CK2 IS THE KEY MOLECULAR SWITCH REGULATING C2C12 CELL DIFFERENTIATION INTO OSTEOBLASTS AND ADIPOCYTES

by

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ABSTRACT

Age related osteoporosis is a medical condition in which bones become weak and can break easily. The quality of patients' life becomes significantly less than average. Existing treatments are costly and not efficient. The majority of the existing drugs target osteoclasts maturation or activity (bisphosphonates and Selective Estrogen Receptor Modulators [SERMS]), but bone remodeling is needed to maintain body homeostasis, and these drugs were reported to cause side effects. Parathyroid hormone (PTH) is the only treatment available that increases bone turnover. Additional treatment which would increase bone mass are in desperate need. Bone Morphogenetic Protein 2 (BMP2) treatment was shown to increase bone mass and is a promising potential treatment for age related osteoporosis. BMP2 has multiple effects on the stem cell differentiation driving Mesenchymal Stem Cells (MSCs) into adipocytes and osteoblasts by a mechanism that is not fully understood. Osteoporotic patients have increased number of adipocytes in their bone marrow, but a decreased number of osteoblasts.

BMP2 signaling is a complex process and begins with BMP2 binding to BMP Receptor Type Ia (BMPRIa) and BMP Receptor Type II (BMPRII). Upon ligand binding, BMPRII phosphorylates BMPRIa. Activated BMPRIa initiates Smad-dependent and Smad-independent signaling including p38, JNK, ERK, PI3K, and NF-kB pathways. BMP receptors are found in caveolae and Clathrin Coated Pits (CCPs) and the current dogma of BMP2-induced signaling states that CCPs are needed for Smad signaling and Smad-independent signals are delivered via cholesterol enriched membrane domain. There is some evidence that CCPs might be inhibitory to Smad signaling. In the present study it was shown that BMP2-induced initial BMPRIa and

Smad phosphorylation takes place in caveolae, revising the current dogma. Membrane localization of BMPRIa and Smad phosphorylation is an important step for designing future therapeutic applications as well as understanding BMP2 induced signaling.

Due to its ability to cause differentiation into osteoblasts and adipocytes, BMP2 treatment could potentially cause a problem for its usage as a treatment for age related osteoporosis. In order to design more specific treatments for targeting osteogenesis, the mechanism of differentiation needs to be fully understood. The present study was designed to address the differences in the BMP2-induced signaling resolving in adipogenesis and osteogenesis. Here, we report that BMP2-induced osteogenesis signals via Smad4 and mTor-AKT pathway, while adipogenesis signals via Smad4, MEK, NF-kB and p38 pathways.

Additionally, it was found that Caspase1 activity causes adipocyte differentiation, but inhibits osteoblast differentiation. Finally, CK2 was identified to be a molecular switch directing C2C12 differentiation depending on the specific site of interaction with BMPRIa. Blockage of CK2.1 and CK2.2 interaction sites with specific peptides led to adipogenesis and blockage of CK2.3 interaction site resulted in osteogenesis. Moreover, CK2.3 peptide, in addition to resulting in differentiation into osteoblasts, inhibited adipocyte differentiation. It was also shown to redirect mature adipocytes into osteoblasts. CK2.3 had the greatest potential to be used in treatment of age related osteoporosis due to its specific action directed towards osteoblasts. Caspase1 inhibitors also present interest for clinical applications, and need further exploration.

Chapter 1

INTRODUCTION

1.1 Bone

Bone is mineralized connective tissue, and together with cartilage makes up the skeletal system. The skeleton supports the body, protects vital organs, and serves as a site for muscle attachment. It is also an important tissue in its function to maintain serum homeostasis as a reserve for calcium and phosphates [1]. Additionally, bone contains bone marrow. Bone marrow includes hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), and endothelial stem cells (EC). HSCs give rise to leukocytes (white blood cells), erythrocytes (red blood cells), thrombocytes (platelets), and osteoclasts. MSCs can differentiate into osteoblasts, chondrocytes, myocytes, adipocytes and fibroblasts (Figure1.1). ECs are sometimes called angioblasts due to their role in angiogenesis or blood vessel formation [2].

Two types of bone make up the skeleton: flat bones and long bones. Flat bones are the skull, scapula, mandible and ileum, and long bones include, among other bones, the tibia, femur, humerus, and ribs. Flat bones develop by intramembranous ossification, whereas long bones develop by endochondral ossification. The cartilage gets replaced by cancellous (spongy) bone at the primary ossification center in the process of development surrounded by the periosteum. As long bones develop further, the primary ossification center gets vascularized and the medullary cavity forms in the diaphysis. The secondary ossification center forms in the epiphysis, which gets separated from the diaphysis by the growth plate (Figure1.2). Bones grow by chondrocyte hypertrophy at the epiphyseal growth plate, cartilage growth and matrix

deposition. Cartilage lays down ground for osteoblastic bone formation during bone growth.

Bone is a very dynamic tissue with constant turnover. About 10% of human skeleton gets remodeled every year. Cells that are responsible for this turnover are osteoclasts (bone resorbing cells) and osteoblasts (bone forming cells) (Figure1.3). Osteoclasts are gigantic multinucleated cells which form by fusion of mononucleated hematopoietic progenitor cells. The early differentiation of HSC into the monocyte/ macrophage progenitor depends on transcription factor PU.1 which 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 k B (RANK). At this stage of differentiation cells are termed pre-osteoclasts. RANK ligand (RANKL), expressed on osteoblasts and stromal cells, is the crucial growth factor for the final stages of osteoclast differentiation. Binding of RANKL to the RANK receptor activates nuclear factor k B (NF-kB) signaling leading to the mononuclear osteoclast expression of tartrate-resistant acid phosphatase (TRAP). Cells mature and fuse, creating multinucleated mature osteoclast. Osteoblasts also secrete osteoprotegerin (OPG), which is soluble and binds to RANKL. Binding of OPG to RANKL does not allow for RANK-RANKL interaction, thus serving as a negative regulator for osteoclast differentiation. Cytokines associated with inflammation play a significant role in osteoclast activity. Interleukin-1 (IL-1), IL-6, M-CSF, tumor necrosis factor alpha (TNF-α), and lipopolysaccharides (LPS) enhance and prolong osteoclast activity [3].

Once activated, osteoclasts release protons and lysosomal hydrolases into the constricted environment of the extracellular space. These hydrolytic enzymes include

cathepsin K and metalloproteases, which degrade collagen and other proteins in the matrix. The dissolution of the mineralized matrix occurs via acidification from the released protons (creating a pH of 4-5). As a result, shallow bay forms under the osteoclast known as the resorption bay or pit. The products of the degraded mineral component of bone such as calcium ions, soluble inorganic phosphates and water, get released into the circulation. Once the resorption of the designated bone is complete, osteoclasts undergo apoptosis (programmed cell death) [1]. After resorption is complete, pre-osteoblasts proliferate and differentiate into osteoblasts which form new bone as discussed below.

Osteoblasts are differentiated MSCs that retain the ability to divide and secrete type I collagen (constituting about 90% of the proteins in bone). The differentiation process begins with the proliferation of osteoprogenitor cells by activation of *c-myc*, *c-fos* and *c-jun* genes encoding for the growth factors FGF, IGF-1, TGF-β, BMPs and cell adhesion proteins such as fibronectin, and type I collagen. As osteoprogenitor cells mature into osteoblasts expression of the genes *Msx2*, AP1 (*c-fos*), helix-loop-helix (*HLH)* decrease and cells enter a stage of extracellular matrix (ECM) maturation. Activation of runt-related transcription factor 2 (Runx2) leads to production of type I collagen, bone sialoprotein, osteopontin, osteonectin, osteocalcin and alkaline phosphatase (ALP) which are responsible for the calcification of bone. 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 [3]. 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 [1].

1.2 Age Related Osteoporosis

Age related osteoporosis is a medical condition in which the bones become weak and can break easily. Treatment of osteoporosis is costly, and quality of life becomes significantly less than average. As of today, about 10 million people in the U.S. already have the disease and about 34 million more are at risk. It is estimated that by 2025 osteoporosis will be responsible for approximately three million fractures resulting in $25.3 billion in costs each year (http://www.nof.org/home). A number of therapies have been developed (bisphosphonates, Selective Estrogen Receptor Modulators (SERMS) and Parathyroid Hormone [PTH]), but these drugs are expensive and can cause concern due to potential side effects, such as osteonecrosis of the jaw[4]. Bisphosphonates inhibit osteoclasts, but as mentioned earlier, bone remodeling is needed to maintain body homeostasis. SERMS target the estrogen receptors, not by building new bone, but by preventing the resorption of old bone by inhibiting osteoclast maturation. PTH is the only treatment available that increases bone turnover and in as little as two years of everyday consumption can reverse the process of bone loss and return bone to its pre-osteoporotic density. PTH treatments have variable results due to their concentration dependency. Doses that are required for trabecular bone can increase bone turnover in cortical bone. New therapeutics which increase osteoblastic activity are desperately needed. Over the years Bone Morphogenetic protein 2 (BMP2) and BMP7 have been studied in preclinical trials due to their ability to induce bone formation [3]. In 2002, BMP2 has been approved by FDA for spinal fusions (www.fda.org), and a shortly after for open long bone fractures, due to its

effect of "turning everything it touches into the bone." Since osteoporosis is a disease of bone loss, and BMP2 is a bone-inducing growth factor, the possibility of BMP2 usage as a treatment in patients is being looked into. *In vivo* studies using osteoporotic mice models show promising results: systemic injections of rhBMP2 reverse the osteoporotic phenotype, increase number of MSCs, increase osteoblast activity and decrease apoptosis [3]. Analysis of osteoporotic patients in a BMP2 study revealed that patients indeed have low serum levels of BMP2 and high titer of BMP antibodies [5]. It also has been shown that variants in the *BMP2* gene elevate likelihood of developing osteoporosis [6, 7]. While BMP2 seems like a good candidate for fracture healing and spinal fusions, there are some consequences to it. In order to work properly, high doses of BMP2 are needed (1.5mg/ml), BMP2 delivery is via repetitive ectopic injections, plus BMP2 has multiple effects on the stem cell differentiation discussed below.

1.3 Bone Morphogenetic Proteins (BMPs)

BMPs are a group of multifunctional growth factors and cytokines that belong to the transforming growth factor beta (TGF-β) superfamily. Most BMPs are synthesized as large precursors of about 400-500 amino acids consisting of an N-terminal signal peptide (directs the protein to secretory pathway), a prodomain (ensures proper folding) and a C-terminal mature peptide [8, 9]. Carboxy-terminal mature proteins are known to be proteolytically cleaved upon dimerization from the prodomain at an Arg-X-X-Arg sequence by serine endoprotease (in the case of BMP4 by Furin, proprotein convertases (PCs) 6 and PC7 [10]) followed by their secretion from the trans-Golgi [11] (synthesis and secretion is summarized in Figure1.4 A). Most active BMPs are composed of 50-100 amino acids with seven cysteines, six of

them forming three intramolecular disulfide bonds, known as cysteines knots. The seventh cysteine is used for dimerization with another monomer by a covalent disulfide bond, thus forming the active signaling molecule [12]. These disulfide bonds could provide unique protection to the BMP ligands making them highly resistant to changes in temperature and pH, allowing for their extraction from the bone without degradation. All BMPs (except BMP15, which lack the seventh cysteine) form either homodimers or heterodimers. Heterodimerization of BMP2/5, BMP2/6, BMP2/7, and BMP4/7 have been observed both *in vivo* and *in vitro*, and appear to be more effective activators of the signaling pathways than their homodimers [9, 13, 14]. Based on their sequence similarity (over 50% highly conserved across species) and known functions, BMPs are typically divided into major four subgroups: BMP2/4, BMP5/6/7/8a/8b, BMP9/10, and BMP12/13/14) [15, 16] (BMP1/3/11/15/17, and BMP18 do not fall into any of these subgroups).

Following cleavage at the primary site by serine endoproteases, the prodomain remains non-covalently associated with the mature active BMP dimer (Figure1.4 A). Recent studies have established that at least BMP4, BMP7, BMP9, BMP10, BMP11, and BMP14 form complexes with their prodomains following cleavage [17-19]. Only the BMP2 prodomain and the short form of the BMP4 prodomain fail to complex with their respective growth factors [17]. Following secretion, the complex is directly targeted to elements of the extracellular matrix (Figure1.4 B), specifically microfibrils, where the prodomain mediates binding to fibrillins [17, 20]. The extracellular matrix itself can act as regulator of the BMP signaling. Heparan sulfate proteoglycans (HSPGs) shape BMP gradient at the cell surface, arbitrate BMP2 internalization and potentiate BMP2 osteogenic activity [21].

All BMPs are released by the conventional secretory pathway, except BMP1. BMP2, BMP3, BMP4, BMP5, BMP6, and BMP7 have been shown to be present in matrix vesicles deposited by growth plate chondrocytes [22] (Figure1.4). Matrix vesicles serve as centers of mineralization and are a subtype of shedding vesicles originating from the plasma membrane [23-26]. BMP2 and BMP4 are known to be constituents of serum and are generally assumed to be in the soluble mature active form [27-29].

One of the regulatory levels of the BMP signaling includes synthesis of extracellular BMP antagonists, which can be stimulated by BMPs, indicating a tight regulation of BMP signaling. Antagonists prevent binding between BMPs and their specific cell surface BMP receptors. There are over 15 antagonists identified to date and they are classified into three subgroups based on the size of their cystine knot: differential screening-selected genes in the Neublastoma (Dan) family with an eight-member ring, twisted gastrulation (Tsg) with a nine-member ring, and chordin (Chd), noggin (Nog), ventroptin (Chrdl1), follistatin (Fst) and FLRG-follistatin-related gene (Fstl3) with ten-member rings. The Dan family is further divided into four subfamilies: 1) Dan (NO3/ Nbl1- neublastoma, suppression of tumorigenesity1); 2) PRDC- protein related to Dan and Cerberus (Grem2), gremlin (DRM- downregulated by *v-mos*/ IHG-2- induced in high glucose-2); 3) Cerberus (Cer1) and coco (Dand5); and 4) USAG-1-uterine sensitization-associated gene-1 (Sostdc1-sclerostin domain containing 1) and sclerostin (Table2).

Although initially identified in bone extracts, hence the name, BMPs are now known to be involved in regulation of cell proliferation, differentiation, chemotaxis and apoptosis in a variety of normal tissues and have recently been linked to cancer

[30]. They also play an important role in a vast number of developmental processes of various tissues, including lung, skin, brain, eye, liver, and kidney (Table1). Due to this importance in the development of many organs, it recently has been suggested to change their name to Body Morphogenetic Proteins instead [31, 32]. Importance of BMPs in early development can be demonstrated by genetic manipulations. BMP2, BMP4, BMP8b, and BMP10 homozygous null mice are embryonic lethal [33-36]. This embryonic lethality did not allow for function in postnatal development and adult tissue homeostasis, so studying conditional knockouts is used instead [37, 38].

In the present study we focus on BMP2 since it stimulates osteoblast differentiation early in the differentiation pathway. Heterozygous null mice for *BMP2* died at embryonic day 7.5-9 from the failure of the proamnionic canal to close and abnormal development of the heart in the exocoelomic cavity [33]. In conditional *BMP2* knockout mice the earliest steps of fracture healing is blocked even in the presence of other osteogenic stimuli. These mice have spontaneous fractures which do not get resolved over time [39].

1.4 BMP Receptors

BMP receptors are transmembrane bound serine/ threonine kinase receptors. They are composed of short extracellular domain with 10-12 cysteine residues, a single transmembrane domain and an intracellular domain with serine/threonine kinase region [40]. In order to signal, BMP has to bind to at least one type I and one type II receptor. Receptors can form both homomeric and heteromeric complexes, but signaling occurs only through the formation of heteromeric complexes with specific type I and type II receptors. There are five known type I receptors: ALK1 (Acvrl1), ALK2 (ActRI), ALK3 (BMPRIa), ALK4 (ActRIb) and ALK6 (BMPRIb); and three

type II receptors: BMPRII, ActRIIa, and ActRIIb [12] (Table3). In addition, an alternative splice variant of BMPRII which lacks most of the C-terminal tail (short form [SF] of BMPRII) has been identified [41]. BMPs bind with different affinity to their specific receptors. BMP2 and BMP4, for example, have higher binding affinity for the type I receptor, but BMP7 has higher binding affinity for BMPRII [42-44].

Type II receptors are constitutively active kinases which, upon binding of BMP to the heteromeric complex, phosphorylate the type I receptor at a glycine/serine-rich juxtamembrane region, called the GS-box. Upon phosphorylation, type I serine/threonine kinase becomes activated and transduces the signal downstream [45-47].

Transmembrane protein BAMBI (BMP and Activin membrane-bound inhibitor) acts as a pseudoreceptor at the cell surface by forming stable complexes with the Type II receptors. It lacks however the intracellular serine/threonine kinase domain, and thus inhibits signaling by titrating available Type II receptors [48].

1.5 Receptor Complexes and Localization in BMP2 signaling

In order to signal, BMP2 need to bind to heteromeric receptor complexes consisting of at least one Type I and one Type II receptor. Here, we focus on BMP2 signaling via BMPRIa and BMPRII. Presently there are two receptor complexes found in the BMP2 signaling: preformed complexes (PFC) and BMP induced signaling complexes (BISC). BMP2 can bind with high affinity to BMPRIa and this interaction will recruit BMPRII to the complex (BISC), or it can bind to a PFC [49, 50]. It is believed that ligand binding to different receptor complexes activate different signaling cascades: BMP2 binding to PFCs induces Smad-dependent, and binding to BISCs induces Smad-independent signaling pathways [51, 52].

BMPRIa and BMPRII are found in caveolae and clathrin coated pits (CCPs). Caveolae are flask shaped invaginations of the plasma membrane enriched in Caveolin-1(Cav1), the scaffolding protein responsible for caveolae shape. There exists two populations of caveolae, one enriched in Cav1αβ (deep invaginations) and other enriched in Cav1β isoform (shallow invaginations) [53, 54]. There is some evidence that Cav1β inhibits BMP signaling, while Cav1αβ inhibition is minor [53, 55]. Interaction between BMPRII and Cav1 was observed and seems to be regulated by Cav1 phosphorylation in the vascular smooth muscle cells [56]. Caveolae seem to be the preferred location for the signaling molecules and CCPs are believed to be centers for the endocytotic signaling for proteins [51]. About 26% of BMPRII is found in caveolae and about 74% is localized to CCPs. BMPRII does not appear to exist outside of these domains. On the other hand about 35% of BMPRIa is found in caveolae, about 45% is found in CCPs and about 15% appears to exist on its own in the plasma membrane. Additionally, following BMP2 stimulation there seems to be redistribution of receptors on the plasma membrane. Seventy one percent of BMPRIa is found localized in CCPs following 90 minutes of BMP2 stimulation, but no significant effect on BMPRII localization was observed [57].

Until recently, the process of shuttling of the receptors was unclear and regulatory proteins involved were not identified. Proteins interacting with BMP receptors could regulate receptors localization.

1.6 Proteins that Interact with BMPRIa and BMPRII

A number of proteins have been identified to interact with BMP receptors. Some of these proteins interact only with BMPRII, such as Cyclic Guanosine 3',5' Monophosphate dependent Kinase I (cGKI) [58], c-Src [59], Related to EPS15

(Epidermal Growth Factor Receptor path Substrate 15 (EPS15R) [51], and Inhibin [60]. Many proteins have been shown to interact with both BMPRIa and BMPRII. These include, but is not limited to Adapter protein 2 (AP2) [57], Caveolin-1(Cav1) [53, 55], Protein Phosphatase 2A (PP2A) [61], and X linked Inhibitor of Apoptosis Protein (XIAP) [62, 63]. Some of these proteins interact only with BMPRIa, including bone morphogenetic protein associated molecule 1 (BRAM1) [64-66], Endofin [67], Smad1/5/8 [68, 69], Tab1 (Tak1 binding protein) [62, 70-72], Tak1 (TGF-beta Activated Kinase 1) [106, 108, 231], Splicosome Associated Protein subunit 4 (SAP49) [73], Splicing Factor 3b subunit 4 (SF3b4) [74], and Casein Kinase II (CK2) [75]. In this study, we focus mainly on CK2. Analysis of potential binding and phosphorylation sites of BMPRIa using a Prosite search revealed three sites at which the CK2 alpha subunit could phosphorylate BMPRIa (Figure1.5 shown in black). Additionally, a Prosite search identified Caspase1 cleavage site on the BMPRIa (Figure1.5 shown in green). Therefore there exists the possibility that CK2 and Caspase1 could be involved in BMP signaling and needs further investigation.

1.7 Casein Kinase 2

Casein Kinase 2 (CK2) is a highly conserved, ubiquitously expressed enzyme with more than 300 substrates that affects cell growth, proliferation, differentiation, apoptosis and tumorigenesis [76]. CK2 is detected predominantly in the nucleus, while fractions have also been isolated from the plasma membrane, Golgi apparatus, endoplasmic reticulum (ER) and ribosomes. In all cases, the activity is localized to the membrane-associated cell fractions [77]. It has been shown that pleckstrin homology domain of CK2 interacting protein-1 (CKIP-1) is responsible for recruitment of CK2 to the plasma membrane [78]. The CK2 complex is a tetramer with two catalytic (α

and/or α′) subunits and two regulatory β subunits (Figure1.6) [79]. The α and α′ subunits are structurally similar but are coded by different genes and the α′ is not conserved throughout the species while α subunit is [80]. The two β subunits are inactive by themselves, but are crucial for the assembly of the tetrameric CK2 complex as well as recruitment and docking of CK2 substrates. They also enhance the catalytic activity of the α and α' subunits [81]. The catalytic subunits of CK2 are responsible for phosphorylation of targeted substrates at serine, threonine and, less favorably, tyrosine residues surrounded by the acidic sequences of proteins. As already mentioned, CK2 has many known protein substrates and this number grows every day. Some of the CK2 substrates are enzymes involved in transcription and translation (RNA Polymerase I and II, DNA topoisomerase I and II, and DNA ligase), signal transduction proteins (PKC, PKA, IRS-1, Insulin receptor, IGF-II receptor, Calnexin, Calmodulin, and many others), transcription factors (c-Myc, c-jun, p53, and many others), protein synthesis factors (eIF3, eIF4B, eIF, and eIF2β), and cytoskeleton and structural proteins (β- Tubulin, both heavy and light Myosin chains, Clathrin, tau, and many more) [82]. Two transcription factors of our interest are being phosphorylated by CK2: NF-kB and C/EBP [83] since they are actively involved in stem cell differentiation into osteoblasts and adipocytes. CK2 is important in the cell cycle and in the cells decisions of life and death, as it tightly regulates proliferation and is involved in apoptosis [84]. Knockouts of either the β regulatory subunit or α subunit is embryonic lethal to mice [85, 86]. A conditional knockout study revealed that deletion of regulatory subunit of CK2 β was lethal to embryonic fibroblasts [76]. Homozygous knockouts of CK2 α resulted in defects in the heart [86] and knockouts of CK2 α′ resulted in infertility of males due to defects in spermatogenesis [87]. CK2 is an

important kinase in a variety of cellular processes including control of cell cycle, cell motility, embryogenesis, cell differentiation and apoptosis. It is not surprising that CK2 activity has been implicated in poor cancer prognosis and tumorigenesis [76].

1.8 BMP2-Induced Signaling Pathway

Signaling is activated when BMP2 ligand binds to its receptors (Figure1.7 A). Different signaling pathways can be activated depending on which receptor complexes the ligand binds to. As discussed previously (section 1.6), there are two different receptor complexes on the plasma membrane, PFCs and BISCs. PFCs are believed to activate Smad-dependent pathway, whereas BISCs initiate Smad-independent signaling [52]. Smad-dependent signaling pathway is the most studied among BMP signaling pathways. Other pathways include, but not limited to, Mitogen Activated Protein kinase (p38), C-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K) and nuclear factor kappa B (NF-kB) [9, 88] (Figure1.7).

1.8.1 Smad-dependent pathway

The Smad-dependent pathway is initiated when BMP2 binds to its receptors that are in PFCs. Upon ligand binding to the Type I receptor in a PFC, the Type II receptor phosphorylates the Type I in the GS box (glycine/serine rich region of the Type I receptor), activating it. Once active, the Type I receptor then initiates Smad-dependent signaling. There are eight members of the Smad family of proteins and they are usually grouped by their function and by the signaling pathways they participate in. Regulatory Smads (R-Smads) consist of Smad2, and 3 (TGFβ signaling pathway), Smad1, 5 and 8 (BMP signaling pathway), Inhibitory Smads (I-Smads) consist of

Smad6 (BMP pathways), and Smad7 (TGFβ pathway), and Common Smad (Co-Smad) is Smad4 [89]. Phosphorylated Smad1, 5 and 8 bind to and activate Smad4. Following activation, the Smad complex translocates to the nucleus where they activate the expression of targeted genes [47] (Figure1.7 B). Smad1, 5 and 8 signaling have been shown to be involved in cell growth, morphogenesis, apoptosis, development and immune responses. Smad signaling also plays crucial role in osteoblast and adipocyte differentiation (Figure1.7 B-Figure1.7C) [90].

1.8.2 Smad-independent signaling

Smad-independent signaling becomes activated when BMP2 binds to BMPRIa and this interaction recruits BMPRII to the complex, named BISC. Once in the complex, BMPRII phosphorylates BMPRIa at the GS box and initiates p38, JNK, ERK, PI3K and NF-kB signaling (Figure1.7 B). The formation of BISC leads to activation of TGF-β-activated Kinase 1 (TAK1), a MAP kinase kinase kinase, and TAK binding protein 1 (TAB1). The TAK1-TAB1 complex can then interact and activate MKK3, MKK6, NF-kB, and MKK4. MKK3 and MKK6 are MAP kinase kinases which in turn activate p38, MAP kinase. MKK4 is a MAP kinase kinase which activates JNK, MAP kinase [12, 70, 91]. Once activated p38 and JNK regulate gene transcription effecting osteoblast differentiation and apoptosis [91] (Figure1.7 B). p38 activation also is linked to adipocyte differentiation due to its activation of ATF-2 [90, 92] (Figure1.7 C). BMP2 stimulation can also lead to the activation of the PI3K, but the mechanism of this activation is not well understood [93]. Activated PI3K then phosphorylates AKT, a serine/threonine kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, gene transcription and cell migration. BMP2 can also activate ERK, but the mechanism is

not known. ERK1/2 further activates MEK1/2 [94, 95]. The involvement of this many different signaling pathways could explain effects of BMP2 signaling of cell survival, differentiation, migration, and apoptosis.

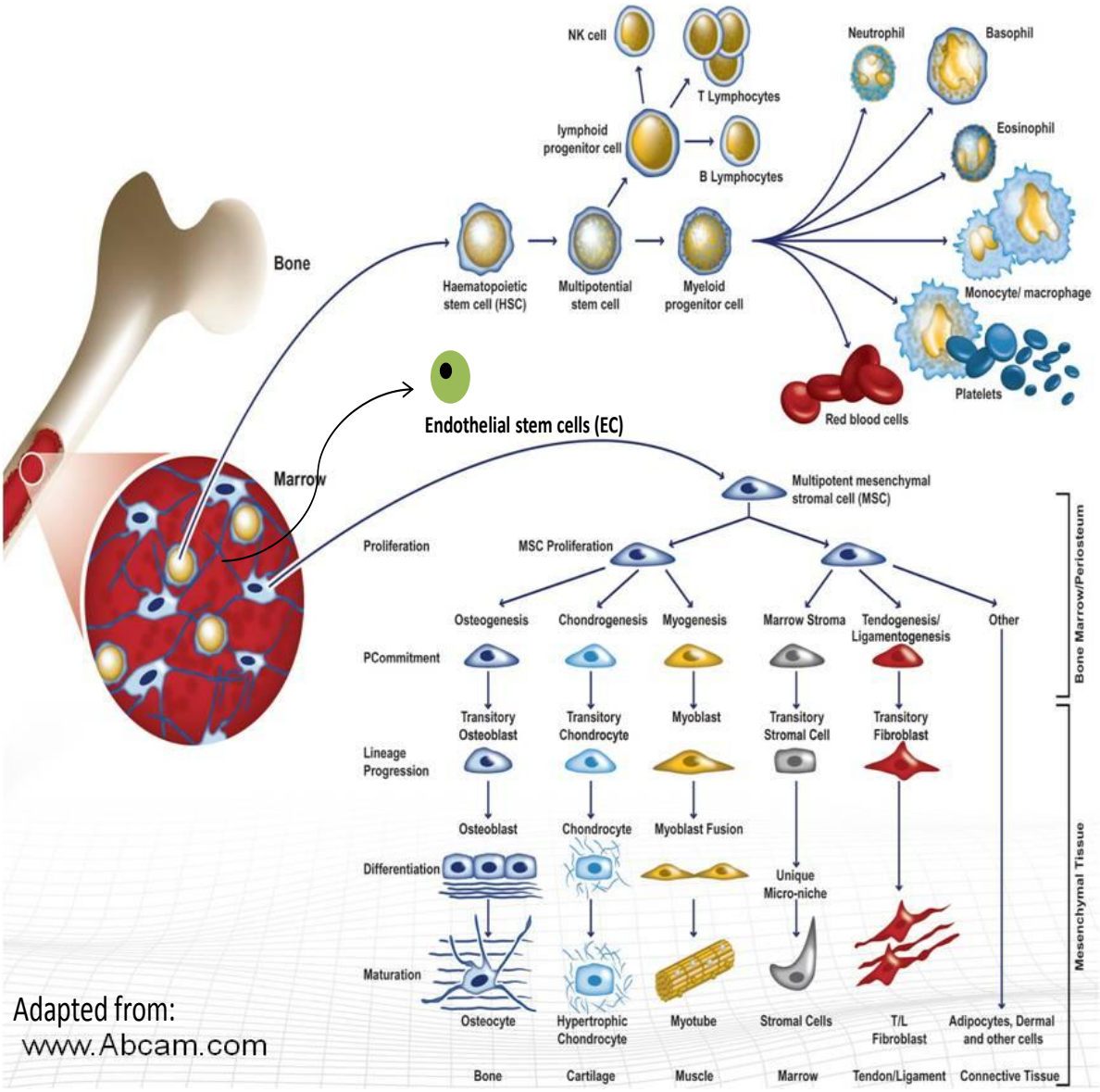
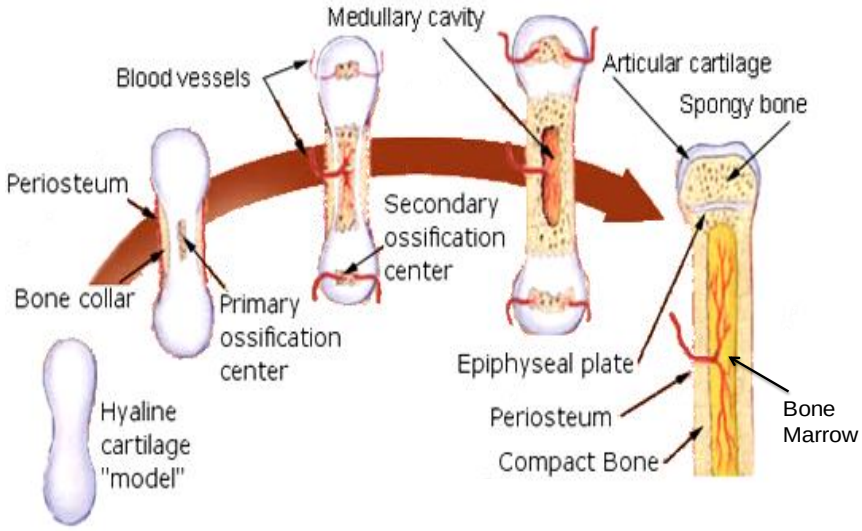


Figure1.1 Stem cells from the bone marrow differentiate into two distinct lineages, hematopoietic stem cells (HSC) and multipotent mesenchymal stromal cells (MSC). HSCs give rise to leukocytes (white blood cells), erythrocytes (red blood cells), and thrombocytes (platelets). MSCs can differentiate into osteoblasts, chondrocytes, myocytes, adipocytes and fibroblasts. Picture adapted from [www.Abcam.com]



Adapted from: http://en.wikipedia.org/wiki/File:Bone_growth.png

Figure1.2 Development and growth of long bones. Long bones develop by endochondral ossification, where cartilage gets replaced by cancellous bone at the primary occification center in the process of development surrounded by periosteum. As long bones develop further, the primary ossification center gets vascularized and medullary cavity forms in diaphysis. The secondary ossification center forms in the epiphysis, which gets separated from diaphysis by the growth plate. Image adapted from [http://en.wikipedia.org/wiki/file:Bone_growth.png]

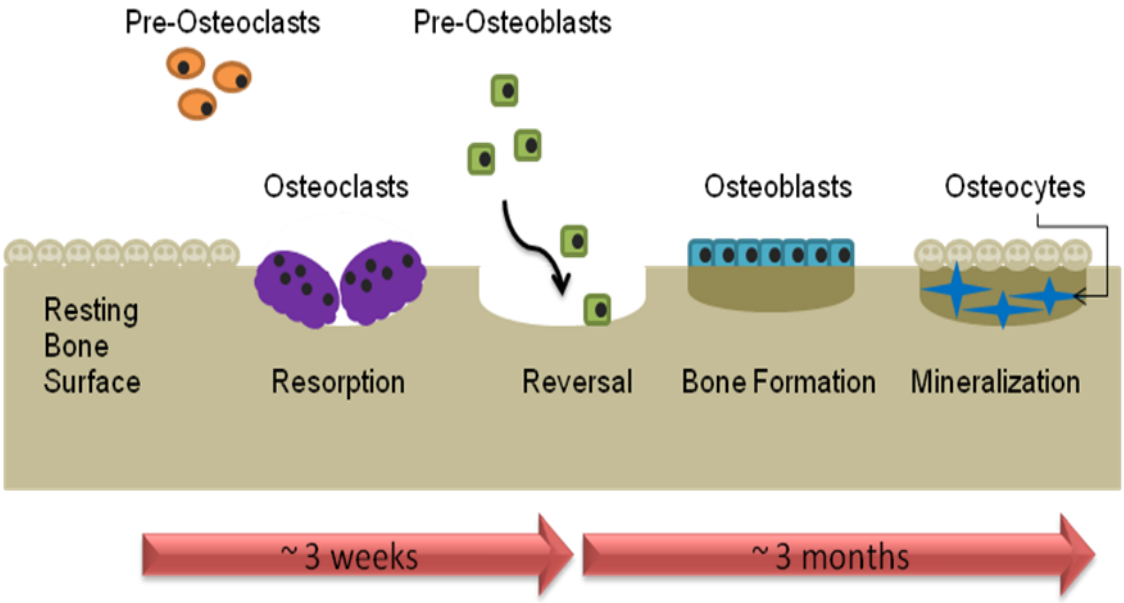


Figure1.3 Simplified cartoon summary of bone remodeling process. Bone remodeling occurs via activity of osteoclasts (bone resorbing cells) and osteoblasts (bone forming cells). Upon stimulation, pre-osteoclasts differentiate and mature into osteoclasts, which release protons and lysosomal hydrolases into the constricted environment of the extracllular space. These hydrolytic enzymes degrade collagen and other proteins in the matrix and protons create low pH to dissolute mineralized matrix. Once resorption phase is complete, pre-osteoblasts differentiate into the osteoblasts in the reversal phase. Osteoblasts are responsible for calcification of the bone. Mature osteoblasts undergo terminal differentiation and become osteocytes embedded in their own matrix. The resorption phase is fairly short, occurring in about 3 weeks, while bone formation and mineralization could take up to 3 months.

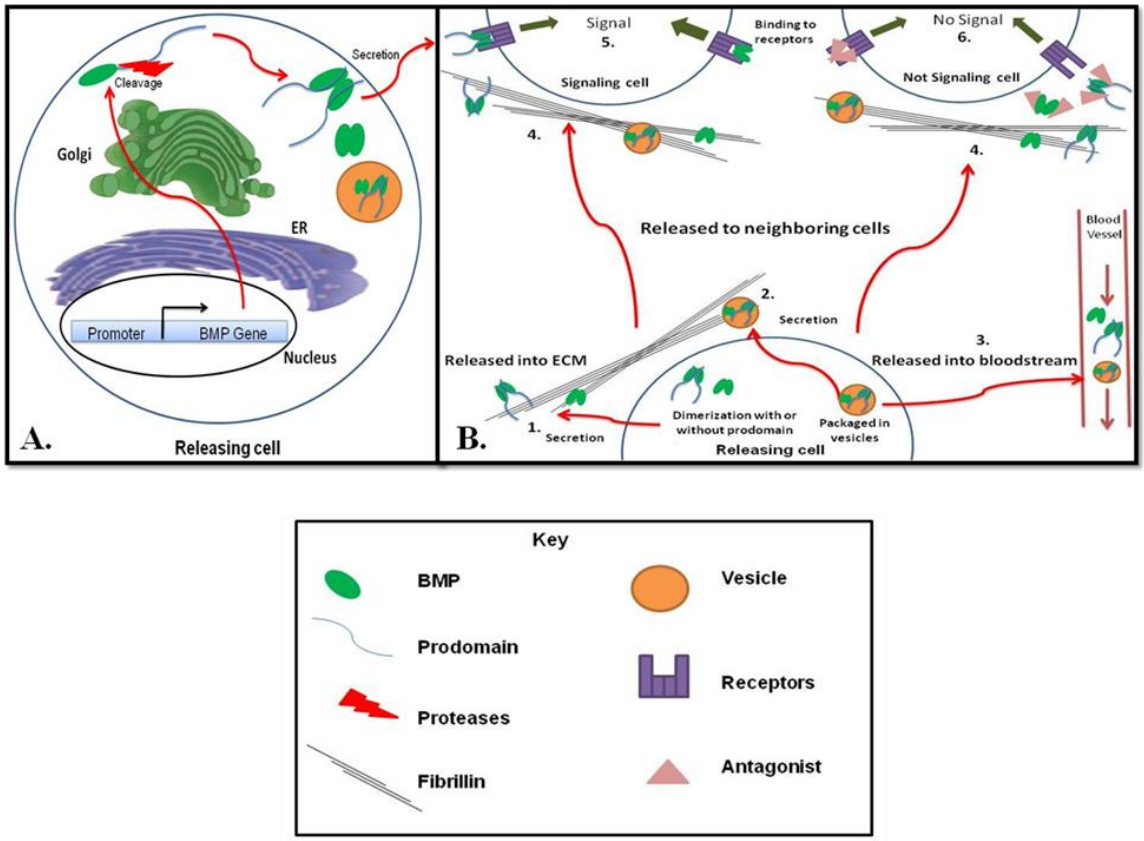


Figure1.4 BMP Bioavailability and Regulation by Extracellular Environment. Summary of the secretion and bioavailability of BMPs and the extracellular regulations. (A) Process leading to the secretion of BMPs. Transcription of BMPs is in the nucleus, followed by translation in the ER, and post-translational modifications in the Golgi. Next is the proteolytic cleavage of the prodomain and dimerization of monomers. Then BMPs are secreted from the releasing cell in dimeric form with or without the prodomain, or packaged into vesicles. (B) Fate of BMP molecules after secretion from the releasing cell. (1) Secretion of dimerized BMPs linked with the prodomain or not, and their interactions with their own ECM (fibrillin). (2) BMPs may be secreted in matrix vesicles. (3) BMPs are secreted into the bloodstream. (4) Secreted BMPs interact with neighboring cells ECM. (5) BMP dimers (associated and non-associated with prodomain) bind to receptors and initiate signaling, while (6) antagonists to BMPs can either interact directly with BMP or its receptor producing no signal. Published in [88].

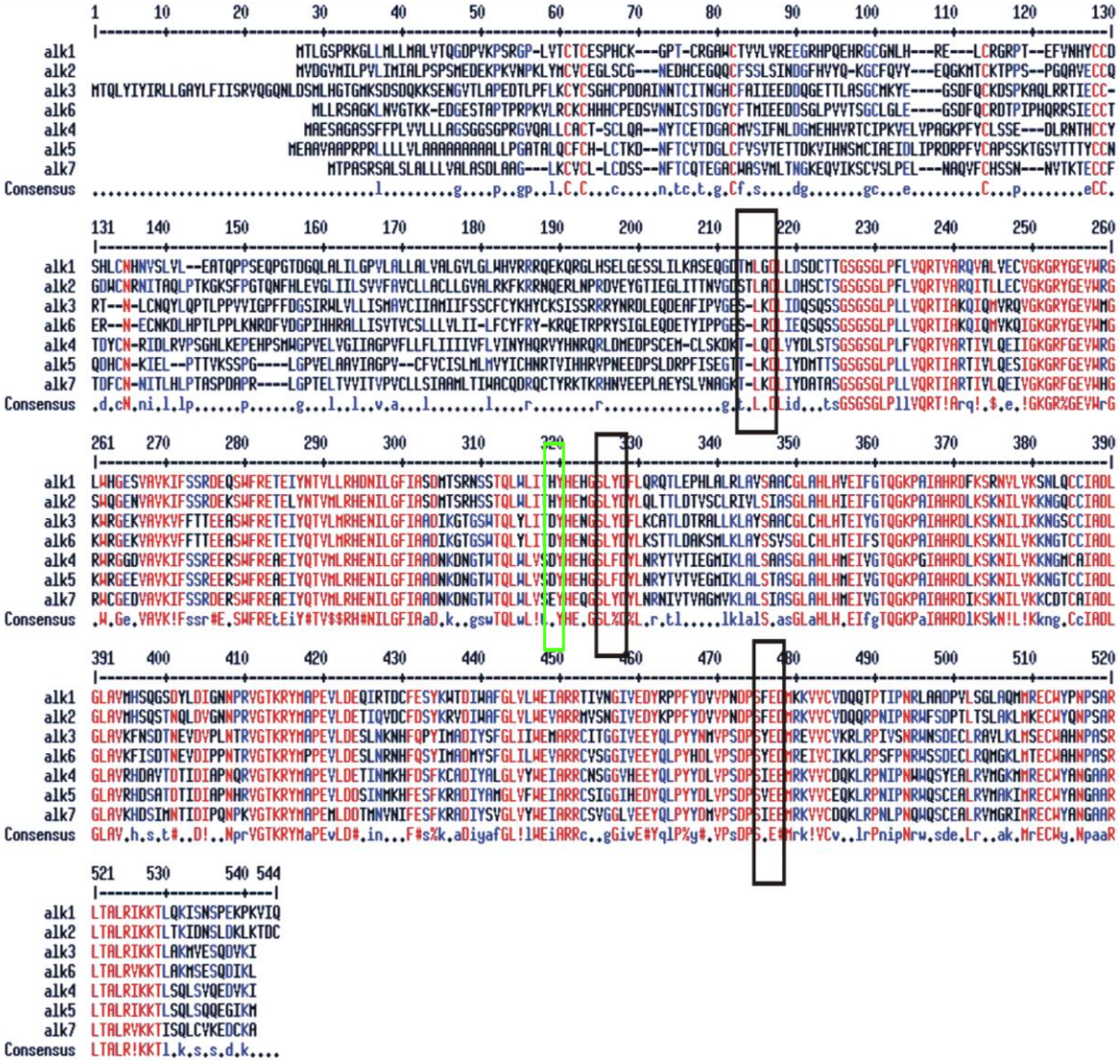
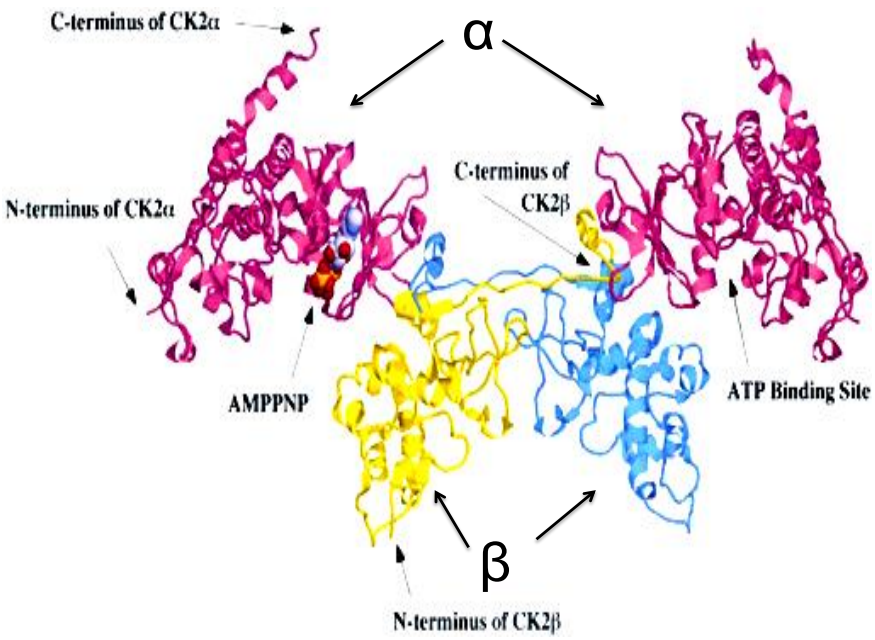


Figure1.5 Alignment of TGF-β Type I Receptors. A Prosite search that included pattern with high probability of occurrence was conducted for the sequence alignment in TGF-β Type I receptors. Search yielded three possible CK2 phosphorylation sites in the Type I receptors located at amino acids 213-217 (SLKD), 324-328, (SLYD) and 475-479 (SYED), shown by black boxes. Search also revealed Caspase1 cleavage site at amino acid 310 shown in green.



Adapted from: Litchfield, DW. 2003. Biochem. J. *369: 1-15*

Figure1.6 Ribbon diagram depicting structure of tetrameric CK2. The catalytic CK2α subunits are illustrated in magenta. One regulatory CK2β subunit is illustrated in yellow and the other CK2β subunit is illustrated in blue. In yellow are the N-termini and C-termini for one CK2α subunit and for the CK2β subunit. One catalytic subunit has ATP binding site and the other one has AMPPNP (nonhydrolysable ATP analog) [79].

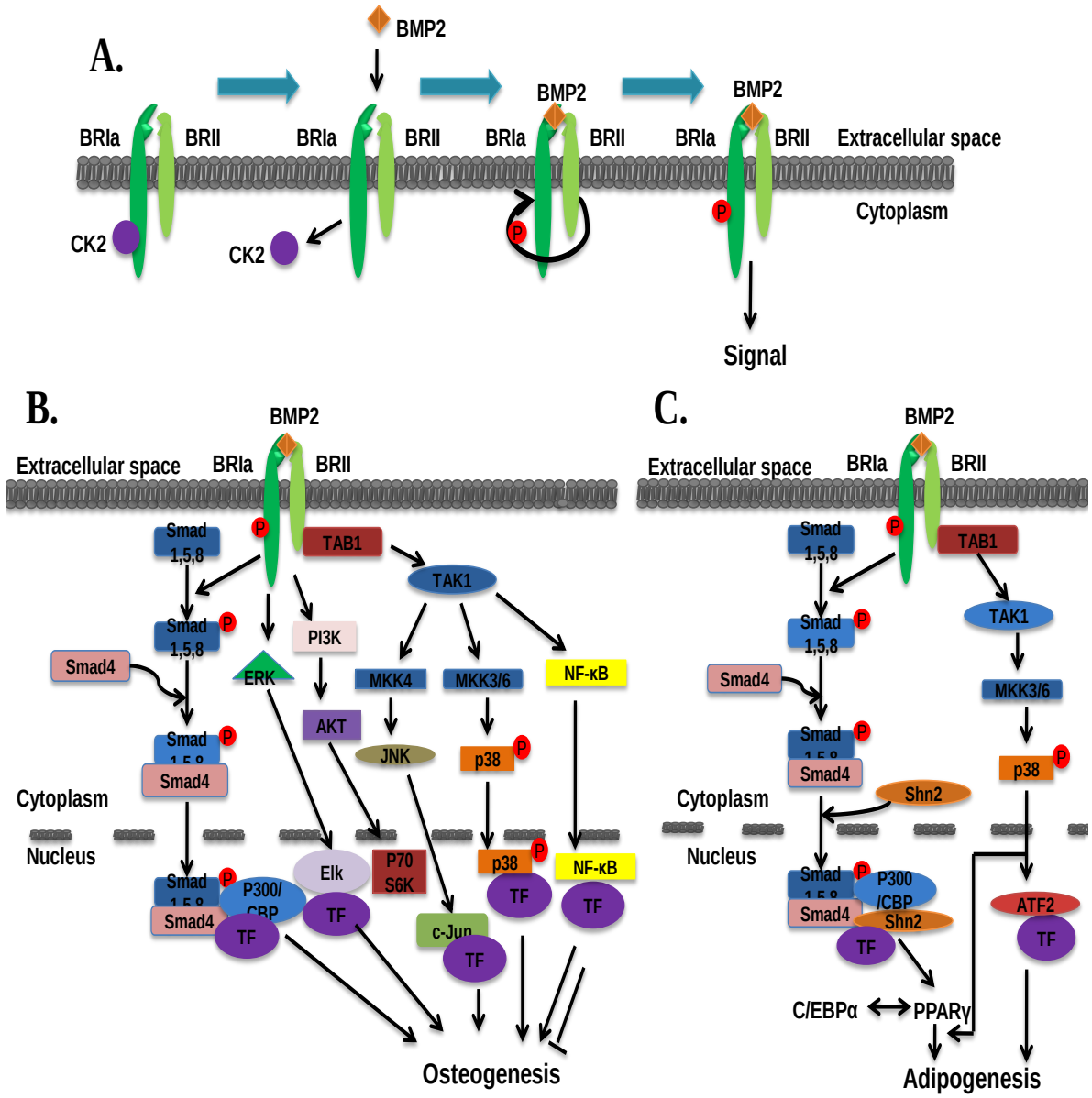


Figure1.7 BMP2 signaling pathway resulting in osteogenesis or adipogenesis. Ligand binding to BMPRIa and BMPRII results in the release of CK2, followed by phosphorylation of BMPRIa by a constitutively active BMPRII, leading to signaling (A). Signaling via Smad1, 5, and 8, ERK, PI3K, JNK, p38, and NF-kB results in osteogenesis (B). Activation of Smad1, 5, and 8 and p38 pathways alone are implicated in adipogenesis (C).

Table1. BMP ligands with their known expression sites, chromosome locations, functions, and mutations in mice. Note. Some information was obtained from the Jackson Laboratory [http://www.informatics.jax.org/] and GeneCards Database [http://www.genecards.org/], where the cutoff for the expression was at 10 of normalized intensity. Published in [88].

BMP	Expression	Functions	Chromosome	Mutations in mice	Ref
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	8p21.3	Heterozygous null: reduced ossification of the skull, persistent herniation of the gut, abnormal collagen fibrils in the amnion, death at birth	[96]
BMP-2	Spleen, kidney, lung, pancreas	Skeletal repair and regeneration/ heart formation	20p12	Heterozygous null: die at embryonic day 7.5-9 with failure of the proamniotic canal to close and abnormal development of the heart in the exocoelomin cavity	[33, 97]
BMP-3 (oste-ogenin)	Thymus, bone marrow, spleen, brain, heart, skeletal muscle, pancreas, prostate	Negative regulator of bone morphogenesis	4q21.21	Homozygous null: increased bone density	[98, 99]
BMP-3b (GDF10)	Brain, spinal cord, skeletal muscle, pancreas, prostate	Cell differentiation regulation/ skeletal morphogenesis	10q11.22	Homozygous null: normal phenotype	[100, 101]
BMP-4 (BMP-2b)	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	Skeletal repair and regeneration/ kidney formation	14q22-q23	Heterozygous null: abnormalities of the kidney and urinary tract. Targeted mutants: embryonic lethality, aberrant mesoderm differentiation, developmental retardation, disorganized posterior structures, failure of the lens induction and lack primordial germ cells	[97, 102-104]
BMP-5	Thymus, bone	Limb development/	6p12.1	Homozygous null:	[105-

	marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, pancreas, prostate	bone and cartilage morphogenesis/ connecting soft tissues		shortened, slightly ruffled external ears due to a defective cartilage framework affecting the whole skeleton. A series of genomic deletions of the region cause embryonic lethality	107]
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	6p24-p23	Homozygous null: delayed ossification in the developing sternum, females smaller in size	[107, 108]
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	20q13	Homozygous null: postnatal lethality, a wide range of skeletal and cartilage abnormalities, renal dysplasia and polycystic kidney, and eye defects	[35, 97, 102, 107-110]
BMP-8a (OP2)	Thymus, bone marrow, spleen, brain, spinal cord, heart, kidney, lung, pancreas, prostate	Bone morphogenesis/ Spermatogenesis	1p34.3	Homozygous null: spermatogenesis defects and germ cell degeneration	[35, 111]
BMP-8b	Bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, liver, pancreas	Spermatogenesis	1p35-p32	Homozygous null: incidents of lethality. Heterozygous and surviving homozygous males: various degrees of germ cell deficiency and infertility	[35]
BMP-9 (GDF2)	Data not found	Bone morphogenesis/ development of cholinergic neurons/ glucose metabolism/ anti-angeogenesis	10q11.22	Data not found	[112, 113]
BMP-10	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, lung, liver, pancreas,	Heart morphogenesis	2p13.3	Homozygous null: embryonic lethality with cardiac dysgenesis	[99, 112]

	prostate				
BMP-11 (GDF11)	Thymus, bone marrow, spleen, brain, spinal cord, pancreas	Pattering mesodermal and neural tissues, dentin formation	12q13.2	Homozygous null: lethality, defects in urinary, renal, nervous system, vision and eye development, skeleton and respiratory	[114, 115]
BMP-12 (GDF7/ CDMP2)	Data not found	Ligament and tendon development/ sensory neuron development	2p24.1	Heterozygous null: defects in nervous system, embryogenesis, shorter life span	[116, 117]
BMP-13 (GDF6/ CDMP2)	Data not found	Normal formation of bones and joins/ skeletal morphogenesis/ chondrogenesis	8q22.1	Homozygous null: multiple joint and skeletal patterning defects affecting the extremities, inner ear, and skull	[117-119]
BMP-14 (GDF5/ CDMP1)	Bone marrow, heart, kidney, liver	Skeletal repair and regeneration	20q11.2	Homozygous null: slightly shorter bones of the limbs, and drastically shorter bones of the feet, with some complete or partial fusions	[97, 117]
BMP-15	None	Oocyte and follicular development	Xp11.2	Homozygous null: female infertility or reduced female fertility and smaller litter size, abnormalities in folliculogenesis, ovulation, and oocyte morthology	[120]
BMP-16	Data not found	Skeletal repair and regeneration	Data not found	Data not found.	[121]

Table2. Antagonists, known BMPs, their functions, expression, and mutations in mice. Note. Information about mutations in mice was obtained from the Jackson Laboratory [http://www.informatics.jax.org/]. Published in [88].

Antagonist	BMPs	Functions	Expression	Null Mutations in mice	Ref
Dan	BMP2 BMP4 BMP7 BMP14	Tumor suppressant/ proliferation/ dorsalization	Embryonic tissues, Spemann organizer	Defects in skeleton and behavior	[122]
PRDC	BMP2 BMP4 BMP2/4 BMP6 BMP7	Regulation of BMP signaling in ovary, brain, and other adult tissues.	Ovary, brain, spleen, osteoblasts	No data found	[123]
Gremlin	BMP2 BMP4 BMP2/4 BMP7	Kidney and limb development. Blocks osteoblast differentiation and function	Kidney, limb, brain, testis, mesenchymal tissues	Neonatal lethality with bilateral agenesis of the kidneys and ureters, oligodactyly, limb skeletal malformations, cyanosis, dispnea, and abnormal lung morphology	[124-126]
Cerberus	BMP2 BMP4 BMP7	Neural tissue formation/ head organizer. Blocks Nodal, BMP, and Wnt signaling	Neural tissues	Homozygous: appear normal. One allele displays behavioral abnormalities and a mild increase in body weight with age	[127]
Coco	BMP4	Neural inducer/ ectopic head in embryos/ left-right axis formation. Blocks BMP/TGF-β and Wnt signaling	Embryonic tissues, ectoderm, mesoderm	Partial neonatal lethality with left pulmonary isomerism, abnormal heart looping, atrial and ventricular septal defects and thoracic situs inversus. Surviving pups display partial premature death with abdominal organ position abnormalities	[128]
USAG-1	BMP2 BMP4 BMP6 BMP7	Kidney disease progression/ teeth development	Kidney, teeth	Craniofacial, abnormalities in nervous system. Spontaneous: craniofacial, abnormalities in skeleton, and digestive system	[129]
Sclerostin	BMP4 BMP5 BMP6	Negative regulator of bone formation	Arteries, brain, kidney, liver, duodenum, stromal cells, osteoblasts, osteocytes, hypertrophic	Sclerostosis, prolongation of active bone forming phase of osteoblasts, with characteristic of excessive bone growth	[130]

			chondrocytes		
Tsg	BMP2 BMP4	As agonist- enhances cleavage of BMP/chordin complex by BMP1/ tolloid (releasing free BMP) As antagonist- binds BMPs, silencing signaling Required to specify the dorsal-most structures in embryo.	Osteoblasts	Homozygous: healthy at birth, but fail to thrive and exhibit dwarfism with delayed ossification and immune system. Premature death.	[131]
Chordin	BMP2 BMP4 BMP7	Neural induction/ mesoderm dorsalization	Chondrocytes, osteoblasts, Spemann organizer	Homozygous for a targeted null: some lethality prior to embryonic day 8.5, but most die perinatally with abnormalities of the skull, mulfanctions of cervical and thorastic vertebrae, cardiovascular defects, and absence of parathyroid and thymus	[132, 133]
Noggin	BMP2 BMP4 BMP5 BMP6 BMP7 BMP13 BMP14	Bone formation/ apoptosis/ important in neural tissues formation/ dorsal ventral axis formation	Neural tissues, Spemann organizer, chondrocytes	Homozygous: joint lesions, axial skeleton abnormalities and lethality. Chordin/ Noggin double mutants lack part of forebrain, eyes, have disrupted mesoderm formation and abnormal left-to-right patterning	[134-136]
Ventroptin	BMP4 BMP5 BMP6	Retinal patterning	Ventral retina, forebrain, diencephalon, limb buds	No data found	[137]
Follistatin	BMP2 BMP4 BMP6 BMP7 BMP11	Neural induction/ growth and weight/ whisker, teeth, and skeletal development/ musculoskeletal system. Inhibits Activin A and BMPs function	During gastrulation in the blastophore, proliferating chordrocytes and osteoblasts	Insufficiencies in skeleton. Homozygous null: retarded growth, reduced diaphragm and intercoastal muscle mass that lead to neonatal respiratory failure, shiny tight skin, defects of the hard palate and thirteenth ribs, and abnormal whiskers and teeth	[138]
FLRG	BMP2 BMP11	Neural induction/ modulated osteoclast differentiation. Inhibits Activin A and BMPs function	Heart, lung, kidney, testis, bone	Defects in cardiovascular, homeostasis, liver, adipose, endocrine, and digestive systems	[139, 140]

Table 3. BMP receptors with their known ligands, expression, chromosome locations, and mutations. Note. Some information was obtained from the Jackson Laboratory [http://www.informatics.jax.org/] and GeneCards Database [http://www.genecards.org/], where the cutoff for the expression was at 10 of normalized intensity. Published in [88].

Name	BMP	Expression	Chromo-some	Null Mutations in mice	Ref
ALK1 (Acvrl1)	BMP9 BMP10	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	12q11-q14	Lethality, serious defects in cardiovascular, including fusion of major arteries and veins, abnormalities in embryogenesis	[112, 141-143]
ALK2 (ActRI)	BMP2 BMP4 BMP6 BMP7 BMP9	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	2q23-q24	Lethality, defects in cardiovascular, embryogenesis, nervous system, skeleton, growth and size	[46, 102, 108, 111, 141, 144, 145]
ALK3 (BMPRIa)	BMP2 BMP4 BMP6 BMP7 BMP10 BMP12 BMP13 BMP14	Thymus, bone marrow, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	10q22.3	Die by embryonic day 9.5, smaller, and form no mesoderm. Conditional KO: gross malformations of the limbs with complete agenesis of the hindlimb	[15, 41, 46, 102, 112, 144-146]
ALK4 (ActRIb)	BMP3 BMP11	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	12q13	Lethality, abnormalities in embryogenesis, nervous and respiratory systems	[98, 142, 145, 147]
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	4q22-q24	Affect shape of the distal limb skeleton resulting in brachydactyly or failure to generate digit cartilage. Female sterility	[15, 41, 46, 102, 112, 142, 144-146]
BMPRII	BMP2 BMP4 BMP6 BMP7 BMP9	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas,	2q33-q34	Arrest at the egg cylinder stage and die before embryonic day 9.5 with failure to form organized structure and lacking	[15, 41, 46, 112, 141,

	BMP10 BMP12 BMP13 BMP15	prostate		mesoderm	144, 145, 148]
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	2q22.3	Skeletal and facial abnormalities, defect in reproduction in adults	[15, 46, 112, 142, 144, 145, 149]
ActRIIb	BMP2 BMP6 BMP7 BMP9 BMP11 BMP14 BMP16	Thymus, bone morrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	2p22	Abnormal left-right axis development, artrial and ventricular septal defects, right-sided morphology of the left atrium and left lung, spleen hypoplasia	[46, 141, 142, 145, 150, 151]

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The cell line C2C12 was purchased from American Type Culture Collection (Manassas, VA, USA). Recombinant BMP2 was obtained from GenScript (Piscataway, NJ, USA). HD (control peptide containing the Antennapedia homeodomain signaling sequence), CK2.1, CK2.2, and CK2.3 specific peptides were purchased from GenScript (Piscataway, NJ, USA). Plasmids encoding BMPRIa-RFP, BMPRIa mutants MCK2.1, MCK2.2 and MCK2.3 constructs were subcloned and mutated by Mutagenex (Piscataway, NJ, USA).

Polyclonal rabbit anti-Caveolin1 antibody was purchased from BD transduction laboratories (San Jose, CA). Monoclonal mouse anti-phosphoserine antibody was purchased from Invitrogen (Camarillo, CA). Phospho-Smad1/Smad5/Smad8 antibody was purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibody against Smad1/5/8, polyclonal goat anti-sera against BMPR-IA, mouse monoclonal antibody against PPARγ, horseradish peroxidase (HRP) conjugated donkey anti-goat IgG, goat anti-mouse IgG-HRP, and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All antibodies were tested for saturation and optimal dilutions were used.

SB203580 (p38), PD98059 (MEK), Rapamycin (mTOR), AKT IV inhibitor, and AKT X inhibitor were purchased from CalBiochem (San Diego, CA). NF-kB inhibitor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and

Z-YVAD-FMK (Caspase1 inhibitor) was purchased from BioVision (Mountain View, CA)

2.2 Cell culture

Murine myoblast cells (C2C12) were grown in Dulbecco's Modified Eagle's Medium (Hy-Clone, Pittsburgh, PA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 0.5% (v/v) L-Glutamine (Cellgro, Manassas, VA) and 1% (v/v) penicillin/streptomycin (Hy-Clone, Pittsburgh, PA). 1X Triton (Cellgro, Manassas, VA) was used to detach cells from the flasks during cell splitting and plating.

2.3 Design of CK2 peptides

A Prosite search including patterns with a high probability of occurrence on BMPRIa yielded three possible CK2 phosphorylation sites. These sites were located at amino acids 213-217 (SLKD), 324-328, (SLYD) and 475-479 (SYED) (Figure1.3). Peptides were designed with the Antennapedia homeodomain signal sequence for cellular uptake and incorporated one of these binding sites, CK2.1 (SYED), CK2.2 (SLYD), and CK2.3 (SLKD). The peptides also included several amino acid residues flanking each side, creating a total length of 27 amino acids (CK2.1) and 29 amino acids (CK2.2 and CK2.3).

2.4 Design of BMPRIa mutants lacking CK2 binding site

Mutant constructs of BMPRIa were generated and verified by DNA sequencing by Mutagenex (Piscataway, NJ, USA). The BMPRIa sequence in the expression vector pcDNA I was subcloned into the ds RFP monomeric vector using the cloning sites Hind III and XbaI (BMPRIa-RFP). The BMPRIa subcloned into expression vector

pcDNA I has previously been described [49]. The BMPRIa mouse sequence contains three predicted CK2 phosphorylation sites, located at amino acids 213-217 (SLKD), 324-328, (SLYD) and 475-479 (SYED) (Figure1.1). The serine amino acid was point mutated to an alanine in each of the CK2 phosphorylation sites. The ds RFP monomeric vector was obtained from Dr. Robert Sikes at the University of Delaware.

2.5 Sucrose Gradient

C2C12 cells were grown to 90% confluence in large flasks and serum starved for 24 hours before stimulation. Cells were then stimulated or not stimulated with 40nM BMP2 for 0, 10, 30 and 45 minutes where noted and lysed using 3 ml of 1% Triton X-100 lysis buffer (1% (v/v) Triton X-100, 1mM EDTA pH7.4, 10mM Tris pH7.4) in the presence of 1mM PMSF, 1mM DTT, 10mM tetrasodium pyrophosphate, 17.5mM β-glucrophosphate and protease inhibitor mix (1mg/ml of each of Leupeptin, Aprotinin, Soybean Trypsin inhibitor, Benzamidine/ HCl, Pepstatin, and Antipain), followed by pulse sonication. Three milliliters of sample were then mixed with 3 ml of 90% (w/v) sucrose in MES-buffered saline (25mM MES pH 6.5, 0.15M NaCl), placed at the bottom of the ultracentrifuge tubes, and overlaid with 3 ml 35% (w/v) sucrose in Mes-buffered saline (0.25M Na_2CO_3)- continuous sucrose gradient. Three milliliters of 5% (w/v) sucrose in MES-buffered saline (0.25M Na_2CO_3) was then placed on the top of the tube- discontinuous sucrose gradient. Tubes were placed in Beckman swinging bucket rotor (SW41Ti) and spun at 39000rpm for 18 hours at 4°C in Beckman L8-55M ultracentrifuge. Immediately after ultracentrifugation was stopped, tubes were removed and 1 ml fractions were collected from the top of the gradient into 2ml eppendorf tubes. 200µl of 100% (w/v) TCA (Trichloroacetic acid- 500g of Trichloroacetic acid was dissolved into 270 ml dH_2O and then volume was brought up

to 500 ml) was added to samples and placed into 4°C for at least 4 hours. After TCA precipitation, samples were centrifuged at 14000rpm for 5 minutes. Supernatant was removed and discarded (pellet was almost never visible at this point, so it was always assumed that it is there). Pellet was then washed twice with ice cold Acetone and air dried. Samples were put in 5X SDS running buffer and run on SDS PAGE gel. Western blotting was performed using a CAV1 antibody (BD Transduction), Smad1, 5, and 8 or phospho-Smad1, 5, and 8 (Cell signaling) antibody followed by a secondary HRP antibody (Santa Cruz, CA, USA). Western Blot was detected by ECL (Pierce).

2.6 SDS-PAGE Gel

SDS-PAGE gels composition consisted of resolving, 15%, gel: 3.75 ml of 40% (w/v) Acrylamide/Bisacrylamide, 2.5ml of 1.5M Tris, 0.1 ml of 10% (w/v) SDS, 3.6 ml dH_2O, 0.05 ml of 10% (w/v) APS, and 0.005 ml TEMED. Stacking, 4%, gel: 1.25 ml of 40% (w/v) Acrylamide/Bisacrylamide, 1.575 ml of 1M Tris, 0.125 ml of 10% (w/v) SDS, 9.525 ml dH_2O, 0.0625 ml of 10% APS, and 0.0125 ml of TEMED. 5X loading buffer (5ml Glycerol, 1g SDS, 2.56 ml β-mercaptoethanol, 2.13ml of 0.5M Tris-HCl pH6.8 and a dash of Bromophenol blue) was added to samples, and samples were boiled for 5 min prior to loading into the gels. 15µl of samples were loaded per well and gels were run at 60V until proteins reached resolving gel, voltage was then increased to 120V. Composition of running buffer used was 1% (w/v) SDS, 250mM Tris base, 2.5M Glycine in dH_2O.

2.7 Western Blotting

After samples were run on SDS-PAGE gels, apparatus was disassembled and gels containing resolved proteins were placed in methanol for 5 minutes. Gels were then placed in transfer buffer (25mM Tris-HCl, and 250mM Glycine in dH_2O) along with membranes and blotting paper for 15 minutes for equilibration. Bio- Rad Trans-Blot SD semi-dry transfer cell (Bio-Rad) was used to transfer proteins from gel onto nitrocellulous membrane at constant 20V for amount of time, dependent on the number of gels, 30 minutes for 2 gels and 45 minutes for 4 gels transferred. Membranes were first checked for presence of proteins by Ponseau S stain (Acros Organics) and blocked in 3% (w/v) BSA/TBS-T for at least 1 hour or overnight. Membranes were then washed twice with TBS-T (1% (v/v) 1M Tris-HCl pH 7.4, 3% (v/v) 1M NaCl, 0.1% (v/v) Tween20 in dH_2O). Primary antibodies in 3% (w/v) BSA were then applied to the membranes for at least an hour and washed 3 times with TBS-T, following secondary antibodies for 1 hour. Membranes were then washed with TBS-T to remove excess of antibodies and ECL (Pierce) was applied before detection.

2.8 Van Kossa

C2C12 cells were grown to 90% confluence and serum starved overnight before treatment. Next day cells were treated as noted per individual experiment. After 4 to 8 days, depending on the conditions of individual experiment, cells were washed with cold PBS pH7.4, fixed using 4% (w/v) parafolmaldehyde for 10 minutes, and washed with cold PBS pH7.4 again to remove remnants of fixative. Van Kossa stain (5% (w/v) Silver Nitrate in dH_2O) was applied to each well and plate was put under the UV light for 10-30 minutes depending on the intensity of individual UV light box. Cells were then washed with dH_2O until dH_2O washed clear. Plates were allowed to

dry and the mineralized area was identified by dark areas. These areas were analyzed by taking random high magnification images of each well of treatment with a Nikon TMS automatic mode with phase 1. Data was then quantified with the use of ImageJ (NIH, Bethesda), where images were converted to 8 bid and threshold was set to the positive control. Same threshold was used for all treatments in an individual experiment. The surface area of the stain was quantified by using the "analyzing particles function", a function of ImageJ that can be used to calculate areas of black which represented mineralization.

2.9 Oil Red O

C2C12 cells were grown to 90% confluence in cell star plates from GBO and serum starved overnight before treatment. Next day cells were treated as noted per individual experiment. After 4 to 8 days, depending on the conditions of individual experiment, cells were washed with cold PBS pH7.4, fixed using 4% (w/v) parafolmaldehyde for 10 minutes and washed with cold PBS pH7.4 again to remove excess fixative. Oil Red O stain was prepared by creating a stock solution consisting of 0.35g of Oil Red O powder dissolved in 100ml of isopropanol. On the day of staining 6 ml of stock solution was mixed with 4 ml of dH_2O which was labeled as working solution and set aside for 20 minutes after which it was filtrated through 0.22µm filter. The working solution was applied to each well for 15 minutes and washed once with dH_2O for a period of 1 minute to avoid washing off lipid droplets. Plates were allowed to dry. The area covered by lipid droplets (identified by the red stain) was analyzed by taking random high magnification images of each well of treatment with a Nikon TMS automatic mode with phase 1. Data was then quantified with the use of ImageJ, where images were converted to 8 bid and threshold was set to the positive control. Same

threshold was used for all treatments in an individual experiment. The surface area of the stain was quantified by using the "analyzing particles function", a function of ImageJ that can be used to calculate areas of dark which here is the lipid droplets. Note: Plates have to be cell star from GBO, others do not support adipocyte growth.

2.10 Co-transfection of C2C12 cells

C2C12 cells were grown to 90% confluence in 24 well plates and serum starved overnight before treatment. Cells were then transfected by Turbofect (Fermentas, Glen Burnie, MD) following manufacture's procedure with 3 µg DNA plasmid encoding MCK2.2 and 200 ρmole of the siRNA against BMPRIa, Cav1, CK2, or Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA) where noted. Cells transfected with MCK2.2 alone were used as controls. Following four hours, the media was exchanged to serum containing media. The next day cells were serum starved for 4 days, when they were fixed and ether van Kossa or Oil Red O staining was performed as previously described.

2.11 Treatment with Inhibitors

C2C12 cells were grown to 90% confluence in 24 well plates and serum starved overnight before treatment. Cells were then transfected by Turbofect (Fermentas, Glen Burnie, MD) following manufacture's procedure with 3 µg of DNA plasmid encoding MCK2.2. Transfected cells with MCK2.2 alone were used as controls. Following four hours, the media was exchanged to serum containing media. The next day cells were serum starved and treated with SB203580 (p38), PD98059 (MEK), Rapamycin (mTOR), AKT IV inhibitor, AKT X, Z-YVAD-FMK (Caspase1),

and NF-kB inhibitor where noted. After 4 days cells were fixed and stained with van Kossa stain or Oil Red O stain as previously described.

2.12 Immunoprecipitation of proteins

Cell lysates were centrifuged at 14,000rpm for 5 minutes and supernatant was collected into a new tube. Samples were normalized to total protein content, measured by Nanodrop. G-sepharose (Invitrogen) was washed with 1ml PBS 3 times prior to usage and resuspended in 1 ml of PBS. 3µl of antibody against a specific protein to be immunopercipitated was added to sample and incubated on the shaker for 2 hours at 4°C to allow for binding to the protein of interest. 30µl of G-Sepharose in PBS was added to each sample (tip of pipette was cut to allow the passage of the beads) and samples were incubated for 1 hour on the shaker at 4°C to allow binding to antibody. Samples were then centrifuged at 14,000rpm for 5 minutes. 25µl of 5X loading buffer was added to beads and samples were run on SDS gel followed by western blotting as previously described.

2.13 Data Analysis

Single factor Anova, followed by Tukey's HSD (Honestly Significant Difference) post-hoc test was used to analyze all data presented. All experiments were repeated three or more times. All data was normalized to control in each experiment as noted. * denotes significantly different ($p<0.05$). ** denotes significantly different ($p<0.01$)

Chapter 3

INITIAL BMP2-DEPENDENT BMPRIA AND SMAD 1, 5, AND 8 PHOSPHORYLATION OCCURS IN CAVEOLAE

3.1 Introduction

BMP2 signaling is a complex process as described in Chapter 1. Binding of BMP2 to the BMPRIa and BMPRII receptors activates receptor complexes, allowing for the phosphorylation of BMPRIa by BMPRII. Once the BMPRIa is phosphorylated, it interacts with downstream effectors to transduce the signal into the cell. The most studied signaling pathway activated by BMP2 is Smad1, 5, and 8. Once activated, Smad1, 5, and 8 (R-Smads) bind to Smad4 (co-Smad) and complex then translocates to the nucleus to regulate gene expression of Runx2 [45]. Other pathways that are activated by BMP2 binding include the p38, ERK, JNK, PI3K, and NF-κB pathways [93, 95, 152] (Figure1.7, reviewed in [88]). The molecular mechanism of cellular regulation of signaling pathways is poorly understood.

BMP2 receptors exist in PFC or BISC which are localized in caveolae and clathrin coated pits (CCPs). Smad activation is induced upon ligand binding to PFCs and p38 activation occurs upon ligand binding to BISCs [49, 50]. The role of these membrane domains in BMP2 induced signaling has been addressed for a number of years by many research groups.

Caveolin-1 (Cav1) was shown to inhibit activation of BMPRIa in PFCs by binding to BMPRII (Nohe et al., 2005). Upon BMP2 binding, Cav1-BMPRII interaction is released and the signal is initiated [55]. Hartung et al. showed that lovastatin treatment did not inhibit Smad signaling, but resulted in an increase in alkaline phosphatase (ALP) production, known marker for early osteogenesis [51].

Lovastatin is commonly used in studies for disruption of caveolae and lipid rafts since it inhibits the function of enzymes involved in protein prenylation necessary for localization to plasma membranes. In the same study inhibition of endocytosis by the K44A (dynamin mutant which inhibits CCP endocytosis) had no impact on Smad phosphorylation, but gene transcription activity was decreased by 65%. From these results, it was concluded that Smad-dependent signaling occurs via CCP mediated endocytosis and ALP production is mediated via both CCP-mediated endocytosis and lipid rafts [51]. This finding is in contrast with a previous study in 2002 by Rauch et al. where faster Smad1 translocation into the nucleus upon disruption of CCPs was observed [153]. A more recent study by Bragdon et al. (2009) demonstrated that disruption of CCPs activates the Smad pathway even in the absence of BMP2, suggesting that CCPs negatively regulate the Smad pathway. ALP expression was also elevated upon disruption of CCPs in this study [57]. Taken together these studies demonstrate importance of both CCPs and caveolae in BMP2-dependent signaling, but the exact location on the plasma membrane where initiation of Smad signaling occurs is unclear. In the present study the localization of initial BMPRIa and Smad1, 5, and 8 phosphorylation was addressed. It was found that initiation of BMP2- induced Smad-dependent signaling occurs in caveolae.

3.2 Results

Recent data from our lab demonstrated that BMP2 binds preferentially to receptors located in caveolae (Bonor, unpublished observations). In this study, BMP2 was covalently linked to an Atomic Force Microscopy (AFM) tip and the binding force of BMP2 to BMPRIa was measured. Simultaneously the same area of the cell was labeled for caveolae and AP2 (adapter protein 2 found in CCPs) and confocal images

were taken. BMP2 was found to bind stronger and with higher frequency to its receptors localized in caveolae than in CCPs or outside of these domains. The location on the plasma membrane where BMP2-dependent Smad signaling takes place was further investigated.

3.2.1 Initial BMPRIa phosphorylation occurs in caveolae.

To determine where on the plasma membrane BMP2-dependent BMPRIa phosphorylation occurs, membrane fractionation based on lipid composition was used. Lipid rafts, including caveolae, are detergent resistant, small and cholesterol-enriched areas of the plasma membrane. These properties allow their separation from the rest of the plasma membrane and membranous organelles by using a sucrose gradient. In accordance with the current literature caveolae on the plasma membrane were detected in fractions 3-5. CCPs are generally found in fractions 7-8, fractions 8-12 correspond to ER and Golgi, while fractions 10-12 correlate with early endosomes [154]. A slight phosphorylation of BMPRIa in caveolae fractions in the absence of BMP2, and heavily phosphorylated BMPRIa in caveolae fractions upon 30 minutes of BMP2 stimulation was found (Figure3.2). These results indicate that initial BMPRIa phosphorylation takes place in caveolae, thus the initial BMP2 signal is initiated in caveolae, and not in CCPs.

3.2.2 Initial Smad 1, 5, and 8 phosphorylation occurs in caveolae.

Since initial BMPRIa phosphorylation occurs in caveolae, it was hypothesized that initial Smad phosphorylation would also take place in caveolae domains. The localization of Smad phosphorylation by fractionation of the membrane based on lipid composition was determined as previously discussed. Phosphorylated Smad1, 5, and 8

was not detected at 0 minutes of BMP2 stimulation (Figure3.3 B). Phosphorylation of Smad1, 5, and 8 occurred 10 minutes after 40 nM BMP2 stimulation in fractions 3, 4, 7 and 8 (Figure3.3 C). After 30 minutes of 40 nM BMP2 stimulation, phosphorylated Smad1, 5, and 8 shifted to fractions 4, 5, 7, 8, 9, and 10 (Figure3.3 D). After 45 minutes of 40 nM BMP2 stimulation, phosphorylated Smad1, 5, and 8 were degraded in the endosomes (Figure3.3 E). These observations demonstrate that BMP2-dependent Smad signaling is initiated in caveolae as early as 10 minutes following BMP2 stimulation.

3.3 Discussion

BMP2 is a crucial growth factor in cell differentiation, skeletal formation and embryogenesis as revealed by genetic manipulations in mice. Heterozygous null mice for *BMP2* died at embryonic day 7.5-9 from the failure of the proamnionic canal to close and abnormal development of the heart in the exocoelomic cavity [33]. In conditional *BMP2* knockout mice the earliest steps of fracture healing was blocked even in the presence of other osteogenic stimuli. Conditional knockout mice have spontaneous fractures which are not resolved over time [39]. These findings suggested a possibility for BMP2 treatment of osteoporotic patients with a decreased mineral bone density. *In vivo* studies using osteoporotic mice models show promising results: systemic injections of rhBMP2 reverse osteoporotic phenotype, increase number of MSCs, increase osteoblast activity and decrease apoptosis [3]. While BMP2 treatment has been approved by the FDA for spinal fusions and for open long bone fractures (www.fda.org), its mechanism of signaling is not fully understood.

The role of caveolae and CCPs in BMP2 signaling was addressed by many studies as previously discussed. According to Hartung et al. Smad-dependent signaling

occurs via CCP mediated endocytosis and ALP production is mediated via both CCP-mediated endocytosis and lipid rafts [51]. Additionally, it has been reported that BMPRIb and BMPRII (not for BMPRIa which is also present in caveolae and CCPs), undergo endocytosis via CCP, but only BMPRII can be internalized via caveolae [51]. In this study, overexpression of HA-BMPRIb and myc-BMPRII in C2C12 and COS7 cells were used. Since C2C12 cells do not normally express the BMPRIb receptor as analyzed by Northern blotting, and only BMPRIa mRNA is expressed in this cell line [155], the relevance of BMPRIb internalization remains questionable. Bragdon et al. reported that disruption of CCPs activates the Smad pathway even in the absence of BMP2, concluding that CCPs negatively regulate the Smad pathway. ALP expression, a known marker for early osteogenesis, was also elevated upon disruption of CCPs in this study [57]. Differences in these findings could be partially explained by differences in disruption methods used. Hartung et al. used K44A (dynamin mutant which inhibits CCP endocytosis), while Bragdon et al. used more specific disruption methods such as EH29 (Eps15 mutant which disrupts the assembly of CCPs), and intracellular potassium depletion (disrupts the coated pits at the surface) treatments in addition to K44A.

These conflicting reports in the current literature on the role of CCPs on Smad signaling raise a number of questions and contradictions. If the clathrin-dependent endocytosis is necessary for the Smad phosphorylation and signal transduction, then it would be expected that no signaling occurs upon their disruption, or at least a significant decrease in Smad signaling exists in their absence. Instead an increase in Smad phosphorylation and quicker Smad translocation into the nucleus is observed [57, 153]. Additionally, BMPRIa and BMPRII were observed to localize to caveolae

and redistribute on the plasma membrane after BMP2 binding (discussed in section 1.5) [57] and caveolae seem to be the preferential sites for ligand binding to BMPRIa (Bonor, unpublished observations).

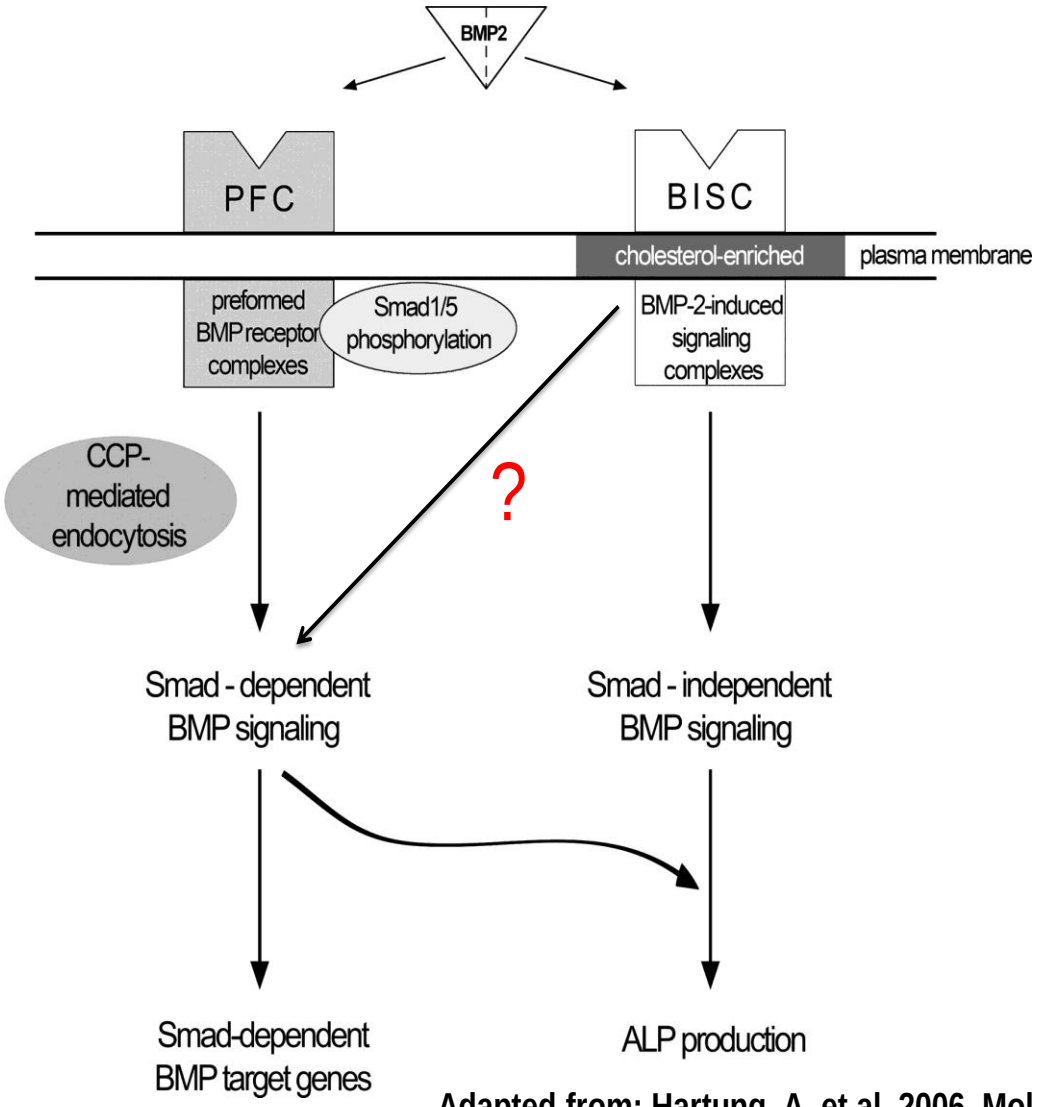
It is hypothesized that Smad signaling might occur in different parts of the plasma membrane instead of or in addition to CCPs and that caveolae play a more important role in BMP2 signaling than currently perceived.

The first step in BMP2 signal transduction is phosphorylation of BMPRIa in the GS box by constitutively active BMPRII upon ligand binding. In the present study, the question of the location of this phosphorylation on the plasma membrane was addressed. The results presented here indicate that initial BMPRIa phosphorylation occurs in DRM and caveolae fractions, as determined by sucrose gradient. Phosphorylated BMPRIa was found in caveolae following 30 minutes of BMP2 treatment (Figure3.2). An unexpected result obtained in this experiment was that BMPRIa is also slightly phosphorylated in the absence of stimuli. This phosphorylation does not appear to be the activating phosphorylation by BMPRII in the GS box, which can be seen upon BMP2 stimulation. It could be an inhibitory phosphorylation, since cells were serum starved for at least 18 hours, and had to be in cell cycle arrest in the absence of growth factors. This phosphorylation could be by CK2, and could explain why BMPRII cannot phosphorylate BMPRIa in the absence of the ligand when receptors are in preformed complexes and certainly needs further exploration.

Upon phosphorylation, BMPRIa serves as a signal transducer into the cell, by interacting, and activating downstream signaling molecules. Smad phosphorylation studies were done at various time points ranging from 10 minutes to 2 days, depending

on experimental conditions when stimulated with 300ng/ml BMP2 in C3H10T1/2 cells [90]. Studies done in C2C12 and COS7 cells show Smad phosphorylation following 30 minutes after the stimulation with 20nM (520 ng/ml) BMP2 [51, 52, 68]. In the present study, the location of Smad phosphorylation on the plasma membrane was examined. Smad1, 5, and 8 phosphorylation were observed 10 minutes after stimulation with 40 nM (1040 ng/ml, concentration previously shown to initiate signaling in our experimental conditions) of BMP2 in DRM fractions. After 30 minutes of stimulation with 40 nM BMP2, phosphorylated Smad1, 5, and 8 had shifted slightly, but remained in DRM fraction. After 45 minutes of stimulation phosphorylated Smad1, 5, and 8 seems to be degraded in the endosomes (Figure3.3). These results are in the agreement with the time dependent study of Smad phosphorylation which revealed that Smad gets phosphorylated as early as 15 minutes after BMP2 stimulation and reaches its peak of activation at 30-60 minutes in P19 cells [156]. The novelty of the result lies in observations that BMP-dependent Smad signaling is initiated in caveolae. These results demonstrate the need of the current dogma of BMP2 signaling to be revised (Figure3.1). The new model would have to take into an account the initiation of Smad-dependent signaling in the DRMs instead of CCPs.

Revelation of BMP2-induced initial Smad-dependent signaling occurring in caveolae could have a potential clinical application of targeting Smad responses in the cells by targeting caveolae (for example by lovastatin treatment).



Adapted from: Hartung, A. et al. 2006. Mol. Cell. Biol.

Figure3.1 Smad-dependent and Smad-independent signaling originate from distinct endocytotic routes of the BMP receptors. Smad-dependent signaling occurs when BMP2 binds to preformed complexes of receptors (PFC) whereas Smad-independent signaling occurs through BMP induced signaling complexes (BISC). Image adapted from [51].

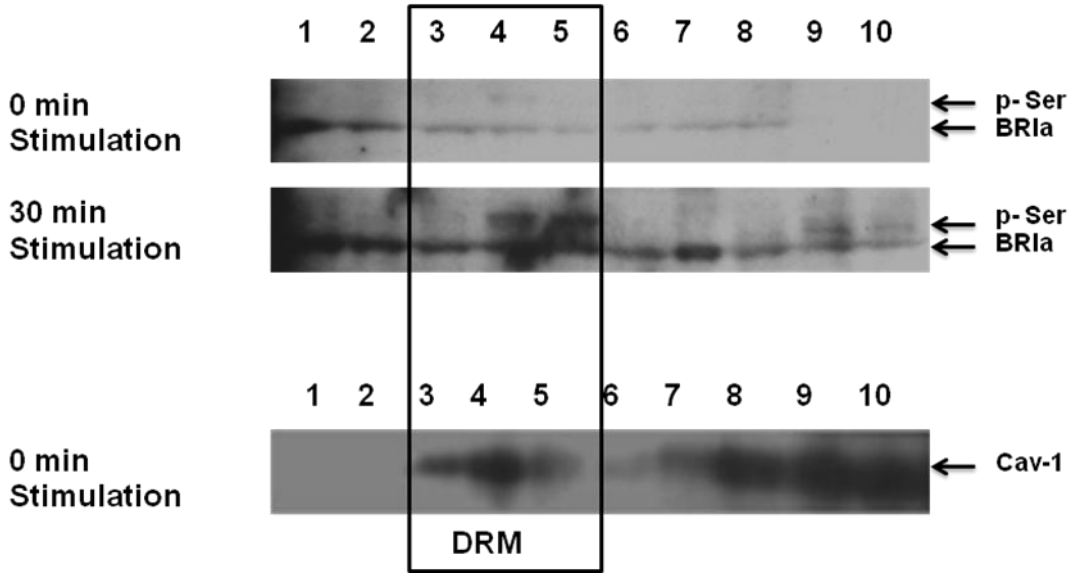


Figure3.2 BMPRIa is phosphorylated in the DRM fractions 30 min after BMP2 stimulation. C2C12 cells were stimulated or not stimulated with BMP2 and lysed using lysis buffer containing 1% Triton X100. Lysates were subjected to sucrose gradients. 1ml fractions were collected from the top of the gradient followed by TCA precipitation for Cav1 blot and IP BMPRIa for p-Ser blot. Western blotting was performed using a Caveolin-1 antibody (BD Transduction), p-Ser antibody (Cell signaling) followed by a secondary HRP antibody. Caveolae on the plasma membrane are detected in fractions 3-5. 8-12 correspond to ER and Golgi, while fractions 10-12 also include endosomes.

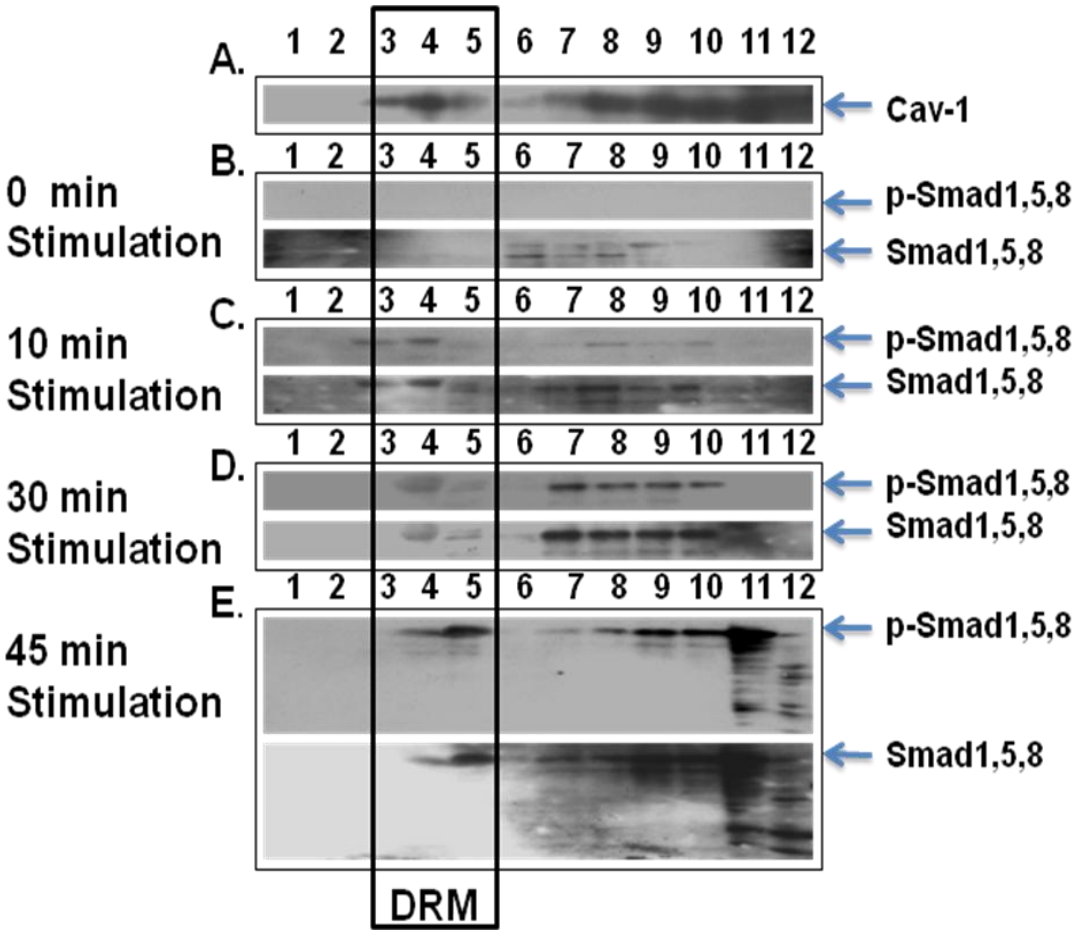


Figure3.3 Smad1, 5, and 8 phosphorylations occur in caveolae followed by BMP2 stimulation. C2C12 cells were stimulated or not stimulated with BMP2 and lysed using lysis buffer containing 1% Triton X100. Lysates were subjected to sucrose gradients. 1ml fractions were collected from the top of the gradient followed by TCA precipitation. Western blotting was performed using a Caveolin-1 antibody (BD Transduction) (A), Smad1, 5, and 8 or phospho-Smad1, 5, and 8 (Cell signaling) (B-E) followed by a secondary HRP antibody. Caveolae on the plasma membrane are detected in fractions 3-5. 8-12 correspond to ER and Golgi, while fractions 10-12 also include endosomes.

Chapter 4

INHIBITION OF CK2 BINDING TO BMPRIA INDUCES BMP2 INDEPENDENT DIFFERENTIATION INTO OSTEOBLASTS AND ADIPOCYTES

4.1 Introduction

Osteoporosis is mainly an age-related process which has a negative effect on the bone mass. As we age, a high number of adipocytes and a low number of osteoblasts are seen in the bone marrow, and while almost no adipocytes are formed in the bone marrow at a young age, their number increases with age, resulting in fatty marrow [157-159]. It was shown that people with increased abdominal fat are at greater risk for osteoporotic hip fractures [160]. Increased body adiposity affects not only bone, but several other diseases have been linked to it: type 2 diabetes mellitus, cardiovascular dysfunction, liver steatosis and cirrhosis, neurodegenerative Alzheimer's disease and even some cancers associated with increased fat body composition [161-164]. An increase in adipocytes poses a concern for public health and mechanisms involved in adipocyte differentiation need to be studied in a great detail. It has been suggested that differentiation of MSC shifts away from osteoblast production to differentiation into adipocytes with age [165]. Both adipocytes and osteoblasts come from the MSC lineage, but the details of mechanism regulating differentiation are not completely understood. One of the key growth factors that have been shown to influence MSC differentiation is BMP2. BMP2-induced signaling pathways utilize the same intermediate molecules leading to adipogenesis and osteogenesis. The present study was designed to identify the key differences in BMP2

signaling pathways to better understand adipocyte differentiation process and how it differs from differentiation into osteoblasts.

BMP2-dependent MSC differentiation into osteoblasts and adipocytes occurs via two types of BMP receptors, the Type I and the Type II receptors. A possibility for the involvement of different receptors in BMP2 signaling has been addressed for years, but no conclusive data have been gathered. Some studies show that BMPRIa is responsible for the differentiation into adipocytes, while BMPRIb is needed for differentiation into osteoblasts [166, 167]. Other studies report that BMPRIa plays a role in osteogenesis and BMPRIb acts in adipogenesis [168].

Two major BMP2-induced signaling pathways include the Smad-dependent (Smad1, 5, and 8) and the Smad-independent pathways (p38, PI3K, ERK, NF-kB, and JNK), and their activation was shown to lead to activation of Runx2 in osteogenesis (Figure1.7 B). In adipogenesis, Proliferator-Activated Receptor gamma (PPARγ) gets activated by Smad1, 5, and 8 and p38 pathways in C3H10T1/2 cell line [90] (Figure1.7 C). PPARγ was reported to be a key regulator and a marker for pre-adipogenesis, since it caused lipid synthesis [169]. Additionally, another intracellular molecule, Schnurri-2 (Shn-2) enters the nucleus, after BMP2 stimulation, and in cooperation with Smad1 and Smad4 complex induces the expression of PPARγ [170] (Figure1.7 C). To date key molecular differences in these pathways have not been identified and an open question remains as to how the same signaling pathways result in different outcomes.

Another possibility for different end points of differentiation utilizing the same receptors could come from different BMP receptor oligomerization. Ligand binding to PFCs might trigger adipogenesis while ligand binding to BISCs might result in

osteogenesis or vice versa. BISCs are localized to caveolae and PFCs are in CCPs [49, 50]. BISCs activation results in p38 signaling, while PFCs initiates Smad signaling [52]. By default then, signals delivered through caveolae would results in p38 activation (ALP production), and signals delivered via CCPs would results in Smad activation.

The present study was designed to examine/test the differences in molecular mechanisms of stem cell differentiation into osteoblasts and adipocytes. A possibility of involvement of a signaling molecule not previously associated with this pathway was addressed by proteomic search for possible interactions with BMPRIa. A Prosite search revealed three potential binding/phosphorylation sites on BMPRIa for CK2 at amino acid residues 213-217 (CK2.3), 324-328 (CK2.2), and 475-479 (CK2.1), which are conserved throughout TGF-beta Type I receptor (Figure1.3, black boxes). It also revealed a potential Caspase1 cleavage site on the BMPRIa at 310aa (Figure1.3, green box). Caspase1 seems to be a reasonable target to study in adipocyte differentiation since it was shown to be upregulated during adipogenesis and its activity is increased both in genetically susceptible and diet-induced obese animal models. *In vivo* treatment of obese mice with a Caspase1 inhibitor significantly increases their insulin sensitivity [171]. Study of involvement of Caspase1 and CK2 in BMP2-induced MSC differentiation would elucidate the mechanism regulating osteogenesis and adipogenesis.

4.2 Results

Numerous reports exist to document that BMP2 induces differentiation of undifferentiated cells into osteoblasts and adipocytes depending on the cell type and on the BMP2 concentration (reviewed in [172, 173]). However, no study has compared

distinct differentiations end points resulting in adipocyte or osteoblast formation in the same cell concurrently to understand the difference between the two. To address the mechanism driving cell type-specific differentiation of MSC the following study was conducted.

4.2.1 BMP2 induces osteogenesis at all concentrations used and at high concentration it induces adipogenesis in C2C12 cells.

The goal of this experiment was to determine whether C2C12 cells differentiate into osteoblasts and mature adipocytes in our experimental conditions, and whether this differentiation is dependent upon BMP2 concentration. C2C12 stimulated or not stimulated (control) with 40pM, 40nM and 200nM of BMP2. After 8 days, cells were fixed and stained for mineralization (van Kossa) and lipid droplets formation (Oil Red O) separately. Mineralization is a marker of mature osteoblasts/osteocytes and lipid droplet formation is a marker of mature adipocytes. Cells were treated equally between experiments and our results indicate that not only C2C12 were able to commit to different lineages, differentiation was dependent on the concentration of BMP2. BMP2 induced differentiation into osteoblasts at all concentrations (Figure4.1 A). However, only the highest concentration BMP2 (200nM) induced adipogenesis (Figure4.1 B).

4.2.2 Specific peptides block interaction of CK2 with BMPRIa

CK2 was identified as an interacting protein with BMPRIa by proteomic search which revealed three potential binding/phosphorylation sites on BMPRIa for CK2. Specific peptides were designed to block this interaction, CK2.1 (aa475-479), CK2.2 (aa324-328), and CK2.3 (aa213-217). Additionally to the CK2 sequence, these peptides contained the Antennapedia homeodomain (HD) signal sequence to allow for

enhanced cellular uptake of peptides through the plasma membrane. HD peptide contains only the Antennapedia homeodomain and was used as a peptide control. Investigation of an interaction between BMPRIa and CK2 was performed with or without BMP2 treatment (40nM), and peptides treatment (100nM). Immunoprecipitation for alpha or beta subunits of CK2 or for BMPRIa was performed and the presence of BMPRIa was observed. Interaction of BMPRIa with alpha and beta subunits of CK2 was observed in untreated cell, and was still present with HD control peptide. When cells were treated with BMP2 or specific peptides, CK2 alpha and beta subunits were released from BMPRIa (Figure4.2). These results demonstrate the specific decrease in the CK2-BMPRIa interaction upon treatment with BMP2 and specific peptides.

4.2.3 Blockage of CK2-BMPRIa interaction induces osteogenesis.

Since stimulation with CK2.1, CK2.2 and CK2.3 resulted in the release of CK2 from BMPRIa, and the release of CK2 in the absence of the ligand leads to BMP2-independent signaling [174], the effect of treatment with the specific peptides on osteoblastic differentiation was determined. In this experiment cells were treated with the specific peptides and the end point differentiation were measured by van Kossa staining. The HD peptide was used as a control peptide at each concentration. Each of the blocking peptides induced osteoblasts differentiation at 100nM (Figure4.3). Additionally, CK2.2 induced mineralization at 500nM (Figure4.3 B) and CK2.3 induced mineralization at 40nM and 500nM (Figure4.3 C). These results suggest that CK2.3 has a broader range of action in osteoblastic differentiation.

To further test these observations, three BMPRIa mutants each lacking one of the three CK2 binding site-MCK2.1 (aa475-479), MCK2.2 (aa324-328), and MCK2.3

(aa213-217) were created, as described in materials and methods. C2C12 cells were transfected with MCK2.1, MCK2.2, or MCK2.3, separately. Transfection with wild type BMPRIa was used as a control. After 5 days, cells were fixed and stained for mineralization with van Kossa stain. MCK2.1 showed slight, but not significant increase, whereas MCK2.2 and MCK2.3 showed significant increase in mineralization compared to the overexpression of BMPRIa (Figure4.4). Since the mutants have only a point mutation of serine to alanine in each of CK2 binding sites, individual mutation results in more specific effects than peptide treatments. MCK2.3 has shown the most mineralization, as did CK2.3. These results illustrate that even though C2C12 differentiation into osteoblasts needs all three CK2 binding sites, the CK2 phosphorylation site located at amino acids 213-217 (CK2.3) shows the greatest effect on cell differentiation.

4.2.4 Blockage of CK2-BMPRIa interaction induces adipogenesis.

To investigate the effect of CK2 blocking peptides have on adipogenesis, C2C12 cells were stimulated with CK2.1, CK2.2, and CK2.3. HD peptide was used as a control peptide for each concentration used. Cells were treated in the same manner as for mineralization experiment and 8 days post stimulation were stained with Oil Red O to visualize lipid droplet formation. Results showed an increase in adipogenesis over the control for all concentrations of CK2.1 (Figure4.5 A). CK2.2 induced adipogenesis only at lower concentrations (Figure4.5 B), whereas CK2.3 seemed to have an inhibitory effect on lipid droplet formation (Figure4.5 C). CK2.1 peptide induced the least amount of mineralization in the previous experiment, but seemed to have the greatest effect on adipogenesis. CK2.3 peptide had the greatest induction of osteogenesis, but an inhibitory effect on adipogenesis. These data were verified with

the use of the BMPRIa mutants, MCK2.1, MCK2.2, and MCK2.3. C2C12 cells were transfected with 3 mg of BMPRIa mutants, MCK2.1, MCK2.2, and MCK2.3. BMPRIa transfected cells were used as a positive control. Obtained results demonstrated that only MCK2.2 had an effect on lipid droplet formation compared to the overexpression of BMPRIa alone, while MCK2.1 and MCK2.3 had no effect (Figure4.6). Since mutants are more specific, conclusion can be drawn that CK2.2 (aa324-328) binding site is crucial for adipocytes differentiation, while both CK2.2 and CK2.3 are needed for osteogenesis.

4.2.5 BMPRIa, Cav1, CK2, and Smad4 are needed for osteogenesis and adipogenesis.

To study the difference of signaling mechanisms involved in the differentiation pathways of osteogenesis and adipogenesis, key players in the BMP2-induced signaling cascade were downregulated. BMP2 signals through Type II and Type I receptors. Since C2C12 cells express only BMPRIa and not BMPRIb, BMPRIa was downregulated. It was demonstrated previously that the initial BMPRIa phosphorylation occurs in caveolae (Figure3.2), thus the effect of Caveolin-1 (Cav1) downregulation was also investigated. The Smad pathway is the most studied pathway and was previously shown to lead to differentiation into osteoblasts and adipocytes; Smad4 was downregulated in this study. CK2 interacts with BMPRIa (Figure4.2), and its release has an effect on both osteogenesis and adipogenesis as was shown previously (Figure4.3-Figure4.6). For this reason the effect CK2 downregulation on differentiation was investigated as well. Since it was shown previously that scramble siRNA does not interfere with signaling (Bragdon et al., unpublished), in this study the siRNAs were used without including control. C2C12 cells were transfected with 3 μg

MCK2.2 and 20 pmoles of siRNA against BMPRIa, CK2, Cav1 or Smad4 where noted. Transfected cells with MCK2.2 alone were used as positive controls, due to its ability to induce both osteogenesis and adipogenesis. After 4 days cells were fixed and stained for mineralization using van Kossa, and lipid droplet formation using Oil Red O. It is not surprising that downregulation of BMPRIa, Cav1, and Smad4 by siRNA significantly decreased cell differentiation into osteoblasts and adipocytes (Figure4.7). Inhibition of differentiation by downregulation of CK2 could be addressed by its inability to bind to BMPRIa due to already mutated interaction site, MCK2.2. More experiments are certainly needed to investigate the role CK2 plays in cell differentiation additionally to its interaction with BMPRIa.

4.2.6 Osteoblastic differentiation occurs through mTor-AKT pathway, and adipocyte differentiation occurs through p38 and NF-kB pathway.

BMP2 signal transduction downstream of receptors utilizes Smad1, 5, and 8, PI3K /AKT, JNK, p38, and NF-κB (Figure1.7 B). According to current literature, Smad and p38 are the two signaling pathways that lead to adipocyte differentiation (Figure1.7 C). Several possibilities could explain why signaling pathways activate the same intermediates but result in two different outcomes. To address the possibility that adipocyte differentiation is initiated by signaling via a different pathway(s) compared to osteoblast differentiation, specific inhibitors were used to shut down specific signaling pathways and the effect on cell differentiation was observed.

To address the question of the optimal concentration of the inhibitors to be used, dose responses of SB203580 (p38), PD98059 (MEK), and Rapamycin were preformed for adipogenesis. C2C12 cells were transfected with MCK2.2 to induce adipogenesis and the next day treated with 1µM, 4µM, and 10µM of SB203580. After

4 days cells were fixed and Oil Red O staining was performed for lipid droplet formation. All concentrations of SB203580 significantly reduced lipid droplet formation (Figure4.8 A). In a separate experiment C2C12 cells were treated with 5µM, 20µM, and 50µM of PD98059 on the next day following transfection with MCK2.2. After 4 days cells were fixed and Oil Red O staining was performed for lipid droplet formation. Only 50µM of PD98059 showed significant inhibition of adipogenesis (Figure4.8 B). Finally, MCK2.2 transfected C2C12 cells were treated with 100nM, 200nM, and 300nM of Rapamycin the day after the transfection in a separate experiment. After 4 days cells were fixed and Oil Red O staining was performed for lipid droplet formation. No significant effect on lipid droplet formation was observed upon treatment with 100nM and 200nM of Rapamycin, but 300nM of Rapamycin significantly inhibited lipid droplet formation (Figure4.8 C).

Next, experiment determining specific inhibitors on differentiation into osteoblasts and adipocytes was done. C2C12 cells were transfected with MCK2.2 to induce adipogenesis and osteogenesis and next day treated with either 1µM SB203580 (p38), 5µM PD98059 (MEK), 300nM Rapamycin (mTOR), 10µM AKT IV inhibitor, 10µM AKT X inhibitor or NF-kB inhibitor (Figure4.9). After 4 days cells were fixed and stained with van Kossa stain to visualize mineralization or Oil Red O staining was performed for lipid droplet formation. Treatments with AKT IV and AKT X inhibitor did not allow for cell survival; they lifted off as a sheet of cells and could not be quantified. Rapamycin significantly inhibited osteogenesis, but MEK and p38 inhibitors had no effect on mineralization, indicating that mTor-AKT pathway is important for osteoblastic differentiation (Figure4.9 A). p38, MEK and NF-κB inhibitors significantly reduced lipid droplet formation, but Rapamycin had no

significant effect on the lipid droplet formation (Figure4.9 B). The difference between this experiment and previous one in the Rapamycin treatment is in the increased sample number (Figure4.8 n=7, and Figure4.9 n=28). Taken together these results show that osteoblast and adipocyte differentiation indeed occur via different pathways in the cell, namely, osteogenesis signals through mTor-AKT pathway, while adipogenesis signal through p38, MEK and NF-κB pathways.

4.2.7 Caspase1 activity enhances adipogenesis, but suppresses mineralization.

Proteomic search revealed a potential Caspase1 cleavage site on the BMPRIa at 310aa. This cleavage site is located between two of the CK2 binding sites, CK2.2 and CK2.3. These two sites seem to be crucial in determining the fate of differentiation of C2C12 cells into osteoblasts and adipocytes. The possibility of Caspase1 activity as a regulator of differentiation was investigated. To test the role Caspase1 has on the C2C12 differentiation, cells were transfected with MCK2.2 to induce adipogenesis and on the next day were treated with different concentrations, 2μM, 4μM, 8μM, 10μM and 12μM, of Z-YVAD-FMK, a specific inhibitor of Caspase1 activity. Transfection with MCK2.2 was used as a control. After 4 days cells were fixed and stained with Oil Red O to visualize lipid droplet formation. Treatment with 2μM of inhibitor did not have an effect on the lipid droplet formation, whereas higher concentrations used, 4μM, 8μM, 10μM, and 12μM of Z-YVAD-FMK had a significant reduction of lipid droplets formation (Figure4.10), demonstrating that Caspase1 plays an important role in adipocyte differentiation.

Next, we investigated if Caspase1 plays an equally important role in osteogenesis as it does in adipogenesis. C2C12 cells were treated with 12μM of Z-YVAD-FMK the next day following transfection with MCK2.2. MCK2.2 transfection alone was used as

a control. After 4 days cells were fixed and stained with van Kossa stain to visualize mineralization (Figure4.11 A) and Oil Red O to visualize lipid droplet formation (Figure4.11 B). Caspase1 inhibitor had a significant effect on both adipogenesis and osteogenesis in this experiment, and the effect was completely opposite. Z-YVAD-FMK treatment inhibited adipogenesis while it enhanced mineralization, illustrating that activity of Caspase1 is a key component for determination of a specific differentiation pathway (osteogenesis vs. adipogenesis).

4.2.8 CK2.3 peptide redirects C2C12 cells from adipogenic lineage to an osteoblastic phenotype.

It is shown that Adipose-Derived Adult Stromal cells (ADAS) from the adipogenic lineage can be redirected into an osteoblastic lineage using BMP2 and retinoic acid (RA) treatment simultaneously [175]. Since the re-directing adipocytes and osteoblasts had been done, the prospect of reversing the differentiation process using specific peptide CK2.3, which induced osteogenesis, but not adipogenesis in C2C12 cells, was investigated. C2C12 cells were transfected with MCK2.2 to induce differentiation to an adipogenic lineage. One or two days following transfection with MCK2.2, cells were treated with the peptide CK2.3 for a total of four days. Treatment with CK2.3 one day after the transfection had no effect on mineralization, but when CK2.3 treatment was done 2 days after the transfection, it increased mineralization and decreased the number of lipid droplets (Figure4.12). These results show that C2C12 cells can be redirected from an adipogenic to an osteoblastic lineage, and that CK2 is the key molecular switch in C2C12 differentiation into osteoblasts and adipocytes.

4.3 Discussion

Age related osteoporosis is a medical condition in which the bones become weak and can break more easily. Increased body adiposity was linked to increased risk for developing age related osteoporosis [160]. With age a shift from osteoblast to adipocyte differentiation is observed in the bone marrow [157-159]. Treatment of bone loss in osteoporotic patients could be designed by finding a way to inhibit adipocyte differentiation and to induce osteoblast production simultaneously. Osteoblasts and adipocytes come from MSC lineage and the mechanism regulating differentiation is poorly understood. One of the key growth factors influencing MSC differentiation is BMP2. While BMP2 has been approved by FDA for spinal fusions, and is demonstrating promising results for reversing the osteoporotic phenotype in mice [3], it also leads to adipocyte differentiation upon treatment. The actual ratio of differentiated osteoblasts to adipocytes upon stimulation was not looked at, and the mechanism of differentiation is unclear. There are some speculations as to why same ligand binding results in two distinct outcomes, but no clear and agreed upon answer was found to date.

BMP2-induced signaling begins with ligand binding to BMPRIa. The possibility of different types of BMP type I receptors being involved is somewhat controversial. Some studies show that BMPRIa is responsible for the differentiation into adipocytes, while BMPRIb is needed for differentiation into osteoblasts [166, 167]. Other studies report that BMPRIa plays a role in osteogenesis and BMPRIb in adipogenesis [168]. While this might be true for some cell lines, C2C12 cell line used in present study does not express BMPRIb mRNA, only BMPRIa mRNA is expressed in this cell line as analyzed by Northern blotting [155]. The ability of C2C12 cells differentiate into osteoblasts and adipocytes (Figure4.1) in the absence of BMPRIb

demonstrates that both differentiation pathways can signal via BMPRIa. Differentiation into osteoblasts vs. adipocytes instead was concentration dependent. This result might be explained in terms of different ligands accessibility to their receptors. It was mentioned before that different receptor oligomerization could be responsible for stem cell differentiation. Receptors exist in preformed complexes (PFCs) which are believed to be located in CCPs and in BMP2-induced signaling complexes (BISCs) which are in caveolae [49, 50]. BISCs signaling believed to result in p38 signaling, while PFCs initiates Smad signaling [52]. As discussed in the previous chapter, there are some conflicts in this proposed signaling model and results presented in Chapter 3 demonstrate the need to revise this dogma. In the new signaling model both CCPs as well as caveolae are involved in Smad-dependent and Smad-independent signaling. Hence, the hypothesis that PFCs signal initiating adipogenesis and BISCs signal activates osteogenesis could not be correct unless PFCs and BISCs are present in mixed populations in caveolae as well as in CCPs.

Casein Kinase 2 (CK2) was identified to be interacting with BMPRIa in the absence of stimuli and this interaction was lost upon BMP2 stimulation (Figure4.2). There are three sites for potential binding/phosphorylation sites on BMPRIa for CK2 at amino acid residues 213-217 (CK2.3), 324-328 (CK2.2), and 475-479 (CK2.1) (Figure1.3), which are conserved throughout TGF-beta Type I receptor. The importance of these interaction sites was tested by designing specific peptides to block this interaction, CK2.1, CK2.2, and CK2.3. Loss of interaction between CK2 and a specific site on the BMPRIa lead to differentiation into osteoblasts and adipocytes depending on the specific interaction site (Figure4.3- Figure4.6). One of the CK2 binding sites on the BMPRIa, CK2.3, was identified to specifically induce

osteogenesis and inhibit adipogenesis. In this, CK2.3 peptide showed some promising results not only as a specific inducer of osteoblast differentiation, but also showed a potential in redirecting adipocytes into the osteoblast lineage (Figure4.12). *In vitro* experiments certainly need to be repeated using an *in vivo* system, but presently, CK2.3 possesses a great potential in therapeutic treatment for osteoporotic patients.

The difference in signaling pathways downstream of receptors was addressed in this study. Presented results demonstrate that osteogenesis utilizes Smad4 and mTor-AKT pathway, while adipogenesis signal via Smad4, MEK, and p38 pathways (Figure4.7-Figure4.8). Extensive research has been done to address differences in Smad4 and p38 pathways in the differentiation already, but this difference seems to be dependent on the cell types. In C3H10T1/2 cells both p38 and Smad were shown to be required for differentiation into adipocytes by inducing and upregulating PPARγ [90]. In trabecular bone-derived osteoblasts p38 and Smad pathways were shown to be important for induction of osteoblast maturation [68]. While PI3K was shown previously to be activated by BMP2 stimulation [88, 93], no solid evidence was presented for the mTor-AKT pathway in the osteogenesis in C2C12 cell differentiation. Additionally, BMP2-induced activation of PI3K/AKT pathway is shown to be implicated in a number of cancers (prostate, gastric, pancreatic, ovarian cancers, and melanoma) by promoting epithelial-mesenchymal transition (EMT) in these transformed cells which results in cancer cells motility and increased invasiveness [176]. In the current study, mTor-AKT pathway showed to be important for stem cell differentiation of C2C12 cells into osteoblasts.

As one of potential regulators of adipogenesis, Caspase1 involvement in differentiation was analyzed. Caspase1 was shown to be upregulated during adipocyte

differentiation and its activity is increased both in genetically susceptible and diet-induced obese animal models. *In vivo* treatment of obese mice with a Caspase1 inhibitor significantly increases their insulin sensitivity [171]. In the presented study, inhibition of Caspase1 inhibited adipogenesis but enhanced osteogenesis demonstrating its important role in cell differentiation (Figure4.9). Caspase1 cleavage site (aa 310) on the BMPRIa is located just below CK2.3 phosphorylation site (aa 213-217), but above the CK2.2 (aa 324-328), and CK2.1 (aa 475-479) sites. The new proposed signalling model of BMP2-induced MSC differentiation lies in the release of CK2 from different CK2-BMPRIa interaction. Release of CK2 from CK2.3 interaction site regulates differentiation into osteoblasts and release from CK2.2 interaction site regulates differentiation into adipocytes (Figure4.13). Once CK2 gets released from the CK2.2 binding site, it is proposed to make a cleavage site available for Caspase1. Caspase1 cleaves BMPRIa at aa 310 and this cleavage results in adipogenesis. Another possibility is that once released from BMPRIa, CK2 can either phosphorylate PPARγ to cause its activation or phosphorylate Caspase1, causing its activation. PPARγ has a potential to be phosphorylated by CK2 at S492, but such phosphorylation has never been observed [177].

The key differences in the cell differentiation pathway leading to osteoblast and adipocyte differentiation were identified. This opened a new chapter of the research related to BMP2-dependent stem cell signaling, and raised questions that hopefully will be answered in a near future.

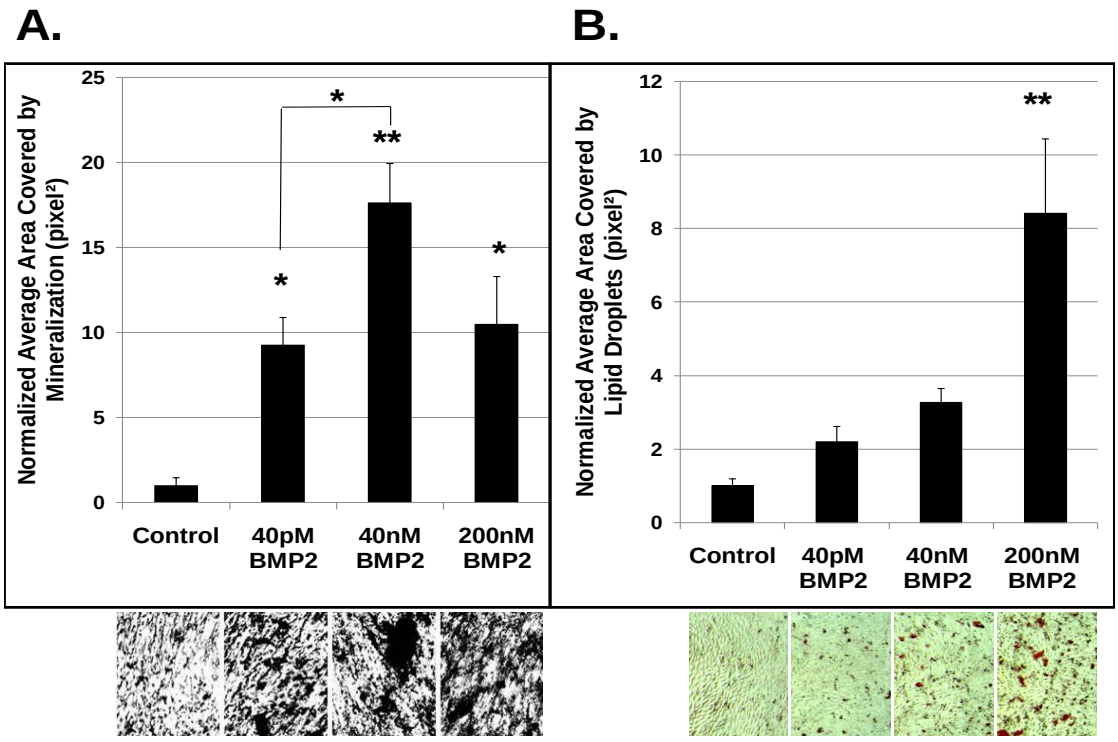


Figure4.1 BMP2 induces osteogenesis and at high concentration adipogenesis. C2C12 cells were treated with 40 pM BMP2, 40 nM BMP2, and 200 nM BMP2 and stained for (A) mineralization using the van Kossa stain (B) or lipid droplet formation using Oil Red O stain. At least 4 independent experiments were performed (n=13) and averages are graphed with SEM as error bars. Data was normalized to control. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to control.

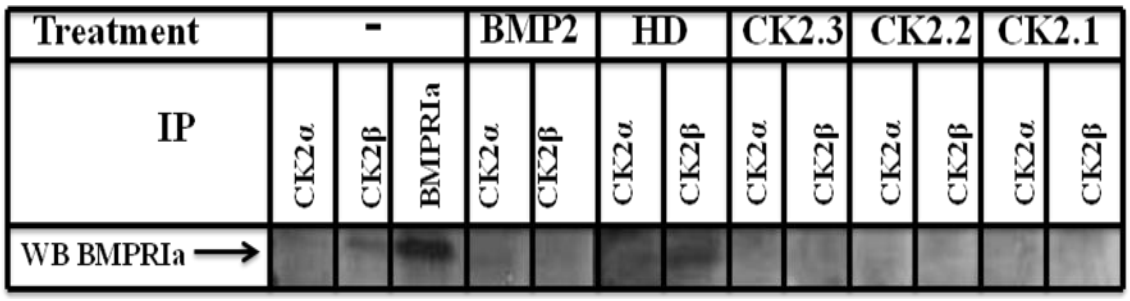


Figure4.2 CK2 Interacts with BMPRIa and gets released upon treatment with BMP2 and peptides CK2.1, CK2.2 and CK2.3. C2C12 cells were either stimulated or not stimulated with BMP2 (40 nM), or HD, CK2.1, CK2.2, CK2.3 (100 nM). Immunopersipitation for alpha or beta subunits of CK2 or for BMPRIa was performed and the presence of BMPRIa was detected. Interaction of BMPRIa with alpha and beta subunits of CK2 is observed in untreated cell, and was still present with HD treatment. When cells were treated with BMP2 or specific peptides designed to block this interaction, CK2 alpha and beta subunits got released from BMPRIa.

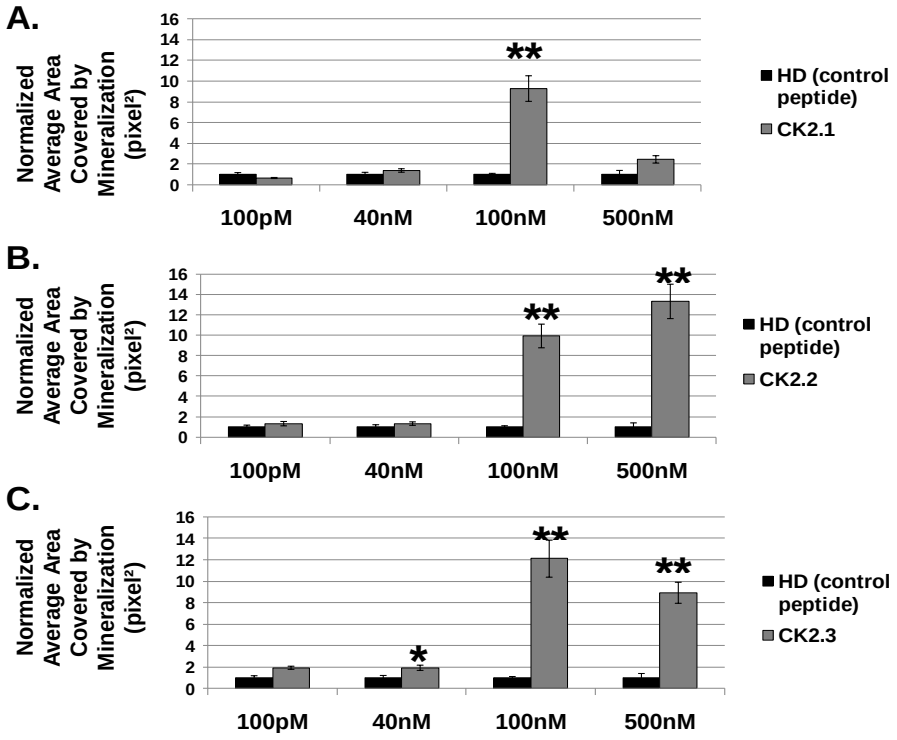


Figure4.3 Blockage of CK2-BMPRIa interaction induces osteogenesis. C2C12 cells were treated with 100 pM, 40 nM, and 500 nM of designed peptides and stained for mineralization using the van Kossa stain. (A) CK2.1 induced mineralization at 100nM, (B) CK2.2 induced mineralization at 100nM and 500nM, and (C) CK2.3 induced mineralization at 40nM, 100nM and 500nM compared to HD (control peptide). At least 4 independent experiments were performed (n=10) and averages are graphed with SEM as error bars. Data was normalized to HD (control peptide) at each concentration. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to HD (control peptide) at each concentration.

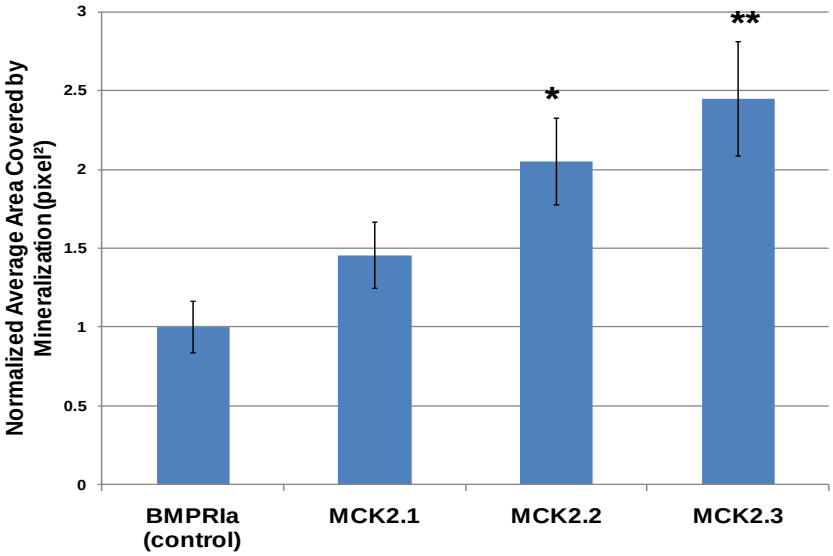


Figure4.4 BMPRIa mutants lacking CK2 binding sites induce osteogenesis. Mutants for each of the CK2 binding sites of BMPRIa were subcloned into dsRFP vector and BMPRIa-RFP was used as a control. Constructs were transfected in C2C12 cells and stained for mineralization using the van Kossa stain. MCK2.2 and MCK2.3 induced mineralization compared to BMPRIa overexpression. At least 4 independent experiments were performed (n=19) and averages are graphed with SEM as error bars. Data was normalized to BMPRIa (control). * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to BMPRIa.

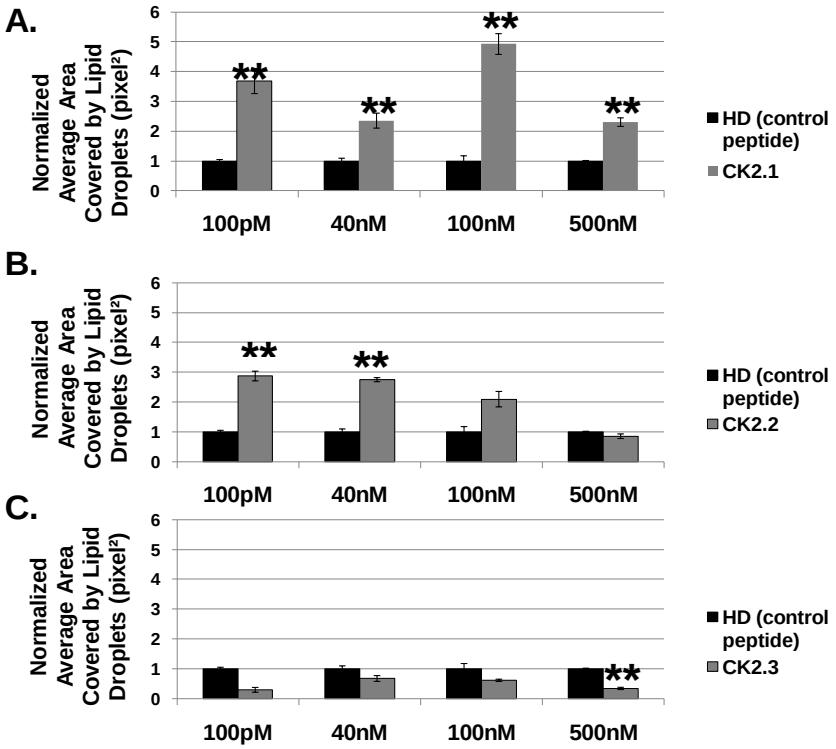


Figure4.5 Blockage of CK2.1-BMPRIa and CK2.2-BMPRIa interaction induces adipogenesis. C2C12 cells were treated with 100 pM, 40 nM, and 500 nM of designed peptides and stained for lipid droplet formation using Oil Red O stain. (A) CK2.1 induced adipogenesis at all concentrations, (B) CK2.2 induced adipogenesis at 100pM, 40nM and 100nM, and (C) CK2.3 inhibited adipogenesis compared to HD (control peptide). At least 4 independent experiments were performed (n=6) and averages are graphed with SEM as error bars. Data was normalized to HD (control peptide) at each concentration. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to HD (control peptide) at each concentration.

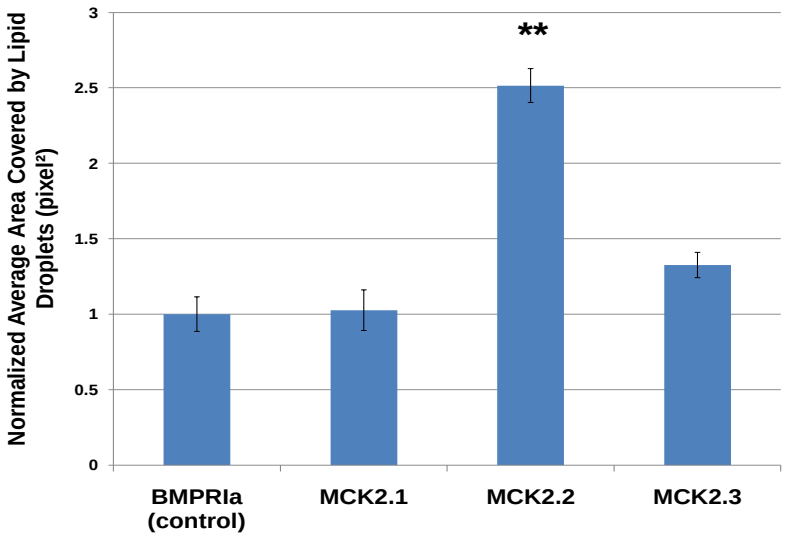


Figure4.6 BMPRIa mutant lacking CK2.2 binding site induces adipogenesis. Mutants for each of the CK2 binding sites of BMPRIa were subcloned into dsRFP vector and BMPRIa-RFP was used as a control. Constructs were transfected in C2C12 cells and stained for lipid droplet formation using Oil Red O stain. MCK2.2 induced adipogenesis compared to BMPRIa overexpression. At least 4 independent experiments were performed (n=18) and averages are graphed with SEM as error bars. Data was normalized to BMPRIa (control). * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to BMPRIa.

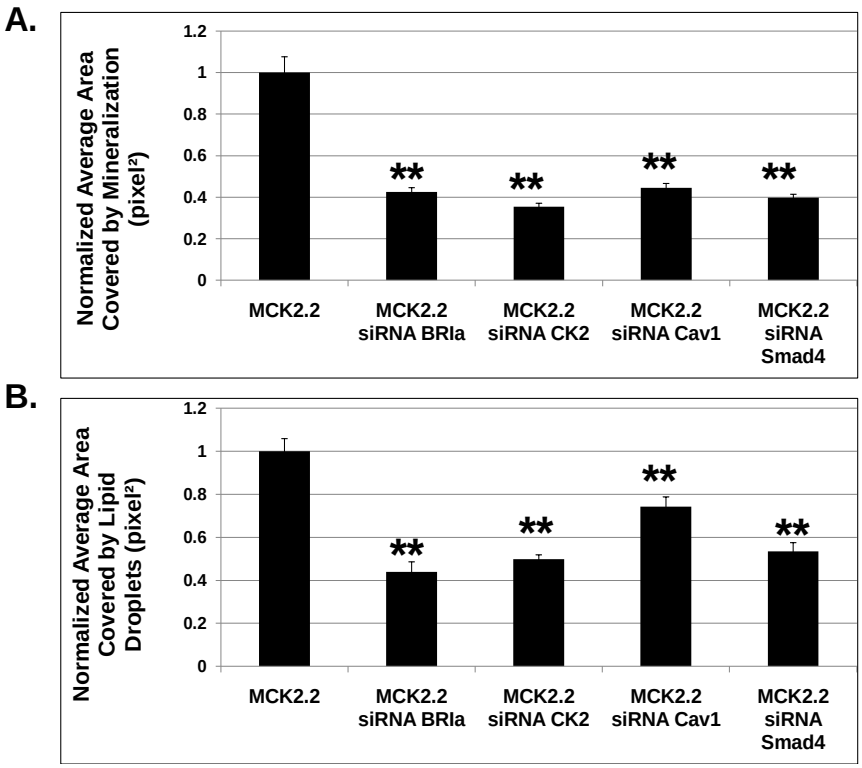


Figure4.7 Downregulation experiment with siRNAs demonstrated that BMPRIa, CK2, Cav1 and Smad4 are important for osteogenesis and adipogenesis. C2C12 cells were transfected with 3 µg MCK2.2 and 20 pM of siRNA against BMPRIa, CK2, Cav1 or Smad 4 where noted. Transfected cells with MCK2.2 alone were used as controls. Cells were stained for (A) mineralization using van Kossa staining or (B) lipid droplet formation using Oil Red O staining. At least 4 independent experiments were performed (n≥15) and averages are graphed with SEM as error bars. Data was normalized to MCK2.2. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to MCK2.2.

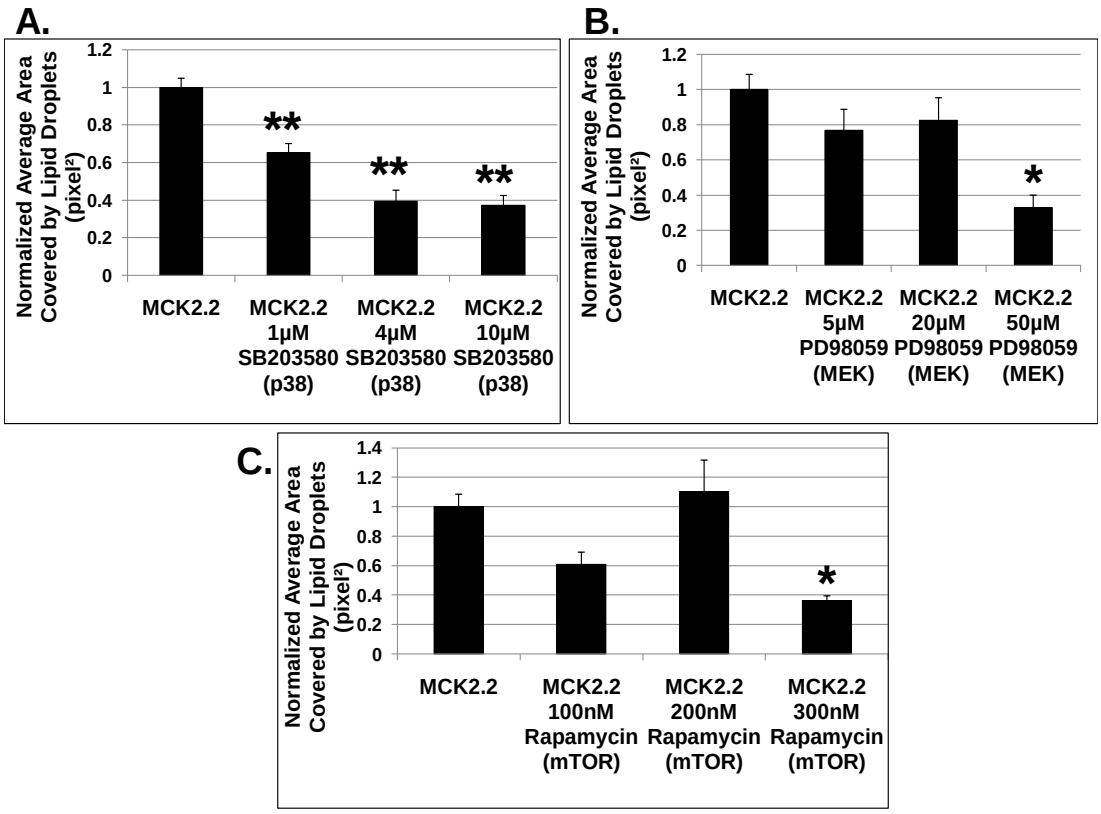


Figure4.8 Effect of SB203580 (p38), PD98059 (MEK), and Rapamycin (mTOR) specific inhibitors on adipogenesis. C2C12 cells were treated with inhibitors and stained for lipid droplet formation using Oil Red O stain. (A) SB203580 shows significant inhibition of adipogenesis with all concentrations used. (B) PD98059 shows significant inhibition of adipogenesis at 50μM concentration. (C) Rapamycin shows significant inhibition of adipogenesis at 300nM. Two independent experiments were performed (n≥7) and averages are graphed with SEM as error bars. Data was normalized to MCK2.2. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to MCK2.2.

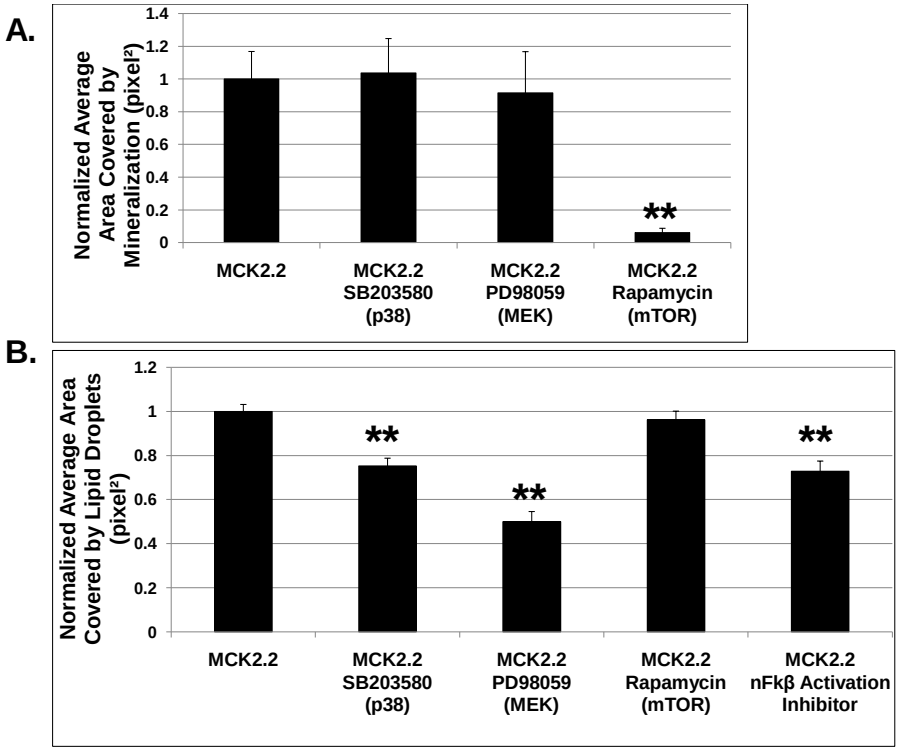


Figure4.9 Osteoblastic differentiation occurs through mTor-AKT pathway, and adipocyte differentiation occurs through p38, MEK and NF-kB pathway. C2C12 cells were transfected with MCK2.2 and treated with 1µM SB203580 (p38), 5µM PD98059 (MEK), 300nM Rapamycin (mTOR), and NF-kB inhibitor where noted. Transfected cells with MCK2.2 alone were used as controls. Cells were stained for (A) mineralization using van Kossa staining or (B) lipid droplet formation using Oil Red O staining. At least 4 independent experiments were performed (n≥19) and averages are graphed with SEM as error bars. Data was normalized to MCK2.2. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to MCK2.2.

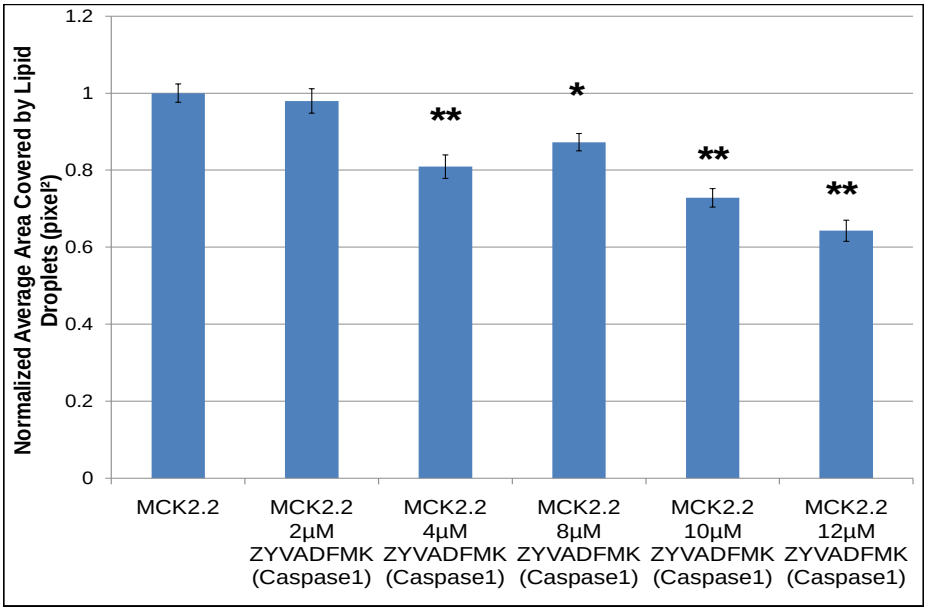


Figure4.10 Concentration effect of Z-YVAD-FMK specific inhibitor for Caspase1 shows significant inhibition of adipogenesis. C2C12 cells were transfected with 3 μg MCK2.2 and treated with 2μM, 4μM, 8μM, 10μM, and 12μM of Z-YVAD-FMK where noted. Transfected cells with MCK2.2 alone were used as controls. Oil Red O stain was performed to visualize lipid droplets formation. At least 4 independent experiments were performed (n≥32) and averages are graphed with SEM as error bars. Data was normalized to MCK2.2. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to MCK2.2.

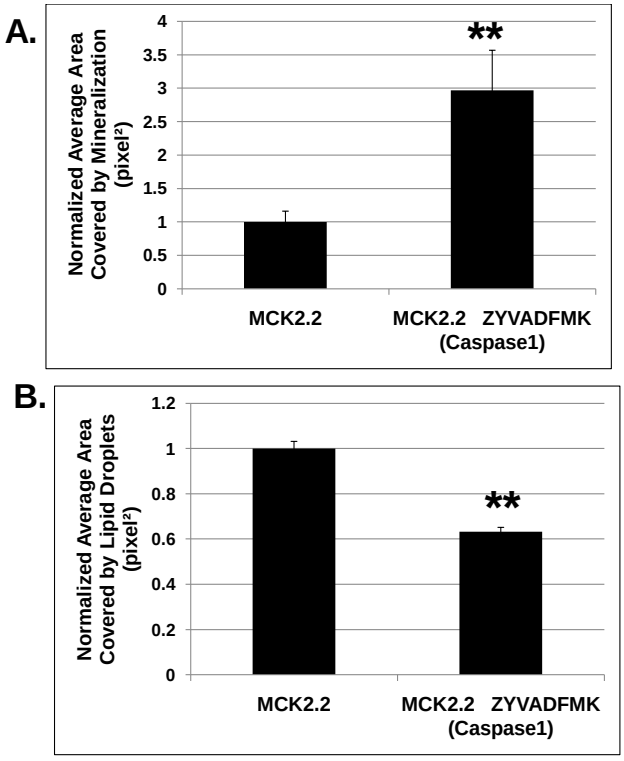


Figure4.11 Caspase1 inhibitor enhances mineralization, but suppresses adipogenesis. C2C12 cells were transfected with 3 µg MCK2.2 and treated with Z-YVAD-FMK. Transfected cells with MCK2.2 alone were used as controls. Cells were stained for (A) mineralization using van Kossa staining or (B) lipid droplet formation using Oil Red O staining. At least 4 independent experiments were performed (n≥17) and averages are graphed with SEM as error bars. Data was normalized to MCK2.2. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to MCK2.2.

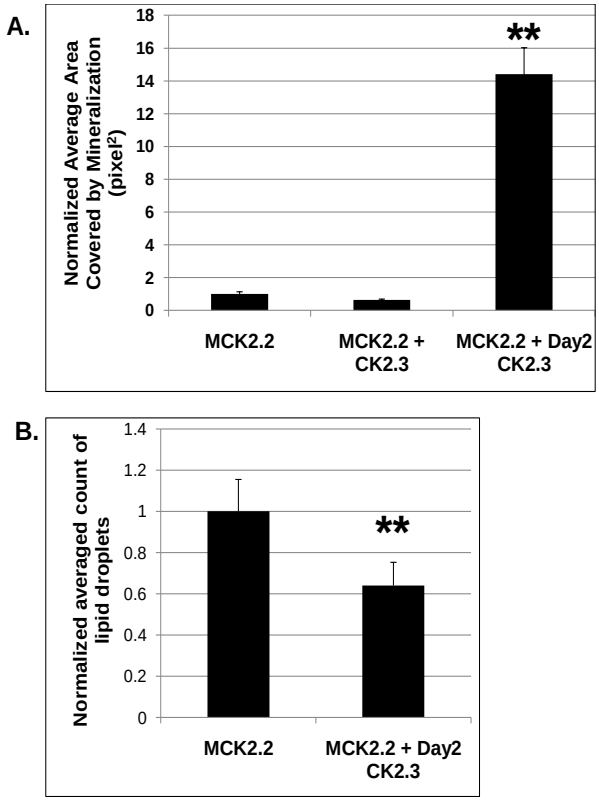


Figure4.12 CK2.3 peptide redirects C2C12 cells from adipogenic lineage to an osteoblastic phenotype. C2C12 cells were transfected with MCK2.2 and treated with CK2.3, where noted, on the next day or two days later. Transfected cells with MCK2.2 alone were used as controls. Cells were stained for (A) mineralization using van Kossa staining or (B) lipid droplet formation using Oil Red O staining. At least 2 independent experiments were performed and averages are graphed with SEM as error bars. Data was normalized to MCK2.2. * significantly different ($p<0.05$) ** significantly different ($p<0.01$) relative to MCK2.2.

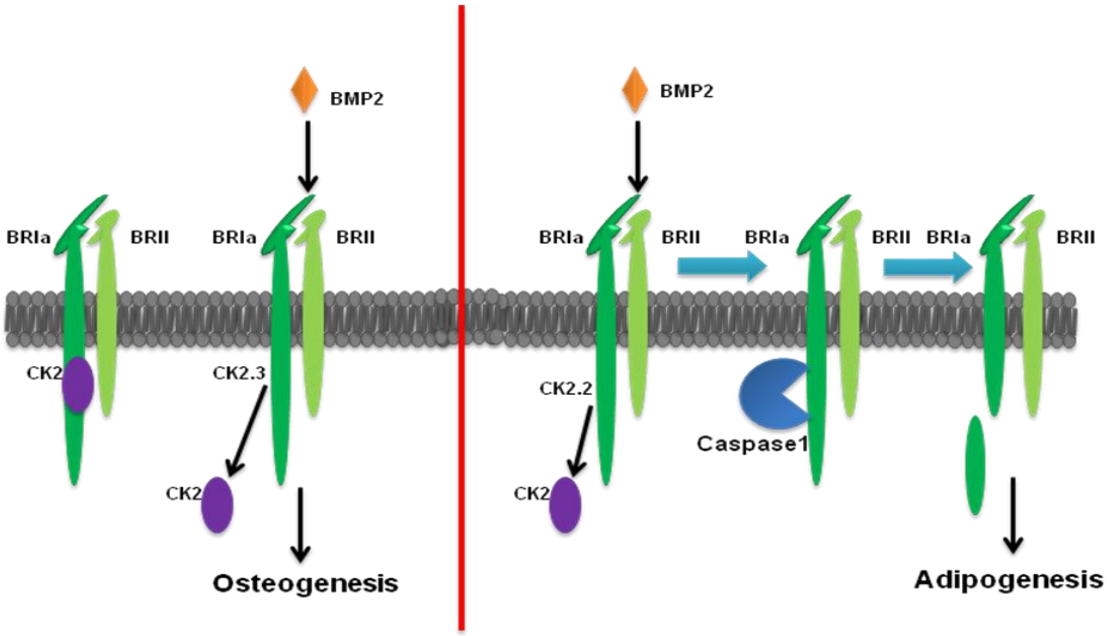


Figure4.13 New proposed model of BMP2-induced MSC differentiation. Release of CK2 from different CK2-BMPRIa interaction sited regulates differentiation into osteoblasts (release from CK2.3, aa 213-217), and adipocytes (release from CK2.2, aa 324-32). Once CK2.2 gets released from CK2.2 binding site, it is proposed to free cleavage site for the Caspase1. Caspase1 cleaves receptor at aa 310 and this cleavage results in adipogenesis.

Chapter 5

SUMMARY AND PROSPECTUS

Age related osteoporosis is a medical condition in which the bones become weak and can break easily. Treatment of osteoporosis is costly, and quality of life becomes significantly less than average. As of today, about 10 million people in the U.S. already have the disease and another 34 million are at risk (http://www.nof.org/home). It is estimated that by 2025 age related osteoporosis will be responsible for approximately three million fractures resulting in $25.3 billion in costs in the U.S. annually (http://www.nof.org/home). Majority of the existing drugs target osteoclasts maturation or activity (bisphosphonates and SERMS), but do not increase bone remodeling which is needed to maintain bone homeostasis. PTH is currently the only treatment available that increases bone turnover, but needs to be administered by daily injections for a period of 2 years at the right concentration to return bone mineral density to its pre-osteoporotic density [178]. Additional treatment which would increase bone mass are desperately needed. Such treatments would be targeted on increasing the osteoblast differentiation from MSCs.

BMPs initially were identified in bone extracts, and are important for bone development. Recently their emerging role is an involvement in the regulation of cell proliferation, differentiation, chemotaxis and apoptosis in a variety of tissues [30]. BMPs play important role in a vast number of developmental processes of various tissues, including lung, skin, brain, eye, liver, and kidney. BMP2 is of a special interest in determining MSCs differentiation into osteoblasts [39], since it was approved by Food and Drug Administration for spinal fusion and fracture repair (www.fda.org).

Due to its ability to drive MSC differentiation towards osteoblasts, it also could be a potential treatment of osteoporotic patients. *In vivo* studies using osteoporotic mice models show promising results: systemic injections of recombinant human BMP2 reverse the osteoporotic phenotype, increase number of MSCs, increase osteoblast activity and decrease apoptosis [3]. Analysis of osteoporotic patients from a BMP2 study revealed that patients indeed have low serum levels of BMP2 and high titer of BMP2 antibodies [5]. It also has been shown that variants in the *BMP2* gene elevate likelihood of developing osteoporosis [6, 7]. However there are drawbacks to BMP2 treatments. In order to work properly, high doses of BMP2 are needed (1.5mg/ml), BMP2 delivery is via injections, plus BMP2 has multiple effects on the stem cell differentiation driving MSCs into adipocytes and osteoblasts by a mechanism yet to be fully understood.

BMP2 signaling cascade begins when BMP2 binds to heteromeric receptor complexes consisting of at least one BMPRIa and one BMPRII. BMPRIIs are constitutively active kinases which, upon binding of BMP2, phosphorylate BMPRIa at a glycine/serine-rich juxtamembrane region, called the GS-box. Upon phosphorylation, serine/threonine kinase domain of BMPRIa becomes activated and transduces signal downstream [45-47]. Presently, there are two receptor complexes found in the BMP2 signaling: PFCs and BISCs. BMP2 can bind with high affinity to BMPRIa and this interaction recruits BMPRII to the complex (BISC), or it can bind to PFC [49, 50]. It is believed that BMP2 binding to PFCs induces Smad-dependent, and binding to BISCs induces Smad-independent signaling pathways [51, 52]. Smad-independent signaling pathways include, p38, JNK, ERK, PI3K, and NF-kB [9, 88] (Figure1.7). The mechanism of activation of different downstream effectors is not known, but it is

hypothesized that different domains on the plasma membrane could influence the availability of intracellular proteins.

BMP receptors are found in caveolae and CCPs. Caveolae are flask shaped invaginations of the plasma membrane enriched in Caveolin-1 (Cav1), scaffolding protein responsible for caveolae shape. Several research groups studied differences of involvement of CCPs and caveolae in distinct signaling cascades, but the current literature seems conflicting. Current dogma of BMP2-induced signaling states that CCPs are needed for Smad signaling and that Smad-independent signals delivered via lipid rafts [51]. Yet in another study it was shown that following BMP2 stimulation, redistribution of receptors on the plasma membrane occurred: over 35% of BMPRIa moved into CCPs [57]. This suggests that in order to signal receptors had to be in caveolae or on the plasma membrane. Also an increase in Smad phosphorylation and quicker Smad translocation into the nucleus was observed when CCPs were disrupted [57, 153]. Such conflicting reports have raised a question of where does BMP2-induced signaling takes place on the plasma membrane.

The first step in BMP2 signal transduction is phosphorylation of BMPRIa in the GS box by constitutively active BMPRII upon ligand binding. In the present study the question of the location of this phosphorylation on the plasma membrane was addressed. Results presented indicate that initial BMPRIa phosphorylation occurs in detergent resistant membrane (DRM) and caveolae fractions, as determined by sucrose gradient. Phosphorylated BMPRIa was found in caveolae following 30 minutes of BMP2 stimulation (Figure3.2). Additionally, slightly phosphorylated BMPRIa was found in caveolae in the absence of stimuli. This phosphorylation does not appear to be the activating phosphorylation by BMPRII in the GS box, which can be seen upon

BMP2 stimulation. Instead it could be an inhibitory phosphorylation. In this experiment, cells were serum starved for at least 18 hours, and had to be in the cell cycle arrest in the absence of growth factors. This phosphorylation could be by CK2, and could explain why BMPRII does not phosphorylate BMPRIa in the absence of the ligand when receptors are in preformed complexes. It could also be phosphorylation by FKBP12, which was shown previously to phosphorylate BMPRIa in MLB13MYC clone 17 (C17), a limb bud-derived cell line [179]. FKBP12 is a negative regulator of BMP2 signaling and is released upon BMP2 stimulation [180]. Further analysis is needed to address this basal level phosphorylation of BMPRIa in the absence of stimuli. To address possibility of BMPRIa phosphorylation by CK2, specific sites of interaction between BMPRIa and CK2 could be blocked and phosphorylation can be detected. Such an experiment could be achieved by immunoprecipitation studies for BMPRIa done in the presence and in the absence of specific peptides (CK2.1, CK2.2, and CK2.3), or by overexpression of mutants lacking interaction sites (MCK2.1, MCK2.2, and MCK2.3). If CK2 phosphorylates BMPRIa in the absence of stimuli, the receptor would not be found phosphorylated when interaction would be blocked. However, if the BMPRIa would be phosphorylated upon blockage of the CK2-BMPRIa interaction, that result would indicate phosphorylation by another kinase and further work on identification of such a kinase would have to be done.

Upon phosphorylation, BMPRIa serves as a signal transducer into the cell, by interacting, and activating downstream signaling molecules. Smad was shown to be phosphorylated as early as 15 minutes after BMP2 stimulation and reaches its peak of activation at 30-60 minutes in P19 cells [156]. In the present study, the location of Smad phosphorylation on the plasma membrane was examined at 0, 10, 30 and 45

minutes following BMP2 stimulation. Smad1, 5, and 8 phosphorylation was observed 10 minutes after stimulation with 40 nM of BMP2 in detergent resistant membrane (DRM) fractions. After 30 minutes of stimulation with 40 nM BMP2, phosphorylated Smad1, 5, and 8 shifted slightly, but remained in DRM fraction. After 45 minutes stimulation, phosphorylated Smad1, 5, and 8 seems to be degraded in endosomes (Figure3.3). These results demonstrate a need for the current dogma of BMP2 signaling to be revised (Figure3.1). The new model would have to take into an account the initiation of Smad-dependent signaling in DRMs.

Membrane localization of BMPRIa and Smad phosphorylation is an important step in the understanding of BMP2-induced signaling. New therapeutic applications could be designed to target Smad signaling by targeting caveolae. Additionally it could explain concentration dependency of BMP2 on MSC differentiation into osteoblasts and adipocytes. It was observed that all tested concentrations of BMP2 (40 pM, 40 nM, and 200 nM) resulted in osteogenesis, but only high concentration (200 nM) of BMP2 resulted in adipogenesis (Figure4.1). BMP2 binds with higher affinity to its receptors in caveolae (Bonor et al., unpublished) and initiation of signal is achieved upon cell treatment with 40 nM BMP2 in caveolae. It could be hypothesized that osteogenesis results from ligand binding to its receptors in caveolae. When all receptors in caveolae are occupied, BMP2 binds to BMPRIa in CCPs and results in adipogenesis. To test these hypothesis disruption studies of caveolae and CCPs could be done, and the effect of cell differentiation could be monitored by measurement of osteoblast and adipocyte formation.

Presently, the difference in the signaling pathways resulting in adipogenesis vs. osteogenesis is not uncovered. This difference must be in the downstream

effectors of BMPRIa. Upon BMP2 stimulation two major signaling pathways are activated, Smad-dependent and Smad-independent, as previously discussed. BMP2-induced signaling pathways are shown to result in adipogenesis, such as Smad1, 5, and 8, and p38 pathway (Figure1.7 C). The same pathways in addition to PI3K, ERK, NF-kB, and JNK induce osteogenesis (Figure1.7 B). In this study, an attempt was made to uncover the differences between osteoblast and adipocyte differentiation and to identify key players. Since BMPRIa was observed to be phosphorylated in the absence of BMP2 stimulation, potential binding and phosphorylation sites of BMPRIa using Prosite search was performed. This search revealed three sites at which the CK2 alpha subunit could phosphorylate BMPRIa at amino acids 213-217 (SLKD), 324-328, (SLYD) and 475-479 (SYED). CK2 is a highly conserved, ubiquitously expressed enzyme with more than 300 substrates that affects cell growth, proliferation, differentiation, apoptosis and tumorigenesis [76]. CK2 is detected predominantly in the nucleus, while fractions have also been isolated from cytoplasm, plasma membrane, Golgi apparatus, Endoplasmic Reticulum (ER) and ribosomes. In all cases, the activity is localized to the membrane-associated cell fractions [77]. To investigate whether there is an interaction between CK2 and BMPRIa, an immunoprecipitation study was preformed and an interaction between the BMPRIa and CK2 in the absence of BMP2 and loss of it upon BMP2 stimulation was observed (Figure4.2). Therefore, there is a strong possibility that CK2 is involved with BMP2 signaling. Peptides were designed with the Antennapedia homeodomain signal sequence for cellular uptake and incorporated one of these binding sites, CK2.1 (SYED), CK2.2 (SLYD), and CK2.3 (SLKD). To verify that these peptides block the interaction between CK2 and BMPRIa, another immunoprecipitation study was

performed and no interaction between CK2 and BMPRIa with peptides stimulation was observed (Figure4.2). Additionally, loss of interaction between CK2 and a specific site on the BMPRIa lead to differentiation into osteoblasts and adipocytes depending on the specific interaction site (Figure4.3- Figure4.6). One of the CK2 binding sites on the BMPRIa, CK2.3, was identified to specifically induce osteogenesis and inhibit adipogenesis. *In vivo* intracranial injections of CK2.3 into mice calvaria of four-week old C57L/6J mice induced significant bone formation over the PBS and HD controls as measured by bone area (Bragdon et al., unpublished). Additionally, CK2.3 peptide treatment resulted in a specific induction of osteoblast differentiation, and showed a potential to redirect adipocytes into the osteoblasts (Figure4.12). Ongoing experiments by Bragdon et al. were designed to address the possibility of CK2.3 peptide to redirect primary adipocytes isolated from bone marrow of B6.C3H-6T mice that mimic osteoporotic phenotype. Bone marrow cells were isolated, primary adipocytes were cultured, treated with CK2.3 peptide and soon the ability of CK2.3 peptide to redirect adipocytes into osteoblasts is going to be measured. Additionally, injections of CK2.3 immobilized in a 1:2 solution of basement membrane Matrigel (successfully used previously in our lab) into bone marrow of B6.C3H-6T mice need be done. After 10 days mice should be sacrificed and bone marrow content of adipocytes and bone area should be measured. *In vivo* experiments would provide useful pre-clinical insights for using CK2.3 treatment on osteoporotic patients.

Furthermore, this study demonstrates that osteogenesis signals via Smad4 and mTor-AKT pathway, while adipogenesis signal via Smad4, MEK, NF-kB and p38 pathways (Figure4.7-Figure4.8). It was previously shown that both p38 and Smad are

important signaling pathways for osteogenesis and adipogenesis [68, 90]. While BMP2-induced activation of PI3K/AKT pathway is shown to increase motility and invasiveness of cancer cells [176], here it is shown to directly influence C2C12 cell differentiation into osteoblasts. The difference in such responses might be explained by different cell lines used, or by utilization of different intracellular molecules downstream of PI3K and needs to be addressed.

Furthermore, a Prosite search revealed a Caspase1 cleavage site (aa 310) on the BMPRIa, just below CK2.3 phosphorylation site (aa 213-217), but above the CK2.2 (aa 324-328), and CK2.1 (aa 475-479) sites. Caspase1 was shown to be upregulated during adipocyte differentiation and its activity is increased both in genetically susceptible and diet-induced obese animal models. *In vivo* treatment of obese mice with a Caspase1 inhibitor considerably increased their insulin sensitivity [171]. Inhibition of Caspase1 significantly reduced lipid droplet formation, marker of adipogenesis, but enhanced osteogenesis demonstrating importance in cell differentiation (Figure4.11). It was previously shown that CK2 phosphorylates Caspase9 and this phosphorylation does not allow for Caspase9 activation by Caspase8 in the Fas ligand induced apoptosis pathway [181]. This inhibition of the extrinsic apoptosis pathway by CK2 could explain why CK2 activity is associated with poor cancer prognosis and tumorigenesis [76]. Also, interaction with another member of caspase family only highlights the importance to study CK2-Caspase1 interactions to better understand the complex process of stem cell differentiation regulation.

Taken together, our results propose a new signalling model of BMP2-induced MSC differentiation. This model is based on the release of CK2 from different CK2-BMPRIa interactions. Release of CK2 from CK2.3 interaction site regulates

differentiation into osteoblasts and release from CK2.2 interaction site regulates differentiation into adipocytes (Figure4.13). Once CK2 gets released from the CK2.2 binding site, it is proposed to make a cleavage site available for Caspase1. Caspase1 cleaves BMPRIa at aa 310 and this cleavage results in adipogenesis. To further study involvement of Caspase1 in differentiation, BMPRIa receptor was mutated (D to E) at the Caspase1 cleavage site (310) and overexpression of this mutant should be studied in its ability to drive osteogenesis and adipogenesis. According to preliminary data, mutation of Caspase1 cleavage site should result in enhancement of mineralization and inhibition of adipogenesis mimicking treatment with the inhibitor. Another possibility is that once released from BMPRIa, CK2 can either phosphorylate PPARγ to cause its activation (PPARγ has a potential to be phosphorylated by CK2 at S492, but such phosphorylation has never been observed [177]), or phosphorylate Caspase1, causing its activation. Additionally, caveolae were identified as regions of the plasma membrane involved in initiation of BMP2 signaling. Our model would have to include caveolae, since they were shown to be sites of insulin signaling pathway that leads to translocation of GLUT4 (glucose transporter) to the plasma membrane [182], thus have to be important not only in the initiation of signaling, but throughout life of adipocyte.

The current study has improved current knowledge of BMP2-induced cell differentiation into osteoblasts and adipocytes. Involvement of CK2 in regulation of MSC differentiation opened a possibility for developing treatments targeted for increasing osteoblasts and decreasing adipocytes, leading to improvement of bone strength. Specific CK2.3 peptide could be used for induction of osteoblast production in osteoporotic patients. It could be delivered to bone by absorbable collagen sponge

(ACS) [183], or by coupling it to fibrin and poly(lactic-co-glycolic acid) (PLGA) nanoparticles for directed release [184]. This targeted differentiation has a potential of not only utilizing MSC pool into bone forming cells, but it could also decrease adipogenesis in patients. Such specific targeting could restore bone homeostasis by restoring bone turnover. About 10% of human skeleton gets remodeled every year, resulting in stronger bones. Bones function lies not only in the body support, but also in calcium and phosphates storage needed for maintaining body homeostasis [1]. By targeting osteoblast production, but not osteoclasts function, body homeostasis can be restored in the presence of healthy bones.

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