CK2.3, A MIMETIC PEPTIDE OF BMPRIA, IS A POTENTIAL ACTIVATOR OF OSTEOBLAST DIFFERENTIATION AND ACTIVITY

by

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ABSTRACT

Osteoporosis, often referred to as OP, is a disease that is characterized by low bone mineral density. This decrease in bone mineral density leaves the bone more fragile and porous. This fragility in bone is due to an imbalance between the bone forming cells, osteoblasts, and the bone reabsorbing cells, osteoclasts. With the bone being more fragile, OP patients are more susceptible to fractures and the risk of fracturing a bone increases with age. Approximately 1 in 2 women and 1 in 4 men over the age of 50 will break a bone due to OP and an estimated 20% of those who suffer hip fractures die within a year due to complications.

Despite the ever-constant presence of osteoporosis, mortality rates for this disease remain unchanged, demonstrating the ineffectiveness of current treatments on the market. The Nohe laboratory has demonstrated previously a novel Bone Morphogenetic Protein Receptor Type Ia (BMPRIa) interaction with Casein Kinase 2 (CK2) and upon BMP2 binding to the serine/threonine kinase receptor complex, CK2 dissociates from BMPRIa allowing the phosphorylation of downstream signals (Bragdon et al., 2010). Using a prosite search, the Nohe laboratory has identified three potential BMPRIa CK2 interaction sites. The Nohe laboratory has developed three mimetic peptides (CK2.3, CK2.2, and CK2.1) that encompass the site-specific sequences that could block the interaction of CK2 with BMPRIa.

In this study, I investigated the ability of CK2.3 to induce the activation and differentiation of preosteoblasts and osteoblasts *in vivo* and *in vitro*. In mice, CK2.3

increased OC and ALP expression levels in a dose dependent manner. This trend was only seen in mice femurs that were extracted 4 weeks post initial injection. It was not seen in 1 week and 2 weeks post initial injection. The increase in the expression of these two markers was not caused by cellular proliferation but rather differentiation and activation.

Also, in this study, I investigated CK2.3s' ability to increase ALP and OC expression intensities in undifferentiated, 1 week differentiated, 2 weeks differentiated and 4 weeks differentiated C2C12 cells. C2C12 cells are a murine myoblast cell that can differentiate into osteoblasts under the proper conditions. Differentiated and undifferentiated cells were stimulated with PBS, CK2.3 or BMP2 and it was hypothesized that CK2.3 would produce results similar to those of BMP2. Through the use of immunofluorescent staining and imaging, I was able to show that CK2.3 does increase the level of these marker in comparison to PBS and in some cases to BMP2 as well.

CK2.3 may regulate BMPRIa downstream signaling for the initiation of bone formation but the signaling cascade still needs to be properly understood. Understanding the signaling pathway could help identify molecular targets that are necessary for inducing bone growth. Given the research I have presented, CK2.3 provides a unique opportunity to identify targets that can be used as a therapeutic treatment for osteoporosis.

Chapter 1

INTRODUCTION

1.1 Bone Morphology and Remodeling Cycle

Bone is a rigid, dynamic tissue that responsible for the formation of the skeletal system of the human body. While the bones have many roles, the most vital is the support and protection of the organs that reside within the body. Other roles include locomotion, support of hematopoiesis in red bone marrow, storage of inorganic minerals (i.e. calcium and phosphate), endocrine regulation and provide a surface for the attachment of muscles (Proff and Römer 2009). Bone tissue is composed of both an organic and inorganic component. The organic component mainly consists of collagen type I, which accounts for 90% of the total bone protein. The other 10% of protein consists of non-structural proteins like growth factors, blood protein, osteocalcin (OC) and alkaline phosphatase (ALP) (Proff and Römer 2009). The inorganic portion of the bone is composed mainly of mineral hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which breaks down into calcium, phosphate and hydroxide (Proff and Römer 2009).

Bone formation is tightly regulated and controlled by three major components; osteoblasts (cells responsible for formation of bone matrix), osteoclasts (cells responsible for resorption of the bone) and osteocytes (cells that regulate bone

formation via endocrine system) (Raggatt and Partridge 2010) (Fig:1.2). There are two main methods of bone formation; intramembranous ossification and endochondral ossification. Intramembranous ossification creates direct bone formation from mesenchymal tissue, which is a process that results in the formation of the flat bones such as the skull, scapula, mandible and ileum (Price, Oyajobi, and Russell 1994). On the other hand, endochondral ossification creates bone from mesenchymal stem cells differentiating into chondrocytes. This differentiation forms hyaline cartilage, which later is replaced by bone formation through bone cell infiltration (Fig: 1.1). Endochondral ossification is responsible for the formation of longs bones, like the femur, tibia, ribs and humerus (Mackie et al. 2008).

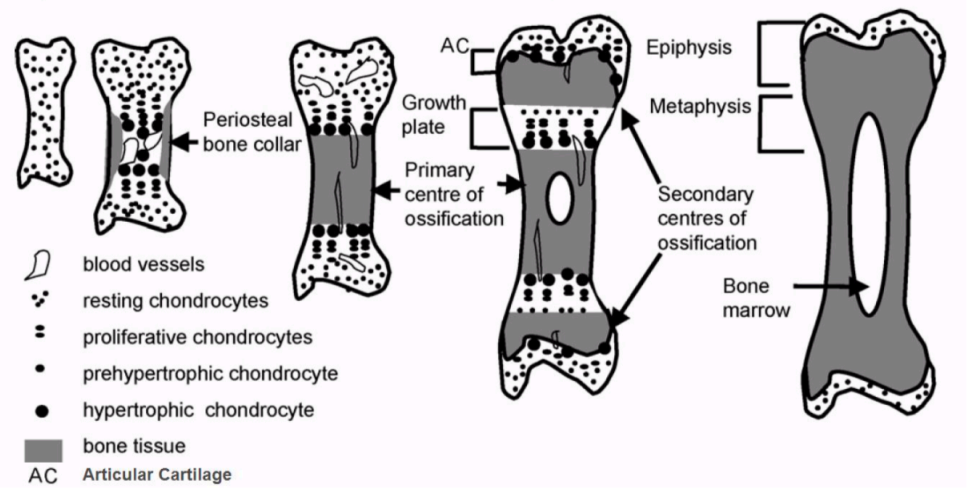


Figure 1.1 **The development of long bones through the process of Endochondral ossification.** Image was modified from (Mackie et al., 2008).

1.2 Function and Lineage of Bone Cells

Bone is considered a form of dynamic tissue that has a constant turnover rate. Every year, approximately 10% of the human skeleton is remodeled, giving rise to a

completely remodeled skeleton in about 10 years. As mentioned previously, the cells that are responsible for this rapid turnover are the osteoblasts and osteoclasts. Osteoclasts are large, multinucleated cells that form through the fusion of several mononucleated hematopoietic progenitor cells (Proff and Römer 2009; Kurihara et al. 1989). The early differentiation of the HSC into the monocyte/ macrophage progenitor depends on a specific transcription factor, PU.1. PU.1 regulates c-fms, the monocyte/macrophage colony stimulating factor (M-CSF) receptor. M-CSF binding to c-fms is required for both monocyte progenitor proliferation and the expression of the receptor activator of nuclear factor kB (RANKL) (Teitelbaum 2000). At this stage of differentiation cells are termed pre-osteoclasts. RANK ligand (RANKL), which is expressed on osteoblasts and stromal cells, is the crucial, final growth factor needed for complete differentiation (Datta et al. 2008; Teitelbaum 2000). The binding of RANKL to the RANK receptor activates nuclear factor kB (NF-kB) signaling, which leads to the mononuclear osteoclast expression of tartrate-resistant acid phosphatase (TRAP). It is at this point that cells mature and fuse, which creates a mature osteoclast. Though osteoblasts are the bone forming cells, they are responsible for secreting osteoprotegerin (OPG), which binds to RANKL (Udagawa et al. 1999; Takahashi, Udagawa, and Suda 1999). The binding of OPG to RANKL does not allow RANK to interact with RANKL, which in turn negatively regulates osteoclast differentiation (Simonet et al. 1997).

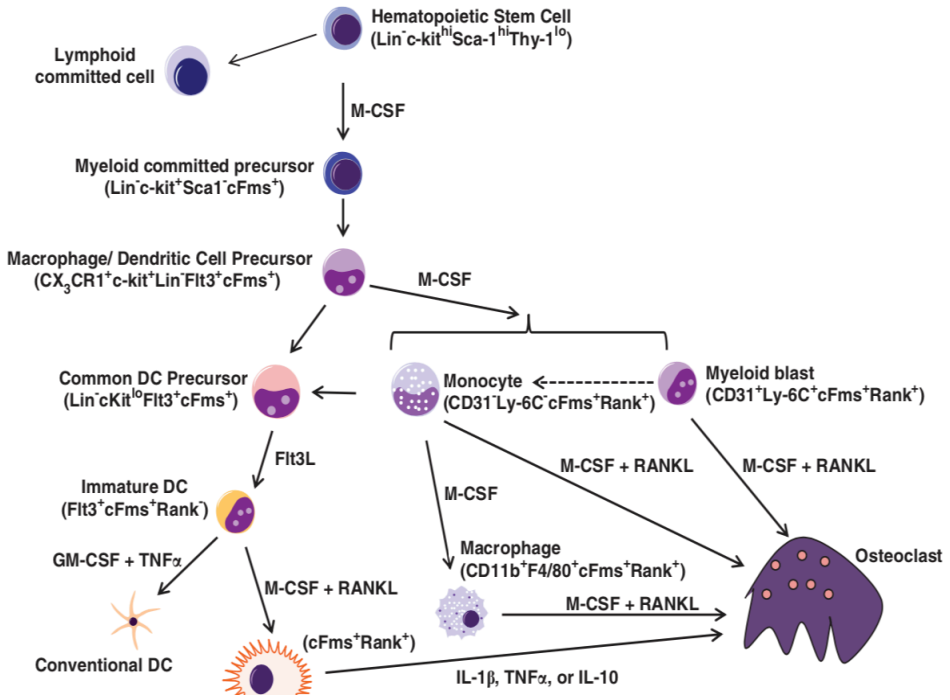


Figure 1.2 **A detailed schematic of the differentiation pathways of HSCs. Taken from (Long and Humphrey 2012).**

Once osteoclasts are activated, they release protons and lysosomal hydrolases into the confined environment of the extracellular space. Some of these hydrolytic enzymes include cathepsin K and metalloproteases, which are responsible for degrading collagen and other proteins in the matrix. The release of protons causes a dissolution of the mineralized matrix via acidification by creating a pH of 4-5. As a result of this acidification, a shallow bay forms under the osteoclast known as the resorption bay or pit (Väänänen and Laitala-Leinonen 2008). The products of the degraded bone such as calcium ions, soluble inorganic phosphates and water, get released back into circulation. Once the resorption of the target bone is complete, osteoclasts will undergo a programmed cell death, known as apoptosis. Once

resorption is complete, pre-osteoblasts will proliferate and differentiate into osteoblasts.

Osteoblasts are differentiated mesenchymal stem cells (MSCs) that retain the ability to divide and secrete collagen type I, which makes up approximately 90% of bone. Osteoblasts are bone forming cells that are known to form bone tissue by secreting ALP, OC, collagen type I, proteoglycans and bone sialoprotein (BSP), which are responsible for the calcification of bone (Wennberg et al. 2000). A number of transcription factors and biochemical factors tightly regulate osteoblast recruitment, function and maturation. Osteoblast differentiation is controlled by the secretion of lipid-modified glycoproteins of the wingless (Wnt) family, bone morphogenetic protein 2 (BMP2) and several other transcription factors. BMP signaling is among the many important signaling pathways that induce osteoblastogenesis and produce essential transcription factors such as runt-related transcription factor 2 (RUNX2) and Osterix. BMPs found in bone influence MSCs commitment to osteoblasts that express specific proteins, such as ALP, OC, and the osteogenic transcription factor RUNX2. Osteoblasts lay down new bone in the pits formed by the osteoclasts, by mineral deposition on the newly formed matrix replacing the old bone completely.

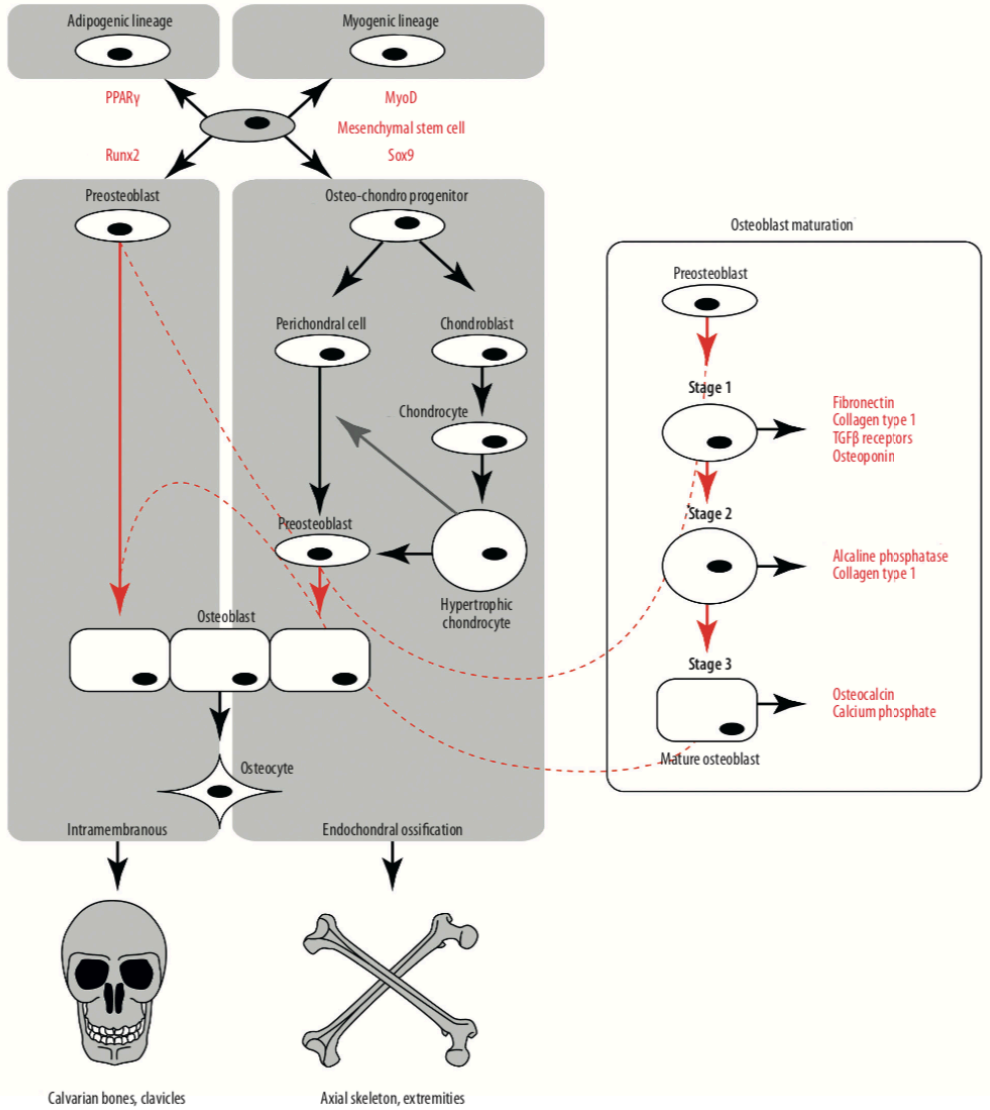


Figure 1.3 **A detailed schematic of the differentiation pathways of MSCs. Taken from (Rutkovskiy, Stensløkken, and Vaage 2016).**

ALP and OC are not just biochemical markers for osteoblast activity. In fact, they play a much larger role in bone formation. Osteocalcin, also known as "bone gamma-carboxyglutamic acid (Gla) protein (BGP)," is the most abundant noncollagenous protein of bone matrix. Once it is transcribed, osteocalcin undergoes

posttranslational modifications within the osteoblast before it can be secreted (Patti et al. 2013). These posttranslational modifications include the proteolysis of a prepropeptide and the carboxylation of three glutamic residues (located in positions 17, 21, and 24) in glutamic acid. Vitamin D directly stimulates osteocalcin transcription (in fact the gene has a "vitamin D responsive element") while vitamin K regulates the carboxylation processes (Patti et al. 2013). Carboxylated Glaresidues are involved in calcium and hydroxyapatite binding, allowing osteocalcin deposition in mineralized bone matrix. On the contrary, noncarboxylated osteocalcin has a low affinity for hydroxyapatite and is more easily released into the circulation. However, both the carboxylated and the undercarboxylated forms are detectable in the peripheral blood, as well as total osteocalcin that is usually measured as a marker of bone formation (Patti et al. 2013). An immunoassay analysis in normal individuals estimated that up to 50% of osteocalcin is undercarboxylated and that this percentage may change in response to fluctuations in intakes of vitamin K on a daily basis. Thus, levels of undercarboxylated osteocalcin are influenced by levels of vitamin K, whereas total circulating concentrations of osteocalcin are influenced by bone cells activity, completely independent of vitamin K (Patti et al. 2013). Although osteocalcin is released by osteoblasts during bone formation and binds with the mineralized bone matrix, its precise function in bone metabolism has not been fully elucidated (Patti et al. 2013).

The metalloenzyme known as alkaline phosphatase (ALP) exists as several tissue-specific isozymes encoded by separate genes. The enzyme, which is expressed

in many species (plants, bacteria and animals), catalyzes the hydrolysis of phosphomonoesters, R-O-PO3, with little regard to the identity of the 'R' group (Golub and Boesze-Battaglia 2007). The catalytic mechanism involves the formation of a serine phosphate at the active site which reacts with water at alkaline pH to release inorganic phosphate from the enzyme (Sharma, Pal, and Prasad 2014). In the presence of high concentrations of an organic alcohol, a transphosphorylation reaction results when the organic alcohol releases the enzyme-bound inorganic phosphate and becomes phosphorylated (Golub and Boesze-Battaglia 2007). In vertebrates, the enzyme is an ectoenzyme, which is attached to the outer face of the plasma membrane through a phosphatidyl inositol-glycophospholipid (GPI) anchor covalently attached to the C-terminus of the enzyme. Humans have four ALP genes corresponding to intestinal, placental, placental-like and liver/bone/kidney (tissue nonspecific; TNAP) gene products (Golub and Boesze-Battaglia 2007). ALP has many different functions in the many organisms and tissues where it occurs. More than 80 years ago, the high level of ALP expression in bone was noted, and the first hypothesis put forward to explain why ALP was important for hard tissue formation. In this formulation, ALP was postulated to increase the local concentration of inorganic phosphate, a concept known as the 'booster hypothesis'. The role of ALP in hard tissue formation has been vigorously debated since then, with two divergent outcomes. As more has been learned about the biology of hard tissues and mineral metabolism, the role of ALP as a marker for osteogenic activity has been consistently solidified. Its' crucial role is now undisputed since it became apparent that the genetic diseases known as

hypophosphatasia (OMIM: 171760) result from mutations in the gene coding for TNAP, and lead to a phenotype characterized by under mineralization of bone (Golub and Boesze-Battaglia 2007).

Mature osteoblasts undergo terminal differentiation and become embedded in their own matrix during the mineralization stage. At this point of differentiation, they are defined as osteocytes and transcription genes such as Osterix, Dlx5, and ATF4 and proliferation genes are turned off (Marcus et al., 2008). Osteocytes are believed to be responsible for bone matrix maintenance and for the response to mechanical forces applied to the bone, and are thus termed mechanotransducers (Ross and Pawlina, 2005). Osteocytes, in bone embedded are proposed to sense microcracks and microfractures triggering the subsequent osteoclast differentiation and bone resorption. Further resorption is carried out by the fully differentiated osteoclasts that resorb bone forming pits and this process allows proteins like BMPs and insulin-like growth factor-1 (IGF-1) to influence mesenchymal stem cells commitment (Bonewald, 2007a; Bonewald, 2007b; Matsuo and Irie, 2008)

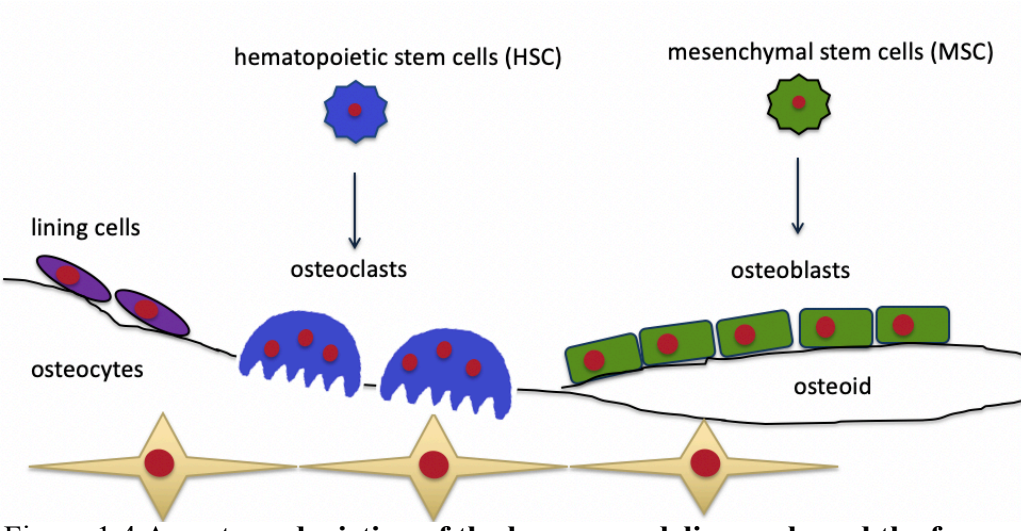


Figure 1.4 **A cartoon depiction of the bone remodeling cycle and the four main components: osteoblasts, osteoclasts, osteocytes and bone lining cells.**

1.3 C2C12 Cells and their Lineage

C2C12 cells are an immortalized murine myoblast cell line. The C2C12 cell line is a sub clone of myoblast cells that were extracted originally by Dr. Davis Yaffe and Dr. Ora Saxel at the Weizmann Institute of Science in Israel back in 1977 (Yaffe and Saxel 1977). This cell line was developed for *in vitro* studies of myoblasts that were removed from the complex interaction of these cells *in vivo*, which makes them useful from a research aspect ("C2C12 Transfection Reagent (Mouse Myoblast Cells)" n.d., 12). C2C12 are capable of rapid proliferation under high serum concentrations and differentiation into myoblasts under low serum concentrations. Mononucleated myoblasts can eventually fuse together to form multinucleated myotubules, leading to the precursors of contractile skeletal muscles in the process of myogenesis ("Working with the C2C12 Cell Line" 2012). C2C12 cells are not only used to study myogenesis but can also be used to study osteoblasts and their biochemical pathways due to their

ability to be differentiated into the osteoblast lineage under proper conditions ("Working with the C2C12 Cell Line" 2012).

1.4 Bone Morphogenetic Proteins

For the bone morphogenetic pathway (BMP), the are several BMP ligands that can activate this pathway. These ligands belong to the transforming growth factor beta (TGF-β) family. In the TGF-β family, BMP 1, 2, 3, 4, 5, 6, 7, 8a, 8b, 9, 10, 11, 12, 13, 14, 15 & 16 all have their respective roles, whether they are classified as a BMP or a GDF. They also play an important role in a vast number of developmental processes of various tissues, including lung, skin, brain, eye, liver, and kidney. Due to this importance in the development of many organs, it recently has been suggested to change their name to Body Morphogenetic Proteins instead (Fig. 1.3) (Reddi, 2005; Wagner et al., 2010).

Table 1.1 **BMP ligands with their known expression locations and functions. Some information was obtained from the Jackson Laboratory [http://www.informatics.jax.org/] and GeneCards Database [http://www.genecards.org/]. Published in (Bragdon et al. 2011).**

BMP	Where it is expressed in the body	Functionality
BMP-1	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	Metalloprotease that cleaves COOH-propeptides of procollagens I, II, and III/ induces cartilage formation/ cleaves BMP2 antagonist chordin
BMP-2	Spleen, kidney, lung, pancreas	Skeletal repair and regeneration/ heart formation
BMP-3 (osteogenin)	Thymus, bone marrow, spleen, brain, heart, skeletal muscle, pancreas, prostate	Negative regulator of bone morphogenesis
BMP-3b (GDF10)	Brain, spinal cord, skeletal muscle, pancreas, prostate	Cell differentiation regulation/ skeletal morphogenesis
BMP-4 (BMP-2b)	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal	Skeletal repair and regeneration/ kidney formation

	muscle, kidney, lung, liver, pancreas, prostate	
BMP-5	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, pancreas, prostate	Limb development/bone and cartilage morphogenesis/ connecting soft tissues
BMP-6 (Vrg1, Dvr6)	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	Cartilage hypertrophy/ bone morphogenesis/ nervous system development
BMP-7 (OP1)	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	Skeletal repair and regeneration/ kidney and eye formation/ nervous system development
BMP-8a (OP2)	Thymus, bone marrow, spleen, brain, spinal cord, heart, kidney, lung, pancreas, prostate	Bone morphogenesis/ Spermatogenesis
BMP-8b	Bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, liver, pancreas	Spermatogenesis
BMP-9 (GDF2)	Data not found	Bone morphogenesis/ development of cholinergic neurons/ glucose metabolism/ anti- angiogenesis
BMP-10	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, lung, liver, pancreas, prostate	Heart morphogenesis
BMP-11 (GDF11)	Thymus, bone marrow, spleen, brain, spinal cord, pancreas	Pattering mesodermal and neural tissues, dentin formation
BMP-12 (GDF7/ CDMP2)	Data not found	Ligament and tendon development/ sensory neuron development
BMP-13 (GDF6/ CDMP2)	Data not found	Normal formation of bones and joins/ skeletal morphogenesis/ chondrogenesis
BMP-14 (GDF5/ CDMP1)	Bone marrow, heart, kidney, liver	Skeletal repair and regeneration
BMP-15	None	Oocyte and follicular development
BMP-16	Data not found	Skeletal repair and regeneration

Even though there are numerous ligands that activate this pathway, BMP2 is the most prevalent and studied of these ligands (Utturkar et al. 2013). BMP2 is a growth factor that is known to induced several signaling processes like osteoblastogenesis, osteoclastogenesis, adipogenesis and many others. For these pathways to be activated, BMP2 binds to the receptors BMPRIa and BMPII, which are

dimerized. This binding causes the type II receptor to phosphorylate the type I receptor at the GS domain. The GS domain is a short but highly conserved regulatory sequence found on the cytoplasmic side of the type I receptor . This phosphorylation leads to the release of the interacting protein called casein kinase 2 (CK2), which in turn induces a signaling cascade that activates several pathways. The two major pathways that are activated that affect bone composition are the SMAD dependent and SMAD independent pathways (Fig:1.3). The SMAD dependent pathways initiates when SMAD 1/5/8 are phosphorylated and then recruit SMAD 4. SMAD 4 is a regulatory SMAD that is essential for this pathway. Once this complex is bound together, they will translocate into the nucleus and induce osteogenesis and adipogenesis (Hata et al. 2003). The SMAD independent pathway is initiated through the activation of the TAB/TAK1 pathway. Once phosphorylated and activated, TAB/TAK1 activate MEK1/2, MKK3/6 and PI3K (Nohe et al. 2004; Guicheux et al. 2003; Moseychuk et al. 2013; Rahman et al. 2015). This activation further leads to the activation of the p38, ERK and AKT pathways, which then translocates into the nucleus to induce osteogenesis, adipogenesis and apoptosis. The activation of these pathways is dependent on the release of casein kinase 2 (CK2).

Table 1.2. **BMP receptors with their known ligands and expression locations. Some information was obtained from the Jackson Laboratory [http://www.informatics.jax.org/] and GeneCards Database [http://www.genecards.org]. Published in (Bragdon et al. 2011).**

Receptor Name	BMPs that bind to specific receptor	Expression
ALK1 (Acvrl1)	BMP9 BMP10	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate
ALK2 (ActRI)	BMP2 BMP4 BMP6 BMP7 BMP9	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate
ALK3 (BMPRIa)	BMP2 BMP4 BMP6 BMP7 BMP10 BMP12 BMP13 BMP14	Thymus, bone marrow, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate
ALK4 (ActRIb)	BMP3 BMP11	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate
BMPRII	BMP2 BMP4 BMP6 BMP7 BMP9 BMP10 BMP12 BMP13 BMP15	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate
ALK6 (BMPRIb)	BMP2 BMP4 BMP6 BMP7 BMP10 BMP12 BMP13 BMP14	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate
ActRIIa	BMP2 BMP3 BMP4 BMP6 BMP7 BMP10 BMP11 BMP12 BMP13 BMP14	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate
ActRIIb	BMP2 BMP6 BMP7 BMP9 BMP11 BMP14 BMP16	Thymus, bone morrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate

1.5 Casein Kinase 2 and Casein Kinase 2.3 as a Possible Treatment

Casein Kinase 2 (CK2) is a ubiquitously expressed, highly conserved enzyme that interacts with more than 300 different substrates. The interaction of CK2 with these substrates affects cell growth, proliferation, differentiation, apoptosis and tumorigenesis (Duncan and Litchfield 2008). The CK2 complex is a tetramer with two catalytic subunits (α and/or α′) and two β regulatory subunits (David W. Litchfield 2003). The two catalytic subunits are structurally similar but are coded for by different genes (D. W. Litchfield et al. 1990). The two regulatory subunits are inactive alone

but are essential for the formation of the CK2 complex as well as recruiting and docking CK2 substrates (Bragdon et al. 2010). The catalytic subunits of CK2 are essential for the for the phosphorylation of targeted residues (David W. Litchfield 2003). These residues include serine, threonine and in a less favorable manner, tyrosine. Some of the substrates that interact with CK2 are enzymes that are involved in transcription and translation, signal transduction proteins, transcription factors, protein synthesis factors, and cytoskeleton structure proteins (Allende and Allende 1995).

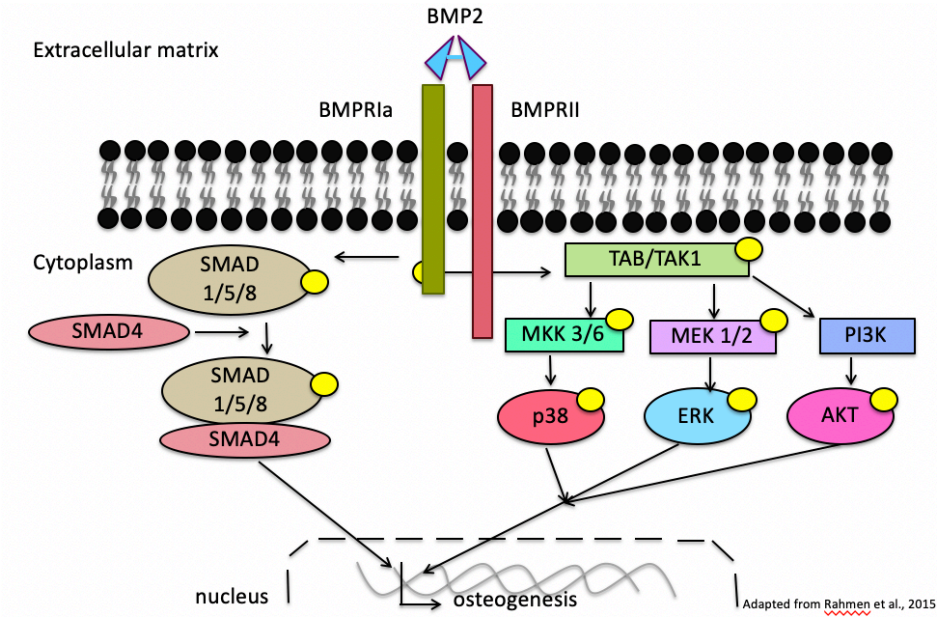


Figure 1.5 **Schematic representing the BMP2 activated signaling pathway that induces osteogenesis.**

It was discovered that the BMPRIa has three potential phosphorylation sites which were respectively called CK2.1, CK2.2 and CK2.3 based on their location on the receptor (Fig:1.4). CK2.3 corresponds to the amino acid sequence SKLD and binding spans amino acid residues 213-217. CK2.3, the only peptide used for my

experiments, was designed to block the interaction of CK2 and the third phosphorylation site on the BMPRIa. This peptide incorporates the antennaepedia homeodomain, which is important to aid in the cellular uptake of the peptide. The peptide was also designed with specific amino acid residues flanking each end of the phosphorylation site sequence to aid with the peptide binding to the correct phosphorylation site. Akkiraju et al. previoulsy showed that CK2.3 induced osteogenesis in primary calvarial cells as well as bone marrow stromal cells (BMSCs) (Akkiraju et al. 2015). Akkiraju et al. also demonstrated that CK2.3 had significantly increased ALP and OC serum levels in mice that were systemically injected with CK2.3 compared to mice that were treated with BMP2 or PBS (Akkiraju et al. 2015) . These measurements were taken 1 week, 2 weeks and 4 weeks post initial injection. Finally, Akkiraju et al. was able to demonstrate that mice that were systemically injected with CK2.3 and BMP2 had significantly higher trabecular bone mineral densities (BMD) (Akkiraju et al. 2015). Along with showing that ALP, OC and BMD increased with CK2.3 injections, it was also shown that CK2.3 decreased serum levels of TRACP 5b, which is indicative of decreased osteoclast activity (Akkiraju et al. 2015). Nguyen et al. successfully demonstrated that CK2.3 increased OC and ALP expression in murine femurs in a dose dependent manner four weeks post initial injection (Nguyen et al. 2018). Along with showing a dose dependent increase in ALP and OC, it was shown that CK2.3 decreased the number of osteoclast cells 1 week, 2 weeks and 4 weeks post initial injection. Finally, it was shown that CK2.3M increased femoral shaft stiffness 4 weeks post initial injection. Finally, Vrathasha et al. were

able to demonstrate that the treatment of C2C12 cells with CK2.3 led to the upregulation of Osterix, ALP and OC (Vrathasha, Weidner, and Nohe 2019). All of the research previously performed indicates that CK2.3 could be a possible therapeutic in the treatment of osteoporosis.

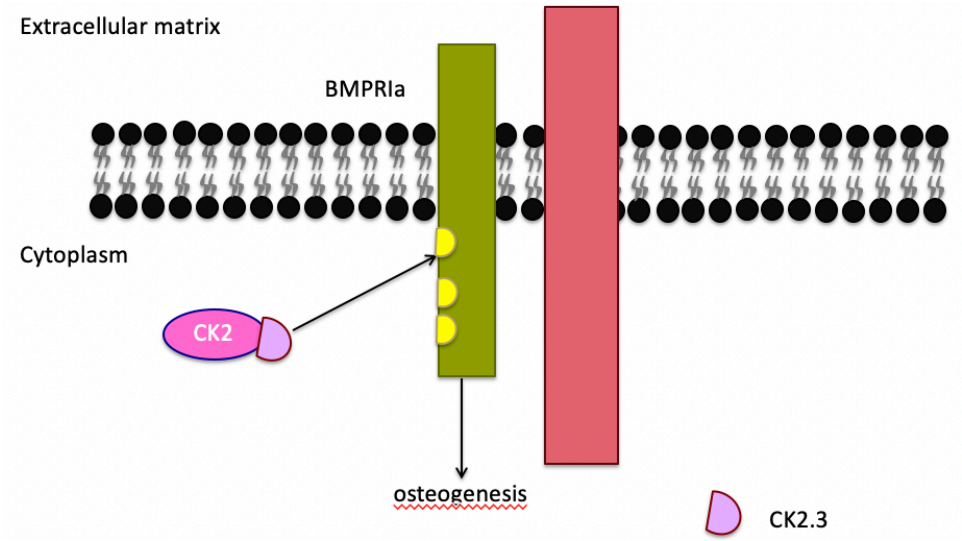


Figure 1.6 **Cartoon depiction of CK2.3 interacting with the specific binding location on BMPRIa**

1.6 Osteoporosis and Current Treatments

Osteoporosis is characterized as a skeletal disease that affects a large portion of the aging population. Approximately one in two women and one in four men aged 50 and older will break a bone due to osteoporosis related complications (Vrathasha, Weidner, and Nohe 2019). Osteoporosis occurs when the pores that are located within the bone become enlarged and in turn decrease bone density. Osteoporosis is caused by an imbalance within the bone remodeling. This imbalance lies within either the osteoblasts or the osteoclasts. This imbalance is caused by either an increase in

osteoclast activity or a decrease in osteoblast activity. In osteoporotic patients, there is a significant decrease in the bone mineral density, which is attributed to an increase risk of bone fractures. The most prevalent type of osteoporosis is type II, age related osteoporosis, which is caused by decreased osteoblast activity. Type II osteoporosis affects more women than men aged 70 and older. Type I osteoporosis, also referred to as postmenopausal osteoporosis, is caused by an increase in osteoclast activity. Type I osteoporosis mainly occurs in women aged 50-70 after menopause, due to a significant decrease in estrogen levels. Women are at risk for developing either type I or type II where as men are only at risk for developing type II osteoporosis. Currently, osteoporosis is diagnosed through the use of a central DXA scan (Dual Energy X-ray Absorptiometry) (Tu et al. 2018). This scan will look at the total hip, femoral neck and lumbar spine. Based on these results, a T-score will be determined for the patient. A T-score shows how much the bone density of the patient is higher or lower than a healthy, 30-year-old adult. If the T-score of a patient is lower that means that the patients' bone mineral density is also lower than it should be (Tu et al. 2018). It is recommended that female patients that are 65 and over and male patients that are 70 years and older should obtain regular bone screening through a DXA scan (Tu et al. 2018). If a patient receives a T-score that is below or equal to a -2.5, they are diagnosed with osteoporosis. Current treatments on the market for osteoporosis are listed in Table 1.1.

Table 1.3 **Table outlining the 5 different current medications used in the treatment of osteoporosis**

Treatment	Type	Common Names	Side Effects
bisphosphonates	Antiresorptive	Boniva, Fosamax	Osteonecrosis of the jaw, atypical femoral fracture, esophageal/stomach irritation
selective estrogen receptor modulator (SERM)	Antiresorptive	Evista	Blood clots, pulmonary embolism, deep vein thrombosis
calcitonin	Antiresorptive	Miacalcin	Liver cancer, severe bone pain, impaired thinking
RANKL inhibitor	Antiresorptive	Prolia	Osteonecrosis of the jaw, endocarditis, severe bone pain, serious skin, lower abdomen, bladder or ear infections
synthetic PTH	Anabolic	Forteo	Osteosarcoma, gout, high blood calcium levels

The current treatments on the market for osteoporosis are not ideal, seeing that they produce several, less than desirable side effects. Treatments can be grouped into two separate categories, antiresorptive and anabolic. As seen in Table 1.1, a majority of treatments on the market are antiresorptive. Antiresorptive treatments focus on inhibiting the activity of osteoclasts, therefore decreasing bone resorption. The most common antiresorptive drug prescribed for the treatment of osteoporosis is bisphosphonates (Tu et al. 2018). Bisphosphonates are recommended to be taken alone, first thing in the morning, on an empty stomach. Due to the way the medication is administered, it is considered an inconvenience. Currently on the market, there is only one anabolic treatment available for osteoporosis, which is synthetic PTH. (Tu et al. 2018). Anabolic treatments focus on producing more bone rather than inhibiting bone resorption. Synthetic PTH can only be taken for a maximum of two years due to the fact that negative side effects outweigh the positive effects that it has (Miller 2008). It has been shown in rats, that the prolonged exposure to synthetic PTH increased the risk of developing osteosarcoma (Miller 2008). There is currently no

treatment on the market that not only focuses on decreasing osteoclast activity but also focuses on increasing osteoblast activity (Tu et al. 2018; Jensen et al. 2010; Kaneko et al. 2000; Yang et al. 2013). There is a greater need to understand the underlying causes of the disease so better therapeutics can be developed. BMP2 is a growth factor that increases osteoblast activity and decreases osteoclast activity. Human recombinant BMP2 (rhBMP2) is approved by the FDA for the healing of long bone fractures. However, the long-term use of BMP2 has been show to increase osteoclastogenesis, making it a nonviable option for the treatment of osteoporosis (Jensen et al. 2010).

1.7 Hypothesis and Experimental Aims

CK2 is important in the activation of osteogenesis. Our lab has previously shown that treatment with CK2.3 increased the expression of ALP and OC serum levels in calvarial cells and bone marrow stromal cells (BMSC) (Akkiraju et al.2014). With this data, we predict that CK2.3, a mimetic peptide of BMPRIa, is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro*. To confirm these predictions, I accomplished the following specific aims:

Aim 1: Analyze osteoblast activity *in vivo*.
- 1a: Conduct immunofluorescent staining on paraffin embedded mice femurs.
- 1b: Determine OC and ALP staining intensity in Week 4.
- 1c: Observe OC and ALP staining intensity in Week 1 and 2.

Aim 2: Stimulate undifferentiated and differentiated C2C12 cells with PBS, CK2.3 and BMP2
- 2a: 1 week grown in differentiation media
- 2b: 2 weeks grown in differentiation media
- 2c: 4 weeks grown in differentiation media

Aim 3: Confirm biochemical marker expression in Western Blot

Chapter 2

METHODS AND MATERIALS

2.1 Mouse Injections

Retired breeders of female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). At 6 months of age, female mice (n=6/group) were injected in the tail vein once a day for five consecutive days with CK2.3 at, 0.76 μg/kg (low), 2.3 μg/kg (medium), and 6.9 μg/ kg (high) per mouse, or 50μl of PBS as a vehicle control. The choice of low, medium, and high concentrations was based on FDA guidelines. According to the guidelines, an accepted drug should have less than a twofold difference in minimum lethal dose and minimum effective dose values. Thus, the tested concentrations should be twofold less and twofold higher than the minimum effective dose value. However, in order to be ensure the safety of CK2.3 we used threefold less (low concentration) and threefold higher (high concentration) than the minimum effective dose (medium concentration). The choice of medium concentration was based on our previous publication where we found 2.3 μg/kg per mouse was the effective dose. At one week, two weeks, and four weeks after the initial injection, mice were sacrificed, and femurs were isolated.

2.2 Histology Sample Preparation

Left femurs were fixed in 10% neutral buffered formalin for 48h and decalcified in 14% EDTA for 3-4 weeks. Chemical end-points were tested with ammonium hydroxide/ammonium oxalate (1:1 v/v). Paraffin embedding and

sectioning were performed by the Histochemistry and Tissue Processing Core at Nemours/Alfred I. duPont Hospital for Children (Wilmington, DE). Right femurs were fixed using 10% neutral buffered formalin for 24-48 hours at 4°C and embedded in methylmethacrylate (MMA) as previously described. Once the femurs were polymerized into blocks, they were sectioned using a Buehler Diamond Watering Blade (10.2cm x 0.3mm) and sanded down.

2.3 Immunostaining

Bone Samples

The paraffin embedded of left femur samples were immersed in two changes of 100% xylene to deparaffinize. Then, samples were rehydrated with washes of 100% ethanol, 96% ethanol, 70% ethanol for 5 min each, and deionized water for 30 seconds. Antigen retrieval was performed by incubation with testicular hyaluronidase at 37°C for 30 minutes. After antigen retrieval the samples were incubated with 3%BSA for one hour at room temperature. Then they were fluorescently labeled overnight at 4°C with a mixture of primary antibodies goat polyclonal IgG osteocalcin (Santa Cruz Biotechnology, Dallas, TX) and rabbit polyclonal IgG alkaline phosphatase (Santa Cruz Biotechnology). This was followed by a mixture of secondary antibodies with AlexaFluor 488 donkey anti-goat IgG (Life Technologies, Waltham, MA) and Alexafluor 594 chicken anti-rabbit for one hour at room temperature. All antibodies were diluted in a 3% BSA solution. Bisbenzimide (Sigma-

Aldrich, St. Louis, MO) was used as a nuclear stain for ten-minute incubation. The coverslips were mounted using Airvol, as previously described. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ.

C2C12 Cells

C2C12 cells were grown in 12 well plates on cover slips until 90% confluency was reached. Cover slips were washed 3x in 1x PBS. Coverslips were then placed in a holder and incubated in ice cold methanol for 5 minutes. The holder was then removed and placed into a separate beaker containing ice cold acetone. Cover slips were incubated in ice cold acetone for 1 minute and 30 seconds. After fixation, the cover slips were placed into a new 12 well plate and washed with ice cold 1x PBS. After washing was complete, cover slips were incubated with 3% BSA for 1 hour at room temperature. The cover slips were then fluorescently labeled for 1 hour at room temperature with a mixture of primary antibodies goat polyclonal IgG osteocalcin (Santa Cruz Biotechnology, Dallas, TX) and rabbit polyclonal IgG alkaline phosphatase (Santa Cruz Biotechnology). This was followed by a mixture of secondary antibodies with AlexaFluor 488 donkey anti-goat IgG (Life Technologies, Waltham, MA) and Alexafluor 594 chicken anti-rabbit for one hour at room temperature. All antibodies were diluted in a 3% BSA solution. Bisbenzimide (Sigma-Aldrich, St. Louis, MO) was used as a nuclear stain for ten-minute incubation. The

coverslips were mounted using Cytoseal. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 400x total magnification and analyzed in ImageJ.

2.4 Cell Culture

Growth, Maintenance and Differentiation

C2C12 cells were used as they are an immortalized murine myoblast cell line. This cell line can be used to study preosteoblasts and osteoblasts. These cells were purchased from American Type Culture Collection (CCL-26) (Manassas, VA) and monolayer cultures were maintained in T-75 flasks grown in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gemini Bioproducts, West Sacramento, CA), 0.5% (v/v) L-Glutamine (Mediatech, Manassas, VA), and 1% (v/v) penicillin/streptomycin (100IU/ml penicillin, 100 μg/ml streptomycin (Fisher Scientific, Pittsburg, PA). Cultures were incubated at 37°C and 5% CO2, and cells were passaged at 90% confluency with 0.05% Trypsin-EDTA (Gemini Bioproducts, West Sacramento, CA). For differentiation, C2C12 cells were grown in differentiation media. Differentiation media consisted of alpha MEM (Caisson Labs, Smithfield, UT), 10% (v/v) Fetal Bovine Serum (FBS) (Gemini Bioproducts, West Sacramento, CA), 8% β-glycerol phosphatase (Sigma Aldrich, St. Louis, MO), 2% ascorbic acid (Fisher Chemical, Fair Lawn, NJ, USA) and 1% (v/v) penicillin/streptomycin (100IU/ml penicillin, 100

μg/ml streptomycin (Fisher Scientific, Pittsburg, PA). C2C12 cells were grown in this media for 1 week, 2 weeks or 4 weeks and then stimulated.

2.5 Stimulation and Cell Lysate Collection

C2C12 cells were grown in T-75 flasks to a 90% confluency in DMEM media containing 10% FBS and 1% penicillin/streptomycin for undifferentiated cells. For differentiation, C2C12 cells were grown in alpha MEM, 8% β-glycerol phosphatase, 2% ascorbic acid, 10% FBS and 1% penicillin/streptomycin. Once 90% confluency was reached, cells were serum starved with DMEM media supplemented only with 1% penicillin/streptomycin for 18 hours. Following starvation, cells were either left unstimulated or stimulated with 40nM BMP2, 100 nM of CK2.3 or PBS as a control for 5 days. After the 5-day stimulation, cells were washed with ice cold 1X PBS (Corning, Manassas, VA, USA) 3 times and then incubated RIPA lysis buffer (150 mM NaCL (Fisher Chemical, Fair Lawn, NJ, USA), 50mM Tris (Fisher Bioreagents, Fair Lawn, NJ, USA), 1.0% Triton X (Fisher Bioreagents, Fair Lawn, NJ, USA), 0.1% SDS (Fisher Bioreagents, Fair Lawn, NJ, USA) and 0.1% Sodium Oxalate (Fisher Chemical, Fair Lawn, NJ, USA) in the presence of protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After 1 hour on ice, cells were scraped from wells and transferred into prelabeled tubes. Cells were sonicated at 36% for 10 seconds twice. After sonicating the cells, cell debris was removed by centrifugation at 13,000× *g* for 20 min and the supernatant containing the cytoplasmic extract was transferred to a new, prelabeled tube. Protein content of the cell lysates were

determined using the BCA assay (Thermo Scientific, Rockford, IL, USA) and were normalized to equal protein content.

2.6 Western Blotting

Whole cell extracts were fractionated by 15% SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 3% BSA in PBST for 60 min, the membrane was washed once with PBS and incubated with antibodies against ALP (1:2000), OC (1:2000) or beta actin (1:5000) at 4 °C for 12 h. Membranes were washed three times for 10 min and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated anti-goat or anti-rabbit antibodies for 1 hour. Blots were washed with PBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols.

2.7 Image Analysis

The images collected were analyzed in ImageJ through the "Analyze Particles" function. Briefly, images were converted to 8-bit, then the threshold was adjusted in order to create a black and white image to eliminate excess background fluorescent staining from the bone. Three regions of interest that corresponded to bone or marrow cavity area were selected and measured for "mean" pixel intensity of each bone or marrow cavity region per image. The image area was selected based upon bone or marrow cavity availability. Pixel intensity was shown to be equivalent to fluorescent staining intensity; therefore, calculating the "mean" pixel intensity would directly

correspond to the intensity of the OC stain within either the bone or marrow cavity region. Once intensities were measured, mean pixel intensities of each respective region (trabecular bone or marrow cavity) were calculated and averaged per respective concentration (PBS, Low, Medium, and High). Secondary control "mean" averages were subtracted from the "mean" averages of the samples in order to obtain an overall value that could be compared across the different concentrations for OC and ALP staining intensity of the bone or marrow cavity.

2.8 Statistical Data Analysis

All data presented was analyzed using single factor analysis of variance (ANOVA), followed by Tukey-Kramer post-hoc test. All experiments were repeated three or more times and normalized to control. Error bars represent standard error of the mean (SEM).

Chapter 3

RESULTS

3.1 CK2.3 Has a Negative Effect on ALP Expression 1 Week Post Initial Injection

Mice were treated over a five-day period with either PBS, CK2.3L, CK2.3M or CK2.3H. One week after the initial injection, the mice were sacrificed and femurs were collected to analyze osteoblast activity by quantifying ALP expression. Results from the quantification of ALP expression intensities in bone marrow cavities and trabecular bone are negatively impacted in mice femurs 1 week post initial injection. CK2.3L, CK2.3M and CK2.3H all decreased ALP expression in comparison to PBS. To confirm the decrease, a one- way anova followed by a Tukey Kramer test was conducted to show significance. A p-value of less than 0.05 denotes that the difference is statistically significant. A: PBS; B: CK2.3L; C: CK2.3M; D: CK2.3H. If a treatment is statistically significant to another, it is labeled with the letter that corresponds to the treatment it is statistically significant to. All of the following experiments used the same statistical analysis.

CONCLUSIONS: This experiment does not support our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.*

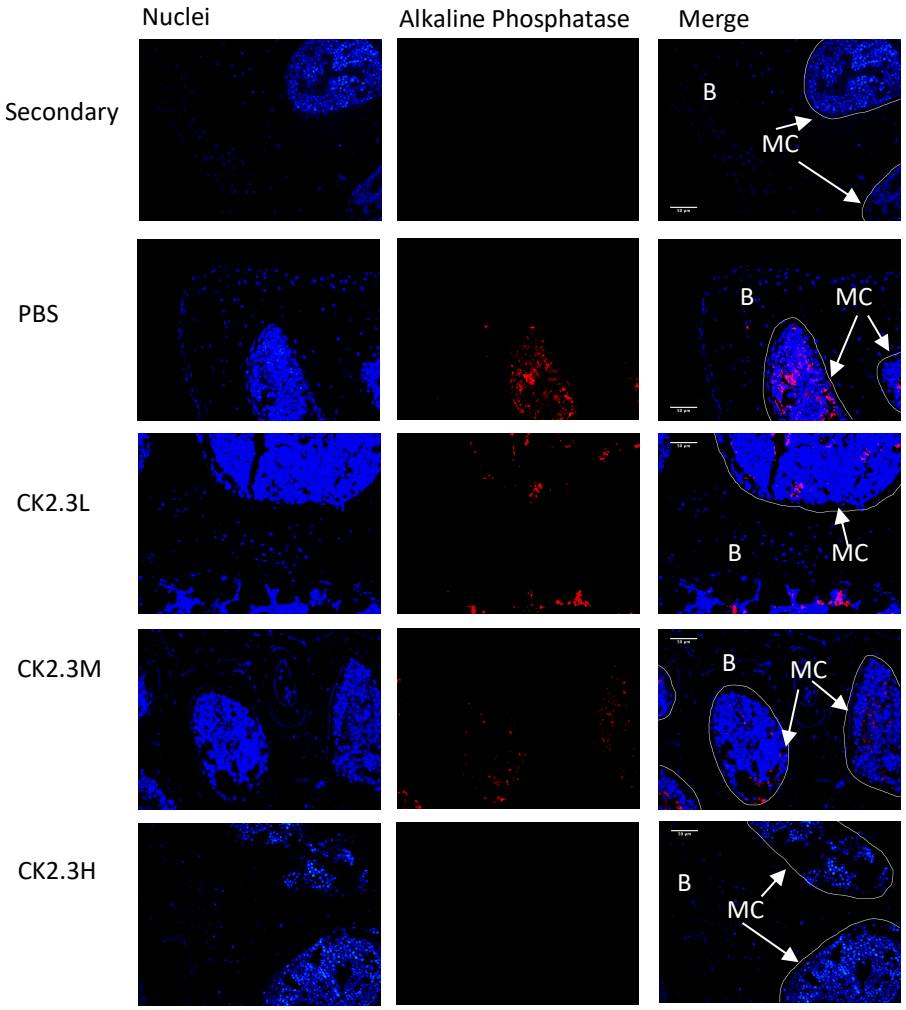
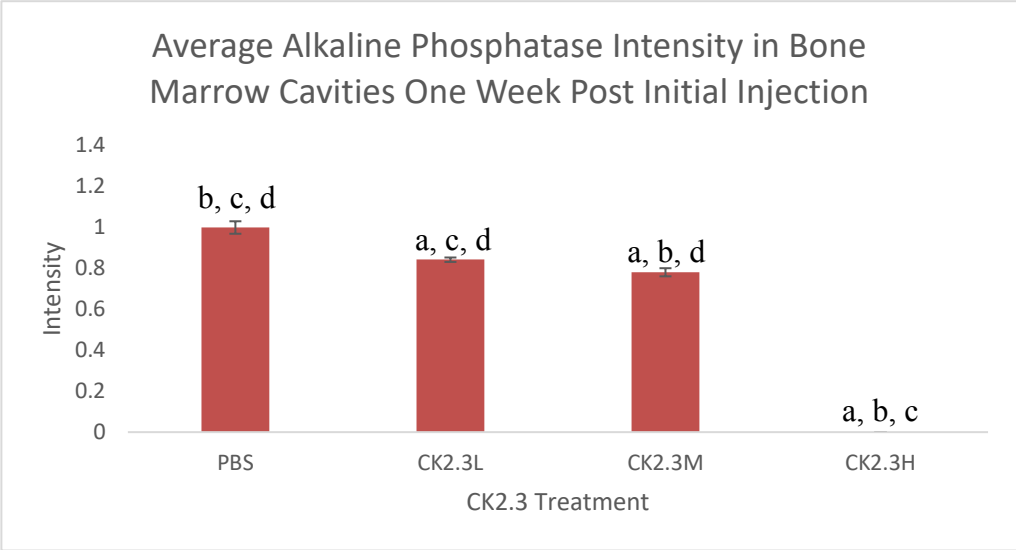


Figure 3.1a Visual representation of fluctuating ALP expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs one week post initial injection. CK2.3 did not elevate expression levels in the CK2.3L, CK2.3M or CK2.3H treatments. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ. MC: marrow cavity; B: Trabecular bone.



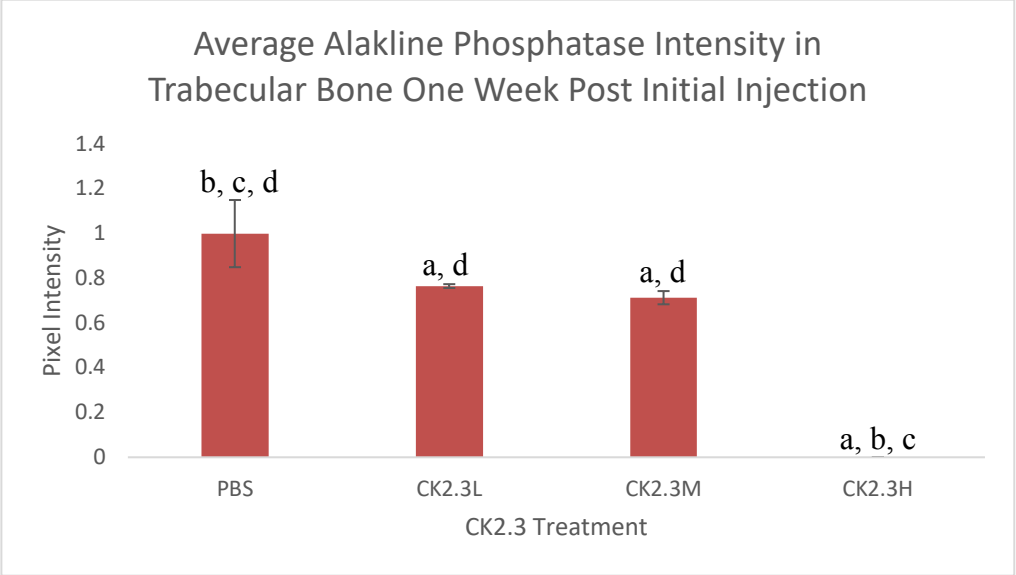


Figure 3.1b Graphical representation of ALP expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs one week post initial injection. Six mice were used per treatment group. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

3.2 CK2.3 Has a Negative Effect on OC Expression 1 Week Post Initial Injection

Mice were treated over a five-day period with either PBS, CK2.3L, CK2.3M or CK2.3H. One week after the initial injection, the mice were sacrificed and femurs were collected to analyze osteoblast activity by quantifying OC expression. Results from the quantification of OC expression intensities in bone marrow cavities and trabecular bone are negatively impacted in mice femurs 1 week post initial injection. CK2.3L, CK2.3M and CK2.3H all decreased OC expression in comparison to PBS, with CK2.3M having the largest impact.

CONCLUSIONS: This experiment does not support our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.*

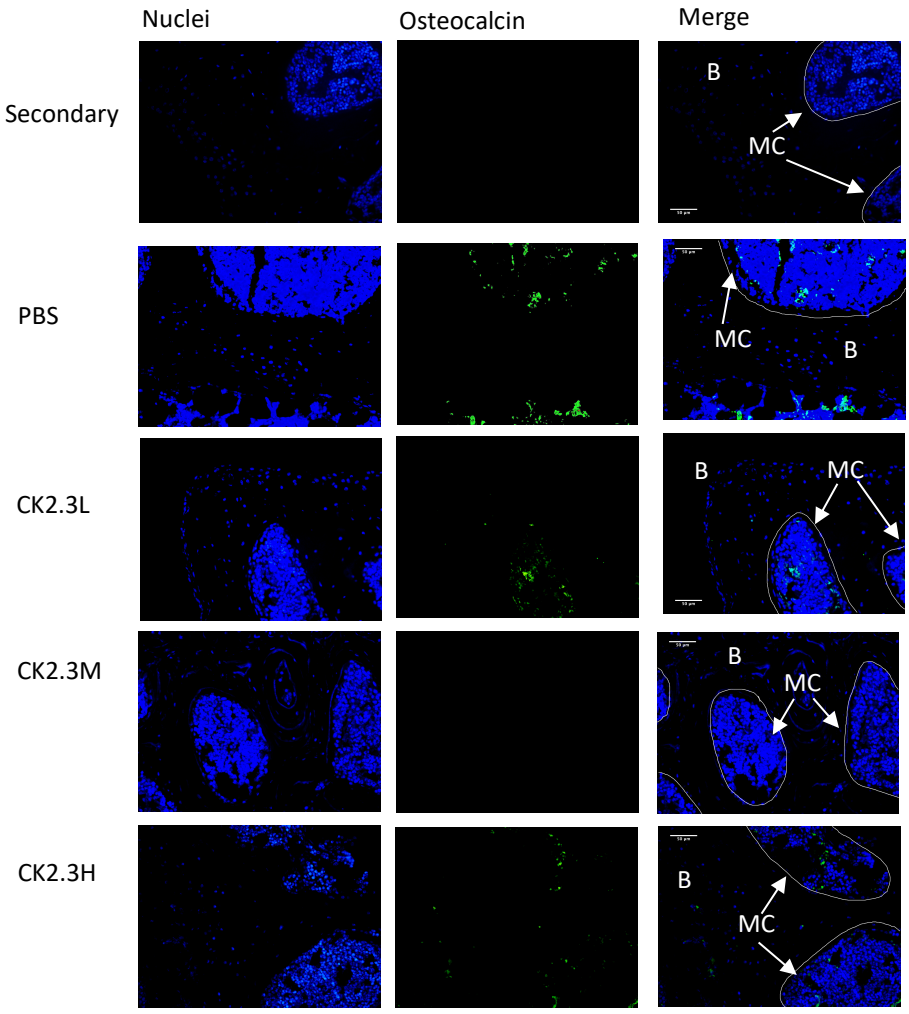
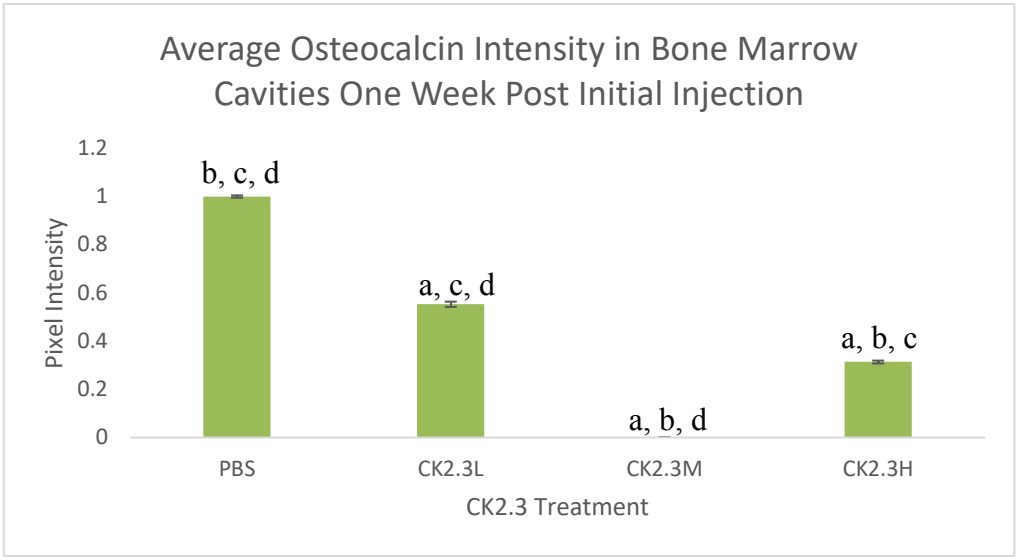


Figure 3.2a Visual representation of fluctuating OC expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs one week post initial injection. CK2.3 did not elevate expression levels in the CK2.3L, CK2.3M or CK2.3H treatments. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ. MC: marrow cavity; B: Trabecular bone



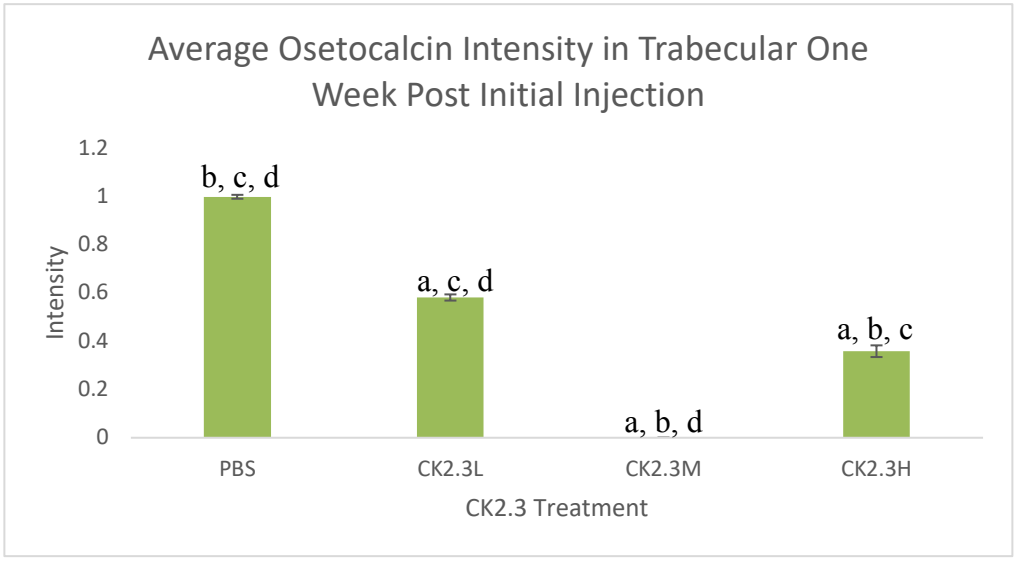


Figure 3.2b Graphical representation of OC expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs one week post initial injection. Six mice were used per treatment group. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test. All treatment groups were significant to one another.

3.3 CK2.3H Has a Positive Effect on ALP Expression 2 Weeks Post Initial Injection

Mice were treated over a five-day period with either PBS, CK2.3L, CK2.3M or CK2.3H. Two weeks after the initial injection, the mice were sacrificed and femurs were collected to analyze osteoblast activity by quantifying ALP expression. Results from the quantification of ALP expression intensities in bone marrow cavities and trabecular bone are negatively impacted in mice femurs 2 weeks post initial injection, with the exception of CK2.3H. CK2.3L and CK2.3M decreased ALP expression in comparison to PBS while CK2.3H increased ALP expression in comparison to PBS.

CONCLUSIONS: This experiment partially supports our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.* CK2.3L and CK2.3M had a negative effect while CK2.3H had a positive effect.

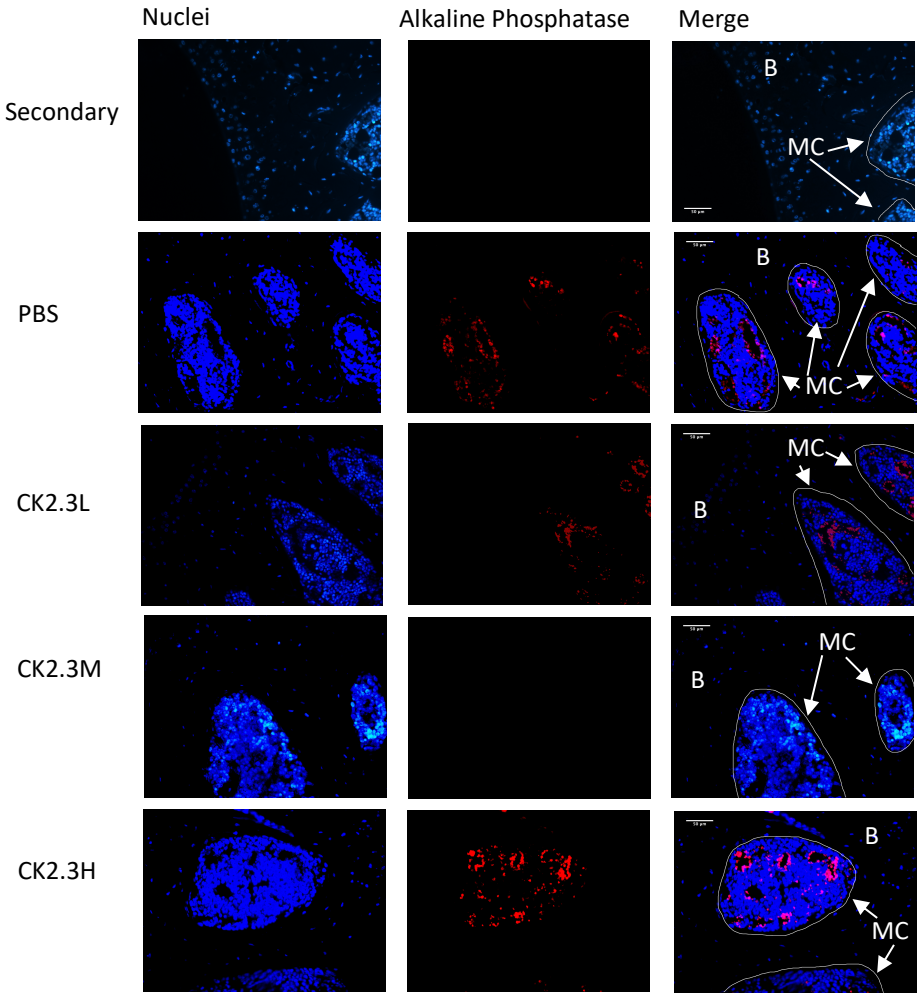
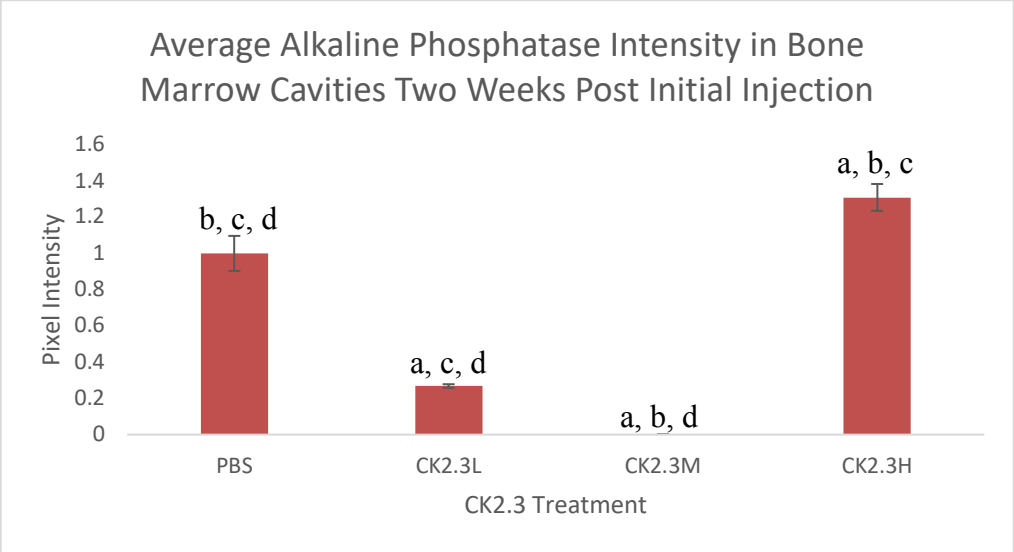


Figure 3.3a Visual representation of fluctuating ALP expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs two weeks post initial injection. CK2.3 did not elevate expression levels in the CK2.3L or CK2.3M treatments but did seem to have a positive effect in the CK2.3H treatment. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ. MC: marrow cavity; B: Trabecular bone.



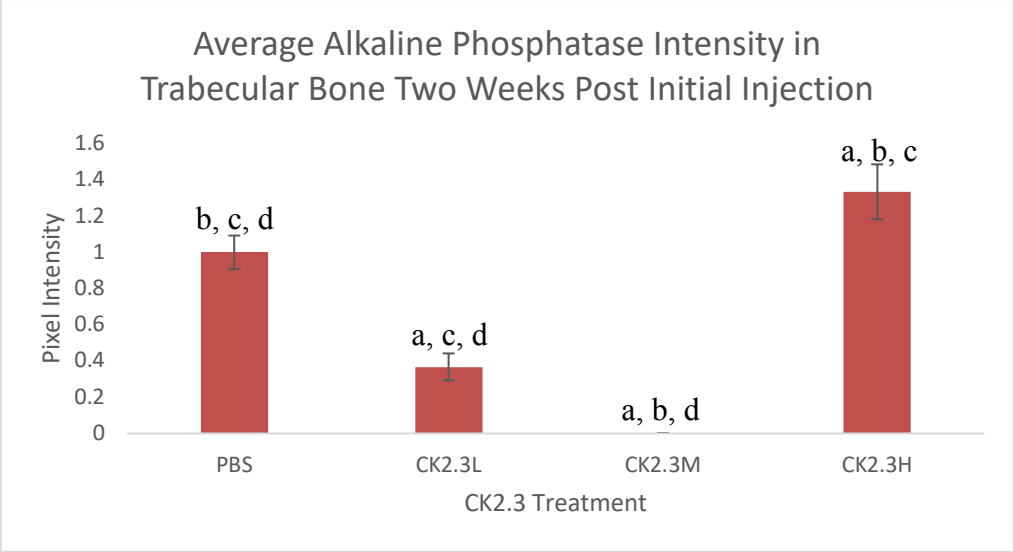


Figure 3.3b Graphical representation of ALP expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs two weeks post initial injection. Ck2.3H was the only treatment that had a positive effect on ALP expression. Six mice were used per treatment group. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test. All treatment groups were significant to one another.

3.4 CK2.3H Has a Positive Effect on OC Expression in Bone Marrow Cavities while CK2.3L and CK2.3H have Positive Effects on OC Expression in Trabecular Bone 2 Weeks Post Initial Injection

Mice were treated over a five-day period with either PBS, CK2.3L, CK2.3M or CK2.3H. Two weeks after the initial injection, the mice were sacrificed and femurs were collected to analyze osteoblast activity by quantifying OC expression. Results from the quantification of OC expression intensities in bone marrow cavities is negatively impacted in mice femurs 2 weeks post initial injection, with the exception of CK2.3H. In trabecular bone, CK2.3L and CK2.3H increases OC expression in comparison to PBS while CK2.3M decreased OC expression in comparison to PBS.

CONCLUSIONS: This experiment partially supports our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.*

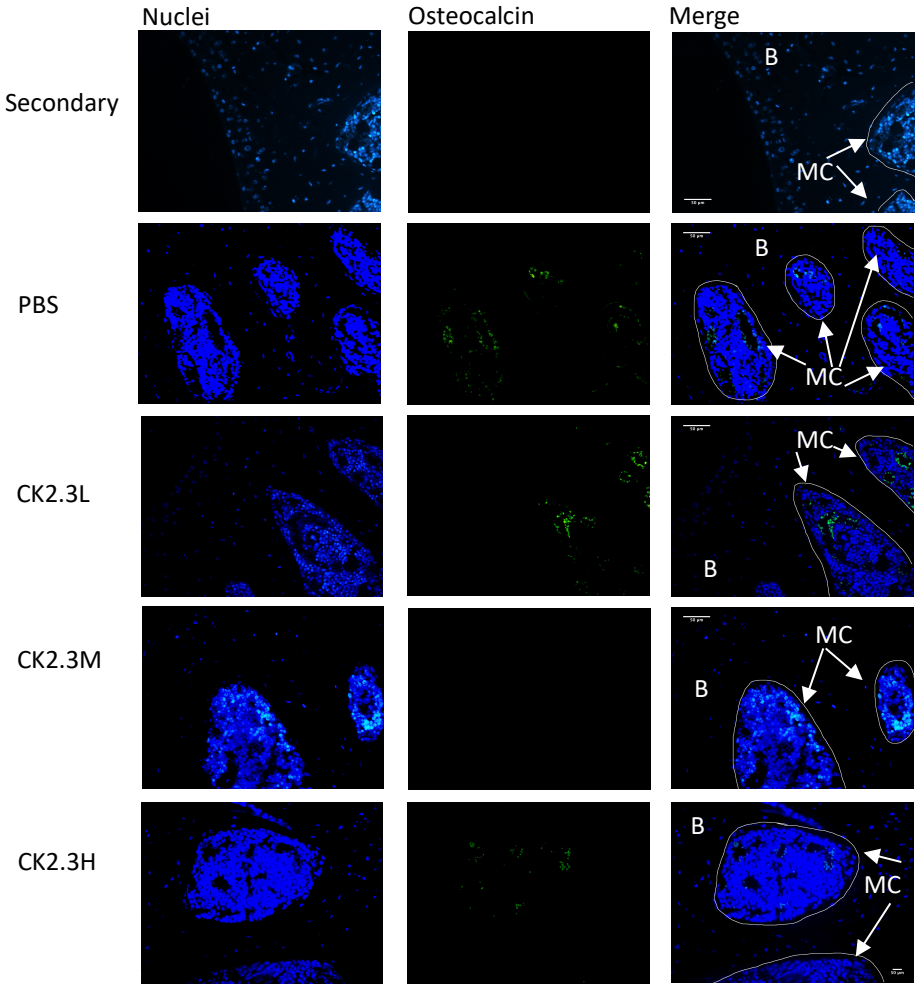


Figure 3.4a Visual representation of fluctuating OC expression seen in osteoblasts situated in bone marrow cavities of murine femurs two weeks post initial injection. All treatment levels had no effect on the osteoblasts in the bone marrow cavities. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ. MC: marrow cavity; B: Trabecular bone.

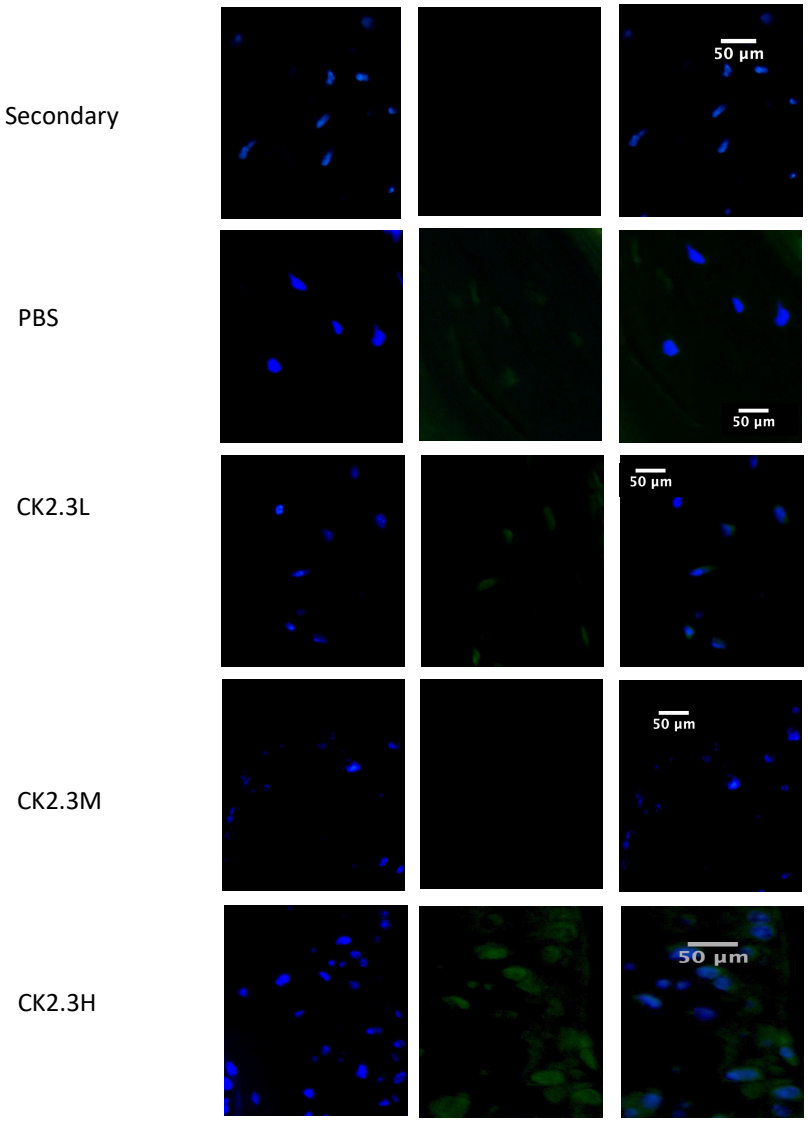
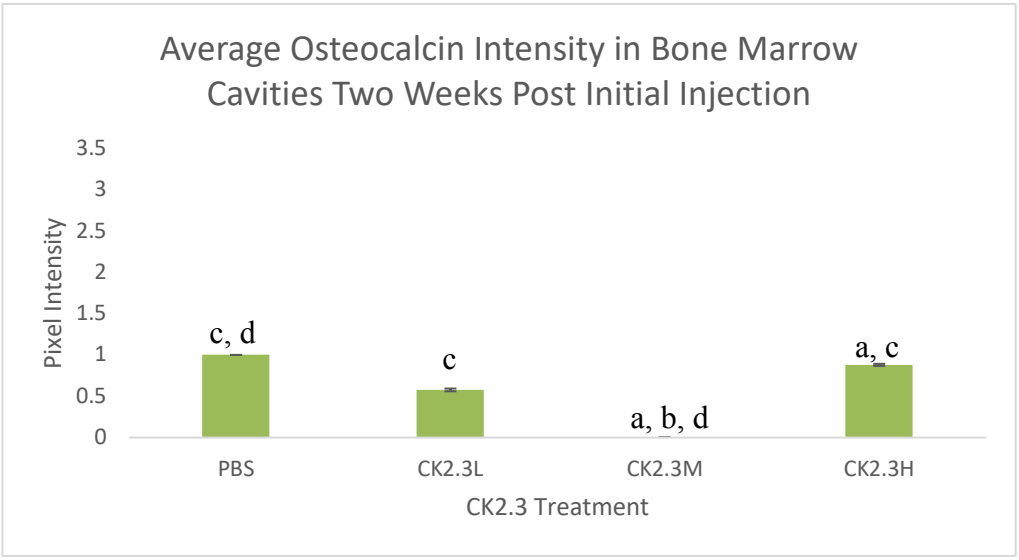


Figure 3.4b Visual representation of fluctuating OC expression seen in osteoblasts situated in trabecular bone of murine femurs two weeks post initial injection. CK2.3L and CK2.3H both had a positive effect on OC expression while CK2.3M had a negative effect on osteoblasts in the trabecular bone. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ. MC: marrow cavity; B: Trabecular bone.



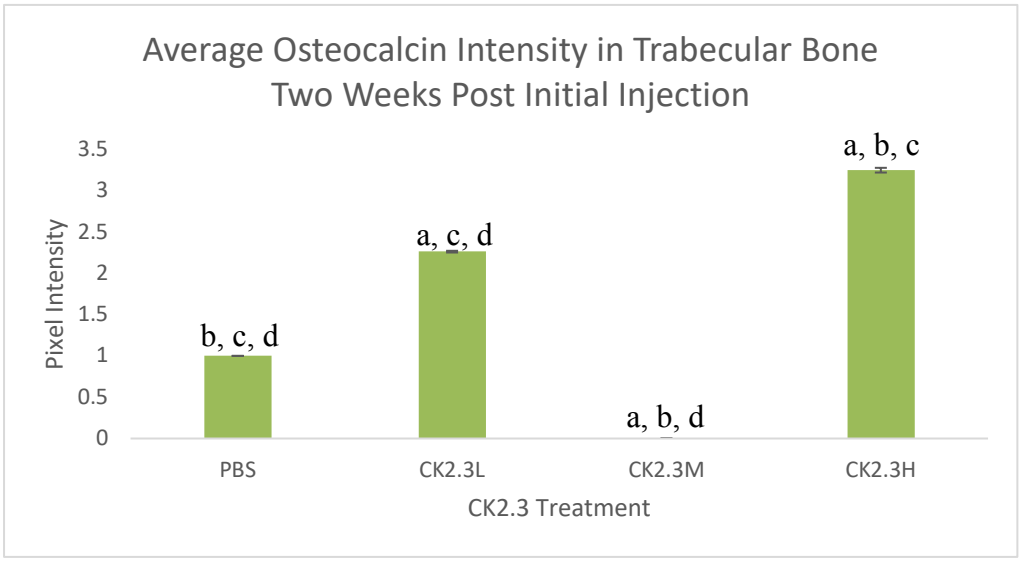


Figure 3.4c Graphical representation of OC expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs two weeks post initial injection. Six mice were used per treatment group. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

3.5 CK2.3 Increases OC Expression in a Dose Dependent Manner Until CK2.3H 4 Weeks Post Initial Injection

Mice were treated over a five-day period with either PBS, CK2.3L, CK2.3M or CK2.3H. Four weeks after the initial injection, the mice were sacrificed and femurs were collected to analyze osteoblast activity by quantifying OC expression. Results from the quantification of OC expression intensities in bone marrow and trabecular bone shows that CK2.3 increases OC expression intensity in a dose dependent manner in mice femurs 4 weeks post initial injection. All doses of CK2.3 increase OC expression intensity.

CONCLUSIONS: This experiment fully supports our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.*

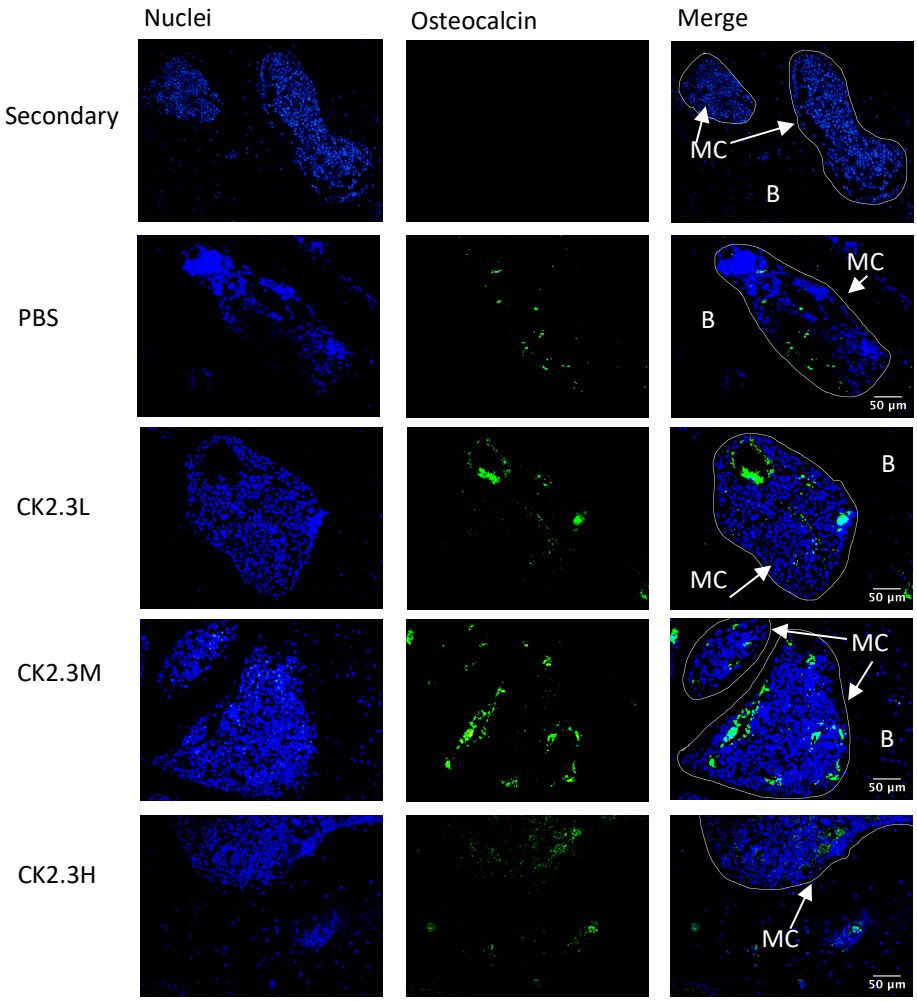


Figure 3.5a Visual Representation of elevated OC expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs. CK2.3 elevated OC expression in the CK2.3L and CK2.3M treatments 4 weeks after the first CK2.3 injection. CK2.3 was the most effective treatment level. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ. MC: marrow cavity; B: Trabecular bone. Adapted from Nguyen et al. 2018.

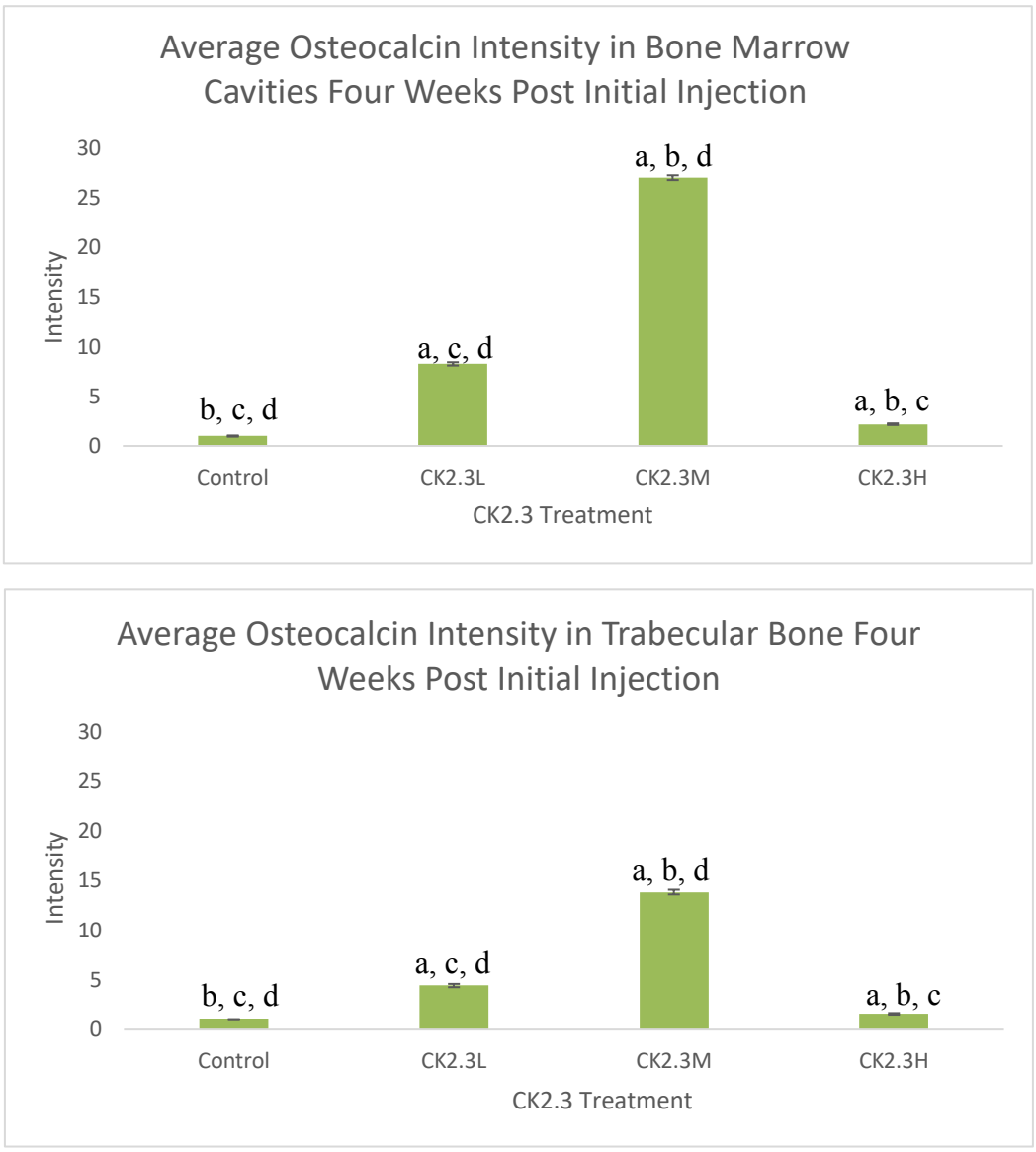


Figure 3.5b Graphical representation of elevated OC expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs. CK2.3 elevated OC expression in the CK2.3L and CK2.3L treatments 4 weeks after the first CK2.3 injection. CK2.3M was the most effective treatment level. Six mice were used per treatment group. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test. Top graph is adapted from Nguyen et al. 2018.

3.6 CK2.3 Increases ALP Expression in a Dose Dependent Manner Until CK2.3H 4 Weeks Post Initial Injection

Mice were treated over a five-day period with either PBS, CK2.3L, CK2.3M or CK2.3H. Four weeks after the initial injection, the mice were sacrificed and femurs were collected to analyze osteoblast activity by quantifying ALP expression. Results from the quantification of ALP expression intensities in bone marrow trabecular bone shows that CK2.3 increases ALP expression intensity in a dose dependent manner in mice femurs 4 weeks post initial injection. All doses of CK2.3 increase ALP expression intensity in comparison to PBS, with the exception of CK2.3H in trabecular bone.

CONCLUSIONS: This experiment supports our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.*

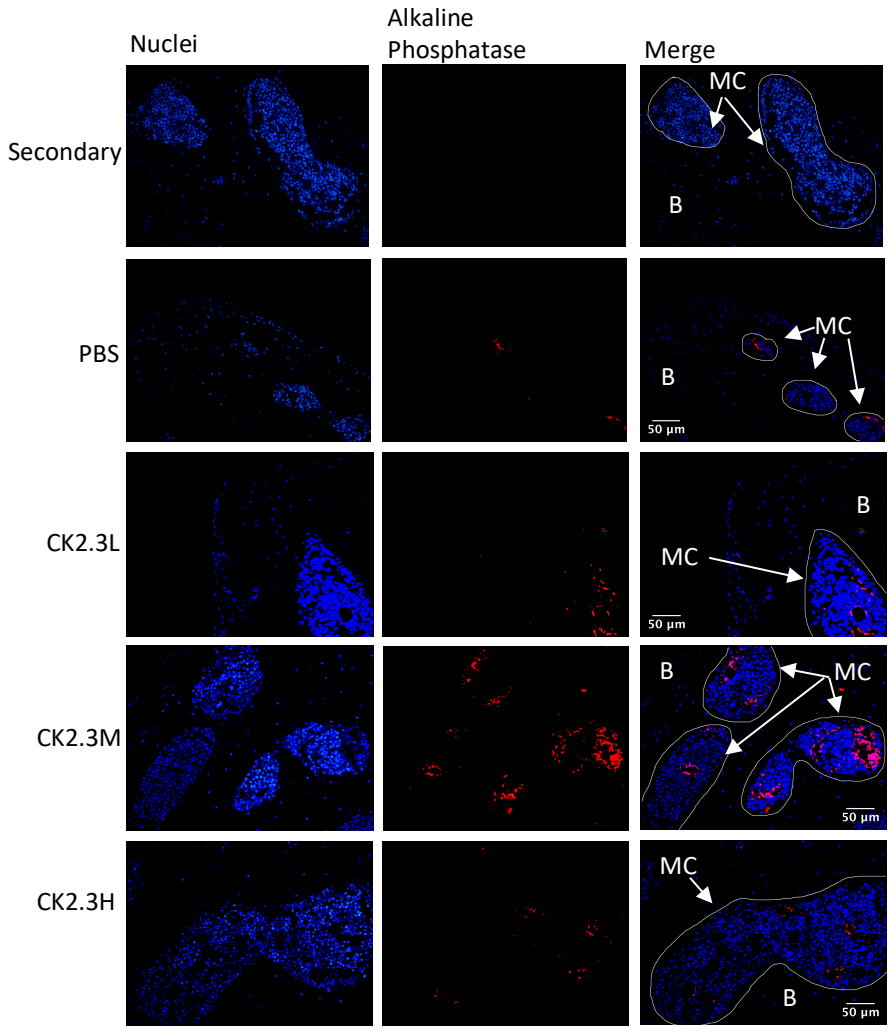


Figure 3.6a Visual Representation of elevated ALP expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs. CK2.3 elevated ALP expression in the CK2.3L and CK2.3M treatments 4 weeks after the first CK2.3 injection. CK2.3M was the most effective treatment level. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 200x total magnification and analyzed in ImageJ. MC: marrow cavity; B: Trabecular bone. Adapted from Nguyen et al. 2018.

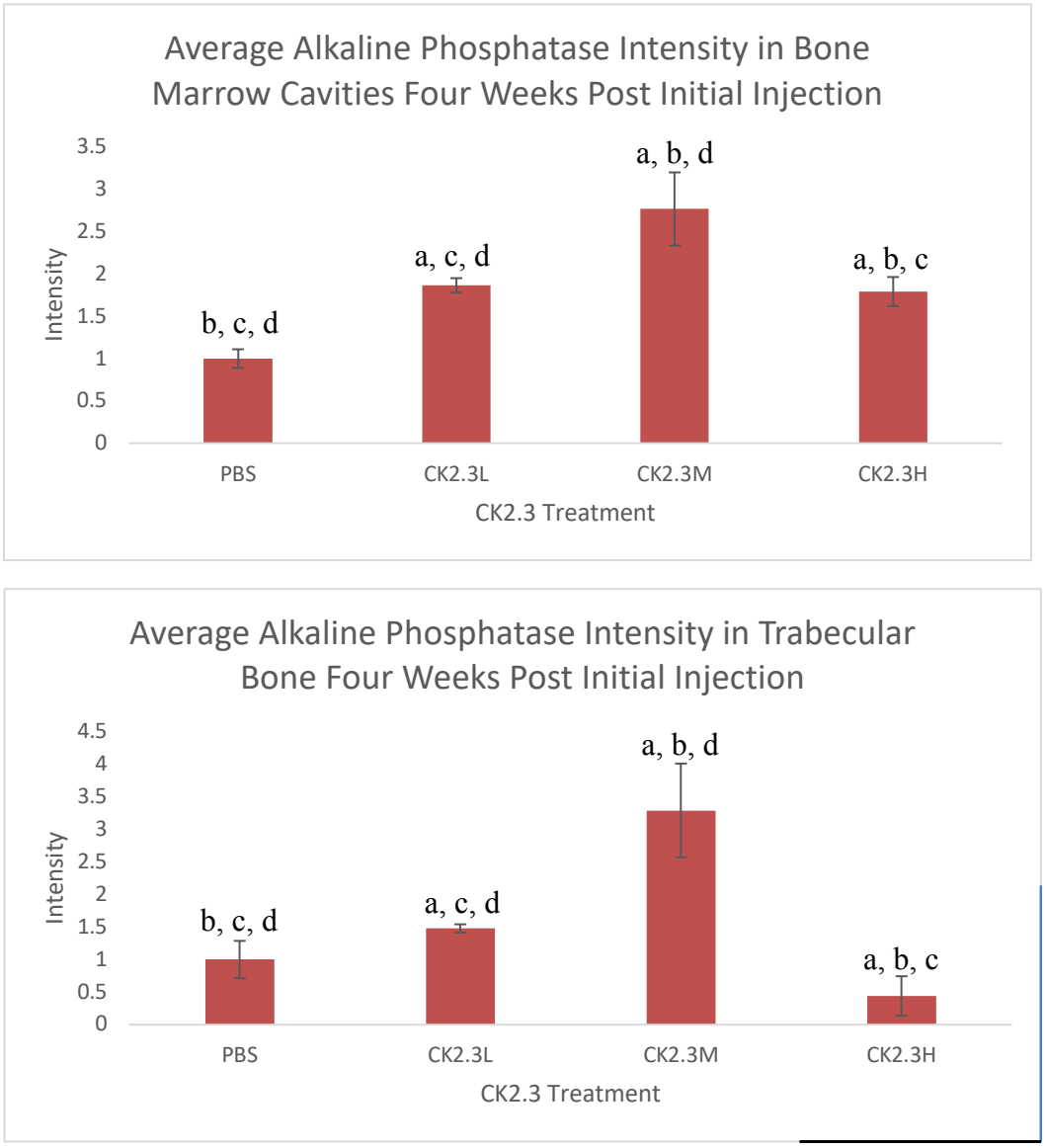


Figure 3.6b Graphical representation of elevated ALP expression seen in osteoblasts situated in bone marrow cavities and trabecular bone of murine femurs. CK2.3 elevated ALP expression in the CK2.3L and CK2.3M treatments 4 weeks after the first CK2.3 injection. CK2.3M was the most effective treatment level. Six mice were used per treatment group. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test. Top graph is adapted from Nguyen et al. 2018.

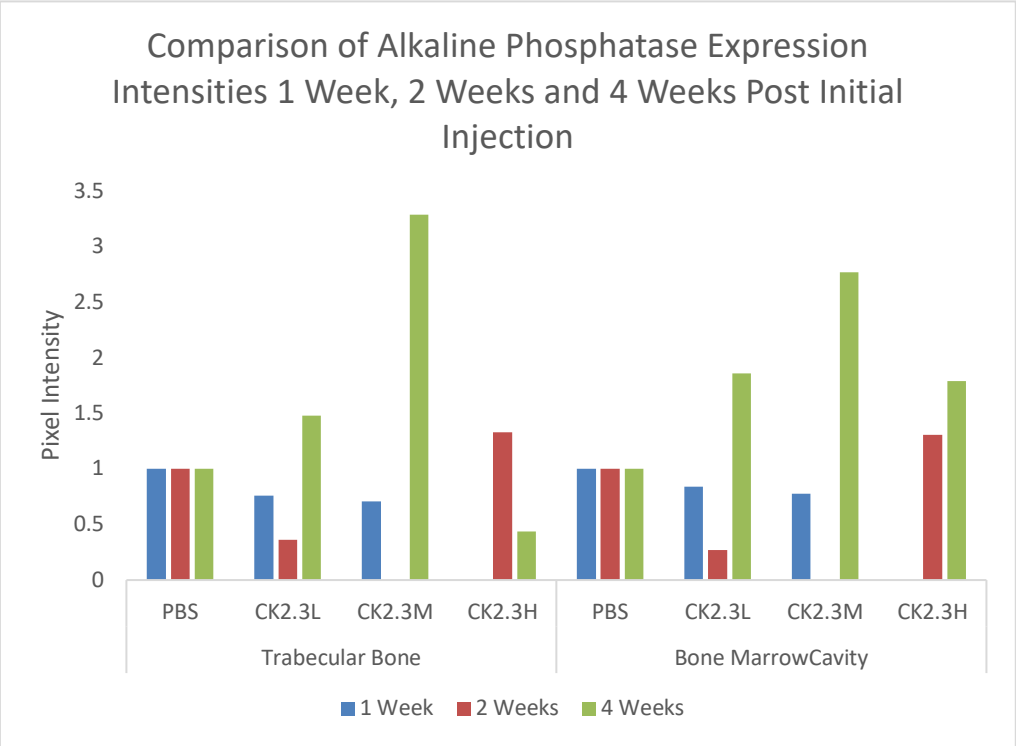


Figure 3.7a Average ALP intensity variation among 1 week, 2 weeks and 4 weeks post initial injection. The left part of the graph compares the intensities that were measured in the trabecular bone while the right side of the graph compares intensities within the bone marrow cavities.

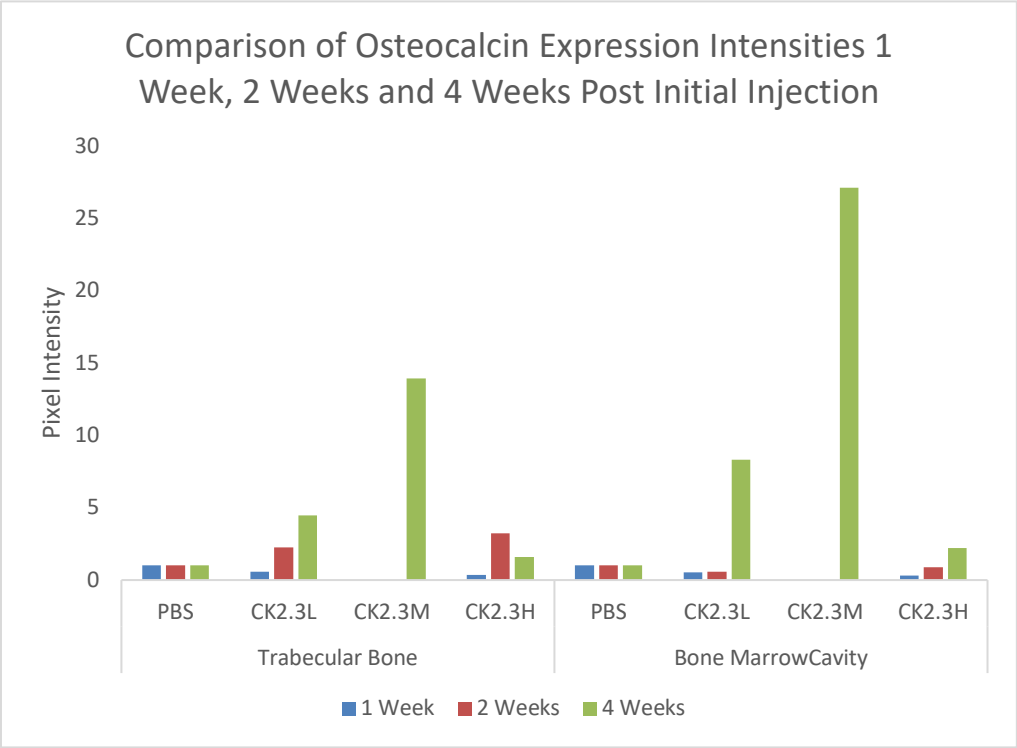


Figure 3.7b Average OC intensity variation among 1 week, 2 weeks and 4 weeks post initial injection. The left part of the graph compares the intensities that were measured in the trabecular bone while the right side of the graph compares intensities within the bone marrow cavities.

3.8 CK2.3 Increases ALP Expression in Undifferentiated, 1 Week Differentiated and 4 Week Differentiated C2C12 Cells

After analyzing the femurs of mice that were treated with varying doses of CK2.3, the direction of the experiment changed direction and the focus shifted to differentiating C2C12 cells and how CK2.3 effects the expression of ALP on a cellular basis. Results from the quantification of ALP expression intensities in undifferentiated, 1 week, 2 week and 4 weeks differentiated C2C12 cells shows that CK2.3 increased ALP expression in comparison to PBS in all weeks with the exception of the 2 week differentiated cells. The most exciting results collected was that of the undifferentiated C2C12 cells. C2C12 cells should express low levels of ALP but when treated with CK2.3, the cells expressed levels of ALP that were similar to that of BMP2. This indicates that CK2.3 may be inducing differentiation. To confirm these differences, a one- way anova followed by a Tukey Kramer test was conducted to show significance. A p-value of less than 0.05 denotes that the difference is statistically significant. A: PBS; B: CK2.3; C: BMP2. If a treatment is statistically significant to another, it is labeled with the letter that corresponds to the treatment it is statistically significant to.

CONCLUSIONS: This experiment partially supports our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.*

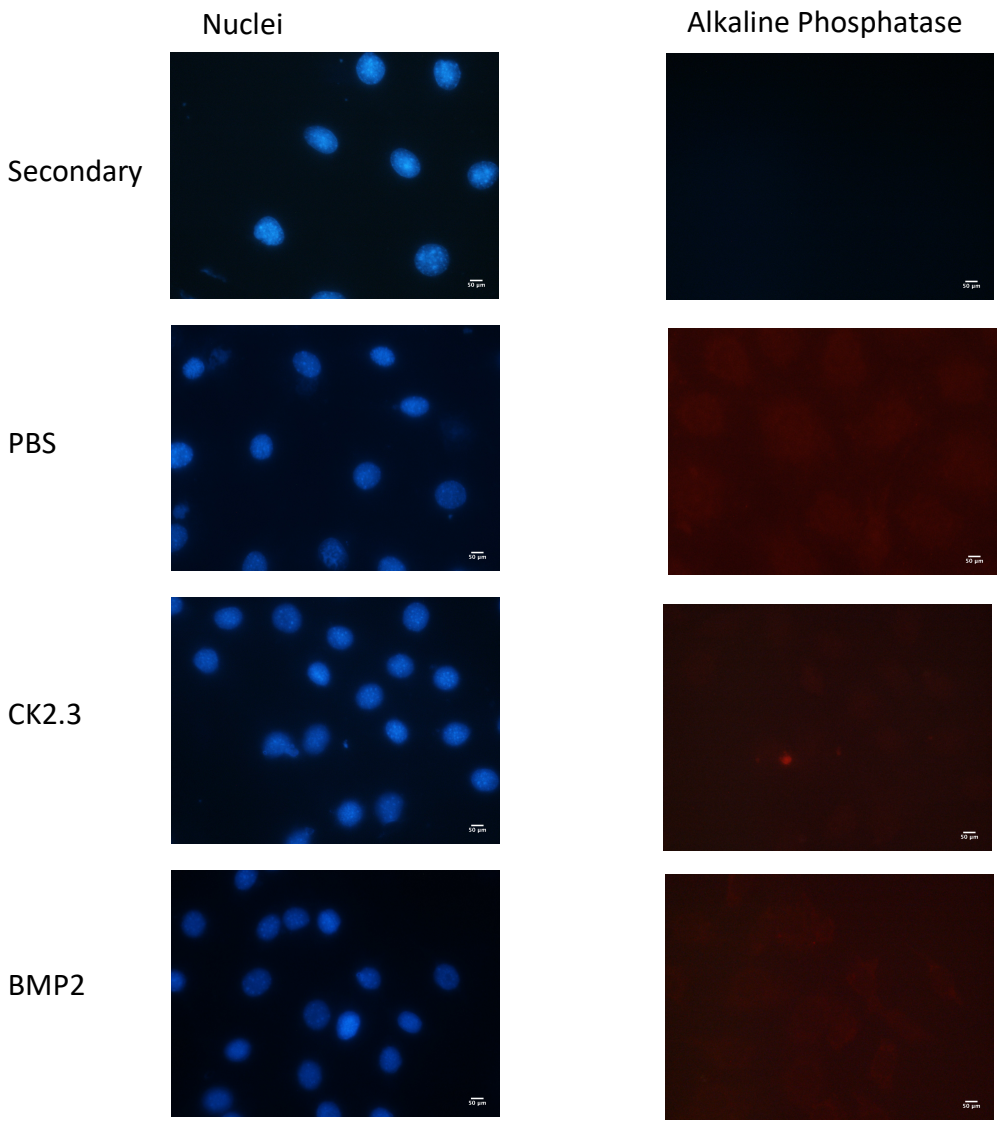


Figure 3.8a Visual Representation of increasing ALP expression in undifferentiated, 1 week differentiated, 2 weeks differentiated and 4 weeks differentiated C2C12 cells. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 400x total magnification and analyzed in ImageJ.

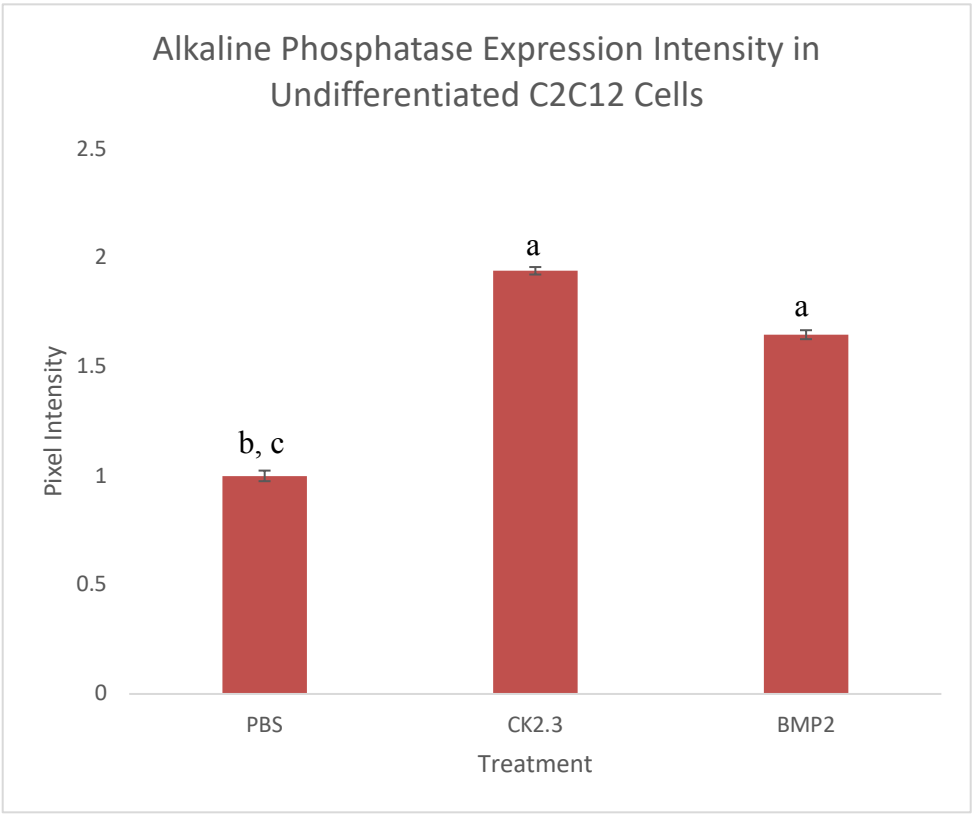


Figure 3.8b Average ALP intensity in undifferentiated C2C12 cells. Both CK2.3 and BMP2 had a positive effect on the intensity levels. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

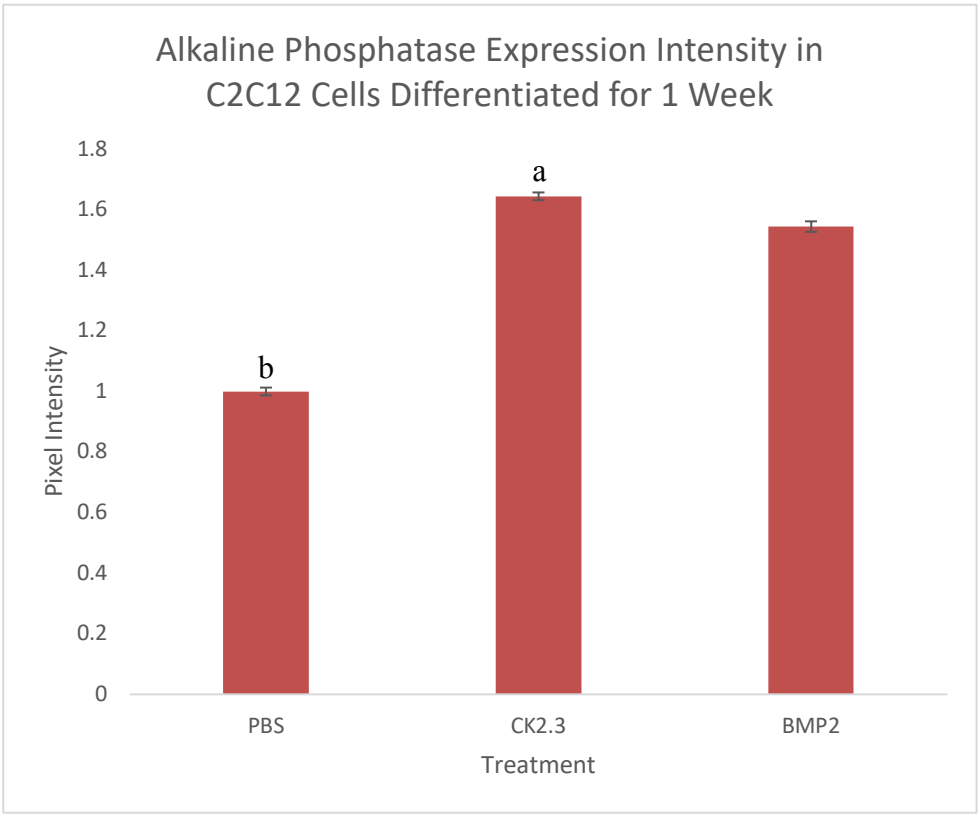


Figure 3.8c Average ALP intensity in C2C12 cells that were differentiated for 1 week. Both CK2.3 and BMP2 had a positive effect on the intensity levels. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

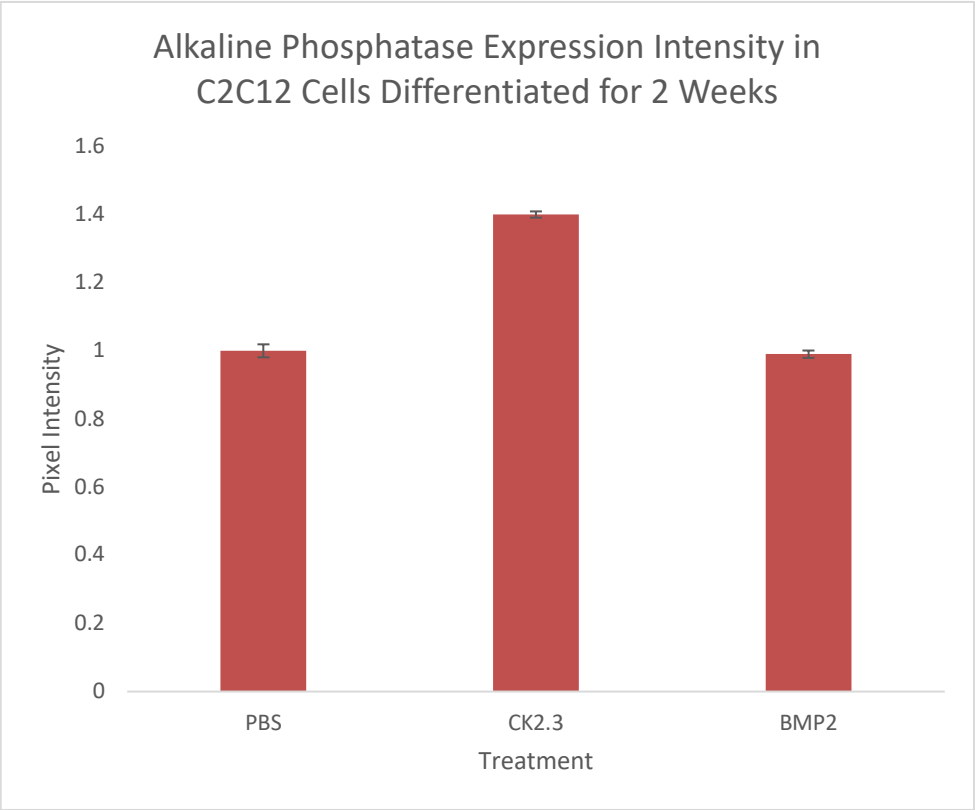


Figure 3.8d Average ALP intensity in C2C12 cells that were differentiated for 2 weeks. CK2.3 was the only treatment in this week to have a positive effect on expression intensity. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

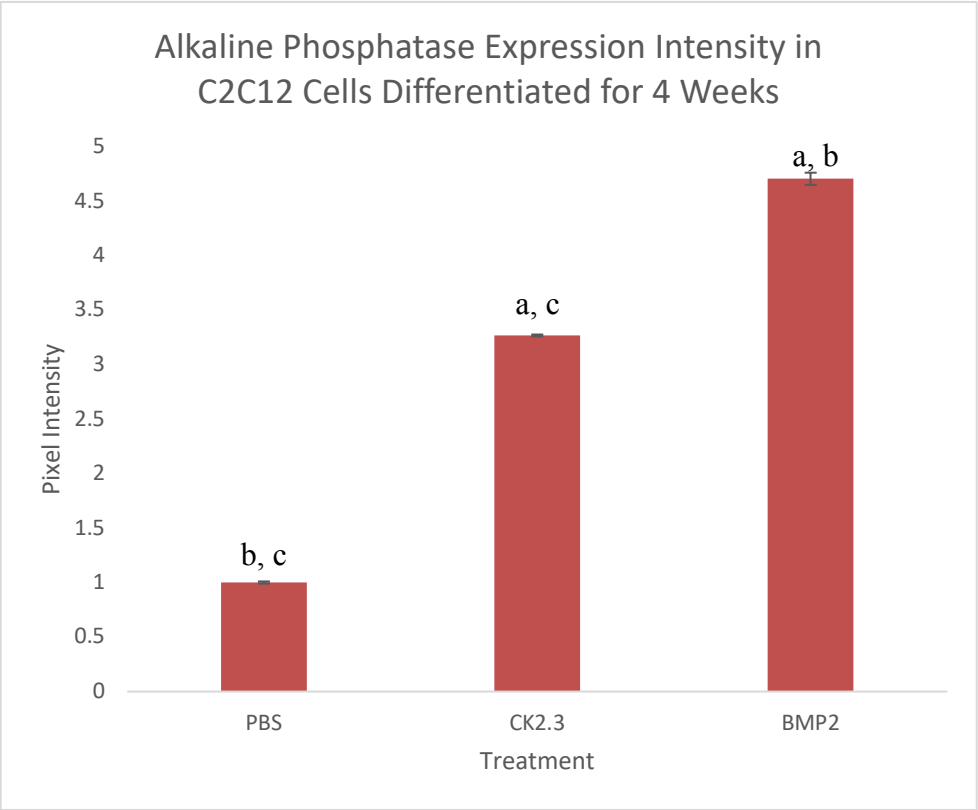


Figure 3.8e Average ALP intensity in C2C12 cells that were differentiated for 4 weeks. Both CK2.3 and BMP2 had a positive effect on expression intensity. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

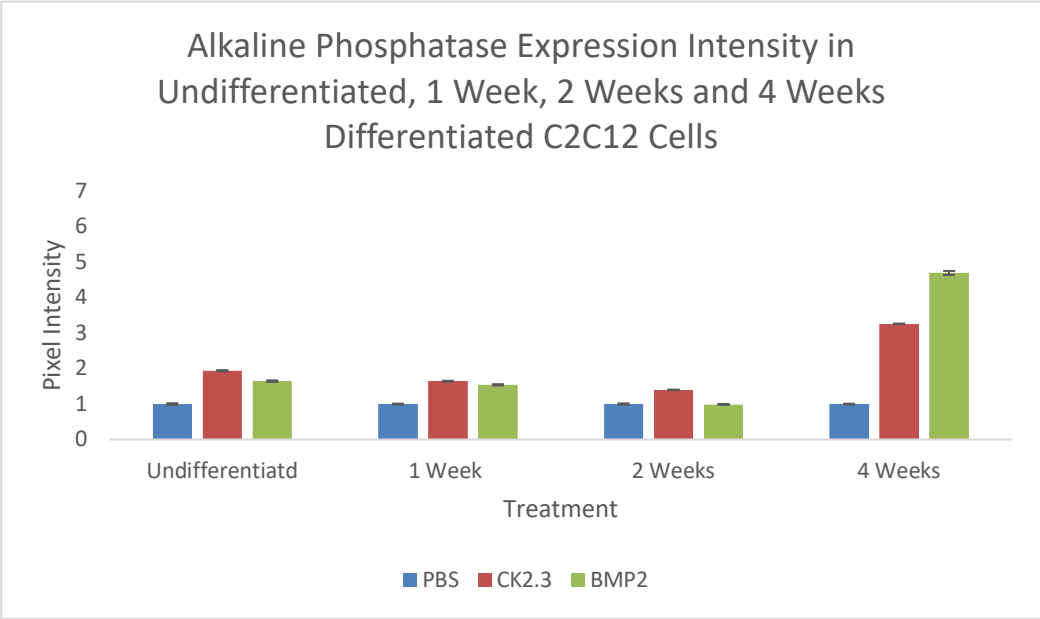


Figure 3.8f Average OC intensity variation among undifferentiated, 1 week differentiated, 2 weeks differentiated and 4 weeks differentiated C2C12 cells.

3.9 CK2.3 Increases OC Expression in Undifferentiated, 1 Week Differentiated and 4 Week Differentiated C2C12 Cells

After analyzing the femurs of mice that were treated with varying doses of CK2.3, the direction of the experiment changed direction and the focus shifted to differentiating C2C12 cells and how CK2.3 effects the expression of OC on a cellular basis. Results from the quantification of OC expression intensities in undifferentiated, 1 week, 2 week and 4 weeks differentiated C2C12 cells shows that CK2.3 increased ALP expression in comparison to PBS in all weeks with the exception of the 2 week differentiated cells. The most exciting result collected was that of the undifferentiated C2C12 cells. C2C12 cells should express low levels of OC but when treated with CK2.3, the cells expressed levels of OC that were similar to that of BMP2. indicates that CK2.3 may be inducing differentiation.

CONCLUSIONS: This experiment partially supports our initial hypothesis that CK2.3 is a potential activator of osteoblast differentiation and activity *in vivo* and *in vitro.*

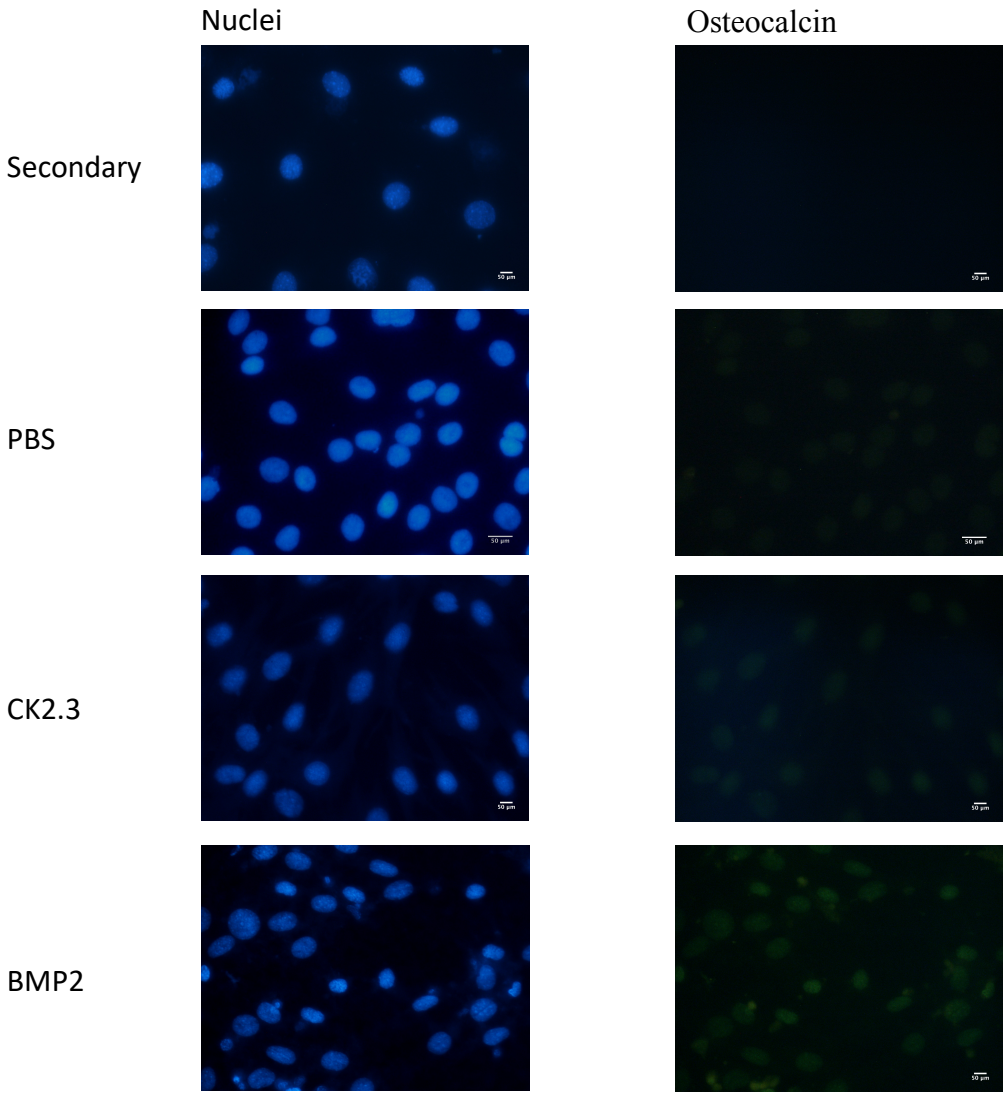


Figure 3.9a Visual Representation of increasing OC expression in undifferentiated, 1 week differentiated, 2 weeks differentiated and 4 weeks differentiated C2C12 cells. Images were taken on Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 400x total magnification and analyzed in ImageJ.

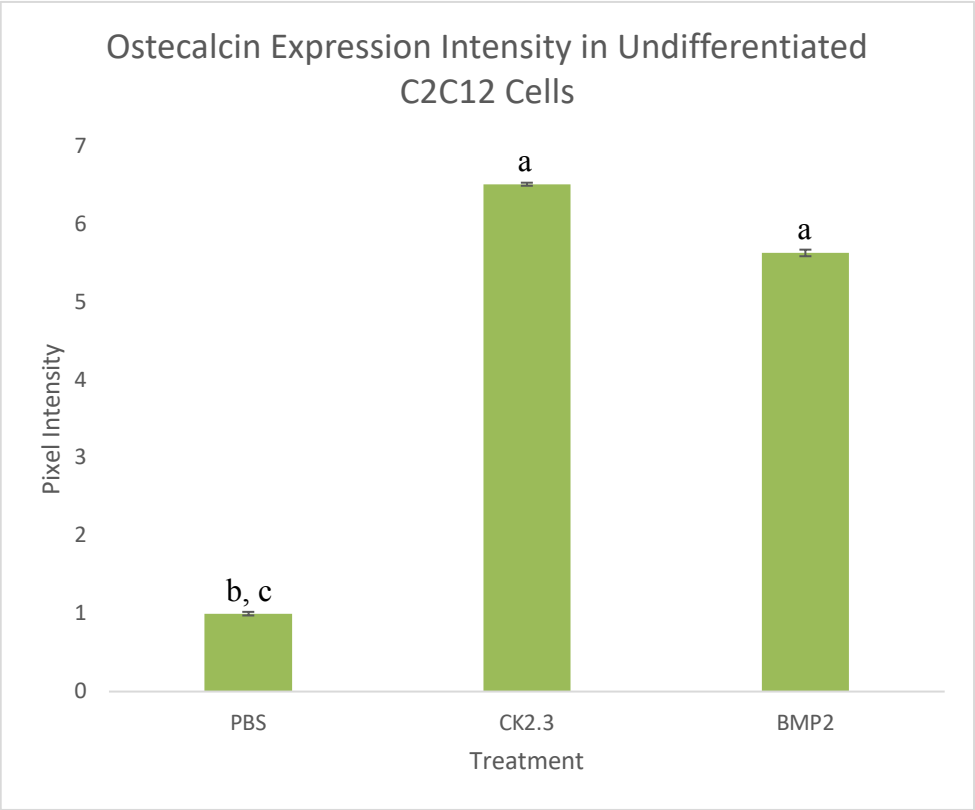


Figure 3.9b Average OC intensity in undifferentiated C2C12 cells. Both CK2.3 and BMP2 had a positive effect on the intensity levels. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

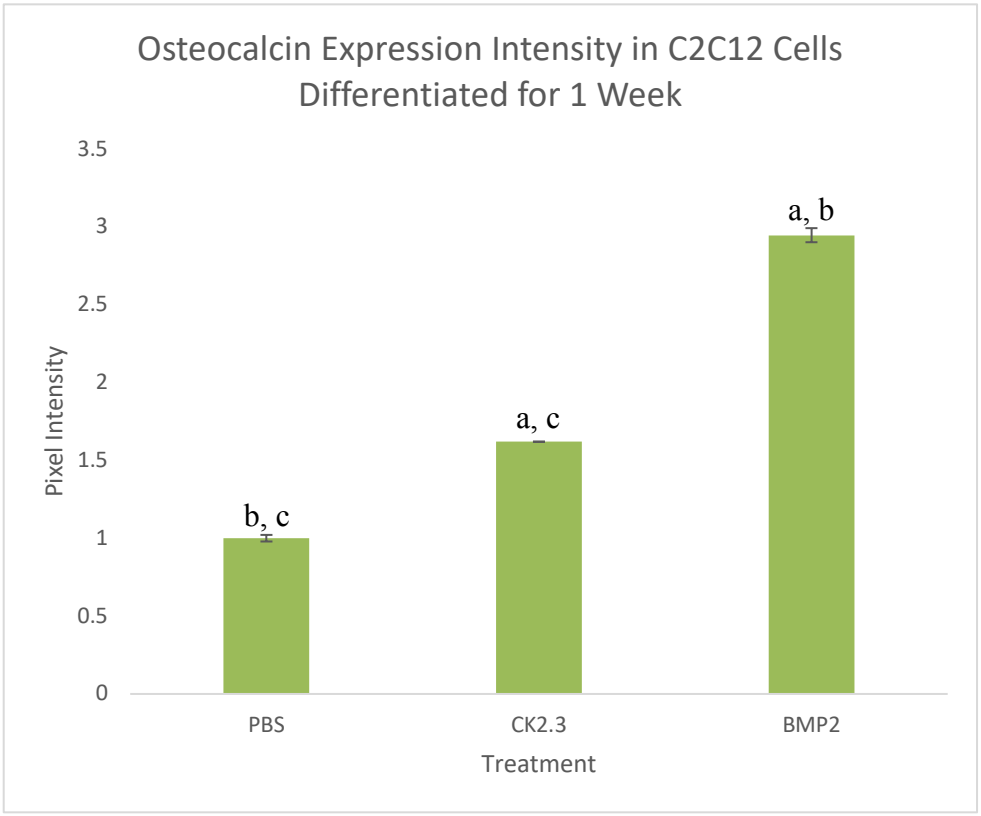


Figure 3.9c Average OC intensity in C2C12 cells that were differentiated for 1 week. Both CK2.3 and BMP2 had a positive effect on the intensity levels. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

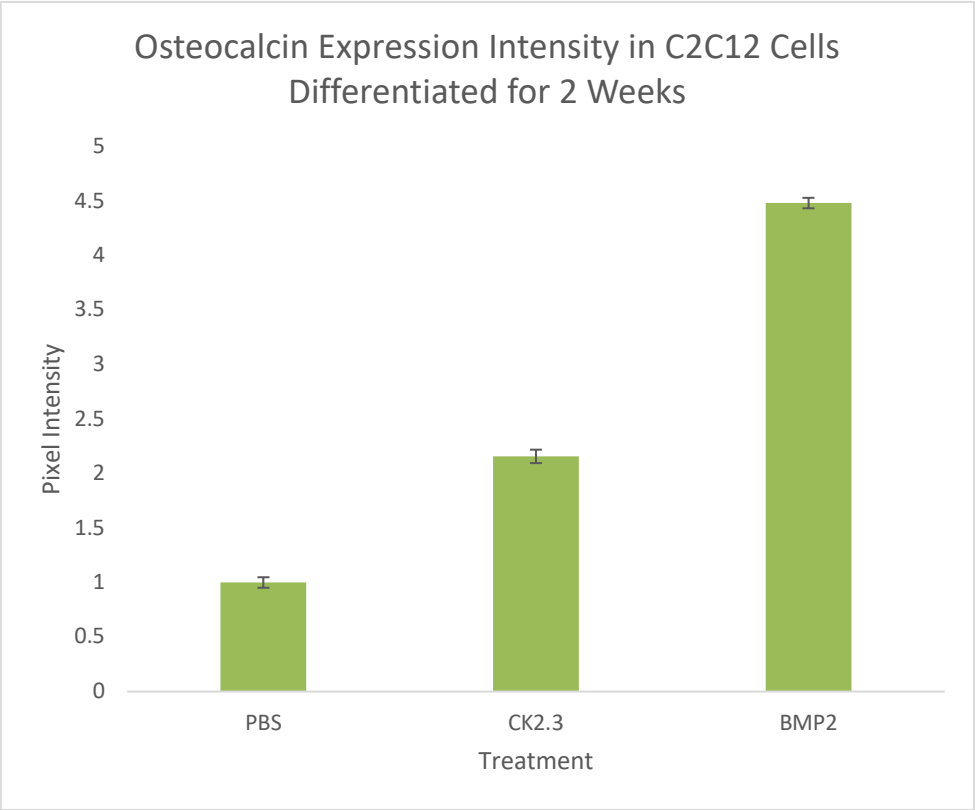


Figure 3.9d Average OC intensity in C2C12 cells that were differentiated for 2 weeks. Both CK2.3 and BMP2 had a positive effect on the intensity levels. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test. No significance was found amongst the data.

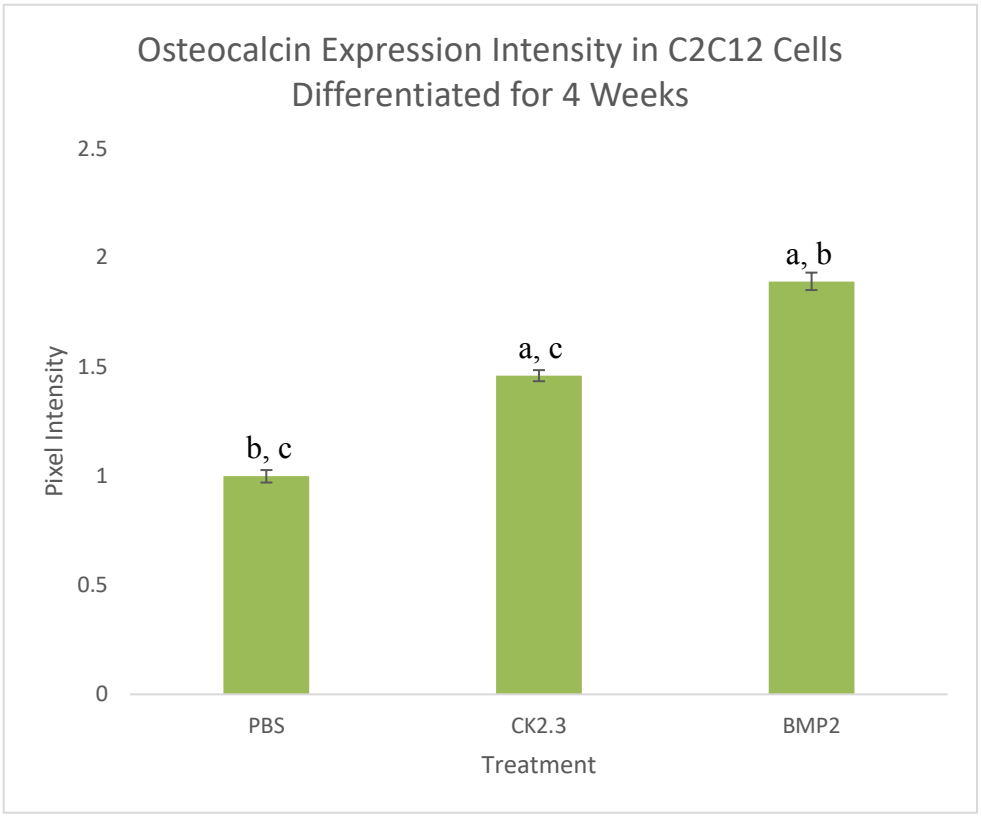


Figure 3.9e Average OC intensity in C2C12 cells that were differentiated for 4 weeks. Both CK2.3 and BMP2 had a positive effect on expression intensity. The experiment was performed a total of three times. A statistical significance test was performed through the use of a one-way ANOVA followed by a Tukey-Kramer test.

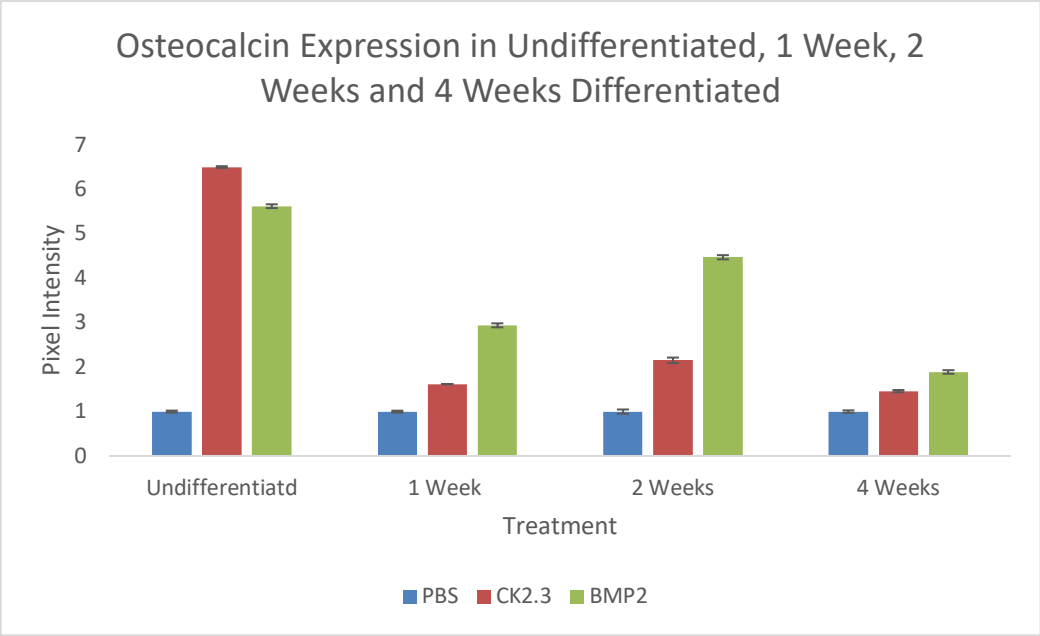


Figure 3.9f Average OC intensity variation among undifferentiated, 1 week differentiated, 2 weeks differentiated and 4 weeks differentiated C2C12 cells.

Chapter 4

DISCUSSION AND FUTURE DIRECTION

Osteoblast differentiation and activity is a critical event needed for the bone remodeling cycle to function properly. Without a properly function bone remodeling cycle, people are at risk for developing osteoporosis. Osteoporosis is commonly observed in the aging population and as of today, the treatments on the market are very limited. Aside from the limits, the treatments that are currently available have detrimental side effects. Therefore, there is a need for a better, more effective treatment. The Nohe lab has designed peptides (CK2.1, CK2.2, and CK2.3) that block the interaction of CK2 with BMPRIa, resulting in the activation of downstream signaling in ligand independent manner. Our labs' earlier studies have revealed that treatment with CK2.3 stimulated ALP and OC expression in serum levels of BMSCs and calvarial cells. A previous study also successfully demonstrated that CK2.3 mediated osteoblastogenesis *in vitro* via the activation of the BMPRIa downstream signaling pathway in absence of BMP2 (Bragdon et al., 2011). Thus, we were interested to look at the role of CK2.3 in osteoblast differentiation and activity activation.

The initial portion of this project focused on analyzing ALP and OC expression in murine femur slices. As explained in the methods and materials, mice were sacrificed 1 week, 2 weeks and 4 weeks after the initial injection with either PBS, CK2.3L, CK2.3M or CK2.3H. Each femur slice of every time point was analyzed for ALP and OC expression in the bone marrow cavities and trabecular bone.

Data collected from these analyses were then graphed and tested for significance. Based on the analysis for 1-week post injection, I found that CK2.3 did not have a positive effect on ALP or OC expression (Fig: 3.1b and 3.2b). A one-way anova followed by a Tukey Kramer test was run on the data collected for this time point and it was noted that all CK2 treatments had a significant decrease on ALP and OC expression in comparison to PBS. The next time point I analyzed was 2 weeks post initial injection (Fig: 3.3b and 3.4b). For ALP expression two weeks post initial injection, CK2.3L and CK2.3M decreased expression in comparison to PBS while CK2.3H had a positive effect (Fig: 3.3b). For OC expression two weeks post initial injection in the bone marrow cavities, CK2.3L and CK2.3M had a negative effect on OC expression while CK2.3H had a positive effect. For OC expression two weeks post initial injection in trabecular bone, CK2.3M was the only treatment that had a negative effect on OC expression while CK2.3L and CK2.3H had positive effect on OC expression (Fig: 3.4b).

Four weeks post initial injection, I began to see results that I expected. Looking at ALP and OC expression levels (Fig: 3.5b and 3.6b), the expression levels increase in a dose dependent manner up until CK2.3H. CK2.3H appeared to have a negative effect on the expression levels of ALP and OC but this is what we expected. Our lab had shown previously that only CK2.3M had increased femoral shaft stiffness four weeks post initial injection (Nguyen et al., 2018). From this data, I am able to say that Figures: 3.1b, 3.2b, 3.3b and 3.4b do not support my initial hypothesis but Figures: 3.5b and 3.6b do support my hypothesis.

Moving on from the *in vivo* work, we wanted to understand what was happening on an individual, cellular basis. The bone slices used contained numerous different cell types like osteoclasts, osteocytes, and bone lining cells, in addition to osteoblasts. As a preosteoblast differentiates into an osteoblast, the levels of ALP and OC expression should increase. So, we wanted to understand what would happen to the expression levels of these proteins not only as the cells differentiate but also as they are stimulated with PBS, CK2.3 and BMP2.

To start this experiment, I stimulated undifferentiated C2C12 cells with PBS, CK2.3 or BMP2 and measured the ALP and OC expression intensity. Data was collected, each timepoint was graphed and a one-way anova followed by a Tukey Kramer test was performed to calculate significance. From the undifferentiated cells, I saw that CK2.3 and BMP2 increased ALP and OC expressions in comparison to PBS (Fig: 3.8b and 3.9b). In week 1 differentiated cells, CK2.3 increased ALP expression in comparison to PBS (Fig 3.8c). OC expression was increased by both CK2.3 and BMP2 in cells that were differentiated for 1 week (Fig 3.9c). For cells that were differentiated for 2 weeks, while it may appear that there is a visual difference in the graph between PBS, CK2.3 and BMP2, there is no statistical significance amongst this data set (Fig: 3.8d and 3.9d). Finally, cells that were differentiated for 4 weeks displayed an overall increase in ALP and OC expression after stimulation with CK2.3 and BMP2 (Fig: 3.8e and 3.9e). From the data collected for this portion of the experiment, I can say Figures: 3.8b, 3.8c, 3.8e, 3.9b, 3.9c and 3.9e support my initial hypothesis while Figures: 3.8d and 3.9d do not.

This study with CK2.3 has opened up many new possibilities to be explored in the future. My studies have primarily dealt with how ALP and OC expression vary *in vitro* and *in vivo*. Based on my results, I've shown that CK2.3M is most effective four weeks post initial injection in murine femur slices and that CK2.3 increases ALP and OC expression in undifferentiated, 1 week differentiated and 4 weeks differentiated C2C12 cells. In the future, it would be interesting to investigate how CK2.3 affects ALP and OC in rats and then possibly move into a human MSC line. In addition, it would also be interesting to elucidate the mechanism of CK2.3 and how it affects the BMPRIa signaling pathway. Finally, the last step to move forward with this project would be to perform a successful western blot, confirming the expression of ALP and OC in the undifferentiated and differentiated cells. A multitude of western blots were performed but to no avail. Many weeks were spent troubleshooting issues I faced but at this time, I have not been successful in completing a viable western blot. Confirming the expression of these two proteins would complete all aims for this project.

To summarize my findings and how they fit into the bigger picture, as mentioned previously, Vrathasha et al., Nguyen et al. and Akkiraju et al. all demonstrated that CK2.3 had a positive impact on osteoblast differentiation and activity (Akkiraju et al. 2015; Nguyen et al. 2018; Vrathasha, Weidner, and Nohe 2019). I was able to successfully demonstrate that CK2.3 had a positive impact on osteoblast activity and preosteoblast differentiation. *In vivo*, CK2.3 did not have a noticeable positive impact in femurs 1 week and 2 weeks post initial injection. In 4

weeks post initial injection femurs, I demonstrated that ALP and OC increased in a dose dependent manner until the dose of CK2.3H. We determined that this increase was due to activation of the osteoblasts and not proliferation (data not shown). Finally, the *in vitro* work I performed demonstrated that CK2.3, like BMP2, differentiated the preosteoblastic C2C12 cells in to mature osteoblasts based off the expression levels of ALP and OC. All of this together supports the hypothesis that CK2.3 can possibly act as a therapeutic drug in the treatment of osteoporosis. It has been shown to increased osteoblastogenesis while also inhibiting osteoclastogenesis, which is a form of treatment desperately needed.

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