [11].

### 1.2.2 Epiphyseal growth plate development

At either end of the developing bone chondrocytes begin to form the epiphyseal growth plates [24]. These chondrocytes form three distinct phases of development: the resting (reserve) zone, the proliferative zone, and the hypertrophic zone [7, 25]. Histologically these zones are distinguishable from one another based on morphology and the expression of specific molecular markers. The resting zone consists of small rounded cells, with low metabolic activity, which are located closest to the articular
cartilage. The resting zone is mainly a reservoir of cells that replenish chondrocytes as they progress into the proliferative zone as the bones develop [26, 27]. While they are relatively quiescent cells, they still proliferate in order to replenish their numbers as they differentiate, albeit at a much lower rate than the proliferative zone [26, 28, 29].

As the cells differentiate, they enter the proliferative zone, where the cells form into a columnar organization of pancake-like cells surrounded by cartilaginous matrix. These cells proliferate in a longitudinal direction towards the diaphysis; this contributes to the lengthening of the developing bone [29, 30]. The proliferation of chondrocytes is highly regulated by the growth factor Indian hedgehog (Ihh) [31-33]. Ihh has been shown to enhance chondrocyte proliferation through Gli3 [34, 35]. Activation of bone morphogenetic proteins (BMPs) have also been shown to be involved in promoting the proliferation of chondrocytes through Smads 1 and 5 [36-39]. Following a finite number of divisions, the cells exit the cell cycle and differentiate into prehypertrophic chondrocytes [26]. These cells begin to lose their flat appearance and become larger than their neighbouring proliferative cells.

The prehypertrophic chondrocytes continue to enlarge as they terminally differentiate into the hypertrophic chondrocytes of the growth plate. The hypertrophic zone is distinctly identifiable by the cells’ large and rounded morphology. The hypertrophic chondrocytes grow to a size approximately five to ten-times larger than their previous stages; this enlargement is responsible for the majority of longitudinal bone growth from the growth plate [40, 41]. The swelling of the cells is due to increased cytoplasm, nucleoplasm, and number of intracellular organelles [28, 42]. The matrix that the hypertrophic chondrocytes secrete includes proteins that facilitate vascular invasion
and the formation of the calcification of the cartilaginous matrix [28]. As endochondral bones develop the primary centre of ossification progresses towards the epiphysis, resulting in a progressively shrinking growth plate until the bone fully matures [5].

**1.2.3 Development of secondary ossification centres**

Following primary ossification and establishment of the epiphyseal growth plates, the secondary ossification centres form. These sites begin to form in middle of the epiphyseal cartilage of long bones [11, 43] through a mechanism that differs from primary ossification. Secondary ossification begins with vascular invasion of the non-mineralized cartilage of the epiphysis [43], followed by hypertrophic chondrocyte mediated calcification [11, 44, 45]. The vascularisation allows recruitment of the osteoclasts that proceed to resorb the mineralized cartilaginous matrix. Following the resorption, osteoprogenitor cells are recruited and differentiate into osteoblasts that deposit the osteoid which mineralizes within the growth plate forming mature secondary ossification centres [45, 46]. The cartilage that is adjacent to the joints becomes the articular cartilage of mature bone. The secondary ossification front begins to grow towards the primary ossification centre. In humans, but not rodents, this results in the eventual replacement of the growth plate with bone [29, 47].

**1.2.4 Bone modeling, remodeling, and repair**

Bone modeling and remodeling are processes whereby bone is resorbed and replaced in order to form the mature tissue and to maintain its structural integrity (Figure 1.2). The resorption of bone is performed by osteoclasts, a multinucleated cell type derived from a mononuclear haematopoietic myeloid cell lineage that becomes the mononuclear osteoclast precursors that fuse to form multinucleated cells. These cells
Figure 1.2 Cellular involvement during bone remodeling. Osteoclast precursors are recruited to sites of mature bone to initiate bone resorption. Osteoclast precursor cells begin to merge, forming large multinucleated cells that differentiate into osteoclasts. When osteoclasts are actively resorbing they develop a ruffled membrane at the bone surface, and they secrete proteases and acidify matrix forming the resorptive pits. The mesenchymal progenitors are then recruited and differentiate into the preosteoblasts. As they move into the resorptive pits they differentiate into osteoblasts. The osteoblasts deposit de novo osteoid that integrates with the neighbouring bone and mineralizes. Osteoblasts then undergo one of three final stages: apoptosis, differentiation into bone lining cells, integration into bone by becoming osteocytes.
are large, around 150-200 µm, and have a large number of vesicles in the cytoplasm. As a pre-requisite for resorption the osteoclast forms a tight seal with the bone surface. The osteoclasts then develop a highly folded membrane, the ruffled border, in order to increase the surface area to maximize secretion and uptake activity. These cells are also identifiable by their expression of tartrate-resistant acid phosphatase (TRAP) and other cell surface markers such as receptor activator of nuclear factor-κB (RANK). Due to the constant stresses on the structure of bone, remodeling is crucial in maintaining its integrity. Both modeling and remodeling is begun by the recruitment of osteoclast precursor cells to the sites of resorption by the osteoblastic and stromal cell secretion of RANK ligand (RANKL) and macrophage colony stimulating factor (MCSF) [48]. RANKL promotes the proliferation of the osteoclast precursors at the site of resorption. Following the proliferation, MCSF promotes their aggregation to form the functional osteoclasts. The fusion of the monocytes results in a multinucleated cell that typically consists of 6-14 nuclei. The osteoclasts begin to resorb the mineralized matrix using proton pumps to acidify and dissolve the mineral in the area to be degraded. Following dissolution of the mineral, the decalcified collagenous matrix is then proteolytically degraded by cathepsins [49]. Osteoprogenitors are then recruited to sites of resorption where they differentiate into osteoblasts and secrete de novo osteoid. The new osteoid seamlessly integrates into the stable bone tissue. The new osteoid then mineralizes, resulting in a fully repaired bone that restores the strength of the tissue.

Following traumatic injury such as a bone fracture, a similar mechanism is activated in order to repair the damaged tissue. In these cases the insult normally results in the release of blood into the region surrounding the break [50, 51]. The blood comes
into contact with bone, resulting in enzymatic release of chemotactic molecules into the area surrounding the site of injury that promote cell recruitment to initiate the repair and remodeling process. The first step in bone repair is the formation of a bone callus. This callus is formed in a mechanism that closely resembles endochondral ossification where first a cartilaginous matrix is formed. The chondrocytes within this cartilage then undergo terminal differentiation into hypertrophic chondrocytes and begin to promote vascular invasion. This, as during endochondral ossification, initiates the recruitment of osteoclast progenitor cells to resorb the tissue. The newly resorbed areas are then filled with de novo osteoid by osteoblasts, forming the repaired bone matrix. This results in the repair of the tissue, but does not necessarily result in a perfectly integrated mineralized matrix with the same shape and/or flexibility as the native, uninjured bone.

1.2.5 Clinical pathologies of bone

Impairment of development at various stages of bone development can result in a wide array of defects. An uncoupling of cell proliferation and terminal differentiation can result in changes that lead to a variety of skeletal dysplasias. These defects often lead to dwarfism and shortening of the appendicular skeleton and/or the axial skeleton [52, 53]. Defects in chondrocyte development, chondrodysplasias, can be caused by: mutations in FGFR3 receptors, resulting in achondroplasia [54]; mutations in SOX9, resulting in campomelic dysplasia [55, 56]; type II collagen mutations leading to type II collagenopathies [5, 57, 58]; type IX collagen mutations leading to epiphyseal dysplasia [5]; and type X collagen mutations leading to Schmid metaphyseal chondrodysplasia [5]. These defects in chondrogenesis account for only a small number of all skeletal dysplasias [5, 59].
Other bone defects include the decoupling of bone deposition and resorption. One such defect, osteoporosis, is caused by the increased resorption by the osteoclasts and decreased deposition by the osteoblasts, resulting in decreased bone mass [60]. The corollary of this is the increased matrix deposition and decreased resorption, resulting in osteopetrosis [61]. Interestingly, both of these diseases result in brittle bones that are more prone to fracture. Osteoporosis alone affects 33% of women and 20% of men. This translates to over $2.3 billion in health care costs in Canada (as of 2010) [62]. Another prevalent pathology of bone, affecting 30-50% of individuals within the United States, is periodontal disease [63-65]. This disease presents as alveolar bone degradation that leads to tooth detachment and loss. Understanding the mechanism of bone development will provide targets for the development of therapeutics to promote the regeneration of bone tissue. This thesis addresses the roles of two proteins during bone development that could be clinically relevant in the regeneration of bone.

1.3 Osteoblasts

Osteoblasts are a cell type derived from a similar mesenchymal progenitor to the chondrocytes [66]. Histologically, the osteoblasts are more difficult to observe as they develop than chondrocytes. A cell culture analysis of osteoblasts provides a method to examine the characteristics of osteoblast differentiation and mineralization [67]. Osteoblastic differentiation is controlled by the master osteogenic regulators Runx2 and Sp7 [68]. As they mature from an osteoprogenitor cell and become osteoblasts, they begin to secrete osteoid, and express osteocalcin and BSP [69]. When the osteoblasts terminally differentiate, they undergo one of three potential pathways of terminal differentiation [67]. First, should the osteoblasts become trapped within matrix of bone,
they develop into osteocytes. The osteocytes are a crucial regulatory cell secreting signaling molecules in order to maintain bone homeostasis. Second, the osteoblasts may also differentiate into periosteal bone lining cells. These cells are, for the most part, inert and await activation from endocrine signaling molecules. Thirdly, the osteoblasts may also undergo apoptosis.

1.3.1 Osteoblast cell culture

The study of osteoblasts in vitro cell cultures systems has been performed using a number of techniques (reviewed in [67]). Osteoblasts can either be primary, such as those isolated from the mesenchymal stromal population or from neonatal calvaria, or they can be a cell line, commonly MC-3T3-E1 or ROS17/2.8 cells. In all cases, the cells are grown in a two-dimensional culture system that is supplemented with ascorbate and glycerol-2-phosphate (G2P). Ascorbate acts as both a signaling molecule to promote osteogenic development, and as a molecule that is required for collagen synthesis. G2P is an important source of organic phosphate that when cleaved by alkaline phosphatase is incorporated into the mineral. These cells are capable of surviving for long periods of culture at a high density, permitting time-course experiments to characterize changes in gene expression and mineralization. The culture of osteogenic cells allows study of specific roles of individual factors related to osteoblastic differentiation and biomineralization.

1.4 Biomineralization

Biomineralization is a complex process by which dissolved salts are precipitated to form highly ordered mineral crystals of hard tissues in living organisms. This is an extremely desirable process as it provides both structure and protection. Undesired
biomineralization results in a number of clinical pathologies covered in Sections 1.2.5 and 1.5.2.3. As such, it has been conserved through all five animal kingdoms, and has been a common feature of animal tissues for approximately 3.5 billion years [70]. The development of the mineralized skeleton occurred at some point during the Cambrian explosion [70]. The primary biominerals that are produced by a variety of organisms include: calcium carbonate for external skeletal carapaces of invertebrates, and hydroxyapatite for the internal skeletal structures of mammals [70]. Highly acidic proteins, such as those in the SIBLING family, have long been thought to be a determinant of biomineral growth [71]. Animals, such as sharks, that have non-mineralized cartilaginous skeletons do not have the SIBLING family of genes supporting their role in directing biomineralization [72].

The rigidity of the mammalian skeleton is due to the carefully regulated deposition of a calcium phosphate mineral, primarily hydroxyapatite (HA), within the collagen lattice. The mineral within the collagen matrix provides the strength for bone to withstand the constant stresses applied to it, while the collagen provides torsional strength to alleviate the brittleness of mineral. Furthermore, citrate has recently been implicated to play an important role in absorbing shock and preventing the aggregation of the HA crystals [73]. The amount of mineral deposited within bone is a process that requires careful balance. Too much mineral results in a brittle tissue; too little mineral and the tissue cannot withstand the stresses applied to it. Interestingly, the localization of mineral is restricted to specific sites within the body, under normal conditions.
1.4.1 Hydroxyapatite

The mineralized portion of mammalian bones is composed primarily of HA \([\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2]\). HA is a stable precipitate of calcium and phosphate. Its precipitation is thought to be regulated by extracellular matrix proteins, as collagen alone has been shown to be unable to promote mineralization [74-77]. HA has a very ordered structure that provides the necessary stability to bone. It propagates longitudinally alongside the collagen fibres \textit{in vivo}. The typical dimensions of HA are 45x25x5 nm [78-80] with the longest face parallel to the collagen. The mineral crystals are tightly packed within the collagen fibres to provide the characteristic rigidity of bone.

1.4.2 Collagen

While the mineral is the key to bone’s rigidity, the organic matrix is the component responsible for its flexibility and stabilizing articular movement. This organic matrix, the osteoid, is composed mainly of type I collagen, although there is a mixture of many non-collagenous proteins, including the SIBLING proteins. The type I collagen provides the basic scaffold of bone, while the other non-collagenous proteins are incorporated with the collagen in order to modulate crystal growth, orientation, and cell recruitment [81].

The main organic component, approximately 90%, of bone is collagen. Of the currently known 28 types of collagen that have been described, bone is composed primarily of type I collagen. Coexpressed with type I collagen in bone are the quantitatively minor collagen types V[82], XI [83], and XXIV [84]. These collagens are thought to be important for controlling the collagen fibril diameter [82, 83] which modulates the fibril strength. As previously mentioned, mineral is required to provide the
rigidity, while collagen is necessary for providing tensile strength. The unique structure of collagen provides properties that are singular in its ability to retain structural integrity and resist denaturation [85].

Type I collagen is a triple-helical molecule formed from two $\alpha_1$ and one $\alpha_2$ chain [86-88]. Both of these chains form a left-handed helix, and when they associate with each other wind into the right-handed conformation of the triple-helix of complete collagen. Each of these polypeptides is of a length of approximately 1000 amino acid residues. When combined, the mass of the triple helical chains is approximately 300 kDa. The specific chains are formed from a repeating pattern of amino acids of $(\text{Gly-X-Y})_n$. Frequently, X is proline, while Y is 4-hydroxyproline. This allows the hydrogen of the side chain of Gly to be centred within the helical structure and permits the $-\text{OH}$ groups on the 4-hydroxyproline to form hydrogen bonds, providing stability to the molecule [89-91].

The triple-helical type I collagen molecules interacts with other type I collagen molecules to form aggregates that are called collagen fibrils. These collagen fibrils assemble in an overlapping pattern such that there are empty regions between each triple helical collagen molecule termed the hole zones. This is what is termed the staggered array of collagen shown in Figure 1.5 [92]. It is in the 67 nm-long hole zones of this array that mineralization is thought to initiate [78, 80, 93-96].

1.4.3 Theoretical models of biomineralization

The process of mineral deposition in bone is an incompletely characterized process that appears to be mediated by a number of highly controlled mechanisms. The body is in a constant state of supersaturation with respect to calcium and phosphate for
HA nucleation [97]. Despite this, the body tends to have no ectopic calcifications. This includes locations of higher levels of ion concentration, such as milk and blood. In order to regulate the process of biomineralization, there is likely an intricate interplay between the proteins and ions within the body. Biomineralization is thought to begin in the aforementioned hole zones within the collagen lattice [94, 95, 98]. This has been demonstrated to be the case in turkey tendon by Arsenault et al [94] and later in human bone [78]. They showed that following the initial nucleation of mineral within the hole zones, the HA crystals then propagate longitudinally parallel to the C-axis of the collagen lattice [99].

The nucleation of mineral is thought to be initiated through one of a few different models. One model proposes that nucleation of mineral is initiated by the acidic regulatory proteins that are localized within these hole zones [78]. It is hypothesized that these highly acidic non-collagenous regulatory proteins bind calcium in order to ultimately form the critical nucleus of HA either through heterogeneous or epitactic nucleation, discussed below. Another model that has been proposed suggests that the biological inhibitors, such as OPN and pyrophosphate, are removed in order to permit the mineral to grow.

Heterogeneous nucleation is a process by which mineral grows on a non-mineral surface [100]. In the case of mammalian skeletons, this is a matrix-mediated system where osteoid is the surface on which the critical nuclei are formed. The surface chemistry of the matrix is required to be capable of sequestration and/or deposition of ions onto the scaffold, or stabilization of the sub-critical nucleus resulting in the crystal growth [101]. One mechanism by which matrix-mediated growth is thought to occur is by
sequestering ions to the surface of the matrix [102]. Following sequestration, the ions are condensed into nano-particles, eventually leading to the formation of a critical nucleus that grows to mineralize the organic matrix.

Epitactic nucleation is a form of crystal nucleation where a surface, provided by an organic system, acts as a mimic of a crystal face [100]. This has been demonstrated in the nacreous layer of a mollusk shell, where it is capable of providing a surface that mimics a crystal face to promote biomineralization [103]. Many of the proteins thought to be involved in the binding and/or nucleation of crystals are inherently acidic, or contain a number of phosphates to provide the negative charge. This is the mechanism by which BSP is thought to act in order to promote biomineralization [104, 105].

1.4.4 Inhibition of mineralization

Within the process of biomineralization, there is a complex interplay between the growth and inhibition of mineral. Inhibition is required in order to regulate the overall speed and the amount of crystal that is formed in hard tissues. Inhibition of growth is also desirable to prevent or slow pathological calcification of soft tissues, such as atherosclerosis and kidney stone formation. Molecules that have been shown to be strongly correlated with the inhibition of mineral growth are OPN [106-108], matrix-gla protein [109], and pyrophosphate [110]. These regulators of growth can act on mineral in a couple of different ways [111]. The first is during the initial precipitation of calcium phosphate nanoparticles whereby these inhibitors bind and block further particle growth. The second is when these molecules bind the surface of growing mineral crystals to competitively inhibit further ion deposition. These mechanisms result in the retardation of the growth of mineral crystals.
1.5 General introduction to the SIBLINGs

The Small Integrin-Binding LIgand N-linked Glycoprotein (SIBLING) family is a group of proteins that includes bone sialoprotein, osteopontin, dentin matrix phosphoprotein (DMP1), dentin sialophosphoprotein (DSPP), which is cleaved into dentin sialoprotein (DSP) and dentin phosphophoryn (DPP), and matrix extracellular phosphoglycoprotein (MEPE). All of these proteins are derived from a common ancestor that is shared with other calcium-binding proteins found in enamel, milk, and saliva [112]. The genes encoding members of the SIBLING family are located in a syntenic gene locus that has been named the “bone gene cluster” located on murine chromosome 5 [113], and human chromosome 4 (4q21) depicted in Figure 1.3 [112]. Genetically, these proteins are localized as single copy genes with similar intron-exon patterns [112]. For example, the first exon is non-coding, the second exon contains the loader sequence and first amino acids of the protein, with one of the final exons containing the RGD integrin-binding sequence. SIBLING proteins are thought to be a crucial regulatory element of biomineralization in animals. Animals with cartilaginous skeletons, such as sharks, lack these genes, and as such lack a mineralized skeleton [72].

These proteins all share similar features in regards to their structure and function. All the members of this family are intrinsically disordered proteins, thus they have a flexible structure [81, 114-118]. This flexibility is thought to be important in their ability to bind multiple motifs while maintaining their function in situ. All of these molecules are highly acidic and may also contain contiguous acidic amino acid residues, either
Figure 1.3: Schematic of human chromosome 4 with the locations of the SIBLEING genes. The SIBLEING genes are located between site 4q21 and 4q23. Each gene encodes a single copy gene. The SIBLEING family and all they share similar intron-exon patterns, structural, and functional characteristics.
glutamic acid or aspartic acid [114, 119-122]. This electronegativity is responsible for binding to the mineral with which they interact. These proteins also contain extensive post-translational modifications, including phosphorylation, glycosylation and sulfation. Phosphorylations have been identified to be a modification that alters the ability to nucleate [123], inhibit, and bind [124] mineral. Finally, all these proteins contain an RGD sequence that binds cell-surface integrins in order to promote cell signaling.

1.5.1 Bone sialoprotein

Bone sialoprotein was first identified as one of the most abundant non-collagenous proteins in bone. BSP was originally isolated as a 23 kDa fragment from bovine cortical bone in the 1960s [120, 125, 126]. Comprehensive characterization of the molecule was not completed until nearly two decades later, when the rat BSP cDNA sequence was elucidated in 1988 [127]. The complete characterization of BSP identified it as an anionic phosphoglycoprotein with a high concentration of sialic acid residues and extensive post-translational modifications [128, 129]. BSP is calculated to have a theoretical molecular mass of 33-34 kDa [120], but following post-translational modification, it tends to run between 60 and 80 kDa on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) [130]. BSP is highly conserved in all species with mineralized skeletons. This includes a wide variety of animals including chicken, frogs, and mammals [131, 132]. BSP’s redundancy and conservation is an indication of its importance in the regulation of biomineralization and bone development. BSP’s anionic characteristics and its prevalence in mineralized tissues suggest that it is one of the primary regulators of biomineralization in bone and teeth. Experiments performed following its primary sequence characterization by Oldberg et al. [127] have
demonstrated that BSP is a potent nucleator of mineralization [105], and it has a strong propensity to bind hydroxyapatite mineral, discussed below.

1.5.1.1 Structure and functional regions

BSP is an intrinsically disordered proteins with little or no secondary or tertiary structure [81, 133, 134]. It contains highly conserved poly-glutamate sequences [131, 132] that are critical for hydroxyapatite (HA) nucleation [105] and binding [128, 135]. BSP contains an amino-terminal collagen-binding sequence, a carboxy-terminal integrin-binding RGD sequence, and two central highly acidic poly-glutamate sequences [120]. A schematic of BSP and its functional regions are shown in Figure 1.4. The collagen-binding sequence mediates the attachment of BSP through hydrophobic interactions [136]. When bound, BSP has been demonstrated to have enhanced nucleation potency [137]. The RGD sequence of BSP mediates cell attachment [138, 139], but it also has been demonstrated to promote osteoblast cell differentiation and mineralization in vitro [140, 141]. BSP acts through binding the cell-surface integrins αVβ1 [142], αVβ3 [143], and αVβ5 [144]. Due to the expression characteristics of BSP and its functional regions, it is postulated to be a potent initiator of biomineralization and mineralized tissue development in vivo.

Cell types involved in bone homeostasis, osteoblasts and osteoclasts, have both been demonstrated to be controlled by BSP-mediated cell-surface receptor activation and intracellular signaling. Overexpression of BSP in MC3T3-E1 osteogenic cells promotes terminal differentiation through the cellular signaling pathway involving focal adhesion kinase and extracellular signal-regulated kinases [140]. This results in an increase in
Figure 1.4 Linear schematic of bone sialoprotein. BSP is approximately 300 amino acids long. The collagen-binding sequence (yellow) is localized at the N-terminus. There are two poly-glutamate sequences (red) that are responsible for binding and nucleation of HA. There is also a C-terminal RGD integrin-binding sequence (blue) that promotes cell adhesion and signaling.
expression of osteoblastic markers such as Runx2, Sp7, and Bglap in MC3T3-E1 cells [141]. In addition, the osteoblasts demonstrate an increase in matrix mineralization, compared to the control, when supplemented with recombinant BSP, or when BSP is overexpressed. Conversely, treating MC3T3-E1 osteoblastic cells with shRNA, to reduce BSP expression, results in a decrease of matrix mineralization and osteoblast marker expression. These studies show that BSP is a promoter of differentiation and mineralization of osteoblasts \textit{in vitro}.

Osteoclasts have also been demonstrated to both express BSP [145] and interact with it [146, 147]. The osteoclastic interaction with BSP appears to be dependent on the phosphorylation state of BSP, as loss of phosphates results in osteoclasts less able to adhere as compared with phosphorylated BSP [148]. Phosphorylation of BSP promotes osteoclastic resorption \textit{in vitro} [149]. BSP also mediates both the influx [150-152] and the efflux [153] of Ca\textsuperscript{2+} from the osteoclasts, suggesting that it may be involved in intracellular signaling. BSP appears to be most important, but not critical, in directing osteoclastogenesis [154, 155] and bone resorption [156], as further discussed below (Chapter 1.5.1.4). Due to BSP being an active regulator of both osteoclasts and osteoblasts, BSP is thought to have an important role in mediating the homeostasis of bone \textit{in vivo}.

\textbf{1.5.1.2 Expression and regulation}

Under normal physiological conditions, BSP is expressed by mineralizing cells such as hypertrophic chondrocytes, osteoblasts, cementoblasts, ameloblasts, and odontoblasts [145, 157, 158]. In bone its expression is mainly restricted to sites of \textit{de novo} bone formation [157, 159]. The only non-mineralized cells that have been shown to
express BSP natively are the trophoblasts during early embryonic development [145] and salivary glands [160]. The expression by trophoblasts is thought to be important for directing cell migration, as well as promoting the implantation of the embryo in utero.

Previous studies investigating the temporospatial expression characteristics of BSP in de novo bone formation have demonstrated that it is expressed by hypertrophic chondrocytes and osteoblasts at a time coinciding with the onset of mineralization [145, 157, 158, 161]. However, BSP expression patterns in developing chondrocytes of the growth plate are still controversial [145, 157]. BSP is also expressed at high levels in other mineralized tissues, including cementum, and dentin [120]. BSP is detectable in the Golgi apparatus of osteoblasts, as it is post-translationally modified and prepared for secretion [161]. These investigations of the expression of BSP suggest that it may be an important regulator of biomineralization as well as cell growth and migration characteristics.

Transcriptional expression of BSP is regulated by a number of different molecules. Basal expression levels are provided by a TATA-like box (TTTATA) and an inverted CCAAT box (GGTTA) [162, 163]. Additionally, transcription factors that have been shown to bind to the promoter sequence include: Ap-1, Cre, and NF-κB [162-164]. Runx2 has also been shown to be a promoter of Bsp transcription in osteogenic [140, 141] and non-osteogenic cells [165]. BMPs are a class of molecules that promote both BSP expression and osteogenic differentiation [166]. Due to BSP’s role as a promoter of biomineralization, its expression is normally silenced. These complex regulatory characteristics are crucial to the maintenance of homeostasis and restricting mineral deposition to desirable areas.
1.5.1.3 BSP-mediated nucleation

BSP has been shown to be a potent nucleator of hydroxyapatite crystals [105]. This is hypothesized to be initiated through a mechanism where the highly acidic sequences attract Ca$^{2+}$, which in turn recruits PO$_4^-$ (Figure 1.5). Eventually this collection of ions forms a critical nucleus, which leads to the collagen being mineralized. The attached phosphates are thought to be an important element regulating the nucleation potency of BSP, and one in particular, serine-136, has been identified to be promote BSP’s nucleation potency [167]. The binding of BSP to collagen also enhances the ability for BSP to nucleate mineral in a steady state collagen-gel system [137].

1.5.1.4 Transgenic BSP mice

Genetic manipulation of the expression of BSP in vivo has resulted in some interesting phenotypes. First, overexpression of BSP cDNA in mice by the cytomegalovirus (CMV) promoter (CMV-BSP) resulted in shortened bones and increased mineralization [155]. The changes in the growth plate described by Valverde et al. showed a decrease of the proliferative zone in the tibia of the CMV-BSP mouse [155]. This decrease of proliferative zone length due to over-expressing BSP is likely due to BSP promoting early cell-cycle exit due to terminal differentiation. This is evidenced by the CMV-BSP mouse demonstrating increased numbers of hypertrophic chondrocytes. Additionally, these mice demonstrate an uncoupling of bone deposition and resorption. The bones of CMV-BSP mice have fewer differentiated osteoblasts and either increased activity or greater numbers of osteoclasts.

Interestingly, ablation of Bsp in a transgenic mouse model (Bsp$^{-/-}$) results in mice that are phenotypically similar to the CMV-BSP animals. These mice also have shortened long
Figure 1.5 BSP-mediated nucleation in the hole zones of the collagen scaffold. (A) Schematic of the staggered array of collagen fibrils with BSP (yellow and red) bound to collagen either adjacent or within the hole zone. The negative charge of BSP promotes the recruitment of the ions to form the critical nucleus. (B) BSP is postulated to stabilize the formation of the critical nucleus within the hole zone of the collagen scaffold, promoting the growth of the calcium phosphate crystals to form HA and mineralize the bone.
bones and increased trabecular bone density at 4-months-of-age compared to their wild-type controls [154]. These phenotypes are likely caused by differing mechanisms. The increased bone density is proposed to be the result of the mice lacking the same bone turnover rate as the wild-type mice. In contrast to this, the $Bsp^{-/-}$ mice have been demonstrated to have decreased mineralization of cortical bone and a significant delay in cortical bone repair following injury [168]. This process of repair in many ways mirrors bone development and this suggests a defect in endochondral bone development, although direct investigation of the developing bone is yet to be performed. What is known of the defect during development is that there is a minor decrease in mineralization of bone in $Bsp^{-/-}$ neonatal mice [154].

As mentioned above, in vitro studies have shown that BSP interacts with osteoclasts, thereby promoting the attachment and mineralized-surface resorption activity [156, 169]. However, Wade-Gueye et al. demonstrated that ovariectomized $Bsp^{-/-}$ mice are still susceptible to significant bone loss or osteoporosis, suggesting that BSP is not a critical requirement for osteoclast attachment and bone resorption in vivo [170]. Previous work investigating BSP’s potential to promote repair of bone defects supports this hypothesis, as BSP has been shown to positively enhance mineralization and repair in vivo [171].

In addition to these defects in bone mineralization, BSP is also involved in cementum formation and is integral to proper tooth attachment [172]. Loss of BSP in this model also resulted in significant tooth resorption and alveolar bone degradation. These studies suggest that BSP is also an important regulatory element in the development and mineralization of periodontal tissues in vivo.
1.5.2 Osteopontin

Osteopontin is an anionic phosphoprotein that is present in many tissues and cell types, including bone and physiological fluids [173]. OPN is also expressed during inflammation, wound healing, and pathological conditions such as cancer [174], discussed below. As such, it was concurrently characterized by a number of independent investigators studying a variety of different tissues. It was first isolated and identified as a 60 kDa transformation-associated phosphoprotein [175]. It was also identified as a 57 kDa molecule that is present in the calcified matrix of bone [176-178]. Shortly thereafter, the primary sequence of OPN was characterized by molecular cloning and sequencing from rat cDNA [119]. OPN is calculated to have a theoretical molecular mass of 33-34 kDa [173, 179], but similarly to BSP, it runs slower at 44 to 75 kDa, by SDS-PAGE. This is likely due to the strong anionic charge as well as extensive post-translational modifications, including phosphorylations, that result anomalous sizes as determined by SDS-PAGE [173, 179]. OPN has been shown to be a potent inhibitor of the formation and growth of calcium oxalate [180] and calcium phosphate in vitro [106, 124].

When OPN was first identified, it was given a number of names that reflect the different tissues from which it was characterized. These include early T-lymphocyte activation, secreted phosphoprotein, uropontin, and bone sialoprotein I. The name bone sialoprotein I was due to it having a high sialic acid content, while the protein currently known as BSP was called BSP-II [177]. The eventual name change to osteopontin, from BSP-I, is derived from its ability to provide a bridge (pons) between the cellular phase and mineral phase (osteo).
1.5.2.1 Structure and functional regions

As mentioned previously, OPN shares a number of functional characteristics with the other members of the SIBLING family. A schematic of OPN is presented in Figure 1.6. First, it is intrinsically disordered and shares the flexible structure of the other family members [181]. It also contains regions of contiguous acidic residues, in this case polyaspartate, required for mineral binding and inhibition [135, 182]. Like BSP, OPN has been shown to be capable of binding collagen [183]. OPN also contains a “QKQ” transglutaminase cross-linking site, important for the formation of polymers with other OPN molecules and other proteins [173]. When isolated from bone, OPN has been shown to be highly cross-linked [184], in contrast with other SIBLING proteins. This polymerization is thought to be important for enhancing OPN’s ability to bind collagen [185]. Additionally, OPN contains cell signaling sequences including the SIBLING-shared RGD sequence [173, 179], multiple CD-44 binding sites [186-189], and a cryptic murine SLAYGLR sequence [190] that is exposed upon thrombin cleavage [191].

Phosphorylation of OPN has been demonstrated to be one of the major mediators of its functions [179]. Varying the number of phosphorylations has been suggested to change its interactions based on cell type. For instance, MDA-MD-435 cancer cells more readily adhered to OPN derived from fibroblasts (low phosphorylation) than osteoblast-derived OPN (high phosphorylation) [192]. In contrast, this study also demonstrated that the mouse ras-transformed 275-3-2 embryonic fibroblasts preferably adhered to the osteoblast-derived OPN than the fibroblast-derived OPN. Furthermore, it has been shown that macrophage recognition of OPN is dependent on its phosphorylation state [189]. Its phosphorylation state is also known to promote the adhesion and spreading of
Figure 1.6 Linear schematic of osteopontin. OPN is approximately 300 amino acids long. The poly-aspartic acid sequence (green) is responsible for binding and inhibition of mineral growth. There is also a central RGD integrin-binding sequence (dark blue) that promotes cell adhesion and signaling, as well as a cryptic SLAYGLR sequence that is activated upon thrombin cleavage.
macrophages, and their migration is augmented upon OPN treatment [189]. It has also been demonstrated that osteoclast activity is dependent on OPN post-translational modification [149]. Thus, the response to the phosphorylation state of OPN is cell-type specific.

1.5.2.2 Expression and regulation

The expression patterns of OPN in normal physiological conditions are highly complex and dependent on a variety of regulatory elements. Opn is transcribed from a single copy gene. It can be spliced forming multiple versions of the protein [193, 194], but its primary expression tends to be full-length OPN. In bone, expression of OPN has been shown to be linked with formation of new bone along the mineralizing front [195]. This high expression level suggests that OPN may regulate the growth of the mineral crystals being deposited during de novo bone formation. The expression of OPN in osteoblasts is dependent on a variety of regulatory factors, including: synthetic bisphosphonates [196], inorganic phosphate [197], and 1,25-dihydroxyvitamin D3 [196, 198]. In addition, studies have suggested that OPN expression is regulated by Ank and PC-1 (gene name Enpp1) in relation to osteoblasts exposure to inorganic phosphate in the cell culture medium [199-201].

The role of OPN expression in osteoblasts presents a controversial story. OPN is a marker of osteoblast development, and its role during osteoblast development is still not fully characterized. The integrin-binding RGD sequence in OPN is predicted to play a role in promoting osteoblast signaling [202, 203], and has been shown to both promote [204] and inhibit [205] osteoblast differentiation. This, in turn, resulted in increased and decreased mineralization in their studies, respectively. Clarification of the effects of OPN
on osteoblast development will help to elucidate the role of this protein in the regulation of biomineralization.

1.5.2.3 Inhibition of mineralization

OPN has been shown to be a potent inhibitor of HA nucleation and growth in vitro [106, 124]. OPN has been studied extensively in order to understand the role it plays in regulating crystal growth. It has long been known that OPN is involved in a variety of pathological conditions including: atherosclerosis [206], and kidney stones [207]. The first motif of OPN shown to inhibit mineralization is that of the highly acidic poly-aspartate regions [124, 208]. Other binding motifs that do not contain poly-aspartate have also been shown to inhibit crystal growth, as reviewed by Azzopardi et al. [209]. These sequences generally include sites with phosphorylations imparting a greater electronegativity. These crystal binding regions of OPN competitively perturb mineral growth by interacting with the positively-charged calcium molecules of the crystal surfaces [210]. Bovine milk OPN has been shown to directly inhibit HA nucleation by binding crystal faces of HA using its multiple crystal-binding motifs [210-212].

There are a number of reports in the literature of OPN being associated with atherosclerotic plaques [206, 213-217], which has lead to experiments investigating its role in cell cultures of vascular smooth muscle cells (VSMCs). OPN is thought to play a role inhibiting mineralization in these cells due to its localization within atherosclerotic plaques [206, 218]. Supplementation of the VSMCs with exogenous OPN isolated from neonatal rat smooth muscle cell cultures resulted in decreased mineral deposition [219]. In a similar system, Opn<sup>-/-</sup> cells were grown under high phosphate conditions to induce calcification, and were shown to have increased mineral deposition relative to WT
controls [220]. These studies suggest OPN inhibits pathological calcifications in atherosclerotic plaques.

Of the aforementioned motifs responsible for crystal growth inhibition, two OPN sequences have been studied in detail to understand their ability to adsorb to mineral and inhibit its growth in vitro [209]. The inhibiting peptides that had high electronegative charge were shown to be capable of strongly binding to HA crystal faces, while more neutral peptides resulted in no crystal growth inhibition. These studies suggested that the negatively charges regions, due to phosphorylation or the contiguous poly-D sequence of OPN, are critical attributes for mineral crystal binding and inhibition.

1.5.2.4 Opn−/− mice

Mice with the Opn gene ablated (Opn−/−) have been reported to have a number of phenotypic anomalies that are not readily apparent unless challenged or probed carefully. They have increased bone mineral density when assayed using Fourier transform infrared (FTIR) imaging [221]. This phenotype develops progressively as the animals age, suggesting a dysregulation between mineral deposition and resorption [221]. Rittling et al. have demonstrated that Opn−/− mice have reduced osteoclast activity [222], suggesting that their increased mineral density phenotype is caused by impaired mineral resorption. Moreover, the mice show many atypical responses to stress. Reduction of the mechanical stress, via tail-suspension unloading, resulted in a lack of osteoclastic response. Without OPN, mechanical signaling is impaired and there is no increase in the resorptive capacity compared with wild-type controls. There is further data to suggest that the Opn−/− cells isolated from spleen and bone marrow readily form osteoclasts. This suggests that OPN is required for osteoclast resorption, while it is not involved in osteoclast formation remains
unchanged [223]. This hypothesis is further supported by the fact that when the $Opn^{+/-}$
mice are implanted with bone discs, only those discs treated with OPN are resorbed
[224]. This could be due to the inability for cells to localize to sites of resorption and/or a
decreased capability for the osteoclasts to act, thus impairing their ability to resorb the
bone. These data propose that OPN is an important regulatory element responsible for
translating mechanical effects to the cells involved in bone homeostasis.

1.5.3 Disorders associated with SIBLING proteins

The majority of abnormalities that arise within mineralized tissues are associated
with the expression and mutations of the collagen genes, and none have been attributed to
aberrations of the SIBLING proteins so far. Interestingly, oral pathologies have been
shown to be caused by mutations within the chromosomal region containing the
SIBLING proteins in humans [225]. These oral pathologies include dentinogenesis
imperfecta and dentin dysplasia [225-227]. These defects in humans are similar to the
phenotypes of mice with the deletion of $Dspp$ [228] and $Dmp1$ [229]. The $Dspp^{+/-}$ mice
demonstrate a phenotype of widened predentin, a defect in mineralization that is similar
in phenotype to dentinogenesis imperfecta type II. The phenotype of the $Dmp1^{+/-}$ mice
includes a number of defects during postnatal tooth development that also match
dentinogenesis imperfecta in humans. As mentioned above, the loss of BSP also resulted
in the loss of acellular cementum in mice [172]. These studies suggest that the SIBLING
proteins are integral to normal odontogenesis, and that aberration of these genes can
result in a variety of dental defects.

Examples of pathological expression of BSP include breast cancer [230-234],
arterial calcifications [235], and kidney stones [236]. The expression of BSP in cancers
has been associated with their calcifications and aggressive metastatic characteristics [233, 237]. This has been specifically identified in breast cancers that have a propensity to metastasize to bone [230]. The BSP expression in metastatic cancers suggests it is a potent chemotactic molecule. This has been confirmed in vitro where decreased BSP resulted in impaired metastasis of MDA-MB-231 breast cancer cells [237, 238].

Dysregulation of BSP also suggests it may be promoting the survival and/or proliferation of these highly aggressive cancer cells. BSP has also been shown to be expressed in other pathological conditions with ectopic mineralization, such as atherosclerosis [235]. Localization of BSP within these plaques suggests that it may have some part in adversely promoting mineralization resulting in arterial stenosis.

OPN has also been identified within similar pathological conditions of cancer, atherosclerotic plaques, and kidney stones, among others. OPN has been identified to be important in mediation of the inflammatory response [239] due to its integrin-binding capabilities [240]. It has been demonstrated that OPN promotes the migration and activity of neutrophils during inflammation. The expression of OPN has been demonstrated to increase in response to certain pathogens [241-244]. It is also worth noting that upon loss of OPN in mice wound healing is altered [245]. These studies all suggest that OPN is a critical factor in the mediation of inflammatory responses.

OPN is studied extensively in cancers as it is expressed in many conditions including: multiple myeloma, leukemia, pancreatic cancer, and melanoma (reviewed in [174]). OPN is strongly correlated with metastatic potential of ras-transformed NIH 3T3 cells [246] and also shown to be upregulated by β-catenin, resulting in increased migration and tumorigenesis in epithelial cells [247]. Interestingly, in contrast to the
phosphorylation dependent responsiveness in osteoclasts and macrophages, cancer cell response appears to be independent of post-translational modification [248]. Similarly to BSP, OPN has been shown to be correlated with vascular invasion. OPN has been shown to promote VEGF-induced cell migration [249] and directly induce angiogenesis in endothelial cells [250].

The exact role of OPN during pathological calcifications is not completely understood, but it appears to be an inhibitor of mineral growth. OPN has been shown to be detected in atherosclerosis [206] and highly expressed during early lesion formation by macrophages and T lymphocytes [216]. Although the Opn⁻/⁻ mouse demonstrates no significant phenotype of atherosclerotic lesions, when it is ablated alongside MGP, plaque growth is exacerbated dramatically [251]. As discussed above, OPN has also been shown to be detectable in kidney stones, and is a potent inhibitor of calcium oxalate growth. These studies confirm that OPN is an important mediator of mineral growth in pathological calcifications.

1.6 Specific objectives and hypotheses

1.6.1 Overview

The goal of this thesis is to delineate the role of the SIBLING proteins BSP and OPN in the mediation of biomineralization and skeletal development. Both of these proteins have been shown to be important for the regulation of mineralization in vitro and in vivo, but their interactions with the cells involved in biomineralization are not completely characterized. This thesis uses a developmental model and osteogenic cell cultures in order to clarify the roles of SIBLING proteins during biomineralization.
The characterization of the results of loss of BSP during embryonic development provides a more thorough understanding of its role during endochondral ossification. The phenotype of the $Bsp^{-/-}$ mouse has only been characterized in adult tissues. The time of onset of the phenotype is not yet known. Characterization of the development of the phenotype will provide evidence to understand the role of BSP during endochondral ossification. Herein I studied the effects of loss of BSP in the developing tibiae. Furthermore, I investigated the phenotype of osteoblasts upon the loss of BSP, and performed adenoviral overexpression to rescue the phenotype of $Bsp^{-/-}$ osteoblasts. This study, along with the $Bsp^{-/-}$ mouse, will identify the role of BSP during osteogenic cell differentiation and their biomineralization.

The characterization of the role of OPN during osteogenic mineralization has not been fully elucidated. In order to address the role of OPN during osteogenic cell differentiation and mineralization, $Opn^{-/-}$ osteoblasts were grown in cell culture and compared to wild-type cells. Once the phenotype was characterized, the cells were then supplemented with exogenous bovine milk OPN and OPN peptides. The characterization of the $Opn^{-/-}$ osteoblasts and subsequent treatment with OPN will identify the mechanism by which it acts to regulate osteogenic-mediated biomineralization.

Herein I identify the importance of BSP during ossification, as well as its role in promoting osteoblast development. In a novel finding, I show that BSP appears to be critical for normal growth plate development and chondrocyte cell-cycle progression. Furthermore, I show that OPN is strictly an inhibitor of mineral growth in osteogenic cell cultures. Additionally, supplementation with exogenous OPN and its functional peptides are capable of rescuing the enhanced mineralization phenotype of the $Opn^{-/-}$ osteoblasts.
without affecting their differentiation characteristics. The identification of the roles of these SIBLING proteins during the process of biomineralization can provide potential targets for prevention or treatment of pathological calcifications.

1.6.2 Study one – Loss of bone sialoprotein impairs endochondral bone development

Hypothesis – BSP is a positive regulator of osteogenic cell development and matrix mineralization.

Specific Aims

1. Determine the phenotype of BSP loss during endochondral ossification.
2. Identify the cells types affected by loss of BSP.
3. Characterize gene expression characteristics upon loss of BSP.

Both chondrocytes and osteoblasts are responsible for promoting matrix deposition and mineralization. Loss of BSP resulted in phenotypic changes that presented as delayed ossification and mineralization. Bsp\(^{-/-}\) mice also demonstrated an aberrant growth plate that likely contributed to their decreased bone length. These studies demonstrated that loss of BSP resulted in multiple cell types having delayed developmental characteristics. There was no significant change in osteoclast activity, suggesting that BSP is not critical to normal osteoclast activity \textit{in vivo}. 
1.6.3 Study two – The role of bone sialoprotein and its functional characteristics in osteoblast differentiation and mineralization

**Hypothesis** – BSP is a positive regulator of osteoblast maturation and mineralization. The functional regions of BSP play independent roles in promoting the differentiation and mineralization of osteoblasts.

**Specific aims**

1. Characterize the phenotype of $Bsp^{-/-}$ osteoblasts over a time-course of development *in vitro*.
2. Perform adenoviral overexpression of BSP to rescue the phenotype of the $Bsp^{-/-}$ osteoblasts.
3. Determine the effects of the functional regions of BSP on osteoblast mineralization and differentiation *in vitro*.

Loss of BSP resulted in delayed osteoblast differentiation and mineralization relative to wild-type controls. I also demonstrated lower metabolic activity of $Bsp^{-/-}$ osteoblast cultures, suggesting these cells have decreased proliferation. Overexpression of BSP resulted in increased expression of markers of osteoblast differentiation and increased mineralization relative to controls. Overexpression of the BSP mutants resulted in increased mineralization. Interestingly, N-terminal truncation and integrin-binding inactivation both resulted in upregulation of different osteogenic genes. This suggests that BSP may have multiple signaling motifs, the RGD integrin-binding sequence and at least one other as yet unidentified signaling motif.
1.6.4 Study three – Osteopontin modulates mineral formation and not osteogenic cell development in vitro

**Hypothesis** – Osteopontin is an important regulatory element in mineralization, and its loss will result in increased mineralization of osteoblast cell cultures.

**Specific aims**

1. Determine the phenotypic changes of primary osteoblasts upon loss of OPN.
2. Supplement the $Opn^{-/-}$ osteoblasts with exogenous OPN to attenuate mineralization in the cell cultures.
3. Discern whether functional peptides of OPN can inhibit mineralization in the $Opn^{-/-}$ osteoblast cell cultures.

I found that loss of OPN resulted in no physiologically relevant changes in expression of osteoblast genes, but that there was increased mineralization in the osteoblast cultures. Supplementation with bovine milk OPN resulted in a dramatic reduction in mineralization while not changing expression of terminal differentiation markers of osteoblasts. Supplementation with functional peptides of OPN rescues the hypermineralization phenotype of the $Opn^{-/-}$ osteoblasts, while also not changing the expression of marker genes of terminal osteoblastic differentiation.
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CHAPTER TWO

LOSS OF BONE SIALOPROTEIN LEADS TO IMPAIRED ENDOCHONDRAL BONE DEVELOPMENT AND MINERALIZATION

1 This Chapter has been reproduced from:
E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, H.A. Goldberg. 2014. Loss of bone sialoprotein leads to impaired endochondral bone development and mineralization. (In revision at Bone August, 2014)
2.1 Chapter Summary

Bone sialoprotein (BSP) is an anionic phosphoprotein in the extracellular matrix of mineralized tissues, and a promoter of biomineralization and osteoblast development. Previous studies on the Bsp-deficient mouse (Bsp\(^{-/-}\)) have demonstrated a significant bone and periodontal tissue phenotype in adulthood. However, the role of BSP during early long bone development is not known. To address this, early endochondral ossification in the Bsp\(^{-/-}\) mouse was studied. Embryonic day 15.5 (E15.5) wild-type (WT) tibiae showed early stages of ossification that were absent in Bsp\(^{-/-}\) mice. At E16.5, mineralization had commenced in the Bsp\(^{-/-}\) mice, but staining for mineral was less intense and more dispersed compared with that in WT controls. Tibiae from Bsp\(^{-/-}\) mice also demonstrated decreased mineralization and shortened length at postnatal day 0.5 (P0.5) compared to WT bones. There was no detectable difference in the number of tartrate-resistant acid phosphatase-positive foci at P0.5, although the P0.5 Bsp\(^{-/-}\) tibiae had decreased Vegfa expression compared with WT tissue. Due to the shortened tibiae the growth plates were examined and determined to be of normal overall length. However, the length of the resting zone was increased in P0.5 Bsp\(^{-/-}\) tibiae whereas that of the proliferative zone was decreased, with no change in the hypertrophic zone length of Bsp\(^{-/-}\) mice. A reduction in cells positive for Ki-67, an S-phase cell-cycle marker, was noted in the proliferative zone. Decreased numbers of TUNEL-positive hypertrophic chondrocytes were also apparent in the Bsp\(^{-/-}\) tibial growth plates, suggesting decreased apoptosis. Expression of the osteogenic markers Alp1, Col1a1, Sp7, Runx2, and Bglap was reduced in the endochondral bone of the neonatal Bsp\(^{-/-}\) compared to WT tibiae. These results suggest that BSP is an important and multifaceted protein that regulates both chondrocyte
proliferation and apoptosis as well as transition from cartilage to bone during development of endochondral bone.
2.2 Introduction

Mammalian long bones develop through a complex and intricately regulated process called endochondral ossification. The formation of bone initiates as a condensation of pluripotent mesenchymal stem cells to form the template of the bone [1]. These precursor cells differentiate into chondroblasts under the regulation of the transcription factors Sox5, 6, and 9 [2, 3]. The chondroblasts deposit a matrix composed mainly of type II collagen and aggrecan before maturing into chondrocytes [4]. These chondrocytes are highly proliferative and develop the early growth plate [5]. The cells within the growth plate terminally differentiate into hypertrophic chondrocytes at the centre of the bone while secreting type X collagen. At this point the cartilaginous anlage calcifies around the late hypertrophic chondrocytes that secrete vascular endothelial growth factor in order to promote blood vessel invasion, and then proceed to undergo apoptosis [6]. This allows recruitment of osteoclasts that resorb the mineralized cartilaginous matrix, as well as secretion of osteoid followed by its mineralization by osteoblasts [7]. The chondrocytes at the ends of the bones continue to proliferate and hypertrophy to regulate longitudinal bone growth, while the osteoblasts terminally differentiate by either becoming trapped in mature bone and becoming osteocytes, or undergoing apoptosis.

Bone sialoprotein (BSP) is an anionic phosphoprotein believed to be one of the primary regulators of mineralization in bone and teeth. It is highly expressed by mineralizing cells such as hypertrophic chondrocytes and osteoblasts [8-10]. BSP is a member of the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family of proteins. This family includes osteopontin (OPN) and dentin matrix phosphoprotein.
(DMP1), both of which are derived from a common ancestor that is shared with other calcium-binding proteins found in enamel, milk and saliva [11]. The members of the SIBLING family are located in a syntenic gene locus that has been named the “bone gene cluster” located on murine chromosome 5 [12]. BSP shares common features with other members of this group, which are intrinsically disordered proteins with little or no secondary or tertiary structure [13-15]. These proteins also contain integrin-binding RGD (Arginine-Glycine-Aspartate) sequences and highly acidic regions composed of (depending on the protein) poly-glutamate or poly-aspartate sequences. In BSP, the poly-glutamate sequences, which are highly conserved [16, 17], are critical for hydroxyapatite (HA) nucleation activity based on in vitro studies [18]. The RGD sequence of BSP mediates cell attachment [19, 20] but also has been demonstrated to promote osteoblastic cell differentiation and mineralization in vitro through cellular signaling pathways involving focal adhesion kinase and extracellular signal-regulated kinases [21]. As such, BSP is postulated to be an important mediator of bone mineralization.

BSP is highly expressed in developing bones during endochondral ossification [22] and is also deposited at the mineralization front of bone and in the cement lines [23]. Overexpression of BSP downstream of a CMV promoter (CMV-BSP) in vivo resulted in mice that are significantly smaller than their wild-type controls [24]. There are also defects in their endochondral bones, with aberrant growth plate formation and development. The growth plates of the CMV-BSP mice have no change in overall length, although there was an increase in hypertrophic zone size and number of hypertrophic chondrocytes. At 8 weeks of age, the mice also show a decrease in the proliferative zone length. This suggests that the overexpression of BSP promotes terminal differentiation in
these chondrocytic cells. In addition, these CMV-BSP mice have increased numbers of osteoclasts in their trabecular bone, suggesting that BSP promotes osteoclast formation [24]. These differences suggest that BSP is also responsible for promoting terminal differentiation in other types of skeletal cells.

Mice deficient in BSP (Bsp^−/−) also have decreased long bone length and cortical bone thickness relative to wild-type (WT) mice at 4 months of age [25]. Conversely, they have a higher trabecular bone density than WT mice, but an apparent lower rate of bone turnover. This decrease in turnover could be due, in part, to a decrease in osteoclast numbers. However, Wade-Gueye et al. demonstrated that ovariectomized Bsp^−/− mice are still susceptible to significant bone loss or osteoporosis, suggesting that BSP is not a critical requirement for osteoclast attachment and bone resorption *in vivo* [26]. Bsp^−/− mice have decreased mineralization of cortical bone and a significant delay in cortical bone repair following injury [27]. This process of repair in many ways mirrors bone development and suggests a defect in endochondral bone development. Malaval et al. noted only a minor decrease in mineralization of bone from Bsp^−/− neonatal mice, and did not report any other changes to skeletal tissues in these mice [25]. To date, there has been no characterization of BSP’s role in early mineralization and bone development processes.

In this study we sought to determine the role of BSP in the development of endochondral bone. We demonstrate that lack of BSP results in delayed patterns of bone development, which can be attributed, in part, to the reduction in the proliferation and apoptosis of the chondrocytes within the growth plate. Additionally, we show that expression of osteoblastic markers is reduced in the Bsp^−/− endochondral bone at postnatal
day (P0.5). Together, these data demonstrate an important role of BSP in normal bone
development and mineralization.
2.3 Materials and methods

2.3.1 Animal protocol

Animal procedures were performed in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and Animal Care and Veterinary Services (ACVS), University of Western Ontario, under protocol number 2008-092. Preparation and genotyping of Bsp homozygous knock-out (Bsp\(^{-/-}\)) and WT mice were described previously [28]. Mice were maintained on a mixed 129/CD1 background and were fed a standard pelleted mouse diet (2018 Tekland Global 18% protein diet, Harlan Laboratories, USA) and tap water ad libitum. Animals used in this study were generated by breeding heterozygous parents to produce litter-matched offspring. For statistical analyses, the number of litter-matched animals used of each time point was as follows: E15.5 n=6, E16.5 n=13, P0.5 n=10, P10.5 n=5.

2.3.2 Histology and immunohistochemistry

Bsp\(^{-/-}\) and WT tibiae were harvested and prepared for histology as previously described [29-31]. Briefly, animals were euthanized and tibiae dissected and fixed in 10% formalin in PBS at 4°C overnight. Tissues were prepared for paraffin embedding using standard histological processing. Longitudinal sections of the tibiae were collected at 5 μm thickness by rotary microtome and mounted on positively charged glass slides. Slides were then deparaffinised in xylene and rehydrated for histological analyses.

Safranin O staining for the cartilage was performed as previously described [29]. Tartrate-resistant acid phosphatase (TRAP) stain was performed to identify osteoclast activity using a commercial kit (TRAP kit, product # 387A, Sigma, Oakville, Canada) as previously described [30]. TRAP-positive foci per scaled unit area (pixels) were
determined. Von Kossa stain, to detect mineral, was performed using 1% silver nitrate under ultraviolet light for 20 min. Unbound stain was removed using 5% sodium thiosulfate for 5 min. The slides were then stained with 1% w/v alcian blue in 3% acetic acid for 20 min, and counter-stained with 0.1% nuclear fast red for 5 min. All chemicals used for staining were acquired from Sigma. Absolute quantification of von Kossa staining was performed by pixel counting of total positively stained area from each section including both trabecular and cortical bone at time points E15.5, E16.5, and P0.5. P10.5 quantification measured the stained trabecular bone or secondary ossification centre relative to total area. Statistical analyses were performed using independent samples t-test (GraphPad Prism software, v4.00).

Immunohistochemistry (IHC) and immunofluorescence (IF) were performed as previously described with some modifications [28]. Antigen retrieval was done by heating samples to 121°C for 30 sec in 1 M sodium citrate (pH=6) in a decloaking chamber (Biocare Medical, Concord, USA). Samples were slowly cooled to 80°C then removed. Slides were then blocked with 5% goat serum in PBS for 1 h. Samples were probed with primary antibody overnight at 4°C at dilutions noted below, or following manufacturer’s guidelines. After washing, secondary antibody was used according to manufacturer’s protocol then developed using peroxidase substrate. Slides were dehydrated through graded ethanol and cleared in xylene prior to mounting.

Reagents for IF and IHC were: rabbit anti-mouse BSP (1:200, courtesy of Renny Franceschi, University of Michigan, Ann Arbor, USA); rabbit anti-Ki-67 (1:200, ab15580, Abcam, Toronto, Canada); In Situ Cell Death Detection Kit (11684795910, version 16, Roche, Laval, Canada); rabbit anti-mouse PECAM-1 (sc-1506-R, Santa Cruz,
Dallas, USA); and rabbit anti-p57 (sc-8298, Abcam). The secondary probe and development was performed using ABC staining system (sc-2018, Santa Cruz) according to manufacturer directions.

2.3.3 Histomorphometry

Proximal growth plates of Bsp/^- and WT tibiae were evaluated by two independent, blinded observers. Ki-67-positive cells were quantified in a 100x100 μm box along the midline of the growth plate at a distance of 300 μm from the end of the hypertrophic zone. Ki-67-positive cells were quantified and normalized to total number of cells per area by ImageJ (v1.46r, NIH, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) quantification was performed by enumerating total numbers of TUNEL-positive cells in the hypertrophic zone using ImageJ. Overall bone length was measured using images of tibiae taken using a LED2500 dissection microscope with integrated EC3 digital camera (Leica Microsystems GmbH, Concord, Canada) and measuring length digitally on ImageJ, followed by converting the length in pixels to μm. Statistical analyses were performed using independent samples t-test (GraphPad Prism).

2.3.4 Microdissection and real-time quantitative PCR

Mouse tibiae were dissected as shown in Figure 2A. Briefly, bones from neonatal mice were separated such that growth plate chondrocytes were pooled into one of two populations: the resting and proliferative zone (RP) or the hypertrophic zone and endochondral bone (HEB). These tissues were pooled and the total RNA was isolated from bones using RNeasy according to the manufacturer’s protocol (74104, Qiagen, Toronto, Canada). Purified RNA was then reverse transcribed using SuperScript II RT kit
according to the manufacturer’s guidelines (18064, Life Technologies). PCR reactions were performed as previously described [32]. We used the two-step Master mix (4440038, Life Technologies) on a 7900 HT system (Life Technologies) according to manufacturer specifications. Probes used for gene expression are shown in Table 1. Beta-2-microglobulin (B2m) was used as a reference gene for normalization of expression and relative quantification. B2m was confirmed as a reference gene by validating its expression using 18s ribosomal RNA (18s) and determining there was no change in expression patterns between tissues and genotypes (data not shown). We performed statistical analysis using independent samples t-test in Prism.
Table 2.1: Genes assayed using real-time quantitative PCR alongside their TaqMan primer codes. **A.** The primers used for assaying osteoblast markers. **B.** Primers used for assaying chondrocyte expression. **C.** Target genes for assaying modeling alongside their associated product numbers.

### A

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2.4 Results

2.4.1 Bone sialoprotein is expressed during development

Expression of BSP in the developing skeleton was assessed in the immature bones of WT mice by immunohistochemistry (IHC). BSP was detected in mineralizing tissues of the WT tibiae from day E15.5, E16.5, and P0.5 and was deposited throughout the diaphysis of the developing bone (Figure 1A, B, &C). We did not detect BSP protein in any of the matched sections from the $Bsp^{-/-}$ mice. We confirmed $Bsp$ expression in the WT tibiae and its absence in the $Bsp^{-/-}$ tissues by performing microdissections to isolate the resting and proliferative zones (RP fraction) from the hypertrophic zone and endochondral bone (HEB) fraction, as depicted in Figure 2A. Following dissection, we performed qPCR on the separate fractions and assayed for expression of osteogenic and chondrogenic markers (see below). $Bsp$ mRNA was detectable in the RP fraction at 20% of that observed in the HEB fraction in WT samples (Figure 2B). Expression of the hypertrophic chondrocyte markers, ColX and Mmp13, confirmed the dissections were performed without significant cross-contamination, i.e., these markers were not detected in the RP fraction (Figure 2C).

2.4.2 Mineralization is delayed in the tibiae of $Bsp^{-/-}$ mice

To determine whether mineralization was affected in the $Bsp^{-/-}$ mice, histological analysis was conducted on the long bones of $Bsp^{-/-}$ mice and control littermates. By von Kossa and alcian blue staining, delays in mineralization were observed at multiple stages of early bone development in the $Bsp^{-/-}$ mice (Figure 3). In E15.5 WT mouse tibiae, mineral deposition was concentrated around the bone collar, with some staining in late hypertrophic cartilage, whereas mineralization was absent in the $Bsp^{-/-}$ tibiae (Figure 3A).
Figure 2.1: BSP is expressed in mineralizing tissues during development, but not in \textbf{Bsp}^{+/−} mice. Immunohistochemical staining with anti-BSP antibodies at E15.5 (A), E16.5 (B), and P0.5 (C) tibiae show where BSP is detected (brown) in the WT tibiae, and confirms its absence in the \textit{Bsp}^{+/−} mice. All sections were counterstained with methyl green. (E15.5 n=6, E16.5 n=13, P0.5 n=10. Scale bar is 100 \textmu m in all panels).
Figure 2.2: Analyses of Bsp expression in neonatal endochondral bones by microdissection. A schematic of a neonatal bone demonstrates where the bones were cut (red lines) (A). This separated the bone into two sections, the central hypertrophic-endochondral bone (HEB), and the resting and proliferative (RP) fraction. Real-time qPCR demonstrated expression of Bsp in RP and HEB fractions of WT bones and its absence in Bsp\textsuperscript{-/-} tissues (B). Confirmation of exclusion of the hypertrophic zone from the RP fraction was done by performing qPCR for ColX and Mmp13 (C). (n=5, error bars are SEM, * denotes p<0.05)
Mineralization was evident at E16.5 in the $Bsp^{-/-}$ bone, but was reduced by 30% (Figure 3D) and more evenly dispersed throughout the diaphysis compared to the well-defined mineralization pattern at the bone collar in WT sections, supporting a delay in mineralization in $Bsp^{-/-}$ bone (Figure 3B). At P0.5 $Bsp^{-/-}$ bone exhibited a weaker staining intensity than the WT bone which, based on analysis of total area stained, translates into a 40% decrease in overall mineral content (Figure 3C & F). P0.5 $Bsp^{-/-}$ bone samples showed a slight but significant 5% reduction in overall tibia length (Figure 3G). The developmental delay persisted throughout secondary ossification, as demonstrated by the well developed pattern observed in WT P10.5 bone compared to that in the age matched $Bsp^{-/-}$ bone (Figure 3G). Quantification of mineral at P10.5 demonstrated a 40% reduction in mineral of the trabecular bone (Figure 3H) and a 60% reduction in mineral content of the secondary ossification centres (Figure 3I) relative to total area.

2.4.3 Modeling of the developing tibiae is unchanged in the $Bsp^{-/-}$ mouse

To determine whether the delay in ossification was related to a modeling impairment, we analyzed tartrate-resistant acid phosphatase (TRAP) activity in the developing bone. In both E16.5 (data not shown) and P0.5 tissue, there was no detectable difference in the number of TRAP-positive foci in $Bsp^{-/-}$ versus WT (Figure 4A & B). To further delineate the characteristics of modeling in these developing tibiae, we investigated expression of markers of bone modeling, $Rankl$, $Opg$, and $Vegfa$ (Figure 4C & D). No significant difference in $Rankl$ or $Opg$ expression was observed in $Bsp^{-/-}$ versus WT bones. Additionally, the $Opg/Rankl$ expression ratio is unchanged between genotypes. $Vegfa$ expression was decreased by 40% in the $Bsp^{-/-}$ as compared to WT bones, suggesting a delay of vascular invasion in $Bsp^{-/-}$ bone.
Figure 2.3: Mineralization is impaired in the developing tibiae of the $Bsp^{-/-}$ mouse.

Alcian blue and von Kossa (black) staining of E15.5 (A), E16.5 (B), and P0.5 (C) tibiae show location of mineral deposition in the WT and $Bsp^{-/-}$ tissues. Sites of secondary ossification of P10.5 $Bsp^{-/-}$ mouse tibiae compared to the WT bones (D). Quantification of mineral content from E16.5 (E), P0.5 (F), P10.5 trabecular bone (H), and secondary ossification centres (I) tibiae of the WT and $Bsp^{-/-}$ bones. Measurements of neonatal tibia lengths from WT and $Bsp^{-/-}$ mice (G). (E15.5 n=6, E16.5 n=13, P0.5 n=10, P10.5 n=5. Scale bar is 100 $\mu$m in all figures. * denotes significance where $p<0.05$)
Figure 2.4: Modeling of the developing bone by osteoclasts appears unchanged in the \textit{Bsp}^{−/−} tibiae. Representative images of P0.5 bones show TRAP-positive foci in the developing tibiae at low (A), and high-magnification (B). Quantification of TRAP-positive foci per scaled unit area (pixels) (C). qPCR of \textit{Rankl}, \textit{Opg}, \textit{Opg/Rankl} expression ratio, and \textit{Vegfα} from HEB fraction of the WT and \textit{Bsp}^{−/−} bones (D). (P0.5 n=5, qPCR n=5. Scale bars are 100 μm. * denotes significance where p<0.05)
2.4.4 Ratios of growth plate zone lengths are altered in the Bsp⁻/⁻ tibiae

Due to the reduction in overall bone length, we next investigated the growth plate for physiological abnormalities. Whereas there was no difference in the overall length of the growth plates between genotypes, detailed analysis indicated that there were significant differences in lengths of the individual zones between the growth plate of P0.5 Bsp⁻/⁻ and WT bones (Figure 5). The resting phase was 20% longer in the Bsp⁻/⁻ versus WT tibiae, with a reciprocal decrease in length of the proliferative zone, and no change in the hypertrophic zone (Figure 5A & B). To determine the extent of the proliferation defect, we quantified cells expressing the S-phase cell-cycle marker Ki-67. There was a 40% reduction in Ki-67-positive cells in the Bsp⁻/⁻ tibiae as compared to WT tibiae, suggesting a marked decrease of proliferating cells within the proliferative zone, or a delay in cell-cycle progression (Figure 5C & D). Finally, we investigated hypertrophic chondrocyte apoptosis and observed a 40% decrease in TUNEL-positive cells in the Bsp⁻/⁻ versus WT tibial sections (Figure 5E & F).

To investigate expression of chondrocyte markers of differentiation, the microdissection fractions previously described were used. The RP fraction was assayed for expression of developmental markers of chondrocyte development Alp1, Col2a1, Acan, Runx2, and Sox9 and no differences were seen between genotypes (Figure 6). As expected, Bsp expression was undetectable in the Bsp⁻/⁻ RP fraction, while present in the WT tissue. As indicated above, there was also no difference in expression of the hypertrophic markers ColX and Mmp13 in the HEB fraction (Figure 2C).
Figure 2.5: The growth plates of $Bsp^{+/}$ neonatal tibiae show significant aberrations and delayed progression through the cell-cycle. Safranin O staining of WT and $Bsp^{-/-}$ P0.5 tibiae show chondrocytes within the proximal growth plate (A). Quantification of the resting, proliferative, and hypertrophic zone lengths of neonate tibiae from WT and $Bsp^{-/-}$ animals (B). Immunofluorescent staining of Ki-67 within the growth plates, denoting cells in S-phase of the cell cycle, high-magnification insets are shown alongside their respective genotypes (C). Quantification of the number of Ki-67-positive cells in the proliferative zone relative to total cell number (D). Immunofluorescent detection of TUNEL in hypertrophic chondrocytes of the growth plate, high-magnification insets are shown alongside their respective genotypes (E). Quantification of the total number of TUNEL-positive cells in the tibiae (F). Scale bar is 100 µm in all figures. n=10, * denotes significance where $p<0.05$. 


Figure 2.6: Expression of chondrocyte marker genes in the neonatal growth plate of the \textit{Bsp}^{−/−} mouse is unchanged. Quantitative real-time PCR probing chondrocyte markers from the RP fraction of growth plate of tibiae from P0.5 animals. (n=5, error bars are SEM, * denotes p<0.05)
2.4.5 Osteoblast marker expression is impaired in Bsp−/− tibiae

Since there was no change observed in the chondrocyte markers, we next examined markers of osteoblastic development in the HEB fraction from P0.5 mouse tibial bone (Figure 7). The expression of many of these markers was decreased in the neonatal Bsp−/− compared to WT tibiae: Col1a1 was reduced to approximately 50%, Sp7 to 40%, Alp1 and Runx2 to 60%, and Bglap to 30%. No differences were detected in Dmp1 or Spp1 transcript levels in these samples at this time point.
Figure 2.7: Expression of osteogenic genes is reduced in the neonatal tibiae of $Bsp^{/-}$ as assayed by qPCR. Quantitative real-time PCR analyses of osteoblast marker expression. Total RNA was isolated from the HEB fraction of tibiae from P0.5 mice. (n=5, error bars are SEM, * denotes p<0.05)
2.5 Discussion

Malaval et al. have characterized the defects in the long bones of $Bsp^{-/-}$ mice [25]. At 4 months of age, there are decreases in mineral density, overall bone length and cortical bone thickness but an increase in trabecular bone density. They suggested that the increase in trabecular bone density was caused by a deficiency in bone turnover by the osteoclasts [25]. Malaval and colleagues also examined the mineral densities of younger and older $Bsp^{-/-}$ bones. In neonatal $Bsp^{-/-}$ mice the mineral density is decreased, whereas at 1 year of age the mineral content appears normal. In our study, we focused on earlier stages of development to determine when and how BSP deficiency first affects skeletal development. We report a similar phenotype of decreased bone length at the early time point of P0.5, and a decrease in mineral content of the embryonic and neonatal bones. We also observed a temporal delay in onset of mineralization during long bone development. $Bsp^{-/-}$ mice do not have detectable levels of mineral in their tibiae at E15.5, in contrast to the substantial level in the WT bone. Even following the initial deposition of mineral in the $Bsp^{-/-}$ bone, it does not appear to be organized around the bone collar as seen in the WT tibia; instead the mineral appeared to be more evenly distributed throughout the $Bsp^{-/-}$ tibia. The delay in development was not restricted to the sites of primary ossification, but also occurred at sites of secondary ossification as seen in the P10.5 tibiae of the $Bsp^{-/-}$ mouse. These delays indicate that BSP plays an important role in controlling the timing of mineral onset and mineral density in vivo.

BSP has previously been shown to be a promoter of osteoclast formation and resorption [24, 33], and the CMV-BSP mice, which overexpress BSP, have enhanced osteoclast activity in vivo [24]. Based on these studies, and the evident delay in
endochondral ossification in the Bsp<sup>−/−</sup> mice, we examined markers of osteoclast activity. We did not observe any difference in the number of TRAP-positive foci in Bsp<sup>−/−</sup> versus WT mice, nor for the ratio of Opg/Rankl expression, known modulators of osteoclast formation, suggesting that there is no impairment in osteoclast formation in the absence of BSP. This is also supported by previous studies in which Bsp<sup>−/−</sup> mice were challenged by tail suspension [25] and ovariectomy [26] with observed loss of bone similar to that in controls. Of note, the observed reduction in Vegfα expression, suggests that BSP may be a promoter of vascular invasion during endochondral ossification, in agreement with previous studies demonstrating that BSP promotes angiogenesis [34].

To better understand and characterize the decrease in length of the tibiae observed in the newborn Bsp<sup>−/−</sup> mice, we examined the stages of chondrocyte development within the growth plate. While there was no difference in the overall length of the growth plate in newborn Bsp<sup>−/−</sup> versus WT mice, in agreement with the first published work on the Bsp<sup>−/−</sup> mice by the Malaval group [25], we observed the resting and proliferative zone lengths were increased and decreased, respectively, with no change in hypertrophic zone length in the P0.5 Bsp<sup>−/−</sup> growth plates. This differs from the recent work published by Malaval’s group<sup>2</sup> where they observed reduced thickness of both the growth plate and the hypertrophic zone length in newborn Bsp<sup>−/−</sup> mice [35]. At present, we cannot explain these apparent differences; however, differences in housing conditions, animal chow, and/or breeding strategies could be responsible. The overexpressing BSP-CMV mouse model demonstrated a similar phenotype of decreased proliferative zone length, although it had

<sup>2</sup>After submission of this manuscript, Reference [35] by the Malaval group was published online.
an increased hypertrophic zone length [24]. The decreases of the proliferative zone length in both the $Bsp^{-/-}$ and CMV-BSP mice appear surprising. However, our data suggest that the reductions in length are due to different mechanisms. In the $Bsp^{-/-}$ mice it may be caused by delayed entry of chondrocytes into the proliferative zone which leads to the observed increased length of the resting zone. In the BSP-CMV mice there may be an accelerated exit of the chondrocytes from the proliferative zone, leading to the observed increased hypertrophic zone length [24].

Analysis of osteoprogenitor development has shown that BSP is expressed at two distinct times: the first during the proliferative immature osteoprogenitor phase, and the second during maturation from preosteoblasts to osteoblasts [36]. Drawing a parallel between the chondrocytes and osteoblasts, we propose the following model for BSP’s role in chondrocyte maturation within the growth plate. Low-level BSP expression in the chondrocytes initiates cell proliferation from the resting phase while also helping to maintain their proliferative capacity as they mature. Increased BSP expression in the hypertrophic chondrocyte phase then promotes terminal differentiation and apoptosis. This model accounts for the differences seen in both the $Bsp^{-/-}$ mice and the CMV-BSP mice.

Understanding the expression characteristics of osteogenic markers in the developing bone provides additional insight into the state of development in the $Bsp^{-/-}$ mouse. Previous work by Malaval et al. showed that the majority of markers are unchanged ($Alp$, $Coll$, $Sp7$, $Bglap$, $Opg$, $Rankl$) in the fully developed femurs of 4 month old $Bsp^{-/-}$ mice [25]. However, $Spp1$ was significantly decreased in the mature $Bsp^{-/-}$ bone. In our study, the mineralized regions of neonatal tibiae from the $Bsp^{-/-}$ mice show a
different picture of osteogenic marker expression during development: a normal expression of *Spp1* but the decreased expression of other osteogenic cell markers (*Alp1*, *ColIa1*, *Sp7*, *Runx2*, *Bglap*) suggesting fewer mature osteoblasts in the *Bsp*−/− mouse. This is similar to the recently published work on young P6 whole bone extracts from the *Bsp*−/− mouse [35]. This discrepancy in expression patterns between developing and mature bone suggests multiple roles for BSP at different stages of development. Mature bone contains significant levels of BSP protein [23], but the *Bsp* transcript is at low or undetectable levels [9]. However, upon challenge it becomes apparent that there are deficiencies in the *Bsp*−/− mouse. Using a long bone defect model, Malaval *et al* demonstrated that bone formation was delayed in 4 month old *Bsp*−/− mice compared to the WT mice [27]. In separate studies, using critical-size calvarial defects in rats, addition of BSP in a collagen-based scaffold enhanced repair [37]. Due to the commonalities between bone repair and development, we can speculate that the loss of BSP results in delayed *de novo* bone deposition similar to our model of delayed endochondral ossification.

BSP is present in a number of other mineralized tissues where its roles are yet to be defined. Recently, we have shown that BSP is involved in acellular cementum formation and is integral to proper periodontal ligament to tooth attachment [28]. In the absence of BSP, the periodontal ligament is disorganized resulting in tooth and alveolar bone resorption. Both the loss of acellular cementum and the phenotype shown herein support the concept that BSP plays a role regulating mineralized tissues and their consequent interactions with the neighbouring non-mineralized tissue. We are currently investigating the implications of BSP loss in these situations.
Previous studies investigating the role of BSP in early bone have provided some valuable insight, but these experiments lacked a temporal characterization of the developmental process. This study has shown that BSP plays an important role in bone development and promotes cell-cycle progression of chondrocytes. BSP promotes normal endochondral bone development, and its absence correlates with delayed initiation of mineralization. Together these data suggest that BSP has a more widespread role in endochondral bone development than previously shown. These differences highlight the need to further our understanding of the roles BSP plays in mineralized tissue development and repair.
2.6 References


CHAPTER THREE

THE ROLE OF BONE SIALOPROTEIN IN OSTEOBLAST
DIFFERENTIATION AND MINERALIZATION
3.1 Chapter Summary

Bone sialoprotein (BSP) is an anionic phosphoprotein expressed by cells within mineralized tissues and is a mediator of biomineralization and osteogenic development. Specific motifs that have been shown to be important for the functional properties of BSP include: a sequence within the N-terminal domain responsible for collagen binding, poly-E containing regions responsible for mineral binding and nucleation, and an RGD integrin-binding sequence that promotes cell attachment and differentiation. However, the importance of these individual motifs has not been investigated in osteoblast cell cultures without endogenous BSP. To address this, adenoviral transduction of BSP and its mutants was performed in osteogenic cells from the $Bsp^{-/-}$ mouse to study their response to overexpressed BSP. Compared to wild-type, $Bsp^{-/-}$ osteogenic cells in culture showed delayed mineralization and expression of osteogenic genes, and decreased metabolic activity. Overexpression of wild-type and functional-domain altered BSP forms in the $Bsp^{-/-}$ osteogenic cells resulted in increased mineralization relative to the empty-vector-treated (EV) $Bsp^{-/-}$ cells. Overexpression of wild-type BSP enhanced gene expression of markers of osteoblasts relative to the EV-treated $Bsp^{-/-}$ cultures, while the collagen-binding deletion and integrin-binding mutation resulted in increased expression of only some of these genes. These studies demonstrate that loss of any individual functional motif of BSP is insufficient to impair its positive differentiation and mineralization activity in osteogenic cultures.
3.2 Introduction

Bone sialoprotein (BSP) is an anionic phosphoprotein expressed in mineralized tissues including bone, hypertrophic cartilage, dentin, and cementum of teeth [1-3]. BSP comprises up to 10% of the extracellular matrix mineral-binding proteins secreted by osteoblasts [4]. Within bone, BSP has been shown to be expressed by mineralizing cells such as hypertrophic chondrocytes and osteoblasts [1-3]. BSP is a member of the Small Integrin-Binding Ligand N-Linked Glycoprotein (SIBLING) group of proteins. Other members include osteopontin (OPN), dentin matrix protein (DMP1), dentin phosphoprotein (DPP), dentin sialoprotein (DSP), and matrix extracellular phosphoglycoprotein (MEPE). The proteins belonging to this group are thought to be mediators of biomineralization and mineralized tissue development.

Members of the SIBLING group of proteins share a number of common features. They are intrinsically disordered proteins with little or no secondary or tertiary structure [5-7]. These proteins also contain the integrin-binding RGD (Arginine-Glycine-Aspartate) sequence responsible for promoting cell adhesion and signaling. Each protein has highly acidic mineral binding regions that may contain either poly-glutamate or poly-aspartate sequences. In BSP, the highly conserved poly-glutamate sequences have been shown to be critical for hydroxyapatite (HA) nucleation in vitro [7, 8].

The functional regions of BSP have been shown to mediate mineral nucleation in cell-free studies and osteogenic development in cell culture. As indicated, the nucleation of hydroxyapatite by BSP is dependent on its highly acidic poly-glutamate sequences [8, 9]. The substitution of these poly-E sequences to poly-A resulted in a 90% loss of nucleation potency [7]. In addition, the phosphorylation of BSP has been demonstrated to
increase its nucleation potency by increasing the electronegative charge [7]. Specifically, serine-135 has been demonstrated to be a critical phosphorylation site that increases the nucleation potency of BSP by ten-fold in a steady state collagen-gel system [10]. It is thought that in order to direct mineralization, BSP should bind to collagen with high affinity. BSP has an N-terminal collagen-binding motif (residues 18-45) [11], and when BSP is truncated in this domain, the resulting protein consisting of residues 28-300 is unable to bind collagen. Additionally, when bound to collagen BSP shows increased HA nucleation potency in vitro [12].

Not only can BSP direct HA nucleation, but it has also been demonstrated to mediate cellular interactions via the integrin-binding RGD sequence [13, 14]. This sequence has been demonstrated to promote osteoblastic cell attachment [15], differentiation [16], and migration [17]. Binding of BSP to the αvβ3 integrins of osteoblasts has been shown to result in signaling through the focal adhesion kinase (FAK) and extracellular signal-regulated kinases (ERK) pathway [18]. These studies demonstrate that BSP has multiple motifs that each have functions in the mediation of osteogenic cell differentiation and mineralization.

Mice ablated for the Bsp gene (Bsp−/−) have decreased long bone length and cortical bone thickness relative to wild-type (WT) mice at 4-months-of-age [19]. Bsp−/− mice also have decreased mineralization of cortical bone and delayed repair following injury [20]. We have documented a delay in mineralization and expression of markers of osteoblast maturation during endochondral bone development (Chapter 2). These studies suggest that loss of BSP impairs the development of osteoblasts in vivo. Similarly, overexpression of BSP in mice (CMV-BSP) has resulted in shortened long bones and
decreased calvarial thickness [21]. The phenotypic differences in both of these mice suggest that BSP is a mediator of both osteogenic cell development and matrix mineralization.

Osteogenic cells have been isolated from transgenic mice in order to study the phenotype upon loss and overexpression of BSP in cell culture. Malaval et al studied mesenchymal-derived $Bsp^{-/-}$ osteoblasts and demonstrated a decreased number of mineralized nodules and delayed expression of osteoblastic markers of differentiation [19]. Similarly, osteoblasts from the CMV-BSP mouse were shown to have increased mineralization and number of bone nodules per well [21]. Both of these studies agree with previous work on MC3T3-E1 preosteoblastic cells showing that adenoviral overexpression of BSP increased mineralization and promoted terminal differentiation in these cells as compared to the empty vector control [22]. The reduction of endogenous BSP expression in this study by shRNA resulted in decreased mineralization and gene expression of osteoblast markers [22]. These studies suggest that BSP is a promoter of both osteoblast development and mineralization.

The role of BSP and its aforementioned functional regions have yet to be elucidated upon overexpression in osteoblasts without interference from endogenous BSP. In this study, we first characterized the phenotype of the $Bsp^{-/-}$ osteoblasts, demonstrating that the loss of BSP results in delayed mineralization, decreased metabolic activity, and delayed osteoblastic marker expression. We then performed adenoviral transductions of wild-type BSP and its functional domain mutants in order to examine their roles in osteoblast differentiation and mineralization. Our experiments demonstrated
that the loss of any single region was insufficient to impair its activity promoting mineralization and osteoblast differentiation \textit{in vitro}.
3.3 Materials and methods

3.3.1 Animal protocol

Animal procedures were performed in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and Animal Care and Veterinary Services (ACVS), University of Western Ontario. Preparation and genotyping of Bsp homozygous knock-out (Bsp^−/−) and wild-type mice were described previously [23]. Mice were maintained on a mixed 129/CD1 background and were fed a standard pelleted mouse diet (2018 Tekland Global 18% protein diet, Harlan Laboratories, USA) and tap water ad libitum.

3.3.2 Cell culture and in vitro assays

Isolation and culture of mesenchymal stromal cells (MSCs) was performed as previously described with minor modifications [19]. Cells were isolated by flushing the femurs of 8-10 week old mice and were allowed to selectively adhere to T-80 flasks (Nunc, Roskilde, Denmark) for 3 days in Iscove’s Modified Dublecco’s Medium (IMDM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (all reagents from Life Technologies, Burlington, ON, Canada). Media was changed every 48 hours. At 70% confluence, cells were passaged into 24- and 96- multi-well dishes (Nunc) at a density of 1 x 10^4 cells/cm². The cells were allowed to proliferate for three days and were then grown in mineralizing media containing alpha minimal essential medium (αMEM) with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and supplemented with 50 µg/mL ascorbate (Sigma-Aldrich, Oakville, Canada), and 2 mM glycerol-2-phosphate (G2P, Sigma-Aldrich). At least three independent experiments were performed with each done in triplicate.
Cell metabolic activity was measured using alamarBlue® (Life Technologies) reagent. The main constituent is resazurin which produces a fluorescently-active metabolite in linear proportion relative to cell number [24, 25]. Fluorescence was measured with excitation and emission wavelengths of 570 nm and 585 nm, respectively, on a Tecan Safire plate reader (Tecan Group Ltd, Männedorf, Switzerland).

Mineralization was assayed using multiple methods: Alizarin red S, calcium quantification, and von Kossa. Cells were fixed in anhydrous ethanol for 10 minutes at -20°C. Alizarin red staining was performed using 500 µL of 1% w/v Alizarin red S dye (Ricca Chemical Company, Arlington, USA) in water and was incubated for 10 minutes at room temperature. Unbound stain was removed by washing with deionized water, and plates were then allowed to dry overnight. Images of wells were taken using a LED2500 dissection microscope with integrated EC3 digital camera (Leica Microsystems GmbH, Concord, Canada). Dye was then extracted using 1 mL of 0.6 M HCl, 0.7M sodium dodecyl sulphate (SDS) for 1 hour while shaking. A volume of 200 µL was transferred to a 96-well plate and absorbance measured at 415 nm on an iMark plate reader (BioRad, Mississauga, Canada). Concentration was determined by standard curve of alizarin red S diluted in extraction solution. Von Kossa and alkaline phosphatase staining was performed as described previously [26]. Briefly, cells were first stained for alkaline phosphatase activity using naphthol and red violet, then washed thoroughly with deionized water. The mineral was then stained using 2.5% w/v silver nitrate and exposed to fluorescent light for 1 hour, and washed with deionized water. Calcium determination was performed using QuantiChrom™ Calcium Assay Kit (DICA-500, Bioassay Systems,
Hayward, CA, USA) according to the manufacturer’s protocol. Spectrophotometric readings were taken at 612 nm on the Safire plate reader.

3.3.3 Adenovirus preparation

Adenovirus was produced using the pAd/CMV/V5-DEST™ Gateway® Vector Kit (Life Technologies) according to manufacturer guidelines. Briefly, Bsp and the mutants that were used were previously prepared in pET28a plasmids [7, 10, 11, 18, 22]. Bsp was then cloned into the pAd/CMV/V5-DEST vector. The vector with BSP was then linearized and transfected in HEK-293 (ATCC, Manassas, VA, USA) cells using FuGENE® HD (Promega, Madison, WI, USA) according to manufacturer guidelines. Cells were then scraped and transferred to a centrifuge tube and repeatedly frozen in liquid nitrogen and thawed at 37°C three times in order to lyse the cells and release the adenovirus. Cell debris was pelleted and the supernatant containing the adenovirus transferred to a clean tube. This crude extract was then used to infect further HEK-293 cells to increase adenoviral concentration. The adenovirus was recovered as described above and then purified using a chromatographic based system, Adeno-X™ Maxi purification kit (Catalogue number 631533, Clontech, Mountain View, CA, USA), according to the manufacturer’s guidelines. Following purification, adenovirus titres were measured using Adeno-X™ Rapid Titer Kit (Catalogue number 632250, Clontech). Adenovirus was then stored at -80°C until use.

3.3.4 Adenoviral transduction of Bsp−/− osteoblasts

MSCs were isolated and cultured as above. Adenoviral transduction was performed in antibiotic-free media during passaging. Viral titration assays were performed and an ideal multiplicity on infection (MOI) was determined to be 300.
Briefly, cells from the T-80 flasks were resuspended in serum-free IMDM following pelleting and 300 adenoviral particles per cell were added. The cells were then transferred into plates at a density of 1 x 10^4 cells/cm^2, and left to transduce for 2 hours. IMDM with 3% FBS was then added to give a final concentration of 2% FBS in IMDM and the cells were left overnight. The following morning the media was removed and the cultures were washed with HBSS three times. The cells were then provided with 10% FBS in IMDM to recover for two days. Following recovery MSCs were induced to begin differentiation using osteogenic media as described above, and this was counted as day 0.

Adenovirus experimental cultures are labelled as follows: WT is untreated osteoblasts from WT mice; KO are untreated cells from the \( Bsp^{-/-} \) mouse; Adenoviral overexpression of BSP and mutants in \( Bsp^{-/-} \) osteoblasts with empty-vector (EV), wild-type BSP (BSP), N-terminal truncated BSP (-28), RGD mutated to KAE (KAE), poly-E to poly-A mutation (EA), and S136A mutation (SA).

### 3.3.5 Real-time quantitative PCR

Total RNA was isolated from cells using TRIzol (Life Technologies) according to manufacturer’s guidelines. PCR reactions were performed using One-step Master mix (4309169, Life Technologies) on a 7900 HT system (Life Technologies) according to manufacturer guidelines. Gene expression assays of \( Alp1, Col1a1, Sp7, Runx2, Bglap, Opn, Dmp1, Ibqp \) were performed using Taqman probes (Life Technologies). \( Beta-2\text{-}microglobulin \) (B2M) was used as a reference gene for normalization of expression and relative quantification. These genes and their protein names are shown in Table 1.
3.3.6 Statistical analyses

Statistical analysis was performed using a one-way or two-way ANOVA with post-hoc Tukey’s test using GraphPad Prism. At least three independent experiments were performed in triplicate.
Table 3.1: Genes and their TaqMan product codes used for real-time quantitative PCR

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<td>Dentin matrix acidic phosphoprotein 1 (Dmp1)</td>
<td>Mm01208363_ml</td>
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Figure 3.1: Linear schematic of BSP forms used in adenoviral overexpression. (A) wild-type BSP, (B) N-terminal truncated BSP unable to bind collagen (-28), poly-E mineral binding and nucleation sites mutated to poly-A (EA), S135 phosphorylation site mutated to A (SA), RGD integrin-binding sequence mutated to KAE.
3.4 Results

3.4.1 Loss of BSP results in impaired mineralization and maturation of osteoblasts

The difference in mineralization between the $Bsp^{-/-}$ and WT osteoblasts was first examined. Alkaline phosphatase (red) and von Kossa (black) staining demonstrated that there was a decrease in mineral and trend of decreasing alkaline phosphatase-positive area in the $Bsp^{-/-}$ cells when compared to the WT cells (Figure 3.2A). A decrease in mineral content was also apparent after staining the $Bsp^{-/-}$ cell cultures with alizarin red S (Fig 3.2A). Extraction of alizarin red dye and spectrophotometric quantification showed that the $Bsp^{-/-}$ cells had a reduction of mineralization of approximately 50% relative to the control cells at weeks 3 and 4 (Figure 3.2B). This defect in mineralization of the $Bsp^{-/-}$ osteoblasts is also apparent at early time points of days 3, 6, and 9 as measured by fluorometric calcium detection (Figure 3.2C). A 10% decrease in metabolic activity of $Bsp^{-/-}$ cells was also observed, suggesting a decrease in their proliferation relative to the WT controls (Figure 3.2D).

3.4.2 Osteogenic marker expression is delayed in the $Bsp^{-/-}$ osteoblasts

Following the determination of decreased mineralization and metabolic activity, we investigated the gene expression of osteoblast markers of maturation. Early markers, $Alp1$ and $Col1a1$ (Figure 3.3A), and transcription factors $Runx2$ and $Sp7$ were unchanged in $Bsp^{-/-}$ mice compared to controls (Figure 3.3B). Figure 3.2C shows that there was a peak in $Opn$ expression in the WT cells at day 14, which is only reached by the $Bsp^{-/-}$ cultures one week later at day 21. The expression of $Opn$ by the $Bsp^{-/-}$ cultures is higher at day 28 compared with the WT cells. $Bglap$ expression is reduced in the $Bsp^{-/-}$ osteogenic cultures at days 11, 14, and 28 relative to the control cells. There was no
Figure 3.2: \textit{Bsp}^{−/−} osteoblasts have reduced mineralization and metabolic activity.

Alkaline phosphatase (red) and von Kossa (black) stains (upper half of A) and alizarin red stains (lower half of A) of osteoblast cultures at weekly time points. (B)

Spectrophotometric quantification of alizarin red dye at 415 nm extracted from \textit{Bsp}^{−/−} (dotted line) and WT (solid line) cultures at weekly time points. (C) Early time point fluorometric calcium quantification at days 3, 6, and 9. (D) Osteogenic culture metabolic activity assayed using alamarBlue when plated and following induction with differentiation medium. (n=5, \*p<0.05)
### A

<table>
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<td><img src="Bsp_Alizarin_red" alt="Images" /></td>
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<td><img src="Bsp_Alizarin_red" alt="Images" /></td>
</tr>
</tbody>
</table>

### B

![Graph](Alizarin_red_concentration_over_time)

### C

![Graph](Calcium_extracted_over_time)

### D

![Graph](Relative_metabolic_activity_over_time)

*Induction*
Figure 3.3: The expression of late osteogenic markers is delayed in the $Bsp^{−/−}$ osteoblasts. Quantitative real-time PCR was performed to assay gene expression of early osteoblast markers (A), transcription factors (B), and mature osteoblast markers (C). The expression of all genes are normalized to $B2m$. (n=5, *p<0.05)
change in Dmp1 expression between genotypes. Bsp expression was undetectable in the Bsp\(^{-/-}\) osteoblasts and it increases over time in the WT cultures, as expected.

In order to confirm that the cells were not undergoing adipogenesis or chondrogenesis, we examined Ppar\(\gamma\) and Sox9 (Figure 3.4). Ppar\(\gamma\) expression is 20%, 40%, and 60% higher in the Bsp\(^{-/-}\) osteogenic cultures at days 14, 21, and 28, respectively. There was no change in Sox9 expression in the Bsp\(^{-/-}\) cultures relative to the WT cells.

### 3.4.3 Adenoviral transduction efficiency and BSP expression

Titration to determine the appropriate multiplicity of infection of adenoviral particles was performed by treating WT MSC-derived osteoblasts with EV adenovirus expressing LacZ. A transduction efficiency of 70% was determined at an MOI of 300 by dividing LacZ-positive cells (blue) by total number of cells (Figure 3.5A). Expression levels at 24 hours post-induction in the serum-free cell culture media was performed by immunoblot for BSP (Figure 3.5B).

### 3.4.4 BSP mutants rescue the Bsp\(^{-/-}\) mineralization defects

Transduction of the Bsp\(^{-/-}\) cells with adenovirus resulted in decreased mineralization when treated with EV relative to their untreated controls (Figure 3.6A). BSP- and mutant-treated cells showed no significant difference in mineralization compared to the EV-treated Bsp\(^{-/-}\) osteoblasts at week 2 (Figure 3.6B). However, at weeks 3 and 4 there is more mineralization in the BSP- and mutant-treated Bsp\(^{-/-}\) cells compared to the EV-treated Bsp\(^{-/-}\) osteogenic cultures (Figure 3.6C, D). Of all the mutant-BSP treatments, KAE-treated Bsp\(^{-/-}\) osteoblasts did not demonstrate as large of an
Figure 3.4: Decreasing *Pparg* and *Sox9* expression confirms osteoblast differentiation. Quantitative real-time PCR was performed to assay gene expression of adipogenic (*Pparg*) and chondrogenic (*Sox9*) markers of maturation. Gene expression was normalized to *B2m*. (n=5, *p<0.05)
Figure 3.5: Adenoviral transduction efficiency of BSP and its mutants in $Bsp^{-/-}$ osteoblasts. (A) LacZ staining (blue) of MSC-derived osteoblasts at a multiplicity of infection of 300. (B) Immunoblot detection of the overexpression of BSP and its mutants by the MSC-derived osteogenic cells is consistent, while none is detected in the EV control. (n=3)
Figure 3.6: Overexpression of BSP and its mutants rescue the impaired mineralization of the EV-treated Bsp<sup>−/−</sup> osteoblasts. Alizarin red staining was performed on tissue cultures and then extracted and assayed spectrophotometrically at 415 nm at weekly intervals beginning at week 2 (A). Quantification of mineralization, relative to WT at week 2, of the untreated WT and Bsp<sup>−/−</sup> cells, and EV-treated Bsp<sup>−/−</sup> cells. Mineralization from the adenoviral-treated cells quantified at 2 (B), 3 (C), and 4 (D) weeks. Representative images of each treatment are shown beneath their respective columns. (n=5, * denotes significance where p<0.05)
increase in mineralization at week 4 as the other BSP variants relative to the EV controls. All treatments were then assayed for metabolic activity to ensure they had maintained normal viability and growth characteristics post-transduction (Figure 3.7). No significant difference between the treatments was determined, suggesting that the cell number is unaffected following adenoviral treatment in this system.

3.4.5 Expression of osteoblast markers of maturation are increased by overexpression of BSP and mutants

In order to determine whether transduction with BSP and its mutants promoted the differentiation of the osteogenic cultures, we examined the expression of osteoblast markers of development at day 28 from EV-, BSP-, -28-, and KAE-treated osteoblasts. Relative to the EV-transduced cells, Alp1 expression increased in the BSP-, -28-, and KAE-treated cells (Figure 3.8A). Coll1a1 expression was increased in the BSP-treated Bsp<sup>-/-</sup> cells compared to the EV-treated controls, while the other treatments demonstrated no change in expression (Figure 3.8B). One major transcription factor, Runx2 was increased in only the BSP-treated cells relative to cells treated with EV (Figure 3.8C). The other major transcription factor examined, Sp7, showed significant increases in BSP-, -28, and KAE-treated cells relative to the EV-treated controls (Figure 3.8D). Bglap expression is increased in the BSP- and KAE-treated cultures relative to the EV-treated control, while expression is unchanged in -28-treated cultures (Figure 3.8E). Dmp1 expression is increased in both the BSP- and KAE-treated osteoblasts while -28-treated expression remains unchanged, relative to the EV control (Figure 3.8F).
Figure 3.7: Cell metabolic activity is unchanged upon viral transduction. Curves from metabolic activity assayed using alamarBlue. Error bars are omitted for clarity. No significant difference in metabolic activity between treatments was detected. (n=5)
Figure 3.8: Real-time qPCR of osteogenic marker expression of $Bsp^{-/-}$ osteoblasts

**overexpression BSP.** Quantitative real-time PCR assaying osteogenic gene expression of the adenoviral treated $Bsp^{-/-}$ osteoblasts at day 28 in culture. $Alp1$ (A), $Colla1$ (B), $Runx2$ (C), $Sp7$ (D), $Bglap$ (E), and $Dmp1$ (F) were assayed. Adenoviral treatment of $Bsp^{-/-}$ osteoblasts included empty-vector (EV), WT BSP (BSP), N-terminal truncated BSP (-28), and RGD mutated to KAE (KAE). (n=5, * denotes significantly different from EV where p<0.05)
3.5 Discussion

BSP is thought to be an important mediator of mineralization and osteoblast development [19, 21, 22]. BSP is expressed during the mineralization phase of MC3T3-E1 cells [27, 28] and primary osteoblast [15, 16] cultures. The Bsp⁻/⁻ mouse demonstrates a phenotype of delayed endochondral ossification (Chapter 2), and osteoblasts derived from the Bsp⁻/⁻ mice have been shown to have decreased mineralization potential and expression of osteogenic markers of development [19]. Similarly, overexpression of BSP has been shown to increase mineralization and terminal differentiation of osteoblasts in both MC3T3-E1 cell culture [22] and osteoblasts derived from the CMV-BSP mouse model [21]. Supplementation of osteogenic cultures with BSP has also been demonstrated to increase expression of osteoblast-related markers [22, 29]. However, the role of BSP and its functional motifs and their mechanism of action have yet to be fully characterized in osteoblasts without interference from endogenous BSP. In this study, we investigated the phenotype of MSC-derived Bsp⁻/⁻ osteoblasts, and the effect of overexpressed BSP and its functional mutants in order to characterize their different functions during osteoblast development.

The mineralization of the Bsp⁻/⁻ osteoblasts demonstrated that loss of BSP results in decreased mineral deposition relative to WT cultures, agreeing with previous work [19]. There was also a decrease in calcium content at days 3, 6, and 9, suggesting that loss of BSP delays early mineral deposition, possibly due to BSP’s hydroxyapatite nucleation activity. The decreased alkaline phosphatase-positive area stained and metabolic activity in the Bsp⁻/⁻ osteoblast cultures suggests that cell proliferation is impaired in the Bsp⁻/⁻ cultures. Interestingly, Valverde et al also noted a decrease in the
growth rate of the osteoblasts when BSP was overexpressed [21]. This defect in proliferation is supported by evidence in the growth plate of decreased chondrocyte proliferation (Chapter 2). Furthermore, it is postulated that BSP expression occurs at two times during osteoprogenitor cell development: during the highly proliferative immature osteoprogenitor phase, and during terminal maturation from preosteoblasts to osteoblasts [30]. This suggests that low-level BSP expression may promote proliferation, while high-level expression promotes terminal differentiation.

In our study, the early markers of osteogenic development and transcription factor expression are unchanged in the $Bsp^{-/-}$ cells compared to the WT controls. The notable differences are delayed $Opn$ and $Bglap$ expression in the $Bsp^{-/-}$ versus WT cells. This suggests that the cells have altered differentiation characteristics. While there was no change in expression of the chondrocyte marker $Sox9$, there was increased expression of adipocyte marker $Pparg$ in the $Bsp^{-/-}$ cells. Due to the similar gene expression patterns and relatively small change in expression, we suspect that the change does not have physiological relevance. These results, alongside the delayed mineralization phenotype, suggest that the loss of BSP results in a delay in both osteoblast differentiation and mineralization in osteogenic cell cultures.

In order to discern which functional regions promote mineralization and/or differentiation, the effect of adenoviral-mediated overexpression of BSP and its mutants on the $Bsp^{-/-}$ osteoblasts was examined. The EV-treatment of the $Bsp^{-/-}$ osteoblasts resulted in some toxicity, as these cells had decreased mineralization compared to the untreated $Bsp^{-/-}$ cells. Adenoviral transduction with BSP, and its mutants, all rescued the mineralization to a similar extent at week 3, as compared to the EV-treated control. At
week 4, BSP, -28, EA, and SA treatments had equivalent effects on promoting mineralization compared to the EV-treated control, whereas the mineral content mediated by KAE-transduction while elevated was not to the same level as the other forms of BSP. If mineralization in these cell cultures was entirely dependent on BSP’s ability to nucleate and promote mineral formation, then a differing mineral content between the mutants and wild-type BSP would be expected. The -28 mutant of BSP may not be able to bind collagen, but it could become trapped within the osteoid maintaining its ability to promote mineralization and bind cell-surface integrins promoting osteoblast development. While the recombinant EA mutant had greatly diminished nucleation potency in the aforementioned cell-free studies [7], this study demonstrated that there was no decrease in mineralization relative to wild-type BSP in cell culture. This may be due to the EA mutant still having an overall negative charge that has been demonstrated to be critical to nucleation of HA [8, 9]. The SA mutant also promoted mineralization equal to the BSP-treated cells, suggesting that the single phosphorylation is not crucial to its ability to initiate nucleation in tissue culture. While this version of BSP still has the collagen-binding domain and poly-E motifs, there is impaired mineralization relative to wild-type BSP, suggesting that the RGD sequence is likely promoting osteoblastic differentiation resulting in increased mineralization. BSP’s role in promoting mineralization in osteoblasts is not solely dependent on any one region and it has multiple mineralizing motifs to ensure its functionally. These results confirm that BSP is a potent nucleator of mineralization.

Previous studies have shown that supplementation with recombinant BSP and overexpression of BSP in osteoblasts promotes osteogenic gene expression in tissue
cultures [22]. While we observed no change in metabolic activity following adenovirus
treatment, there were differences in osteoblast gene expression characteristics. As
expected, the BSP-treated cells demonstrated increased expression of osteogenic markers
relative to the EV-treated control. When treated with the -28 truncated BSP, the
osteogenic cultures had increased \textit{Alp1} and \textit{Sp7} expression. Notably, the genes that were
unchanged in the -28-treated cells relative to the EV-treated controls were markers of
terminal differentiation (\textit{Bglap} and \textit{Dmp1}). Due to the increased mineralization observed
from this treatment, it is unlikely that the mutant was lost in the medium, suggesting that
there could be a cell signaling sequence different from the RGD motif. Other SIBLING
molecules, of which OPN has been most thoroughly characterized, have been shown to
have multiple cell-surface-binding motifs [31-36]. Interestingly, the KAE mutant
demonstrated increased expression of \textit{Alp1}, \textit{Bglap}, and \textit{Dmp1}. This mutant is unable to
bind the cell-surface integrins through its traditional RGD motif, so the increased gene
expression supports the hypothesis that BSP may have a second signaling motif. These
results demonstrate that wild-type BSP promotes osteogenic gene expression as expected,
and that there is likely a second or more signaling motifs in addition to the RGD integrin-
binding motif.

This study investigated the role of BSP during osteoblast differentiation and
mineralization. The loss of BSP resulted in a delay of mineral deposition and maturation
characteristics of osteoblasts. The subsequent treatment of the \textit{Bsp}^{-/-} osteoblasts with
overexpressed BSP and its mutants resulted in increased mineralization and osteoblast
marker gene expression relative to EV-treated controls. These results suggest that the
functional regions of BSP play different roles in mediating osteoblast maturation and
mineralization. The RGD signaling motif appeared to be the predominant functional region of those investigated. However, these data suggest that there is another signaling motif yet to be characterized at the N-terminus. These data confirm that there are multiple redundancies within BSP that work cohesively to mediate mineralized tissue development.
3.6 References


CHAPTER FOUR

OSTEOPONTIN MEDIATES MINERALIZATION AND NOT
OSTEOGENIC CELL DEVELOPMENT IN VITRO

1 This Chapter has been reproduced from:
E. Holm, J.S. Gleberzon, E.S. Sørensen, F. Beier, G.K. Hunter, H.A. Goldberg. 2014.
Osteopontin mediates mineralization and not osteogenic cell development in vitro. (In
revision at Biochemical Journal August, 2014)
4.1 Chapter Summary

Biomineralization is a complex process in the development of mineralized tissues such as bone, and pathological calcifications such as atherosclerotic plaques, kidney stones, gout, and others. Osteopontin (OPN), an anionic phosphoprotein, is expressed in mineralizing tissues and has previously been demonstrated to be a potent inhibitor of hydroxyapatite formation. The OPN-deficient mouse (Opn−/−) displays a hypermineralized bone phenotype starting at 12 weeks post-natally. By isolating and culturing Opn−/− and wild-type (WT) osteoblasts, we sought to determine the role of OPN and two of its functional peptides in osteoblast development and mineralization. Opn−/− osteoblasts had significantly increased mineral deposition relative to their WT counterparts, with no physiologically relevant change in gene expression of osteogenic markers.

Supplementation with bovine milk OPN (mOPN) lead to a dramatic reduction in mineral deposition by the Opn−/− osteoblasts. Treatment with OPN peptides corresponding to phosphorylated OPN220–235 (P3) and non-phosphorylated OPN65–80 (OPAR) also rescued the hypermineralization phenotype of Opn−/− osteogenic cultures.

Supplementation with mOPN or the OPN-derived peptides did not alter the expression of terminal osteogenic markers. These data suggest that OPN plays an important role in the regulation of biomineralization, but does not appear to affect osteoblast cell development in vitro.
4.2 Introduction

Biomineralization is an intricately controlled process that comprises osteogenic cell development as well as the nucleation and growth of hydroxyapatite in calcified tissues such as bone, imparting them with strength and rigidity. Pathological dysregulation of mineralization results in a number of disorders such as atherosclerosis [1], kidney stones [2], and others. A number of mechanisms have been proposed to account for the regulation of mineralization in vivo, including the involvement of proteins responsible for mediating osteogenic cell differentiation and the promotion or inhibition of mineral growth and/or nucleation. One protein implicated in the inhibition of mineral formation is osteopontin (OPN).

Osteopontin is an anionic phosphoprotein that is expressed in many tissues and cell types, and is also found in physiological fluids [3]. OPN is a member of the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family of proteins [4], which includes bone sialoprotein (BSP), dentin matrix phosphoprotein (DMP1), and dentin sialophosphoprotein (DSPP). The genes encoding the SIBLING family of proteins are located in a syntenic gene locus that has been named the “bone gene cluster” on murine chromosome 5 [5] and human chromosome 4 [6]. These proteins are intrinsically disordered, with little or no secondary or tertiary structure [4, 7, 8]. They also contain RGD (Arginine-Glycine-Aspartate) integrin-binding sequences, highly acidic sequences that may include poly-aspartate or poly-glutamate sequences, and are highly post-translationally modified [3]. These post-translational modifications often include phosphorylation, glycosylation and sulfation.
The expression patterns of OPN in different tissues are highly complex and dependent on a variety of regulatory elements. In bone, expression of OPN has been shown to be linked with the mineralization front of osteoid [9-11]. OPN expression has also been associated with pathological calcifications, such as atherosclerosis [1] and kidney stones [12]. The presence of OPN during de novo osteoid deposition and pathological calcification suggests that OPN may be regulating mineral growth in both cases. The expression of OPN in osteoblasts is dependent on a variety of regulatory factors, including: synthetic bisphosphonates [13], inorganic phosphate [14], and 1,25-dihydroxyvitamin D3 [13, 15]. When osteoblasts are exposed to cell culture media containing inorganic pyrophosphate, OPN expression may also be regulated by Ank and Pc-1 (gene name Enpp1) [16-18]. The expression of OPN is also associated with varying degrees of post-translational modification in a tissue- and cell-specific manner, recently reviewed by Ganss and Bansal [3]. OPN’s state of post-translational modification is implicated in its ability to mediate cell signaling and mineral growth inhibition. Increased phosphorylation has been shown to be important in promoting osteoclastic activity [19]. Furthermore, native OPN with post-translational modifications is a more potent inhibitor of HA growth than recombinant OPN lacking these modifications [20].

Mice with the *Opn* gene ablated (*Opn*−/−) have been reported to have increased bone mineral density when assayed using Fourier transform infrared (FTIR) imaging [21]. This phenotype develops progressively as the animals age [21], suggesting a dysregulation between mineral deposition and resorption by osteoblasts and osteoclasts, respectively. Rittling et al. have demonstrated that *Opn*−/− mice have significantly reduced osteoclast activity [22], suggesting that the increased mineral density is due to decreased
resorption. Studies in tissue culture revealed that MSC-derived osteogenic cells from tail-suspended WT mice had decreased mineralization compared to their unsuspended controls. In contrast, osteogenic cells derived from tail-suspended *Opn*<sup>−/−</sup> mice had the same degree of mineralization as their unsuspended counterparts [23], suggesting OPN may be involved in the mechanosignal transduction response of osteoblasts. Currently, there do not appear to be any published studies examining a time course of mineralization and differentiation of osteoblasts derived from the *Opn*<sup>−/−</sup> mice.

Previous work has presented conflicting evidence for OPN’s potential role in osteogenic cell differentiation [24, 25]. Overexpression of OPN in MC3T3-E1 pre-osteoblastic cells resulted in a decrease in the expression of mature osteoblast markers in addition to a decrease in mineral deposition [25]. However, overexpression of OPN in rat bone marrow cells was shown to enhance the expression of osteoblast markers and nodule formation [24]. These contradictory results have yet to be resolved. In order to better understand the role of OPN during bone formation and homeostasis, it is critical to elucidate its function during osteoblast differentiation and biomineralization.

As previously indicated, OPN has also been shown to be a potent inhibitor of hydroxyapatite (HA) nucleation and growth *in vitro* [26, 27]. OPN has been studied extensively in order to understand the role it plays in regulating crystal growth. Motifs of OPN shown to inhibit mineralization include the highly acidic poly-aspartate regions [28] as well as the highly phosphorylated sequences [29]. These crystal-binding regions of OPN competitively perturb mineral growth by interacting with the positively charged regions of the crystal surfaces [30]. Bovine milk OPN (mOPN) has been used extensively in studies of OPN inhibition as it is highly phosphorylated and is readily available in
large quantities [31]. mOPN has been shown to directly inhibit HA nucleation by binding crystal faces of HA using its multiple crystal-binding motifs [30, 32].

Due to OPN’s localization within atherosclerotic plaques [33], Giachelli et al undertook studies using vascular smooth muscle cells (VSMCs) isolated from the $Opn^{-/-}$ mouse. These cells were grown under high phosphate conditions to induce calcification, and were shown to have increased mineral deposition relative to WT controls [34]. Supplementation with exogenous OPN from neonatal rat smooth muscle cell cultures resulted in a decrease in mineral deposition in the same system [35]. These studies confirm that OPN is a potent inhibitor of mineral growth and formation in VSMC cultures.

Of the aforementioned motifs responsible for crystal growth inhibition, we have characterized OPN sequences with potent inhibitory activity [29]. These include OPN 65-80 (SHDHMDDDDDDDDDGD) called OPAR (Osteopontin Poly-Aspartate Region) and OPN 220-235 (pSHEpSTEQSDAIDpSAEK) termed P3, containing 3 attached phosphates. The $Opn^{-/-}$ mouse provides an opportunity to test these peptides without interference from endogenous OPN.

In this study we characterize the phenotype of $Opn^{-/-}$ osteoblasts. We also determined the inhibitory capacity of mOPN and two functional peptides of OPN, namely P3 and OPAR. We demonstrate that $Opn^{-/-}$ osteoblast cultures have an increased mineralization phenotype that is independent of osteoblast differentiation. We also show that upon treatment with mOPN and OPN derived-peptides, there is a reduction of mineral deposition with no alteration of the expression characteristics of terminal
osteoblast markers. These data suggest that the main role of OPN in osteoblasts is to control mineralization, not cellular differentiation.
4.3 Materials and methods

4.3.1 Animal protocol

Animal procedures were performed in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and Animal Care and Veterinary Services (ACVS), University of Western Ontario protocol number 2008-092. Mice were obtained from The Jackson Laboratory (JAX), originally documented by Liaw, et al. [36] (JAX Stock number 004936, Sacramento, USA). Preparation and genotyping of \(Opn^{-/-}\) homozygous mice and WT controls were performed as described by the supplier (JAX). Mice were maintained on a C57BL/6 background and were fed a standard pelleted mouse diet (2018 Tekland Global 18% protein diet, Harlan Laboratories, USA) and tap water \textit{ad libitum}. The \(Opn^{-/-}\) and WT mice breed normally and both can be maintained on a homozygous background.

4.3.2 Milk osteopontin and peptide preparation

Bovine milk OPN, comprising a mixture of both intact and OPN-derived peptides, was purified as previously described by Sørensen and Petersen [37]. Synthetic peptides were prepared as previously described [29]. Briefly, peptides (OPAR - SHDHMDDDDGD, and P3 - pSHEpSTEQSDAIDpSAEK were synthesized using batch method free amino and carboxyl termini using FMOC chemistry. These were then purified using HPLC. Purity and protein content were confirmed using electrospray ionization mass spectrometry and amino acid analysis, respectively.

4.3.3 Cell culture and \textit{in vitro} assays

Isolation and culture of mesenchymal stromal cells (MSCs) was performed as previously described with minor modifications [38]. Cells were isolated by flushing the
femurs of 8-10 week old mice and were allowed to adhere to T-80 flasks (Nunc, Roskilde, Denmark) for 3 days in Iscove’s Modified Dulbecco’s Medium (IMDM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (all reagents from Life Technologies, Burlington, ON, Canada). Cell culture medium was changed every 48 h. At 70% confluence, cells were passaged into 24- and 96- multi-well dishes (Nunc) at a density of $1 \times 10^4$ cells/cm². The cells were allowed to proliferate for three days and were then grown in mineralizing medium containing alpha minimal essential medium ($\alpha$MEM) with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and supplemented with 50 µg/mL ascorbate (Sigma-Aldrich, Oakville, Canada), and 2 mM glycerol-2-phosphate (G2P, Sigma-Aldrich).

To study the effect of OPN on cell development and mineralization, the medium was supplemented with mOPN at final concentrations of 5, 10, or 20 µg/mL, or with the OPN peptides, OPAR and P3, at 20 µg/mL. Supplementation of the culture medium with OPN or its peptides was continued throughout the culture period.

Metabolic activity was measured using alamarBlue® (Life Technologies) reagent. The main constituent is resazurin which produces a fluorescently active metabolite in linear proportion to cell number [39, 40]. Fluorescence was measured with excitation and emission wavelengths of 570 nm and 585 nm, respectively, on a Tecan Safire plate reader (Tecan Group Ltd, Männedorf, Switzerland). At least three independent experiments were performed with each done in triplicate.

Mineralization was assayed using alizarin red S dye (Ricca Chemical Company, Arlington, USA). Cells were fixed in anhydrous ethanol for 10 min at -20°C. These cultures were then stained with 500 µL of 1% w/v alizarin red S dye and incubated for 10
Unbound stain was removed by washing with distilled water, and then plates allowed to dry overnight. Images of wells were taken using a LED2500 dissection microscope with integrated EC3 digital camera (Leica Microsystems GmbH, Concord, Canada). Dye was then extracted using 1 mL of 0.6 M HCl, 0.7M sodium dodecyl sulphate (SDS, Sigma-Aldrich) for 1 h while shaking. An aliquot of 200 µL of dye was transferred to a 96-well plate and absorbance measured at 415 nm on an iMark plate reader (BioRad, Mississauga, Canada). At least three independent experiments were performed with each done in triplicate.

4.3.4 Real-time quantitative PCR

Total RNA was isolated from cells using TRIzol (Life Technologies) according to manufacturer’s guidelines. PCR reactions were performed using One-step Master mix (4309169, Life Technologies) on a 7900 HT system (Life Technologies) according to manufacturer guidelines. Gene expression assays of *Alp1, Colla1, Osterix, Runx2, Sp7, Osteocalcin, Opn, Dmp1*, and *Ibsp* were performed using Taqman probes (Life Technologies). *Beta-2-microglobulin* (B2M) was used as a reference gene for normalization of expression and relative quantification. These genes and their protein codes are shown in Table 4.1.

4.3.5 Statistical analyses

Statistical analysis was performed using a two-way ANOVA for time-course studies, and one-way ANOVA for single time-point experiments. Both were analyzed with post-hoc Tukey’s test using GraphPad Prism v4.00.
Table 4.1: Gene names associated with their protein names and primer codes used for real-time qPCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>qPCR probe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpl</td>
<td>Alkaline phosphatase (Alp)</td>
<td>Mm00475834_m1</td>
</tr>
<tr>
<td>Ank</td>
<td>Ankylosis, progressive homolog</td>
<td>Mm00445040_m1</td>
</tr>
<tr>
<td>Bglap</td>
<td>Osteocalcin (Ocn)</td>
<td>Mm03413826_mH</td>
</tr>
<tr>
<td>B2m</td>
<td>Beta-2-microglobulin</td>
<td>Mm01269327_g1</td>
</tr>
<tr>
<td>Coll1</td>
<td>Type I collagen</td>
<td>Mm00801666_g1</td>
</tr>
<tr>
<td>Dmp1</td>
<td>Dentin matrix acidic phosphoprotein 1 (Dmp1)</td>
<td>Mm01208363_m1</td>
</tr>
<tr>
<td>Enpp1</td>
<td>PC1 (Ectonucleotide pyrophosphatase/phosphodiesterase 1)</td>
<td>Mm00501097_m1</td>
</tr>
<tr>
<td>Ibsp</td>
<td>Bone sialoprotein (BSP)</td>
<td>Mm00492555_m1</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runx2</td>
<td>Mm00501580_m1</td>
</tr>
<tr>
<td>Sp7</td>
<td>Osterix</td>
<td>Mm00504574_m1</td>
</tr>
</tbody>
</table>
4.4 Results

4.4.1 Characterization of $Opn^{-/-}$ osteoblasts

In order to study the effect of OPN on extracellular matrix mineralization, we first assayed the ability of $Opn^{-/-}$ osteoblasts to mineralize in tissue culture. Staining with alizarin red S showed a linear increase in mineralization in the WT osteogenic cultures throughout the time course. The $Opn^{-/-}$ cells demonstrated a similar linear increase in mineralization over time, however there is two-fold greater mineral content relative to WT cells at each time point (Figure 4.1A & B). There was no apparent difference in metabolic activity between the $Opn^{-/-}$ and WT cultures, suggesting that the differences in mineralization are not due to differences in cell number (Figure 4.1C).

Using quantitative real-time PCR, we observed no deviation from WT expression patterns in a number of osteogenic genes from the $Opn^{-/-}$ cells, including: Ank, Alp1, Bglap, and Dmp1 (Figure 4.2). Transcription factors Runx2 and Sp7 (data not shown) were also unchanged. Other osteogenic genes showed some differences. $Col1a1$ expression was reduced by half at day 7, and by 40% at day 14, in the $Opn^{-/-}$ osteoblasts relative to the WT cells. Expression of $Ibsp$ at day 14 was increased by 100% in the $Opn^{-/-}$ cells compared with the WT cultures. The expression of $Enpp1$ was also increased by 60% in the $Opn^{-/-}$ osteogenic cultures at day 28 versus the WT cells. Other than these exceptions, the expression characteristics are indistinguishable between the WT and $Opn^{-/-}$ cells. The overall similarity in expression of multiple genes throughout the time course suggests that OPN does not play a critical role in the maturation of osteoblasts.
Figure 4.1: Biomineralization is increased in the $Opn^{-/-}$ osteoblasts. (A) Quantification of alizarin red dye extracted from $Opn^{-/-}$ (dotted line) and WT (solid line) cultures at weekly time points. (B) Representative images of the alizarin red-dyed experimental wells. (C) Metabolic activity assay of $Opn^{-/-}$ and WT cultures at weekly time points. * denotes significance where $p<0.05$, $n=4$. 
Figure 4.2: Analyses of osteogenic marker expression in $Opn^{−/−}$ osteoblasts show few changes in developmental expression characteristics using real-time quantitative PCR. Quantitative real-time PCR examining markers of osteoblastic differentiation taken at weekly time points. The expression of all genes is normalized to $B2m$. * denotes significance where $p<0.05$, $n=4$. 
4.4.2 Treatment with mOPN

The enhanced mineralization observed in the $Opn^{-/-}$ osteoblasts, in the absence of any large changes in cell metabolic activity or expression of differentiation markers, suggests that OPN functions to primarily inhibit mineral deposition and/or growth. Therefore, we sought to determine whether supplementing the osteoblast cultures with mOPN could attenuate the increased mineralization phenotype. Upon treatment with mOPN, the $Opn^{-/-}$ cultures displayed a significant inhibition of mineralization (Figure 4.3A). The amount of mineral detected in the mOPN-treated $Opn^{-/-}$ osteoblast cultures following treatment with 5 $\mu$g/ml of mOPN was reduced to 10% of the amount in untreated $Opn^{-/-}$ cell cultures. Higher concentrations of mOPN, 10 and 20 $\mu$g/ml, resulted in even lower (~2.5%) mineral deposition. We observed no change in metabolic activity (Figure 4.3C), nor were there any changes in terminal-marker expression (Figure 4.4) upon treatment with mOPN. This suggests that mOPN treatment inhibits mineralization independent of mediating cellular responses with the osteoblasts in our system.

4.4.3 Treatment with OPN peptides

OPN has multiple sequences within the molecule that have been shown to be potent inhibitors of mineral formation by *in vitro* and *in silico* studies [29]. To determine whether some of these peptides affect cell-mediated mineralization, osteoblastic cells from the $Opn^{-/-}$ mouse were cultured in the presence of 20 $\mu$g/mL OPAR and P3. As before, there was increased mineral deposition in the $Opn^{-/-}$ osteogenic cell cultures as compared with the WT cell cultures. The addition of OPAR and P3 reduced the mineralization to half of that of the untreated $Opn^{-/-}$ cells. These peptides did not alter the
metabolic activity characteristics (Figure 4.5B), nor did they change *Ibsp* or *Bglap* expression (Figure 4.6).
Figure 4.3: Supplementation with mOPN attenuates *Opm*−/− osteoblast biomineralization. (A) Quantification of mineralization of cultures at 28 days post-induction. Mineralization was assayed by measuring extracted alizarin red at A415 and normalizing to WT levels. (B) Representative images of the osteoblast cultures stained with alizarin red. (C) Metabolic activity of the osteoblast cultures at day 28. * denotes significance where p<0.05, n=4.
Figure 4.4: Treatment with mOPN does not change expression of terminal osteogenic markers of differentiation of $Opn^{-/-}$ osteoblasts. Real-time qPCR was used to measure expression of $Bglap$ and $Ibsp$ in the untreated and the mOPN-treated osteoblast cultures at 28 days post-induction. The x-axis represents the amount of mOPN supplemented in $\mu$g/mL. * denotes significance where $p<0.05$, $n=4$. 
Figure 4.5: *Opn*<sup>−/−</sup> osteoblast biomineralization is attenuated upon supplementation with P3 and OPAR. (A) Quantification of mineralization of cultures at day 28. Mineralization was assayed by measuring extracted alizarin red at A<sub>415</sub> nm and normalizing to WT levels. (B) Representative images of alizarin red stain of cells treated with OPN peptides. (C) Metabolic activity of the cell cultures at day 28. * denotes significance where p<0.05, n=3.
Figure 4.6: Supplementation of cultures with OPN peptides does not change maturation characteristics of $Opn^{-/-}$ osteoblastic cells. Real-time qPCR of $Bglap$ and $Ibsp$ expression of the cultures treated with 20 $\mu$g/ml OPAR and P3 at day 28. * denotes significance where $p<0.05$, $n=3$. 
4.5 Discussion

The $Opn^{-/-}$ mice were originally characterized to have no significant phenotype [22]. As techniques became more sensitive, and as the mice were challenged, phenotypic differences became apparent. As adults, the $Opn^{-/-}$ mice show increased mineralization of their trabecular bone [21]. This increase in site-specific mineral density was suggested to be due to decreased osteoclast activity [21]. However, other studies suggest that OPN plays a role in promoting osteogenic development. For example, it was recently proposed that OPN interaction with cell-surface integrins promotes osteogenesis of MSCs by virtue of inhibiting adipogenesis [41]. It also has been shown that loss of OPN results in aberrations of signaling pathways in osteogenic cells mediated by, for example, parathyroid hormone (PTH) [42] and alkaline phosphatase (Akp2) [17]. These findings suggest that OPN is a regulator of bone homeostasis. In order to better understand the role of OPN in osteoblast-mediated biomineralization, we characterized a time course of gene expression and mineralization in $Opn^{-/-}$ osteoblasts and confirmed our findings by the addition of exogenous OPN and OPN-derived peptides.

Our studies demonstrate that $Opn^{-/-}$ osteoblasts mediated significantly more mineral deposition in vitro, while the apparent cell number, based on assessment of metabolic activity, and expression of markers for differentiated osteoblasts remained essentially unchanged with a couple of exceptions, as discussed below. This suggests that the increase in mineralization is likely due to growth of the mineral component and not an increase in cell numbers nor enhanced differentiation of osteogenic cells. The potential for OPN to alter the cell-maturation characteristics of osteoblasts is controversial. In a variety of cell types, including cancer cells, OPN’s interaction with cell-surface integrins
or CD44 mediates cell signaling leading to specific responses [3, 43]. As such, OPN is predicted to play a role in promoting osteoblast signaling [44, 45], but, as discussed previously, OPN has been shown to both promote [24] and inhibit [25] osteoblast differentiation. The contradictory results of these studies could be due to a multitude of factors, such as different cell types (primary rat osteoblasts versus MC3T3-E1 pre-osteoblastic cells) or method of OPN gene delivery (adenovirus versus transfection). The method of OPN delivery is important as OPN may be capable of initiating cell-signaling through an intracellular mechanism [46, 47].

In our study, the time course of osteoblast marker expression showed no major overall changes in expression patterns other than \textit{Col1a1}. A decrease in \textit{Col1a1} expression may be interpreted as fewer sites for early mineral formation and thus lower mineral deposition. Despite this, we still observed increased mineralization in the \textit{Opn}^{-/} cells in our study. It was of interest that the expression of \textit{Ank} and \textit{Enpp1} in the \textit{Opn}^{-/} cells, except \textit{Enpp1} at day 28, did not show a difference when compared to WT cells. These genes encode transmembrane proteins that are thought to regulate biomineralization: \textit{Ank} is responsible for the transport of pyrophosphate, a potent inhibitor of mineral formation, out of the cell [48, 49]; \textit{Pc-1} (gene name \textit{Enpp1}) is an enzyme that degrades nucleoside triphosphates to produce pyrophosphate [50-52]. The observed increase in \textit{Enpp1} at day 28 may have been a response to the increased mineralization in these cultures, similar to the expression of \textit{Enpp1} of mineralizing cementoblasts in vitro [53]. In contrast to our studies, previous work has demonstrated that the osteoblasts derived from the \textit{Opn}^{-/} mouse have enhanced expression of both \textit{Ank} and \textit{Enpp1} [17], proposed to compensate for the loss of OPN’s mineral inhibitory
activity. The discrepancies between expression of *Ank* and *Enpp1* in our study and that of Millan’s group [17] may be due to differences in experimental procedures. Our system used stromal-derived in contrast to calvarial-derived osteogenic cells used by Harmey *et al.* [17], thus differing in both cell lineage and stage of development when isolated. The mice from which the cells were isolated came from two independently produced *Opn*<sup>−/−</sup> mouse strains; ours from Liaw *et al.* [36], and the Millan group from Rittling *et al.* [22]. We also used 2 mM G2P, a more physiologically relevant concentration [54, 55], compared to 5 mM G2P used by Harmey *et al.* [17]. It is of relevance that higher G2P concentration in the culture medium has been shown to increase inorganic phosphate, which can result in non-specific calcification [55, 56] and promotion of osteoblast differentiation [57-59]. In our study the loss of OPN does not appear to alter the expression of the major osteogenic markers.

To determine whether the hypermineralization phenotype of the *Opn*<sup>−/−</sup> cells could be rescued, we treated the cells with mOPN. The addition of mOPN resulted in levels of mineralization well below that of untreated *Opn*<sup>−/−</sup> osteogenic cell cultures. The unchanged levels of *Ibsp* and *Bglap* expression suggest that mOPN treatment resulted in no change in osteoblast development. Furthermore, we observed no change in metabolic activity upon treatment with mOPN, suggesting that changes in mineralization were not a reflection of changes in cell number.

Previous work delineating the role of OPN in VSMCs has demonstrated a similar attenuation of mineralization upon addition of OPN [35]. Calcification in the VSMCs was induced using mineralizing medium (including 10 mM G2P), which was supplemented with 0.5-5 µg/mL OPN [35]. This treatment resulted in a decrease in
mineralization similar to that shown in our study on osteogenic cells. This confirms that in mineralizing cell culture systems OPN is a potent inhibitor of mineralization.

To further our understanding of OPN’s mode of mineral inhibition, we investigated OPN-peptides that were previously shown to inhibit the growth of hydroxyapatite [29] and calcium oxalate monohydrate crystals [60]. Upon supplementation with OPAR and the phosphorylated P3 peptide, mineral deposition in \( \text{Opn}^{-/-} \) osteoblast cultures was reduced relative to the untreated \( \text{Opn}^{-/-} \) cells, to a level comparable to the WT osteoblast cultures. Furthermore, the expression of mature osteoblast markers was unchanged by the treatments. Thus, the two OPN-derived peptides, P3 and OPAR, were capable of rescuing the hypermineralization phenotype of the \( \text{Opn}^{-/-} \) osteoblast cultures, with no change in terminal differentiation of the \( \text{Opn}^{-/-} \) cells.

In this study we demonstrated that \( \text{Opn}^{-/-} \) osteoblasts demonstrate greater biomineralization than WT cells \textit{in vitro} and that the hypermineralization is rescued by the addition of mOPN and OPN-peptides. Since there was little change in osteoblast marker expression, we can conclude that OPN mediates its inhibitory effects in the extracellular matrix on the nucleation and growth of mineral crystals. Lastly, the rescue of the hypermineralization phenotype by the inhibitory OPN-derived peptides, without any apparent effect on osteogenic cell differentiation, provides insight into a possible avenue for the development of clinical targets to prevent pathological calcifications.
4.6 References


CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSIONS
5.1 Thesis summary

The aim of this thesis was to characterize the role of the SIBLING proteins BSP and OPN in the processes of endochondral ossification and biomineralization, and provide evidence to understand the resultant phenotypes in mice when these proteins are lost. The mechanisms by which mineral crystal growth is initiated and regulated in vivo remain unclear. BSP and OPN have been demonstrated to be potent regulators of mineral growth in vitro. In this thesis, BSP and OPN were shown to be contributors to the initiation and inhibition of mineralization in cell-based systems. BSP was proposed to be a promoter of osteogenic cell differentiation and chondrocyte cell cycle progression. The characterization of the roles of these proteins, particularly during development, facilitates the understanding of mineralized tissue formation and their roles in normal growth and development.

Endochondral ossification requires complex regulatory mechanisms that mediate the production of ECM, cell development, and biomineralization. When these processes are not perfectly balanced, they result in a variety of skeletal dysplasias in bone [1, 2], and odontogenic defects in oral biology [3-5]. The characterization of the roles of SIBLING proteins during the development of these tissues will help provide insight for the development of novel therapeutics for treatment of these pathologies.

The loss of BSP in endochondral bone development resulted in a delay of primary ossification in the mouse model. This delay during ossification suggested that BSP is important for both the differentiation of osteoblasts and the resulting mineralization of bone. The data from the studies on the Bsp-/- osteoblasts support these findings of delayed mineralization and differentiation characteristics, as in vitro mineralization was also
delayed. This delay was rescued upon re-introducing BSP using an overexpression system. These data suggest that BSP is both a nucleator of mineralization and a promoter of osteoblast development and/or recruitment during endochondral ossification. The loss of OPN in osteoblasts resulted in increased mineralization, while there was no overall change to their gene expression characteristics. Thus, OPN is directly involved in mineral growth inhibition, although it does not appear to be involved in osteoblast differentiation. Overall, these data confirm the importance of BSP and OPN during mineralization in vivo, and provide understanding into the developmental roles of SIBLING proteins during development.
5.2 Contributions and significance of findings

5.2.1 BSP and endochondral ossification

Prior to undertaking this study, BSP was shown to be a positive regulator of mineralization and osteoblast differentiation [6]. In this thesis the loss of BSP in mice was demonstrated to result in decreased neonatal bone length and mineralization, in agreement with findings by Malaval et al. in 4 month old Bsp^/-^ animals [7]. BSP was also shown to be a promoter of endochondral ossification and cell cycle progression of growth plate chondrocytes. The impaired cell cycle progression of chondrocytes within the growth plate suggests that this contributes to the decrease in bone length of the Bsp^/-^ mouse. Primary ossification was delayed during early development; neonatal Bsp^/-^ mice demonstrated decreased mineralization and expression of osteogenic markers. These results suggest that ossification is delayed leading to the phenotypes observed in the Bsp^/-^ mice.

The Bsp^/-^ mouse has impaired repair mechanisms following injury, specifically delayed mineralization of newly formed bone in a cortical gap model [8, 9]. The mineralization defects observed during development may be applicable to the mechanism by which bone repair is delayed. The study presented in this thesis suggests that osteogenic differentiation and/or recruitment is delayed which could lead to impaired callus formation following injury, and delayed mineralization of the repaired tissues. This suggests that BSP is an important molecule directing bone repair, although during development its loss may be compensated for by other SIBLING group members, or other proteins with similar functionalities.
The loss of BSP resulted in delayed development and shortened bones, however as the mice age their bone lengths become normalized relative to their controls [7]. Interestingly, the loss of BSP also results in a deficiency of acellular cementum of teeth in adult mice [10]. This suggests that BSP is an important regulator of mineralized tissue formation, but in bone the loss of BSP is compensated for by another mechanism that is not recapitulated in acellular cementum. Thus, this study demonstrates that BSP has a variety of functions in the skeleton, which differ both temporally and spatially.

### 5.2.2 SIBLING proteins regulate osteogenic mineralization

In this thesis the SIBLING proteins BSP and OPN were shown to be capable of directing biomineralization and growth. BSP is known to be a potent nucleator of mineralization in both cell-free [11] and osteoblast studies [12, 13]. BSP-mediated mineralization is potentiated by binding to collagen [14, 15], and its efficacy as a nucleator is dependent on the contiguous glutamate sequences [16], and its phosphorylation status [16, 17]. The RGD integrin-binding sequence has also been shown to promote osteoblast differentiation, resulting in increased mineralization [13]. However, characterization of these functional motifs has not been conducted in a cell-based system.

In order to ultimately study the role of the functional motifs of BSP, the untreated \(Bsp^{-/-}\) osteogenic cultures required characterization. Therein \(Bsp^{-/-}\) osteoblasts were demonstrated to have delayed mineralization and differentiation (Chapter 3). Additionally, the osteogenic cultures had decreased metabolic activity, suggesting that cell proliferation is reduced. The delayed mineralization and differentiation is similar to the delayed endochondral ossification \textit{in vivo} in the \(Bsp^{-/-}\) mouse (Chapter 2). This lead to
the conclusion that the loss of BSP results in delayed osteogenic development and differentiation that contributes to the phenotype of $Bsp^{-/-}$ mice.

Overexpression of BSP in the $Bsp^{-/-}$ osteogenic cultures resulted in promoting mineralization and osteogenic gene expression compared to the EV control. These results agreed with previous work where overexpression of BSP in wild-type osteogenic cells promotes mineralization and osteoblastic differentiation [13]. Similarly, siRNA knockdown of $Bsp$ expression resulted in decreased mineralization and decreased gene expression of osteoblast markers $Runx2$, $Sp7$, and $Bglap$ [13]. These previous studies implicated the RGD motif as a critical promoter of osteoblast differentiation, thereby promoting mineralization. Our studies investigating the overexpression of BSP in $Bsp^{-/-}$ osteogenic cultures demonstrated that loss of any of the functional-domains of BSP was insufficient to completely ablate its mineralization and signaling capabilities (Chapter 3). Even the RGD to KAE BSP mutant retained similar mineralization capabilities at week 4 as wild-type BSP. This is contrary to previous findings suggesting that the RGD sequence is crucial to the functionality of BSP. These results also suggested that BSP may contain a secondary osteoblast signaling motif that is not yet identified. These findings support previous work, both cell- and cell-free-based, that have shown BSP to be a potent promoter of biomineralization.

Similar to BSP, OPN has been examined in cell-free studies and determined that it is a potent inhibitor of mineralization [18-20]. OPN is also predicted to be a promoter of cell differentiation due to RGD-integrin interactions [21, 22], although its signaling capabilities in osteoblasts is still contentious. Conflicting studies suggested that OPN either promotes [23] or inhibits [24] differentiation, resulting in increased or decreased
mineralization. The loss of OPN was demonstrated to result in increased mineralization in osteogenic cultures, while having no overall change in gene expression characteristics (Chapter 4). Furthermore, supplementation of the cultures with exogenous mOPN resulted in no change in terminal marker expression or metabolic activity. These results contribute evidence to suggest that the role of OPN in osteogenic cell cultures is strictly the modulation of mineralization.

These studies present data that contribute to the understanding of SIBLING protein function in skeletal development and homeostasis. The Bsp<sup>-/-</sup> mouse demonstrates delayed ossification during endochondral bone development that could be caused, in part, by delayed in osteoblast differentiation. Similarly, the Opn<sup>+/−</sup> mouse has increased bone density that could partly be due to mineral growth being less encumbered. The results from these studies provide insight into the roles of these proteins regulating matrix mineralization in vivo.
5.3 Limitations of research and future directions

5.3.1 Limitations of the Bsp−/− mouse model

This thesis has presented novel findings regarding the role of BSP in endochondral ossification. While loss of BSP during development appears to directly result in a delay of mineralization, the mechanism by which this delay occurs has yet to be elucidated. It is still unclear whether the delay is due to impaired osteogenic cell differentiation, impaired recruitment of these cells, or a combination of the two. The mechanism of cell cycle delay of growth plate chondrocytes is also not entirely clear. Delineation of these mechanisms would provide a more complete understanding of the function of BSP in the development and repair of endochondral bone.

In order to investigate the underlying cause of delayed endochondral ossification, mechanistic characterization of the developing tibiae must be performed. This can be accomplished by performing microdissections to isolate and characterize the resting, proliferating, and hypertrophic zones of developing long bones individually at E15.5 [25]. This analysis will specifically identify the genes that may be altered upon the loss of BSP, and identify which developmental stage is directly affected by the loss of function. Specifically, the use of next-generation RNA sequencing will provide substantially more data regarding the expression changes upon loss of BSP. This could also be applied to investigate the phenotype of OPN loss during development. The complete characterization of the expression patterns of these cells during endochondral ossification would provide evidence as to which pathways are impaired, and whether a compensatory mechanism is in place.
Another shortcoming of the mouse model is that the role of BSP is elucidated only in the context of a whole-body loss of function. This makes separating the primary and secondary effects of BSP loss difficult to isolate. It is impossible to determine if a growth plate defect is due to direct loss of function within the chondrocytes, or due to loss of expression of BSP from the osteoblasts or other cell types. A conditional mutant where the _Bsp_ gene can be deleted in either cell type specifically will more readily enable characterization of the specific roles of BSP. A conditional mutant would also provide interesting evidence to examine the mechanism of the loss of cementum in the _Bsp_-/- mouse. These mutants can be achieved by crossing either a cartilage- [26] or bone- [27] specific Cre recombinase with a floxed allele of _Bsp_.

A number of the SIBLING group of proteins are closely related in expression and function during mineralized tissue development. The limited phenotypes observed in all loss of function mice, including _Bsp_-/- and _Opn_-/- mice, are likely due to a compensatory mechanism provided by the related proteins, or those related in function. Thus, the deletion of multiple genes in the same model would characterize the potential interplay between these proteins. Insight into which genes would be viable targets would include those that were identified in the RNA sequencing experiment, described above. For example, the _Opn_-/- mouse has been demonstrated to have a mild phenotype, but when matrix gla protein (MGP) is ablated alongside OPN, the phenotype of atherosclerosis is exacerbated dramatically [28]. Similarly, when the hypomineralized _Akp2_ ablated mouse is crossed with the _Opn_-/- mouse, the mineralization phenotype is partially corrected [29]. This suggests that other proteins with similar functionalities may be performing compensatory mechanisms in the _Opn_-/- mouse. Similarly, the deletion of either _Bsp_ [7,
or *Dmp1* [30, 31] results in impaired development of endochondral bone and teeth. The deletion of both genes may exacerbate the phenotypes resulting in improperly formed bones. Due to the SIBLING genes all being within a syntenic gene locus, the deletion of multiple genes within the group is nearly impossible through cross-breeding. Therefore to study the loss of function of any two genes from the SIBLING group would require homologous recombination and insertion into a blastocyst from one of the currently derived models. Nevertheless, this would present an approach to understand the interplay between the proteins involved in mineralization and ossification.

### 5.3.2 Osteogenic cell culture models

In this thesis, osteoblast cultures were used to further the understanding of SIBLING proteins in the regulation of mineral formation and growth in a cell-based system. The studies of these cells in culture can be extended to characterize the phenotypes of the loss of function in mice. These cells were isolated from bone mesenchyme of 8 to 10 week old mice and were grown in a monolayer culture system. The cells proliferate and are grown at high-density, simulating physiological levels of cell to cell contact to replicate the conditions *in vivo* [32]. These mesenchymal-derived cells contain pluripotent progenitors that are capable of differentiating into cartilage, bone, adipose, and muscle [33]. In order to induce osteogenic differentiation the cultures were treated with ascorbate and glycerol-2-phosphate. While these cells are removed from the microenvironment of neighbouring tissues, it permits direct study of the phenotype upon loss of the *Bsp* or *Opn* gene.

The gene expression patterns following isolation demonstrated that the cultures had expected patterns of osteogenic marker expression, and decreases in both chondro-
and adipogenic marker expression. This demonstrated that the isolated cells differentiated along the osteoblast lineage, but it is impossible to confirm that the cultures were a homogenous population of osteoblasts. It is possible there were other cell types within the isolation, such as fibroblasts, adipocytes, and chondrocytes. Additionally, these cells are very sensitive to confluence and subculturing. If they are grown to a high confluence, or more than one subculture prior to induction, the cells lose their osteogenic phenotype and tend to become senescent (unpublished data). This can result in increased variability within the system if the cells are not handled identically in culture.

While this cell culture system is a more physiologically relevant model than the cell-free studies, it does have its own limitations in regards to data interpretation and how it applies in vivo. The mechanism of mineralization in vivo involves many variables; there is interaction with other cell types, different ECMs, and a multitude of signaling molecules including those from mechanical stress and the endocrine system. This is supported by the osteogenic cultures demonstrating large differences in mineralization upon loss of BSP and OPN compared to WT cultures in vitro, while the phenotype of the mouse is not severe. This is likely due to any number of the other aforementioned compensatory factors within a living animal.

The overexpression of BSP using adenovirus in osteogenic cultures permits expression containing post-translational modifications. The post-translational modification of this overexpressed BSP is predicted to be equivalent to that which is secreted by osteoblasts in vivo. The limitations of adenoviral transduction in this system are mainly due to the toxicity. The very high MOI required to transduce a sufficient number of cells resulted in impaired mineralization upon EV treatment. There also
appeared to be a trend downwards in the metabolic activity upon treatment with the adenovirus, which may have been rescued by BSP overexpression. WT cells would need to be treated with adenovirus in addition to the $Bsp^{-/-}$ osteogenic cultures to distinguish between the effects of the virus and BSP. Overexpression of proteins may also result in a number of differences in cell metabolism and differentiation. This resulting overexpression of BSP occurs at an earlier time and at a higher level than during typical osteoblast differentiation [34]. This may result in BSP-mediated cell signaling earlier than what would happen in normal differentiation, subsequently resulting in premature terminal differentiation.

In order to address many of the shortcomings of an adenoviral system, future experiments should be performed using a lentivirus to mediate gene transduction. Lentiviruses will more readily transduce primary mesenchymal cells than an adenoviral vector [35, 36]. This system will also incorporate the gene of interest directly into the genome, preventing dilution due to cell division which occurs in cells that are transduced with adenovirus. The genes will also be controlled under a different promoter than the CMV promoter. The CMV promoter, used in the adenovirus system, constitutively expresses a very high level of protein that may alter the availability of cellular machinery for production of the normal proteins and express the protein(s) at non-physiological levels. In contrast, treatment with a lentiviral vector normally results in a consistent lower level of expression. These studies should address many of the limitations from the adenovirus vectors used in this study and also provide an opportunity to perform stable integration and expression in vivo, such as the experiment proposed below.
The \( Bsp^{-/-} \) mouse demonstrates a loss of acellular cementum, leading to an acute periodontal tissue phenotype that we can attempt to rescue. Due to this phenotype, functional motif mutants of BSP can be expressed \textit{in vivo} in order determine their importance during acellular cementum formation. A similar phenotype \cite{37, 38} and rescue has been performed with the \( Akp2^{-/-} \) mouse \cite{39, 40}. The investigators attached a deca-aspartate sequence of amino acids to promote the binding of TNAP to mineralized tissues such as acellular cementum. Since BSP contains a strong negative charge, the rescue by intraperitoneal injection of neonates should permit it to bind to the mineralized tissues in a similar manner. This system should result in a rescue of the phenotype of loss of acellular cementum, which will be confirmed by histological assays and microcomputed tomography. This will provide an \textit{in vivo} model that BSP and its functional motifs could be studied.

In the study characterizing the role of OPN (Chapter 4), the cultures were supplemented with exogenous, fully phosphorylated bovine milk OPN (28 phosphorylations). This amount of phosphorylation differs from normal OPN expressed by osteoblasts, which is approximately 30\% phosphorylated (10-11 phosphorylations) \cite{41}. Phosphorylation of OPN has been demonstrated to modulate its signaling capabilities \cite{42}. In order to understand whether OPN is a signaling molecule in osteoblasts, additional studies would need to be performed. Supplementation of the osteoblast cultures with native rat OPN isolated from bone would provide a more physiological post-translationally modified protein as a control. Determining whether supplementation would result in inhibition similar to the milk OPN model would bolster evidence for OPN as an inhibitor of mineralization, and not a promoter of cell signaling,
in a culture-based system. To control for the phosphorylated native OPN, nonphosphorylated recombinant rat OPN may be used. Using recombinant OPN with a mutation of the integrin-binding RGD motif to the KAE would also delineate how OPN inhibits mineralization by eliminating the confounding effects of integrin-mediated signaling. These experiments would provide conclusive results to whether OPN promotes or inhibits osteoblast differentiation. There is also data to suggest that intracellular OPN is a promoter of differentiation [43, 44]. In order to confirm whether this is a characteristic of OPN in osteoblasts, lentiviral transduction of wild-type and functional domain mutants into \textit{Opn}\textsuperscript{-/-} mice could provide insight similar to that proposed for BSP.

The future experiments characterizing the roles of BSP and OPN in osteoblasts and in mice would provide valuable insight into their roles during development. This will improve the understanding into the mechanisms of how mineralized tissues are calcified and how the cells that regulate their development function. The characterization of normal development will further our understanding of skeletal disease, thereby providing insight into the development of novel therapeutics for the treatment of pathological mineralization.

\textbf{5.3.3 Future directions investigating BSP in osteoarthritis}

Recently BSP has been demonstrated to be highly expressed in pathological osteoarthritis (OA) in humans [45]. During OA, the articular cartilage of joints undergoes a number of changes that ultimately result in its degradation. The development of OA has demonstrated that articular chondrocytes begin to undergo differentiation that resembles chondrocyte hypertrophy [46]. As mentioned previously, hypertrophic chondrocytes express BSP, MMPs, and vascular growth factors. This induces the mineralization of the
articular cartilage, degradation of the tissue by MMPs, and promotes vascular invasion of a normally avascular tissue (reviewed in [47]). As BSP is a promoter of matrix mineralization and hallmark of hypertrophic chondrocytes, determining its role in OA could provide insight into the mechanism of pathological development.

A number of methods designed to study OA in mice have been developed (reviewed in [48]). These surgeries result in the destabilization of the knee joint that induces OA. One popular method in mice involves destabilization of the medial meniscus by medial meniscotibial ligament transection [49, 50]. Following surgery, OA develops between four to ten weeks. As BSP has been implicated to be highly expressed in OA [45, 51], examination of this model in the Bsp-/- mouse presents an opportunity to investigate OA without endogenous BSP expression. Histological characterization of the pathogenesis of OA would demonstrate the function of BSP during articular cartilage degradation. The correlation with BSP and OA suggests that the loss of BSP will provide a protective effect with less severe OA being present. Characterization of the pathogenesis could implicate BSP as an instigator of OA.
5.4 Conclusions

These studies have demonstrated that BSP and OPN are important regulators in the formation and growth of biomineral within the skeleton. This thesis has identified specific roles for BSP in primary ossification during endochondral bone development. This provides insight into bone biology and normal development, with a focus on proper regulation of mineralization. The loss of BSP \textit{in vitro} also recapitulated the phenotype of the BSP loss of function mouse. This thesis also identifies OPN as a potent regulator of mineralization, which does not appear to function through signalling pathways in osteogenic cell development. The characterization of these proteins during mineralization provides evidence to suggest these could be valuable clinical targets for the prevention or treatment of diseases associated with pathological calcifications.
4.6 References


Appendix A: Statement of Permission for the Use of Animals for Experimental Research.

All animal experimentation was performed in compliance with the animal use protocol 2008-092. This protocol is held by Dr. Harvey Goldberg, a principal investigator at the Schulich School of Medicine and Dentistry and the department of Biochemistry at the University of Western Ontario in London, Ontario, Canada.

2008-092::5:

**AUP Number**: 2008-092  
**AUP Title**: Functional Characterization of Bone Sialoprotein Using the BSP-null Mouse  
**Yearly Renewal Date**: 11/01/2013

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2008-092 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
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AWARDS AND SCHOLARSHIPS
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Summer 2014  
**NCOHR Poster award**

*Title*: “Delineating the role of bone sialoprotein in bone development and mineralization” (Poster presentation)

*Authors*: E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, and H.A. Goldberg

School of Dentistry
University of Alberta, Edmonton, ON

Fall 2008 –  
Fall 2013  
**Western Graduate Research Scholarship**

*(Value: ~$8,000/year, covers cost of tuition each year for 5 years) – Accepted*

Schulich School of Medicine & Dentistry
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PUBLICATIONS

Holm, E., Gleberzon, J.S., Sørensen, E.S., Beier, F., Hunter, G.K., Goldberg, H.A.  
Osteopontin mediates mineralization and not osteogenic cell development *in vitro*. *(Submitted August 2014)*

Holm, E., Aubin, J.E., Hunter, G.K., Beier, F., Goldberg, H.A. Loss of bone sialoprotein leads to impaired endochondral bone development and mineralization. *(Submitted August 2014)*


CONFERENCE ABSTRACTS
(SELECTED FIRST AUTHOR SUBMISSIONS)

Network for Canadian Oral Health and Research Canadian Dental Student Workshop, Edmonton, AB, Canada. June 2014. Title: “Delineating the role of bone sialoprotein in bone development and mineralization” (Poster presentation) Authors: E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, and H.A. Goldberg Role: Contributed to research, prepared abstract and presented research

Canadian Connective Tissue Conference, London, ON, Canada. June 2014. Title: “Delineating the role of bone sialoprotein in bone development and mineralization” (Oral presentation) Authors: E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, and H.A. Goldberg Role: Contributed to research, prepared abstract and presented research

Canadian Connective Tissue Conference, Montreal, QC, Canada. May 2011. Title: “Delineating the role of bone sialoprotein in bone development and mineralization” (Poster presentation) Authors: E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, and H.A. Goldberg Role: Contributed to research, prepared abstract and presented research

Margaret Moffat Research Day, London, ON, Canada. March 2011. Title: “The characterization of bone sialoprotein in osteoblast differentiation and matrix mineralization” (Poster presentation) Authors: E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, and H.A. Goldberg Role: Contributed to research, prepared abstract and presented research

Great Lakes Mammalian Development Meeting, Toronto, ON, Canada. April 2010. Title: “The characterization of bone sialoprotein in osteoblast differentiation and matrix mineralization” (Poster presentation) Authors: E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, and H.A. Goldberg Role: Contributed to research, prepared abstract and presented research

Great Lakes Mammalian Development Meeting, Toronto, ON, Canada. April 2008. Title: “The characterization of bone sialoprotein in osteoblast differentiation and matrix mineralization” (Poster presentation) Authors: E. Holm, J.E. Aubin, G.K. Hunter, F. Beier, and H.A. Goldberg Role: Contributed to research, prepared abstract and presented research