

**OSTEOGENESIS AND ADIPOGENESIS CONTROL IN THE BMP
SIGNALING PATHWAY**

by

Joyita Dutta

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Joyita Dutta

Approved: _____
Anja Nohe, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Randall L.Duncan, Ph.D.
Chair of the Department of Bioical Scienes

Approved: _____
George H.Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____
Charles G. Riordan, Ph.D.
Vice Provost for Graduate and Professional Education

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TABLE OF CONTENTS

LIST OF TABLES	viii
LIST OF FIGURES	ix
ABSTRACT	xii
Chapter	
1 INTRODUCTION.....	1
1.1 Bone: The Dynamic Mineralized Structural Tissue.....	1
1.2 Osteoporosis	3
2 BONE MORPHOGENETIC PROTEINS RECEPTORS AND PATHWAYS	5
2.1 Bone Morphogenetic Proteins (BMPs)	5
2.2 BMP Receptors.....	7
2.3 Coreceptors of the BMP Signaling Pathway	9
2.4 Receptor Activation.....	13
2.5 Caveolae and Clathrin Coated Pits (ccps)	15
2.6 Proteins interacting with the Receptors	20
2.7 Casein Kinase 2 (CK2).....	21
2.8 Smad dependent Pathway	24
2.9 Smad independent Pathway.....	26
2.10 Ligand independent Pathway	27
3 ADIPOGENESIS	32
3.1 Adipocyte Differentiation.....	32
3.2 BAT, WAT and BRITE.....	33
4 MATERIALS AND METHODS	43
4.1 Materials	43
4.2 Primer Sequences	43
4.3 Cell Culture	44
4.4 Design of CK2 Peptides	44
4.5 Design of BMPR1a lacking CK2 binding site	45
4.6 Isolation of RNA by Triazol method.....	45

4.7	Isolation of RNA by Ambion RNA Mini Prep.....	46
4.8	Reverse Transcription Reaction	47
4.9	PCR.....	47
4.10	Van Kossa.....	48
4.11	Oil Red O.....	48
4.12	Co-transfection of C2C12 Cells	49
4.13	Double stimulation of cells.....	50
4.14	Treatment with Inhibitors	50
4.15	Data Analysis.....	50
5	RESULTS.....	51
5.1	Introduction	51
5.1.1	MCK2.1 mediated osteogenesis is dependent on caveolae and smad.....	51
5.1.2	Osteogenesis mediated by MCK2.3 occurs independent of caveolae and smad.....	51
5.1.3	Osteogenesis via MCK2.3 is mediated by the ERK/MEK signaling pathway.....	52
5.1.4	BMPR1b expression is upregulated in C2C12 cells treated with 40nM CK2.2 only.....	52
6	DISCUSSION.....	54
7	TYPE III RECEPTOR ENDOGLIN.....	58
7.1	Endoglin (CD105)	58
7.2	Endoglin Structure:.....	60
7.3	Endoglin interacts with various Proteins.....	61
8	RESULTS.....	63
8.1	Introduction	63
8.1.1	Expression of Endoglin in C2C12 cells is regulated by stimulation of cells by CK2.1 only.....	63
8.1.2	Stimulation of cells by peptides CK2.2 and CK2.1 leads to expression of PRDM16 and TCF21	64
8.1.3	Time course RNA isolation shows that cells stimulated with CK2.2 express PRDM16 at day1 and day2 while those stimulated with CK2.1 do not.....	64
8.1.4	Treatments with Rapamycin upregulates PRDM16 expression in C2C12 cells	65

8.1.5	Treatments with MEK inhibitor or p38 inhibitor does block adipocyte formation in C2C12 cells	65
8.1.6	Endoglin expression downregulates osteogenesis in C2C12 cells.....	65
8.1.7	Endoglin expression upregulates adipogenesis in C2C12 cells ..	66
8.1.8	Adipogenesis induced by CK2.1 is independent of Endoglin.	67
8.1.9	CK2.3 has no effect on adipogenesis induced by Endoglin.	68
9	DISCUSSION.....	69
9.1	Future Work.....	72
10	CONCLUSION.....	73
10.1	Summary and Prospects	73
	REFERENCES	96

LIST OF TABLES

Table1: Major pathways of the BMP signaling pathway	29
Table2: Expression and function of major adipogenic markers	35
Table3: Summary table showing the different pathways of BMPR1a mutants	57

LIST OF FIGURES

Figure 1:	Osteoporosis caused by an imbalance in the bone formation and bone resorption process.	74
Figure 2:	Principal transcription factors involved in the differentiation of osteoblasts and osteoclasts.	75
Figure 3:	BMPR1a receptor structure.....	76
Figure 4A:	Mineralization area covered by cells transfected with MCK2.1 or MCK2.2 and treated with either siCAV1 or siSmad4 or siCK2. Lower panel shows representative wells for each treatment.....	77
Figure 4B:	Mineralization area covered by cells transfected with MCK2.3 and treated with either siCAV1 or siSmad4 or siCK2. Lower panel shows representative wells for each treatment.....	78
Figure 5A:	Mineralization area covered by cells transfected with MCK2.3 and treated with either p38 inhibitor or Rapamycin (mtor inhibitor) or MEK inhibitor. Lower panel shows representative wells for each treatment.	79
Figure 5B:	Mineralization area covered by cells transfected with MCK2.1 and treated with MEK inhibitor or p38 inhibitor or Rapamycin. Lower panel shows representative wells for each treatment.....	80
Figure 6:	Model for the mutants used in the present study..	81
Figure 7:	Transcription factors involved in differentiation of adipocytes.....	82
Figure 8A:	Endoglin as a key control in the mechanism of adipogenic control in the BMP signaling pathway	83
Figure 8B:	Endoglin structure and isoforms.	84
Figure 9A:	RT-PCR results for C2C12 cells treated with different concentrations of CK2.3.....	86
Figure 9B:	RT-PCR results for C2C12 cells treated with different concentrations of CK2.1.....	86

Figure 9C:	RT-PCR results for C2C12 cells treated with different concentrations of CK2.2.....	86
Figure 9D:	RT-PCR results for Day1 and Day2 treatment of C2C12 cells with CK2.2 and CK2.1.....	87
Figure 9E:	RT-PCR results for treatment of C2C12 cells with Rapamycin and subsequently with CK2.2 and CK2.1 for 3 days.....	87
Figure 9F:	RT-PCR results for treatment of C2C12 cells with MEK inhibitor and subsequently with CK2.2 and CK2.1 for 3 days.....	89
Figure 9G:	RT-PCR results for treatment of C2C12 cells with p38 inhibitor and subsequently with CK2.2 and CK2.1 for 3 days.....	89
Figure 10A:	Mineralization area covered by C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin post Van Kossa staining.....	89
Figure 10B:	Mineralization area covered by C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin and subsequently treated with CK2.1 post Van Kossa staining.	91
Figure 10C:	Mineralization area covered by C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin and subsequently treated with CK2.2 post Van Kossa staining.	91
Figure 10D:	Mineralization area covered by C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin and subsequently treated with CK2.3 post Van Kossa staining.	91
Figure 11A:	Area covered by lipid droplets for C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin post Red Oil O staining.....	92
Figure 11B:	Area covered by lipid droplets for C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin and subsequently treated with CK2.1 post Red Oil O staining	94
Figure 11C:	Area covered by lipid droplets for C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin and subsequently treated with CK2.2 post Red Oil O staining	94
Figure 11D:	Area covered by lipid droplets for C2C12 cells transfected with Δ C Endoglin, S-Endoglin and L-Endoglin and subsequently treated with CK2.3 post Red Oil O staining	94

Figure 12A: Model for Endoglin and BMPR1b mediated adipogenesis..... 94
Figure 12B: Model for Endoglin mediated osteogenesis 95

ABSTRACT

According to NIH around 50% of women over age 50 are affected by Osteoporosis increasing the risk of fractures and injuries. Osteoporotic conditions may arise from either slow bone formation or increased bone resorption or both. Recent studies showed that the bone marrow of osteoporotic patients have increased accumulation of adipocytes. These adipocytes may either migrate into the bone when the bone becomes brittle or stem cells may differentiate into adipocytes instead of osteoblasts. One of the major growth factors that can determine the cell fate of mesenchymal stem cells into adipocytes or osteoblasts is Bone Morphogenetic Protein 2 (BMP2). We recently identified new protein-protein interaction. We identified that Casein Kinase II binds to the BMP2 type IA receptor. There are three potential CK2 binding sites on the receptor and we designed three peptides blocking specifically the interaction of CK2 with one specific binding site on the receptor. We have also synthesized three mutant varieties of the BMPR1a each having a point mutation at each of these CK2 phosphorylation sites. In this study we have deduced a model for osteogenesis mediated by the mutants. In order to investigate the mechanisms of stem cell differentiation by these peptides we identified Endoglin. Endoglin is a co receptor in the BMP signaling pathway and was identified as a marker for cell fate differentiation. Previous studies have shown that low expression of Endoglin is linked with increased osteogenic potential, while high Endoglin expression is linked with high adipogenic

potential. Using RT-PCR we found that Endoglin is upregulated during adipogenesis. Additionally overexpression of Endoglin in mesenchymal cells led to increased adipogenesis suggesting that Endoglin itself is not only a marker, but involved in the mechanism of adipogenesis. Endoglin was specifically upregulated in adipocytes of brown fat origin, since the adipocytes expressed PRDM16 which is a known marker for brown fat. These data reveal a new mechanism of adipocyte differentiation and may assist in the development of new therapeutics decreasing the number of adipocytes in the bone marrow and increasing the number of osteoblasts.

Chapter 1

INTRODUCTION

1.1 Bone: The Dynamic Mineralized Structural Tissue

Bone is the primary mineralized tissue that confers structural rigidity as well as flexibility to the body. Bones are involved in skeletogenesis that is generation of the skeleton and perform a variety of mechanical as well as biological functions. Bones primarily constitute of the osteoblasts and osteocytes or the bone forming cells and osteoclasts that is the bone resorbing cells.[1] A fine balance between the activities of these two distinct classes of cells is critical to maintaining cellular homeostasis. (Fig:1)

Osteoblasts belong to the mesenchymal cell lineage and are genetically and morphologically identical to the fibroblasts. Osteoblasts production can be determined by expression of markers like *Cbfa1* and *Osteocalcin* that can inhibit osteoblast activity.[2, 3] Embryonic development can promote osteoblast differentiation by endochondral ossification or intramembranous ossification. *Cbfa1* has been identified as one of the most critical early pre-determinants of osteoblast differentiation. (Fig: 2) Homologs of *Cbfa1* found in *Drosophila* and *C.elegans*. *Cbfa1* is a highly conserved transcription factor and plays a key role in determining the fate of mesenchymal cells destined to form osteoblasts or chondrocytes. Studies show that *Cbfa1* deficient mice lack proper skeletal formation as well as osteoclast generation ability.[2] Other primary

transcription factors involved in differentiation of osteoblasts are summarized in Figure 2.

Bone formation and resorption is a highly dynamic process that is closely controlled by both the osteoblasts, osteocytes and osteoclasts. Osteoclastogenesis is dependent on osteoblasts, macrophage colony stimulating factor (M-CSF) and receptor for activation of nuclear factor $\kappa\beta$ ligand (RANKL). These factors are sufficient for inducing osteoclastogenesis in stromal cells and osteoblasts. An inhibitor of osteoclastogenesis is Osteoprotegerin (OPG). Expression and availability of these ligands and proteins dictate the degree of bone resorption. Bone resorption is a sequence of steps that involve RANKL, M-CSF, OPG, osteoblasts and parathyroid hormone receptors. Osteoblasts express the osteoclastic RANKL and M-CSF and induce the differentiation of the pre cursor macrophage cells to the osteoclasts. The osteoclasts can then lodge in the mineral matrix of bones and initiate the resorption process by secretion of acidic cations.[3] (Fig: 1)

Increased mineralization caused by decreased osteoclast activity is called osteopetrosis whereas osteosclerosis is caused by increased osteoblast activity. Paget's disease is caused on the other hand by stimulated bone resorption giving rise to fragile bones prone to bending and breaking. Several cancers can also invade bones thus making the tissue prone to malignancy, including breast cancer and prostate cancer. Humoral hypercalcemia of malignancy is caused by dysfunctional calcium balance as well as increased osteoclast activity.[4-8]

Several inflammatory diseases have also been associated with bone. Rheumatoid arthritis and periodontal disease are both inflammatory bone disorders that are caused by destruction of the cartilage, by heightened osteoclast activity, and the degeneration of the periodontium, by accumulating bacteria, respectively.[4]

1.2 Osteoporosis

Osteoporosis is a medical condition in which the bones become increasingly fragile with age and are prone to fractures. According to the 2010 statistics from National Osteoporosis Foundation, nearly 53 million Americans over the age of 50 are osteoporotic or have low bone density putting them at higher risk for developing the disease. About 12 million of them have osteoporosis now, an estimated 13.9 million people will have osteoporosis by 2020 and, about 80 percent of those affected are women. In the recent years, a growing number of potential treatments like bisphosphonates, Selective Estrogen Receptor Modulators (SERMs) and Parathyroid Hormone [PTH] have been introduced, but these drugs are all expensive and have harmful side effects as osteonecrosis of the jaw. [9] Bisphosphonates can inhibit bone resorption by downregulating osteoclasts, but is not enough to maintain bone homeostasis. While estrogen receptors are primary targets of SERMs, they are unable to build new bone. PTH therapy is the only treatment till date that increases bone buildup and daily consumption can stimulate bone formation. Other recent medicines like Denosumab, Cathepsin K inhibitor and Sclerostin inhibitor can act on a wide range of fractures and bone injuries. But they have shown poor compliance or severe side effects in many cases. Thus novel therapeutics that can restore bone homeostasis

and maintain bone mineral density are in high demand. Over the years anabolic therapies as Bone Morphogenetic protein 2 (BMP2) and BMP7 emerged in preclinical trials due to their ability to induce bone formation. [10] In 2002, BMP2 was approved by FDA for spinal fusions, and open long bone fractures. Since osteoporosis involves bone loss, and BMP2 is a bone-inducing growth factor, BMP2 has potential for acting as a new treatment. In vivo studies using osteoporotic mice models show that systemic injections of rhBMP2 can increase osteoblast generation and thus stimulate bone formation. Studies also show that polymorphisms in the BMP2 gene can increase the possibility of developing osteoporosis [11, 12]. Although BMP2 is projected as a good candidate to overturn bone loss, there are some side effects that cannot be overlooked. To work properly, BMP2 must be administered in doses as high as 1.5mg/ml, BMP2 delivery is via repetitive ectopic injections and BMP2 affects stem cell differentiation

In 2009, Canalis reported that Notch signaling is also involved in the osteoblast differentiation program. Studies of expression of Notch under the control of Typ1 I Collagen promoter show osteopenia, low osteoblastogenesis and leads to the formation of woven bone.

Chapter 2

BONE MORPHOGENETIC PROTEINS, RECEPTORS, AND PATHWAYS

2.1 Bone Morphogenetic Proteins (BMPs)

Bone morphogenetic proteins (BMPs) are multifunctional cytokines that belong to the TGF- β superfamily. They have been shown to be involved in critical cellular processes as regulation of cell proliferation, migration, differentiation and apoptosis in a host of tissues. BMPs also act as morphogens that are key players in development of mesoderm, neural patterning, and development of lungs, skin, liver, and kidney as well as skeleton and limb formation. Recent studies have linked BMPs to cancer [13]. Role of BMPs in early development has been shown to be critical. Genetically manipulated BMP2, BMP4, BMP8b, and BMP10 homozygous null mice have been found to be embryonic lethal [14-17]. Since lethality did not allow postnatal development or adult tissue homeostasis, conditional knockouts have been studied instead. [37, 38][18, 19] (Table1)

Synthesis of BMPs occurs as large precursor molecules consisting of about 400-500 amino acids. The precursors at their N-terminus contains a signal peptide that directs the protein to the secretory pathway, a prodomain to ensure proper folding and a C-terminus mature peptide [20, 21]. Upon secretion from the trans-Golgi the BMP-4 precursor molecules are proteolytically cleaved when dimerization occurs in the prodomain at an Arg-X-X-Arg

sequence. This proteolytic activity is brought about by serine endoproteases. For example studies show that *Furin* and *PC6* are convertases that act redundantly to cleave BMP4 in *Xenopus* oocytes. Another site-specific protease that emerges as a key player is *PC7* that cleaves BMP4 at the optimal motif. [22] [23] Post cleavage by serine endoproteases, the mature active BMP4 dimer forms a covalent association with the prodomain to form a latent complex and is secreted into the extracellular matrix. The extracellular matrix then acts as a platform where the prodomain associated with the active BMP4 can interact with various components present in the matrix specifically microfibrils like fibrillin-1.[24] [25]. Other components of the extracellular matrix also can potentially play critical roles in the BMP signaling cascade. For example heparan sulfate proteoglycans (HSPGs) directly interact with BMP2, regulate BMP2 internalization as well as can control BMP2 mediated osteogenic activity in a concentration dependent manner. In C2C12 cells heparan binding to BMP2 and BMP7 has also been shown to downregulate downstream smad signaling. [26].Also heparan sulfate has been shown to be increasing BMP2 induced osteogenic activity in C2C12 cells.[27] BMPs like BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, and BMP7 are released into matrix vesicles from the cytoplasm of the upper hypertrophic chondrocytes. [28]. BMP1 is the only known exception that has been shown to be not secreted in the traditional pathway

Monomeric BMP consists of seven cysteine residues six of which are involved in intramolecular bonding. Active BMPs are formed by dimerization when the seventh cysteine residue forms a covalent disulfide bond with another

monomer [29] . Heterodimers have also been reported and in some cases for example heteromers of BMP2/6 have been shown to be more potent inducers of differentiation in human embryonic stem cells.[30] These disulfide bonds help BMPs defend fluctuations in temperature, pH etc. BMPs usually act as homodimers or heterodimers and it has shown that heterodimers are more potent ligands as opposed to homodimers. BMP15 on the other hand lacks the seventh cysteine residue and as a result is unable to dimerize.[21, 31, 32]

BMP2 is our protein of interest in the current study. BMP2 has been shown to induce osteoblast formation and BMP2 homozygous null mice died at embryonic day 7.5-9. The homozygous mutant embryos also showed failure to close the proamniotic canal and malformation of the heart in the exocoelomic cavity [14]. The conditional BMP2 knockout mice showed the earliest steps of fracture healing are obstructed even in the presence of other osteogenic stimuli. These mice also develop spontaneous fractures which do not get resolved over time [33]

2.2 BMP Receptors

BMP receptors are serine threonine kinase type receptors that are divided into two broad classes:

Type I:

These are primarily the activin receptor like kinases (ALK) and based upon their structural similarities can be further sub grouped under:

1. BMP Receptor type I (BMPRI) group (BMPRIA /ALK3 and BMPRIb /ALK6)
2. ALK I group (ALK1 and ALK2)
3. TGF β R I group (ALK4 /Act r1B,Alk 5/ T β R1 and ALK 7)
4. ALK8

While BMPRIA and ALK2 are expressed in a wide number of tissues, BMPRIb has more restricted expression and ALK 1 has limited expression in endothelial cells and certain other cells.[34, 35] ALK8 has been recently discovered in zebra fish embryos. BMP signaling induced by ALK8 has been reported to influence pancreas development in zebra fish.[36, 37]

Type II

In mammalian tissues primarily three types of type receptors have been found to be widely expressed and bind all types of BMPs.

1. BMPRII
2. Act RII
3. Act RIIB

In most cases the capacity of BMPs to bind to type II receptors is seen to dictate their binding affinity to the type I receptors. [38]

2.3 Coreceptors of the BMP Signaling Pathway

BMP signaling also involves co-receptors that control the downstream signaling pathways. The co-receptors interact with several adapter molecules, effector proteins as well as accessory molecules and have the ability to modulate the downstream signaling.

RGM family of coreceptors

RGM proteins are the first known family of high affinity co-receptors that are specific for BMPs. They were first identified in chick embryos and to date four members of the RGM family have been identified in vertebrates: RGMa, RGMb also known as Dragon, RGMc also known as Hemojuvelin and RGMd which has been identified in fishes. All RGM proteins have a common N-terminal signal peptide, a partial von Willebrand factor type D domain (vWF-type D), that includes a highly conserved proteolytic cleavage site, a hydrophobic domain of unknown function, and a C-terminal GPI-anchor.[39]

Originally discovered as GPI-anchored proteins, at least some RGMs have been demonstrated to be expressed in lipid rafts [40]. Unlike DRAGON, RGMa and Hemojuvelin also possess an RGD (Arg-Gly-Asp) motif, which may be involved in cell–cell adhesion [41]. RGMs have been implicated in mouse models of diseases and in some human pathologic states.

RGMa

RGMa expression is not only involved in axonal guidance during development but also in adults. RGMa and RGMb function in distinct areas in the central nervous system and this is maintained even in the adult state.[42]. RGMa is expression is also observed in the developing mouse cochlea, lung, limb primordia [43], heart, brain, lung, liver, skin, kidney, testis [44]. In addition to its function as a repulsive axon guidance molecule, RGMa has also been shown to play a role in neural tube closure. The RGMa knockout mouse model exhibits defects in neural tube closure. RGMa also acts as an anti-apoptotic factor by inhibiting neogenin. RGMa has been shown to promote neuronal differentiation in the embryonic chick mid- and hind-brain through its receptor neogenin [45, 46] .RGMa has also been shown to associate with BMPR1a and BMPR1b receptors as well as with type II receptors as ACTRIIA and BMPRII. Their association has been shown to activate the downstream SMAD1/5/8 cascade. The underlying molecular mechanism by which RGMa induces BMP2 and BMP4 mediated signaling and the structure of the active BMP ligand/RGMa/type I receptor/type II receptor complex at the cell surface are not known[47].

RGMb/ Dragon

DRAGON is the first identified co-receptor for the BMP signaling pathway and binds to BMP ligands and receptors to enhance BMP signaling. It is a 436-amino acid containing GPI-anchored protein and is an enhancer of BMP

signaling. [48] DRAGON interacts directly with specific BMP ligands in the absence and presence of BMP type I and type II receptors. The action of DRAGON, like other GPI-anchored proteins, may depend on localization in lipid rafts and in this way contribute to the assembly and trafficking of the receptor complex in microdomains. [41]

DRAGON is highly conserved across species in both vertebrates and invertebrates. Orthologs have been identified in human, *Xenopus*, chick, Zebrafish, mouse and *C. elegans*. [48]. It has been shown that DRAGON has extensive maternal expression, and its expression in the nervous systems of pre-implantation embryos suggests that, DRAGON may be a critical role player in the BMP signaling pathway. Over expression of DRAGON leads to the formation of ectopic neurons and decreases neural crest cells in *Xenopus* embryos. This indicates that DRAGON, being a facilitator of BMP signaling, probably modulates the response of developing neurons to BMP ligand gradients thus influencing patterning and differentiation in the developing embryo. [41]

RGMc/Hemojuvelin

In 2003, the gene associated with human juvenile hemochromatosis was identified by positional cloning strategy. The gene was named HFE2 and the protein product was called hemojuvelin [49]. Later the name was changed to hemojuvelin and given a new designation of *HJV*. Juvenile hemochromatosis is an autosomal-recessive disorder characterized by systemic overload of iron in

the early years leading to accumulation of iron in the heart, liver, endocrine glands, joint, and skin. The heart and endocrine glands, being more susceptible to iron toxicity, succumb to its effect earlier. Cardiomyopathy and hypogonadism is also part of the prognosis. Left untreated, this disease can be fatal before the fourth decade of life. HJV can also mediate BMP signaling by binding directly to BMP ligands such as as BMP2/4/6.[50]

RGMd

Recently a new gene belonging to the RGM family, named RGMd, has been identified only in zebra fish [51].

BAMBI

BAMBI or BMP and Activin Membrane-Bound Inhibitor is a 260amino acid transmembrane glycoprotein which is conserved in vertebrates from fish to humans. It is closely related to the type I receptors of the TGF β family in the extracellular domain but has a shorter intracellular domain that exhibits no enzymatic activity.[52-54] Studies show that *Xenopus* BAMBI functions as a general antagonist of TGF β family members by acting as a pseudo receptor to block the interaction between signaling type I and type II receptors.[54]

BAMBI is co-expressed with BMP4 during the embryo development of zebrafish, *Xenopus*, bird and mouse, and its expression is also induced by BMP4.[52, 53] Therefore, BAMBI is believed to act as a negative feedback regulator of BMP signaling during embryo development [54, 55]

Studies show that human BAMBI (hBAMBI) integrates different cellular signaling pathways. It has been shown that hBAMBI can promote Wnt signaling by interacting with the Wnt receptor Frizzled-5 and the downstream mediator Dishevelled-2, [56]

Betaglycan

Betaglycan is another co-receptor for primarily the TGF- β superfamily. Studies in several cell lines, including rat L6 myoblasts that lack endogenous betaglycan, it significantly increases the affinity of T β RI and T β RII for TGF- β thereby enhancing downstream signaling.[57] Various cell lines that express membrane-bound betaglycan also release soluble betaglycan into the medium. Betaglycan is expressed in various animal tissues and cultured cells. Betaglycan is involved in modulation of the TGF β signaling pathway and the structural attributes of betaglycan play a critical role in influencing its functional aspects. [58]

2.4 Receptor Activation

In the absence of ligand stimulation, small fractions of type II and type I receptors are present in preexisting complexes as homodimers and heterodimers on the cell surface. Ligand binding increases oligomerization of the BMP receptors and signal transduction in receptors, which induce conformational changes of the receptor molecules.[59] The intracellular domains of type I receptors have a characteristic GS domain (glycine and serine-rich domain)

located N-terminal to the serine-threonine kinase domains. (Fig: 3) The type II receptor kinase is constitutively active in the absence of BMP. Upon ligand binding, the type II receptor kinase phosphorylates the GS domain of type I receptor, which is a critical event in signal transduction by the serine threonine kinase receptors.

Three-dimensional analysis of the intracellular domain has been reported for TGF β R-I. The inactive conformation of the T β R-I kinase is maintained by interaction between the GS domain, the N-terminal lobe and the activation loop of the kinase [60]. Upon phosphorylation of the GS domain by the type II receptor kinase, the T β R-I kinase is converted to an active conformation. Mutations of Thr-204 in T β R-I and the corresponding Gln in BMP type I receptors to acidic amino acid residues (Asp or Glu) lead to constitutive activation of the type I receptors. The constitutively active type I receptors transduce intracellular signals in the absence of ligands or type II receptors. Type I receptors thus act as a downstream component of type II receptors, and determine the specificity of the intracellular signals. FKBP12, an immunophilin has been reported to regulate leaky downstream signaling in the absence of a ligand thus assuring specificity and tight control. Huse et al showed that FKBP12 binds to GS box of the TGF β -II receptors thus shielding the potential phosphorylation sites and inhibiting downstream signaling. [60]

In the kinase domain, type I receptors have the L45loop between kinase sub domains IV and V, which protrudes from the kinase domain [60] and

specifically interacts with receptor-regulated Smads (R-Smads). Amino acid sequences of the L45 loop are conserved in each type I receptor subgroup, but diverge between different subgroups. The L45 loop of the BMPR-I group is more similar to that of the TGF β RI group than that of the ALK1 group. Interestingly however, receptors of the BMPRI and ALK1 groups activate a similar set of Smads, Smad1/5/8, while those of the TGF β RI group activate a distinct set of Smads, Smad2/3. It has been reported that the region above 29 amino acids is essential for the association of BMPRII-SF (which is a splice variant of BMPRII) with BMPRIb into preassembled complexes and indicates that the interactions of BMPRIa and BMPRIb with BMPRII in the absence of ligand are not identical. On the other hand, the BMPRII mutants were effectively recruited by BMP2 into heteromeric receptor complexes with both BMPRI subtypes.

2.5 Caveolae and Clathrin Coated Pits (ccps)

Localization of BMP receptors in the plasma membrane has been shown to play a critical role in receptor activation as well as downstream signaling. Receptor shuffling occurs under particular conditions that lead to various downstream events. Studies have shown that depending upon where the receptors are housed there may be different degrees of receptor activation as well as triggering of diverse pathways.[61, 62] BMP receptors are localized on the

plasma membrane in distinct microdomains. Two such domains are CCPs (enriched in AP2, EPS15 and Clathrin) and detergent resistant membranes (DRMs). DRMs are further subgrouped into caveolar and non caveolar fractions. [61] Distinct proteins named Caveolin-1, -2, and -3 have been shown to be major components of caveolae. Caveolin-1 exists in two isoforms, Caveolin-1 α and Caveolin-1 β . [63] Recently another family of proteins called cavins has revealed additional structural complexity of caveolae.[64]

The role of these domains in BMP signaling are extensively studied, however it is still controversial. BMP receptors type I and type II clearly localize to these regions. Studies in A431 and C2C12 cells showed that BMPRIa is localized to caveolae and shuttling between caveolae composed of the Caveolin-1 β isoform to caveolae containing both isoforms enhances Smad signaling. Overexpression of Caveolin-1 β in these cells had an inhibitory effect on Smad signaling, while overexpression of Caveolin-1 α had little to no inhibitory effects. Additionally, studies in smooth aortic muscle cells show that BMPRII interacts with Caveolin-1 and this interaction is necessary for Smad signaling. [65] In addition ectopic localization of mutant BMP receptors in caveolae has been reported in Pulmonary Arterial Hypertension. This leads to decreased smad phosphorylation and decreased smad signaling. [61]

Internalization and dephosphorylation of BMPRII by Dullard result in degradation of BMPRII in caveolae, pointing to the importance of these domains in Smad signaling. [66] Another domain in BMPRIa, discovered

recently is the NANDOR (No Activating Non-Down-Regulating) Box that is important for type I receptor down regulation. Situated near the C-terminus end of the type I receptor, this domain plays an important role in receptor internalization and endocytosis. It is an 11 amino acid sequence conserved among all the type I receptors of the TGF- β family, but this motif does not fall into the most well categorized groups of internalization signals. [67]

At the same time, BMP receptors are found to localize in CCPs in A431 and C2C12 cells. Disruption of the pits enhanced smad signaling in C2C12 cells. Supporting these data is that the disruption of CCPs decreases the time it takes for Smad1 to translocate to the nucleus.[61] On the other hand this effect could not be detected in SHED cells and disruptions of CCPs with non-specific agents were not able to reproduce this result showing that cells respond differently to CCP disruption. Recent data also indicate that smad signaling is initiated in CCPs themselves in response to BMP2.[61]

In conclusion these data demonstrate that receptor localization to both membrane domains/pits as well as outside regions on the plasma membrane are able to signal via smad activation. Using Image Cross Correlation Spectroscopy, it is shown that only the type I receptor without BMPRII is present in the absence of BMP2 on the plasma membrane, raising the question how this receptor can activate smad signaling. [61] BMP2 binding to BMPRIa can occur either to preformed complexes (PFC) or BMP induced signaling complexes (BISCs).[68, 69] Studies show that PFCs and BISCs trigger

different pathways. While PFCs are locales for Smad dependent signaling, signaling by BISCs mainly occur through Smad independent pathways.[70, 71] Nohe et al in 2005 showed that Cav-1 inhibits BMP signaling via PFCs in the caveolae [72] while Hartung et al showed that caveolae disruption by Lovastatin facilitated downstream BMP signaling.[70] A recent study by Bragdon et al shows that smad signaling is facilitated by ccp disruption in the absence of BMP2.[62] Additionally, using Fluorescence Resonance Energy Transfer (FRET) technique, it was shown that BMP binds with high affinity to BMP receptors in caveolae. [61, 62] Although conflicting, the results suggest that both caveolae and ccps are critical for initiation and downstream signaling in the BMP pathway.

Recently, the importance of BMP receptor dynamics for smad signaling and mineralization was shown in bone marrow stromal cells (BMSCs) derived from three different mice skeletal phenotypes. The B6.C3H-6T (6T) is a congenic mouse with decreased bone mineral density (BMD) with increased marrow adipocytes and decreased osteoprogenitor proliferation, while the B6.C3H-1-12 (1-12) congenic is a mouse model with increased BMD and osteoblast mineralization. In the experiments C57BL/6J (B6) mice are used as controls since only a segment of Chromosome6 or only 4Mb of Chromosome 1 from the C3H/HeJ mouse was backcrossed to a C57BL/6J background. B6 mice are a mouse strain that already exhibit relatively low bone mineral density.

In BMSCs isolated from the B6 control mouse, knockdown of Caveolin-1 leads to a significant reduction in BMP2 dependent smad signaling and mineralization. These results point to the importance of caveolae in these processes. In the absence of BMP2 down regulation of Caveolin-1 in BMSCs isolated from 6T mice resulted in activation of the Smad signaling pathway, while in BMSCs from 1-12 mice, downregulation of caveolin-1 leads only to smad signaling in the presence of BMP2.

Using Image Correlation Spectroscopy, BMPR1a aggregation on the plasma membrane can be correlated to the extent of BMP signaling and mineralization. Therefore, aggregation of BMPR1a could be used as an indicator for osteogenic potential. Studies showed that BMPR1a is aggregated in BMSCs isolated from 1-12 mice, while it failed to aggregate in response to BMP2 stimulation in BMSCs isolated from 6T mice. Aggregation in response to BMP2 is observed in BMSCs isolated from B6 mice.

Further data obtained indicate that the movement of BMP receptors out of caveolae of the β isoform into caveolae enriched in the alpha and beta isoforms is crucial for enhanced smad signaling and mineralization. BMSCs from low peak bone mass mice, B6 and 6T fail to express BMPR1a in caveolae enriched in the beta isoform, while the high peak bone mice have BMPR1a localized in caveolae composed of this isoform. Upon BMP2 stimulation BMPR1a shuttles between caveolae of different isoforms for enhanced signaling in BMSCs from 1-12 mice.

Interestingly, the release of BMPR1a from caveolae of the alpha beta isoform in BMSCs isolated from 6T mice leads to increased smad signaling and mineralization, while in BMSCs from B6 or 1-12 this effect is not observed. However, disruption of caveolae clearly enhances BMP2 response in BMSCs isolated from 1-12.

Taken together the results obtained in primary cells confirm the conclusions from C2C12 and A431 cells that 1) BMPR1a shuttling between caveolae is important for smad signaling (A431 cells), 2) disruption of caveolae can lead to increased smad signaling and mineralization (C2C12 cells) and 3) trapping of BMP receptors in caveolae and inhibition of shuttling leads to decreased smad signaling (A431, C2C12 cells). It also shows that signaling in C2C12 cells may reflect BMSCs isolated from mice with very low BMD, while results from A431 cells mimic the response of cells with a high peak bone phenotype. However, these data seem to indicate that once a certain threshold of BMP2 signaling is achieved this signal cannot be potentiated; suggesting that negative feedback mechanisms activate at this time. [61, 73, 74]

2.6 Proteins interacting with the Receptors

Extensive studies have been done over the years to reveal the status of protein- protein interactions with the BMP receptors. While some proteins like Adapter protein 2 (AP2) interact with the receptor to direct them to ccps [62], proteins like Dragon [47] and BAMBI [75] decrease downstream signaling by

binding to regulatory smads. BRAM1 and XIAP1 are two proteins that have been associated with inducing SMAD independent downstream signaling pathways. While BRAM1 [76-78] has an inhibitory effect upon binding BMPR1a, XIAP1 [79, 80] acts as a linker between the type I receptor and the TAB1-TAK1 complex. Proteins like FK506 [59] and FKBP12 [81] have been shown to interact with BMPR1a to regulate the downstream signaling. c-Src [82], EPS15R (Related to EPS15) [70] and cGK1 (cyclic guanosine 3', 5' monophosphate dependent kinase I) [83] have been shown to interact exclusively with the type II receptor. While Trb3 (tribbles like protein-3) binding to type II receptor is negatively regulated by BMP4 [84], Tctex-1 binds to BMPR1a, BMPR1b as well as BMPRII [85]. Other lesser known proteins like LIMK1 (Lim Kinase I) [86], Inhibin [87], SAP49 (splicosome associated protein subunit 4) [88], Tob (transducer of ErbB2) [89] and SF3b4 (splicosome factor 3b subunit 4) [90] interact with the receptors but the exact mechanism of regulation are yet to be determined.

2.7 Casein Kinase 2 (CK2)

Recently Casein Kinase2 (CK2) has been discovered to be associated with BMPR1a. CK2 is a universally expressed, highly conserved enzyme that can act on a host of substrate molecules like 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,), transcription factors (c-Myc, c-jun, p53),

protein synthesis factors (eIF3, eIF4B, eIF, and eIF2 β), and cytoskeleton and structural proteins (β - Tubulin, both heavy and light Myosin chains, Clathrin, tau) [91]. Transcription factors like NF- κ β and C/EBP are also phosphorylation targets of CK2 [92]. Activity of CK2 has been detected in both cytosolic as well as cellular fractions. The tetrameric holoenzyme consists of two catalytic subunits (α and α') and regulatory subunit β . The catalytic subunits preferentially phosphorylate substrates at serine, threonine residues and, less favorably at tyrosine residues that are surrounded by the acidic sequences of proteins. The α and α' subunits are distinct polypeptides derived from different genes. [93]. Free CK2 β subunits alone can upregulate or downregulate activity of other kinases as A-Raf, c-mos and p90RSK [94]. Thus CK2 β in addition to CK2 α can regulate a number of kinases [95]. Free CK2 α subunit has also been reported to interact competitively with the catalytic subunit of protein phosphatase 2A (PP2A) increasing the phosphatase activity. [96] In addition the holoenzyme too can recruit and complex with many other proteins many of which act as CK2 substrates.[94].

The CK2 β subunits form inactive tetrameric complexes with the CK2 α subunits. This assembly however is critical for recruitment and docking of CK2 substrates. The catalytic activity of the α and α' subunits are also up regulated by this association.[97]. CK2 is a key player in cell cycle as it tightly regulates cellular proliferation as well as apoptosis [98]. While α , β or β' knockout mice are embryonic lethal [99, 100] a conditional knockout study revealed that

deletion of regulatory subunit, CK2 β , was lethal to embryonic fibroblasts [101]. Homozygous knockouts of CK2 α led to defects in the heart [100] and knockouts of CK2 α' resulted in infertility of males due to defects in spermatogenesis [102]. It is not surprising that CK2 activity has been implicated in poor cancer prognosis and tumorigenesis [101].

CK2 has been proved to be indispensable for numerous crucial cellular and physiological processes as development, differentiation, migration, and apoptosis. As mentioned before studies have shown that association of CK2 with BMR1A in the absence of BMP2 elucidates its role as negative regulator of downstream signaling in the BMP signaling pathway. Upon stimulation with BMP2, CK2 dissociates from BMPR1a that leads to activation of downstream signaling by subsequent phosphorylation of SMAD or the TAB1/TAK1 complex. CK2 thus seems to inhibit phosphorylation of BMPR1a by the constitutively active BMPRII in the absence of a ligand. A Prosite search revealed that there are three possible interaction sites for CK2 on BMPR1a and peptides were synthesized to block these interactions. Each of these peptides named CK2.1, CK2.2 and CK2.3 have been shown to induce mineralization mediated by BMPR1a in the absence of ligand. (Fig: 3) Further to validate the effects of the blocking peptides and understand the specificity elicited by the peptides, Plasmids encoding BMPR1a with single point mutations were synthesized in our lab and when C2C12 cells were transfected with these plasmids ,the cells overexpressed these mutant proteins. Two of the mutants,

MCK2.2 and MCK2.3 induced osteoblast formation in C2C12 cells. (Fig: 3) In vivo studies performed with the blocking peptide, CK2.3, further showed that it is capable of inducing osteoblast formation and bone mineralization. Thus CK2 has the tremendous potential to be used as therapeutic target for various bone related fractures, spinal fusion as well as a drug for osteoporosis. [61, 103]

2.8 Smad dependent Pathway

BMP2 triggers the Smad-dependent pathway as it binds to BMPRIa. Upon ligand binding, the Type II receptor phosphorylates the Type I in the GS box region that is rich in glycine and serine residues. Phosphorylation activates the Type I receptor which then initiates Smad-dependent signaling. The canonical BMP signaling pathway involves the phosphorylation of SMAD 1, 5 and 8 and the subsequent translocation to nucleus in association with SMAD4. There are eight known members of the Smad family of proteins and they participate in host of signaling pathways. Regulatory Smads (R-Smads) consist of Smad2, and 3 that participate in the TGF- β signaling pathway and Smad1, 5 and 8 are regulators of the BMP signaling pathway. Inhibitory Smads (I-Smads) are Smad6 that are a part of the BMP signaling, and Smad7 that are participants of the TGF- β pathway. Smad4 is Common Smad or Co-Smad [104]. Once phosphorylated Smad1, 5 and 8 bind and to Smad4. The Smad complex then translocates to the nucleus where they activate the expression of targeted genes

[105]. Smad1, 5 and 8 signaling have been shown to be key players regulating various critical processes as cell growth morphogenesis, development and immune responses. Smad translocation to the nucleus next leads to activation of various proteins like C/EBP α or ATF2 that act as transcriptional regulators of genes controlling osteoblast and adipocyte differentiation [106].

Endofin, a FYVE domain containing protein has been reported to play a critical role in facilitating SMAD1 activation. *Shi et al* showed that the serine residue in the MH2 domain of SMAD1 interacts with Endofin to facilitate phosphorylation of the protein. This interaction is distinct from the interaction of other FYVE domain proteins like SARA that binds an asparagine residue in the MH2 domain of SMAD2. As BMP2 binding to the receptors, trigger the downstream signaling, Endofin employs the Smad proteins facilitating their phosphorylation, subsequent nuclear translocation and downstream gene expression. In this perspective Endofin acts as a smad anchor. In a negative feedback, Endofin also recruits PP1c that dephosphorylates Smad1 thus shutting off the downstream signaling.[107] Several other phosphatases like PDPs, SCP1-3 and PPM1A dephosphorylate Smad and thus inhibit downstream signaling. Phosphorylation of the linker of Smad mediated by MAPK inhibits downstream Smad signaling. *Bengtsson et al* showed PP2A is a phosphatase that removes the MAPK phosphorylation thus promoting SMAD signaling. [108] In the nucleus the SMAD proteins act as transcription factors regulating various vital genes that control processes ranging from embryogenesis and

neurogenesis to adipogenesis and osteogenesis. Some BMPs can also elicit downstream signaling by phosphorylating SMAD2 and 3.

2.9 Smad independent Pathway

Smad independent pathways are initiated by the phosphorylation of the TGF- β activated kinase 1 (TAK1) TAK binding protein1 (TAB1) proteins. As BMP2 binds to BMPRIa, BMPRII is transphosphorylated triggering the Smad independent pathway via the TAB1/TAK1 proteins. This leads to the triggering of signaling cascades like ERK, JNK, NF κ β and p38, PI3K and PKC. Activation of TAK1, a MAP kinase kinase kinase, and TAB1 leads to a complex formation that then interacts and activates MKK3, MKK6, NF- κ β , and MKK4. Other MAP kinases such as MKK3 and MKK6 trigger p38 while MKK4, a MAP kinase kinase, activates JNK [29, 109, 110]. Activated p38 and JNK can then potentially regulate various osteogenic and adipogenic genes. [110] p38 can also contribute to adipocyte differentiation by the activation of ATF-2. [106] [111] PI3K is also stimulated via BMP2 binding but the mechanism of this activation is not well understood [112]. Activated PI3K can phosphorylate AKT, a serine/threonine kinase that plays a key role in a host of cellular processes such as glucose metabolism, cell proliferation, cell migration and apoptosis. Another pathway activated is ERK, but the mode of activation is still unknown. ERK1/2 can activate MEK1/2 [113, 114]. This complex interplay of signaling cascades can perhaps explain why BMP2 is involved in regulating so many cellular processes ranging from proliferation, growth, migration, invasion to apoptosis.

Several key proteins are also involved in the initiation of the SMAD independent pathways. Proteins like BRAM1 (Bone morphogenetic protein Receptor Associated Molecule 1) and XIAP1 (X-linked inhibitor of apoptosis protein) initiate the phosphorylation of the TAK1 or TAK1/TAB1 complex by associating with the BMP receptors and thus trigger the downstream events by protein–protein interactions. BRAM1 directly binds to the cytoplasmic tail of BMPRIa to link it to TAB1 whereas BMPRIa employs XIAP by to link it with TAB1–TAK1 complex. The interaction between XIAP and TAB1 is critical for the activation of TAK1. It is not known as to whether there is a difference between the mode of recruitment between BRAM1 and XIAP by the TAB1–TAK1 complex. Also it is unknown if XIAP or BRAM1 associates with other activated type I receptors. [61]

2.10 Ligand independent Pathway

Although normal initiation of the BMP signaling pathway occurs only after ligand (BMP) binding of the receptors, recent studies have shown evidence that there are cases when the receptor activation takes place in the absence of the ligand. Several mechanisms inherent to the BMP signaling pathway exist, that prevent this activation leading to ectopic effects.

A single point mutation in the GS box in AcvR1, a BMP type 1 receptor, results in constitutive stimulation of the receptor even in the absence of the ligand.

This leads to disastrous effects as abnormal osteogenesis, chondrogenesis and also development of fused joints. This particular degenerative genetic condition

is called FOP or *Fibrodysplasia Ossificans Progressiva*. [115] Additionally a nine amino acid sequence in the kinase domain of the type 1 receptors called the L45 loop imparts further specificity to the signaling pathway. This domain in the type 1 receptors can dictate the phosphorylation of smads as well as control subsequent genetic responses. (Fig.3)

Release of CK2 from BMPR1a leads to activation of the smad signaling pathway. In other studies CK2 association to ALK1, a TGF beta type I receptor, was shown to be necessary for Smad signaling. [116] Additionally BMP7 signaling is enhanced by CK2. These data demonstrate multiple facets of CK2 in BMP signaling dependent on the receptor and the ligand. [61]

Table 1: Major pathways involved in BMP signaling pathway

Pathways	Signaling	Reference
ERK/MEK	ERK1/2 pathways are inhibited in cells with high level of Caveolin-1 whereas iron induced pathway IKK is activated through activation of ERK/MEK pathway in the caveolae. In lung tissue however it shows that inhibiting Caveolin-1 leads to an increment in the ERK/MEK pathway.	[117-121]
PI3K	Cholesterol depletion downregulates PI3K pathway. Iron stimulation on the other hand causes activation of PI3K in caveolae. Studies in SCLC cells that are devoid of caveolae, showed that PI3K is present in lipid rafts.	[120, 122, 123]
PKC	PKC binding to caveolae directly	[124]

	inhibits signaling downregulating the kinase activity of the kinase as well as autophosphorylation,	
NF- κ β	Lipid rafts recruit the NF- κ β pathway mediated by various adapter molecules. Depletion of cholesterol on the other hand led to the downregulation of the signaling cascade. NF- κ β is also involved in dynamin and clathrin mediated cellular endocytosis. Pathway can be activated in ccps.	[125-127]
p38	Activation p38 by association of Cav-1 leads to an anti- inflammatory process leading to increased downstream signaling.	[128]
Jnk	Studies in a number of cell lines have shown activation as well as inhibition of Jnk pathway when associated with	[117, 125, 129]

	cholesterol .lipid rafts or associated with various adapter proteins as Traf6.	
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Chapter 3

ADIPOGENESIS

3.1 Adipocyte Differentiation

A single fertilized egg gives rise to nearly 200 different cell types that make up the multiple developmental lineages and multicellular organism. The sequence of developmental events that lead to the formation of pre-adipose tissues from the fertilized egg is unknown. Mesodermal fibroblasts have the ability to differentiate into pre-adipocytes, cartilage, bone or muscle tissue. Human pre-adipocytes differentiate into adipose tissue primarily after birth. In rat and mouse, preadipocytes mature into adipose tissue only after birth. Differentiation of pre-adipocytes to adipocytes occurs in animals throughout their life span depending on the energy requirements. However in vivo studies of pre-adipocyte differentiation have been difficult. In animals adipocytes populate approximately one third of the fatty tissues while remaining two thirds is a combination of small blood vessels, nerve tissue, fibroblasts and pre-adipocytes in various stages of development. The 3T3-L1 cell line has been the most well studied model for pre-adipocyte differentiation to adipocytes. The expression of *c-fos*, *c-jun*, *junB*, *c-myc* and CCAAT/enhancer binding proteins C/EBP β and C/EBP δ are observed at various stages of development. (Fig:7) Upon treatment

of pre-adipocytes with a cocktail of differentiation inducers (*i.e.*, MDI which is a mix of Dexamethasone (DEX), Methylisobutylxanthine (MIX) and insulin), induce the cells to go to adipocytes by expression of a host of adipogenic genes. [130]

3.2 BAT, WAT and BRITE

Adipose tissue is a complex, active endocrine organ that secretes hormones and adipokines such as leptin, adiponectin, tumor necrosis factor- α , interleukin-6 (IL-6), retinol binding protein-4 (RBP-4), Plasminogen activator inhibitor (PAI)-1 and macrophages and monocytes chemoattractant protein (MCP-1) that are critical for regulating the balance between food intake and energy consumption as well as maintaining optimal circulating insulin level [131]. Mammalian adipocytes can be divided into two distinct groups, white adipose tissue (WAT) and brown adipose tissue (BAT). While white adipocytes are large spherical cells (30–200 μ m) that accumulate lipids in unilocular droplets, brown adipocytes are smaller polyhedral cells (20–40 μ m) containing multilocular lipid droplets and high number of mitochondria [132]. Mitochondria in BAT have the cell-specific protein uncoupling protein-1 (UCP-1) that is inserted into the mitochondrial membrane. UCP-1 is critical in driving the proton-motive force to generate heat in BAT. [133, 134] Another zinc finger protein PRDM16 (PR domain containing 16) preferentially

facilitates brown fat differentiation. Myf-5 is a key transcription factor of the MyoD family that determines skeletal muscle differentiation. In vivo Myf5-positive cells are induced by PRDM16 to generate brown fat cells. Kajimura et al showed that PRDM16 forms an active transcriptional complex with C/EBP β that is sufficient and critical for driving a brown fat program in naïve fibroblasts.[135]

Various adipose tissue depots have been identified over the years and they have been primarily divided into WAT and BAT. Gene expression analyses as well as visual examination have revealed interscapular, axillary, cervical, mediastinic and intercostal depots of BAT. WAT deposits have been found inguinally, retroperitoneally, mesenterically, epididymally. In addition some sites have been found to be “BRITE” which are not classically white adipocytes since they have the ability to express UCP-1. [136]

In humans older than 30 years of age, approximately 70% of marrow space of the appendicular skeleton is occupied by fat. With increasing age, the red marrow is converted into yellow marrow fat that occupies most of the femoral cavity. Interestingly this conversion occurs most prominently during the age when humans attain their peak bone mass.[137, 138] There are conflicting roles regarding the physiological functions of this marrow fat. Some studies have shown that yellow fat is metabolically inactive while some show that they have the potential to stimulate BMSCs to adipocytes.[139, 140]

Table 2: Expression and Function of primary adipogenic markers

Transcription factors	Expression	Activation/Function	Reference
C/EBP α	Forced expression of this isoform in 3T3-L1 preadipocytes stimulates adipogenesis in the absence of any hormonal induction	<p>C/EBPα is a pleiotropic transcriptional activator of adipocyte-specific genes. Promoters from numerous adipocyte genes contain C/EBP regulatory consensus sequences and are trans-activated by C/EBPα. Initially, C/EBPα is transcriptionally activated by C/EBPβ and C/EBPδ via a C/EBP regulatory element in the proximal promoter. Once C/EBPα is expressed, it appears the expression is maintained through auto activation; Phosphorylation of C/EBPα occurs at three sites in 3T3-L1 adipocytes. Two of these sites, Thr222 and</p>	[141]

		<p>Thr226, are insulin-sensitive sites phosphorylated by</p> <p>GSK-3. The third site, Ser230, is phosphorylated by an unidentified kinase. Thr222 and Thr226 become de-phosphorylated in response to insulin treatment through inactivation of GSK-3.</p>	
C/EBP β and C/EBP δ	<p>early regulators of preadipocyte differentiation; over-expression of either protein in 3T3-L1 preadipocytes accelerates adipogenesis; C/EBPβ can also promote adipogenesis to a limited extent in the multipotent NIH-3T3 cell line; C/EBPβ and C/EBP</p>	<p>Transcriptional activation of C/EBPα is controlled by cAMP response element binding protein (CREB). C/EBPα contains two cAMP responsive element-like cis regulatory sequences in the proximal promoter, and these sites are necessary in maintaining transcriptional activation; A number of kinases can phosphorylate C/EBPα ex vivo including protein kinase A, protein kinase C, MAP Kinase, Ca²⁺-calmodulin dependent</p>	[141]

	<p>δ function in the increase in the number of adipocytes</p>	<p>kinase II, cdk2, and GSK-3; Phosphorylation is an important posttranslational modification of C/EBPβ that leads to the acquisition of DNA-binding function as preadipocytes</p> <p>traverse to the G1-S checkpoint at the onset of mitotic clonal expansion</p>	
CHOP-10	<p>originally identified in a Chinese hamster ovary cells; ectopic expression of CHOP-10 blocks adipogenesis</p> <p>in 3T3-L1 cells</p>	<p>CHOP-10 is unique in that proline and glycine residues in the DNA-binding region interfere with its ability to bind DNA but do not alter its capacity to form heterodimers. Thus, CHOP-10 acts as a dominant negative isoform; CHOP-10</p>	[141]

		<p>heterodimerizes with</p> <p>C/EBPβ and prevents acquisition of DNA-binding</p> <p>to C/EBP regulatory elements</p>	
PPAR γ	<p>Stimulates differentiation of pre-adipocytes in 3T3-L1 cells.</p> <p>PPARγ is a mediator of the recruitment process in brown adipose tissue, by itself or in combination with other factors. The recruitment process in brown adipose tissue</p>	<p>PPARγ is a key regulator</p> <p>of differentiation in both brown and white adipocytes.</p>	<p>[136] [142]</p> <p>[143]</p>
Uncoupling protein 1 (UCP1)	<p>Found in interscapular, cervical, axillary, mediastinal and epididymal adipose tissues depots</p>	<p>UCP1 generates</p> <p>heat by dissipating the proton gradient that is established</p> <p>across the inner mitochondrial membrane</p>	<p>[136]</p>

		during the passage of electrons along the respiratory chain	
PRDM16 (MEL1)	Expressed in interscapular but not in epididymal WAT	Loss of PRDM16 from brown fat cells caused an increase in myogenic gene expression and bona fide skeletal muscle differentiation. Increased PRDM16 expression converted both immortalized and primary skeletal muscle myoblasts into brown fat cells action of PRDM16 is almost certainly dependent on its interaction with PPAR- γ because PRDM16 stimulated adipogenesis in a PPAR- γ -ligand-dependent; ability of PRDM16 to stimulate a brown fat phenotype in white fat precursors was through its association with PGC-1 α and PGC-1 β .	[136, 144]
MicroRNA 206 (miR-206)	expressed in cultured brown adipocytes		[136]

<p>Myosin regulatory light chain (MyIpf)</p>	<p>MyIpf gene is reasonably well expressed in</p> <p>brown adipose tissue from wild-type mice, although the level of MyIpf was still about 30 times lower than in muscle</p>		<p>[145]</p>
<p>Homeobox C9 (Hoxc9)</p>	<p>in white adipocyte</p> <p>cultures, Hoxc9 mRNA levels are significantly upregulated by</p> <p>rosiglitazone treatment (that promotes brite adipocytes); Within the adipose tissues, Hoxc9 was found in the brite</p> <p>depots, but it was not increased by cold acclimation, so its</p>		<p>[136]</p>

	<p>expression did not parallel the induction of UCP1 in the brite</p> <p>depots. It was less expressed in white depots. Hoxc9 was not expressed at all in brown depots; Short stature homeobox 2 (Shox2) being nearly selectively expressed in iWAT.</p>		
Transcription factor 21 (Tcf21)	white preadipocytes express the bHLH transcription factor Tcf21	<p>Member of the bHLH family of transcription factors. Regulates BMP4 expression that in turn can control white adipocyte formation.</p> <p>a transcription factor that has been shown to suppress myogenesis and nuclear receptor activity.</p>	Yuan et al 2011;[146]
Carbonic anhydrase3	Carbonic anhydrase 3 (Ca3) is found in BAT (and muscle) and is downregulated in mouse BAT by cold	A functional role for this abundant protein has still not been identified	[136]

	exposure; It has been stated that Ca3 protein may constitute 24% of the total protein content of white adipocytes		
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Chapter 4

MATERIALS AND METHODS

4.1 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).

SB203580 (p38), PD98059 (MEK) and Rapamycin (mTOR) were purchased from CalBiochem (San Diego, CA).

Taq polymerase enzyme was purchased from Genscript, DNTps and MgCl₂ buffer from Invitrogen and primers for Endoglin, PRDM16, TCF21, BMPR1b, GAPDH and BMPR1a were purchased from Integrated DNA Technology.

4.2 Primer Sequences

The following primer sequences were used:

BMPR1b-F-ATACCAGCTTCCCTATCACGACCT

BMPR1b-R-TGAAATTCTTGCTCTGTCCACAAGTA

PRDM16-F-CAGCACGGTGAAGCCATTC

PRDM16-R-GCGTGCATCCGCTTGTG

GAPDH-F-TGCTGGTGCTGAGTATGTCCG

GAPDH-R-CAAGCAGTTGGTACAGG

TCF21-F-CCACCTCAAACCCAACAC

TCF21-R-GTTCCCAGACTCGCACCT

BMPR1a-F-CAGAATCTGGATAGTATGC

BMPR1a-R-TGAGTCCAGGAACC

4.3 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.

4.4 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 are located at amino acids 213-217 (SLKD), 324-328, (SLYD) and 475-479 (SYED) . 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).

4.5 Design of BMPRIa 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). 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.

4.6 Isolation of RNA by Triazol method

C2C12 cells were grown to 90% confluence. The growth media was then removed and 7 ml Triazol reagent was added to each flask. The flasks were incubated for 3-5 minutes at room temperature to allow complete dissociation of nucleoprotein components. The lysed cells were transferred into 20ml sterile tubes and the genomic DNA was sheared with 2 passes through a 26 gauge needle. 0.2 ml chloroform/ml of Triazol was then added to each cell lysate and shaken vigorously for 15seconds. Next the tubes were centrifuge at 12,000g for 15 minutes at 2-8°C. Centrifugation allows the mixture to separate into lower

phenol chloroform phase, an interphase and a colorless upper aqueous phase. The aqueous phase is then transferred into a new tube. In the next step 5-10 μ g RNAase free glycogen was added that acts as a carrier of RNA to the aqueous phase. Next 0.5ml of isopropyl alcohol /ml Triazol was added to each tube. Samples were incubated at 15-30°C for 10 minutes and then centrifuged at 12,000g for 10 minutes at 2-8°. Post centrifugation, supernatant was removed, the pellet was washed with 75% Ethanol adding 1ml of 75% Ethanol/ml of Triazol. Samples were mixed by vortexing. Samples were then centrifuged at 7,500g for 5 minutes at 2-8°. Pellets were then allowed to dry completely and dissolved in 100 μ l RNAase free water.

4.7 Isolation of RNA by Ambion RNA Mini Prep

C2C12 cells were grown to 90% confluence in 6 well plates and the Ambion RNA miniprep was used to isolate RNA from the cells. Cells were transferred to RNAase free tube and centrifuged at 2000xg for 5 minutes at 4° to pellet. To this was added 0.3ml lysis buffer with 2—mercaptoethanol and then vortexed to lyse the cell walls. Cells were next transferred to 1.5ml tubes and passed 5-10 times through an 18 gauge needle attached to a syringe. In the next step 70% ethanol was added to the cell homogenate and vortexed again to disperse any precipitate. 700 μ l of the homogenate was added to a spin cartridge attached to a collection tube and centrifuged at 12,000xg for 15 seconds at room temperature. 700 μ l wash buffer was then added to the spin cartridge and centrifuged again at 12,000xg for 15 seconds. The elute was discarded. Next the spin cartridge was spun at 12,000xg for 15 seconds at room temperature to

dry and the elute was discarded again. 100µl RNAase free water was then added to the spin cartridge and incubated at room temperature for 1 minute. The cartridge was again centrifuged for 2 minutes at $\geq 12,000xg$ at room temperature to elute the RNA in to a new collection tube. Purified RNA was stored in at-20°C for future use.

4.8 Reverse Transcription Reaction

Concentration of RNA obtained by RNA-miniprep or by Triazol method was obtained by the Nanodrop. For Reverse Transcription reaction , 2µl DNTps, 2µl of oligoDT, 2µl MgCl₂ buffer ,0.5µl of RNAase inhibitor,4µl of RNAase free water and 0.75µl of Reverse Transcriptase enzyme purchased from IMPROM .To this is added 1µg of RNA and the volume is made up to 20µl with RNAase free water. Next the samples are incubated in a water bath at 42°C. Post incubation, the cDNA obtained was stored at -20°C or directly proceed to PCR reactions.

4.9 PCR

cDNA obtained from the Reverse Transcription reaction was added into PCR tubes with 4.5µl RNAase free water,1µl MgCl₂ buffer, 1µl of forward primer, 1µl of reverse primer, 1µl DNTps and 0.5µl of Taq polymerase enzyme purchased from Genscript.

PCR program was run with the following protocol:

4.10 Van Kossa

C2C12 cells were grown to 90% confluence and serum starved overnight before treatment. Next day cells were treated as per requirement of individual experiment. After 3 days, depending on the conditions of individual experiment, cells were washed with cold PBS pH7.4, fixed using 4% (w/v) paraformaldehyde 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 dH₂O) 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 dH₂O until dH₂O 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 bit 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.

4.11 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) paraformaldehyde 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 dH₂O 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 dH₂O for 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 bit 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. Plates have to be Cell Star from GBO; others do not support adipocyte growth.

4.12 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 manufacturer’s procedure with 3 µg DNA plasmid encoding MCK2.2 and 200 pmole 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 either van Kossa or Oil Red O staining was performed as previously described.

4.13 Double Stimulation of cells

Cells were grown to 90% confluency in 6 well plates and serum starved overnight before treatment. Cells were then treated with the peptides (CK2.2 or CK2.1 as per requirement of experiment). The peptides were then removed and treated with Inhibitors of MEK or ERK or mTOR, or siRNA for CK2 or siRNA for BMPR1a.

4.14 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 μ g 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), After 3 days cells were fixed and stained with van Kossa stain or Oil Red O stain as previously described.

4.15 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 ($p < 0.05$).

Chapter 5

RESULTS

5.1 Introduction

Study with the mutant varieties of BMPR1a is critical to understanding the pathways triggered downstream. Also since localization of the receptors plays an important role in receptor activation, it is important to investigate if the receptors are housed in caveolae or on plasma membrane itself.

5.1.1 MCK2.1 mediated osteogenesis is dependent on caveolae and smad

To understand the role of membrane domains in mediating osteogenesis, C2C12 cells were transfected with the mutant BMPR1a, MCK2.1. Previous results from our lab have shown that MCK2.2 induces osteogenesis via BMPR1a, CK2 and CAV1. To appreciate similar effects of MCK.1, transfected cells were fixed and stained and analyzed with ImageJ. It was found that MCK2.1 mediated osteogenesis depends on both smad and caveolae. Downregulation of CK2 however had no effect on osteogenesis.

5.1.2 Osteogenesis mediated by MCK2.3 occurs independent of caveolae and smad

Similarly to elucidate the role of MKC2.3 in mediating osteogenesis, cells transfected with MCK2.3 were fixed, stained and analyzed with ImageJ. Our

results show that MCK2.3 can induce osteogenesis and mineralization independent of caveolae, smad and CK2.

5.1.3 Osteogenesis via MCK2.3 is mediated by the ERK/MEK signaling pathway

As it was stated previously that downstream signaling via three different CK2 interaction sites of BMPR1a takes place through different pathways, our next goal was to look at which signaling cascades were activated. For this cells were transfected with MCK2.3 and then treated with Rapamycin and inhibitors for MEK and AKT. Cells were fixed and stained 4 days later and the extent of mineralization was analyzed by ImageJ.

Our results show that mineralization is significantly decreased in cells treated with inhibitor of MEK while Rapamycin and inhibitor of AKT has no significant effect.

5.1.4 BMPR1b expression is upregulated in C2C12 cells treated with 40nM CK2.2 only

C2C12 cells were grown to 90% confluence in medium flasks and then serum starved overnight. Next the synchronized cells were stimulated with 40nM CK2.2 peptide. Post 3 days stimulation cells were treated with Triazol reagent to lyse the cell membranes and RNA was isolated. cDNA was prepared from RNA and this cDNA was used to perform PCR. PCR results show a band for BMPR1b with 40nM

There are conflicting reports about BMPR1b expression in C2C12 cells. But expression of BMPR1b by stimulation of CK2.2 probably suggests a role in CK2.2 mediated adipogenesis.

Chapter 6

DISCUSSION

A critical growth factor for bone homeostasis is BMP2 and its signaling pathways. A Prosite search revealed that CK2 binds to BMP type 1A receptor at three possible sites (aa 205-208, aa 316-319 and aa 466-469) and this association prevents the constitutively active BMP type II receptor from transphosphorylating the type 1A receptor. From this perspective CK2 acts as a negative regulator of the BMP signaling pathway.[61, 103]. Additionally Nohe et al has showed that C2C12 cells express BMPR1b on the cell surface.[69] Studies done in C3H10T1/2 cells reveal that they are committed to adipogenesis and this is mediated by the BMPR1b receptors.[147] In the current study we found out BMPR1b expression is upregulated in C2C12 cells treated with 40nM CK2.2 only. And since our previous results show that CK2.2 also induces adipogenesis in C2C12 cells, we predict a link between adipocyte formation and BMPR1b expression in C2C12 cells. Also BMPR1b expression occurs with only 40nM stimulation of CK2.2 and not with any higher concentrations. This could be due to a transient expression of BMPR1b in a concentration dependent manner that is triggering the adipogenesis in the cells.

To study the CK2 interactions, peptides were designed by our lab to block the specific CK2 interaction sites. These peptides named as CK2.1, CK2.2 and CK2.3 prevent the CK2 interaction with BMPR1a at the specific sites that they are named after. To understand the effects induced by the

peptides more specifically, mutants of BMPR1a were synthesized. The mutants, MCK2.1, MCK2.2, and MCK2.3, each had a single point mutation at the CK2.1, CK2.2 and CK2.3 binding sites. When these mutants were expressed in the C2C12 cells it was observed that only MCK2.2 and MCK2.3 induced osteogenesis in the cells while MCK2.1 did not have any significant effect. On the other hand when looking at adipogenesis, MCK2.3, did not form lipid droplets while MCK2.2 increased lipid droplet production as evident by Oil Red O staining. MCK2.1 had no effect on adipocyte formation. (Moseychuk et al; unpublished).

Microdomains in the plasma membrane like caveolae and the clathrin coated pits (ccps) are associated with BMP signaling. Studies have been done to elucidate the control mechanism by which BMP2 signaling is regulated by these domains. While some studies suggest that lipid rafts or ccps facilitate the signaling some others suggest that they have an inhibitory effect.[61, 103, 148] Caveolae associated proteins such as CAV-1 has shown to play key roles in the signaling cascade.[103] Our studies with cells transfected with MCK2.2 and MCK2.1 show osteoblast formation and this is dependent on caveolae and smad signaling. However the expression of MCK2.3 in C2C12 cells increased mineralization independent of smad or caveolae. (Fig: 4A and 4B)

BMP2 induces different signaling pathways downstream as smad, p38, NF κ β , ERK and AKT [149-151].The current dogma shows that osteogenesis occurs through the Smad4 and mTOR-AKT pathway, while adipogenesis utilizes the Smad4, MEK, and p38 pathways.[110] Depending on cell lines both p38 and Smad signaling induce adipogenesis by the upregulation of PPAR γ

[106]. On the other hand trabecular bone-derived osteoblasts revealed a role for p38 and Smad pathways during osteoblast maturation [152]. PI3K has also been shown to be stimulated in the presence of BMP2 ligand [61] [112], but the mTOR-AKT pathway role has not been elucidated to date. Additionally, BMP2 can also induce the PI3K/AKT pathway in a number of cancerous cell lines (prostate, gastric, pancreatic, ovarian cancers, and melanoma) [153]. Studies using MCK2.2 demonstrated that the mTOR-AKT pathway is the key inducer of osteogenesis. The ERK/MEK pathway acts via MCK2.3 (Fig: 5A) to induce osteoblast production in a non-smad, non caveolae environment. (Fig: 4B)

In conclusion a novel model for the BMP2 signaling pathway can be formulated from the data. The mutants MCK2.1, MCK2.2 and MCK2.3 signal via different pathways to induce either osteogenesis or adipogenesis. Smad and mTOR induced osteogenesis when triggered via MCK2.2 housed in the caveolae. MCK2.3 however signal independent of caveolae via ERK. MCK2.1 again induced osteoblast formation when located in caveolae through phosphorylation of smad proteins. (Fig: 5) This interplay of different pathways being mediated by the BMPR1a mutants can be utilized to control mineralization of cells. Also striking a balance between osteoblast production and adipocyte generation is key to prognosis of osteoporosis. Since these different pathways and the various routes are adopted by the mutants, it can serve as a novel way to develop therapeutics for osteoporosis.

Table 3: Summary table showing the different pathways of BMPR1a mutants

Mutants	Caveolae dependence	CK2 dependence	Pathways involved in osteogenesis	Pathways involved in adipogenesis
MCK2.1	Caveolae dependent	CK2 independent	SMAD	---
MCK2.2	Caveolae dependent	CK2 dependent	SMAD, mTOR	p38, MEK-ERK
MCK2.3	Caveolae independent	CK2 independent	MEK-ERK	---

Chapter 7

TYPE III RECEPTOR ENDOGLIN

7.1 Endoglin (CD105)

Endoglin or CD105 is a Type I homodimeric glycoprotein, expressed in a host of cell lines. Endoglin was first discovered in the HOON pre B cell line.[154] Endoglin expression has also been confirmed in activated macrophages, normal pro-erythroblasts derived from adult bone marrow,[154] adult endothelial cells, monocytes, (87) vascular endothelial cells and smooth muscle cells.[155]

Low levels of Endoglin expression has been reported in resting endothelial cells, but it is highly expressed in vascular endothelial cells that are active sites of angiogenesis, during embryogenesis [156] [157], in inflamed tissues and healing wounds [158], inflamed synovial arthritis [159], sites of vascular injury [160], and in tumor vessels [161-164]. Conditions like ischemia, reperfusion in the kidney [165], hind limb [166], and heart [167] as well as organs undergoing fibrosis as liver [168] and kidney [169] also induce Endoglin overexpression. In contrast to normal smooth muscle cells [170], in vascular smooth muscle cells (VSMCs) of human atherosclerotic plaques [171] Endoglin expression is higher. Endoglin expression in cardiac fibroblasts controls the profibrogenic

actions of angiotensin II [172]. Human fetuses with cardiac defects show an altered expression of Endoglin [173]. Fourth week onward, human embryos also show expression of Endoglin in the vascular endothelium and has been reported to be critical for all the stages of development of the cardiovascular development. Transient upregulation of Endoglin is also observed in mesenchymal tissue during heart septation. [174]

The extracellular domain of membrane-bound Endoglin can be proteolytically cleaved, releasing a circulating form of Endoglin named soluble Endoglin (Sol-Eng). Increased levels of Sol-Eng are linked to the pathogenesis of severe vascular diseases, such as systemic sclerosis and pre-eclampsia. Increased Sol-Eng levels correlate with metastasis in breast cancer and colorectal cancer.[175] Sol-Eng levels were decreased in patients receiving chemotherapy, which reduces the utility of Sol-Eng as a prognostic marker to the long-term follow-up of cancer survivors who are not treated with chemotherapy.[175]. In prostate cancer, high levels of serum Sol-Eng correlate with advanced stages of tumor progression and urinary Sol-Eng seems to be a useful marker for diagnosis.[176] High levels of Sol-Eng are also present in myeloid malignancies that are characterized by a high cellular proliferation rate, such as acute myeloid leukemia and chronic myeloproliferative disorders [35].

7.2 Endoglin Structure:

Endoglin is an integral membrane protein composed of 561 amino acid extracellular domain and 47 amino acid long cytosolic domain.[177]. Structural analysis of Endoglin reveals that it belongs to the Zona Pellucida (ZP) family of proteins, thus being composed of 260 residue Zona Pellucida domain that also comprises of 8 conserved cysteine residues.[178] . The three-dimensional structure of the extracellular region of Endoglin shows that Endoglin comprises of antiparallel-oriented monomers enfolding a cavity. A high-resolution structure of Endoglin also indicates that each Endoglin subunit comprises three well-defined domains, two ZP subdomains and one orphan domain, which are organized into an open U-shaped monomer [179] The ZP domain in Endoglin is again subdivided into two ZP subdomains that are critical for the oligomerization of the receptor. Additionally different mammalian species show the existence if a highly conserved potential protease cleavage site in the extracellular region of Endoglin, located between the two subdomains of the ZP domain. (Fig.8B). It is hypothesized the ZP domain could be involved in a mediating protein- protein interactions but there is no direct evidence yet.

Post translational glycosylation in Endoglin leads to four N-linked glycosylation as well as an O-glycosylated domain that is rich in serine and threonine residues.[177] Also in the extracellular domain of human Endoglin, there is an Arg-Gly-Asp or RGD motif that has been proposed to be crucial for interaction with integrins and RGD-binding receptors. [180]

Endoglin primarily exists in the long L-form and an alternatively spliced short S-form. The structurally distinct forms differ from each other in their lengths of the cytosolic domains. While the L-form contains the full length

47 residue cytoplasmic domains, the S-form only possesses a 14 amino acid cytoplasmic domain.[181]. Lastres et al has showed that both the isoforms can be phosphorylated by serine threonine kinases as well as TGF β receptors.[180] Also a degree of phosphorylation in L-Endoglin has been found to be greater than that in S-Endoglin since the L-form contains 19 Ser/Thr residues as opposed to only two in the S-form.(Fig:8B)

The carboxyl terminus of the cytoplasmic domain of Endoglin comprises of a consensus postsynaptic density 95/Drosophila disk large/zonula occludens-1 (PDZ)-binding motif (Ser-Ser-Met-Ala) the facilitates interaction with several PDZ domain-containing proteins as well as phosphorylation of distal threonine residues.[161]

7.3 Endoglin interacts with various Proteins

The LIM domain proteins

The cytoplasmic domain of Endoglin has been reported to interact with *Zyxin* and other potential *LIM* domain containing proteins in human umbilical vein vascular endothelial cells. *Zyxin* has also been reported to interact with other proteins like CRP2 at the LIM1 domain [182] while it binds to h-warts/LATS1 and p130^{cas} at LIM1 and LIM2 domains.[183, 184] CRP2 binds to the LIM1 domain of *Zyxin* [185, 186], and h-warts/LATS1 and p130cas bind to LIM domains 1 and 2 [183] [184]. Both *Zyxin* and *Zyxin*-related protein-1 (ZRP-1) interact with p130^{cas}. The ZRP-1 [186]interaction with p130cas has been reported to be between LIM1 and Lim2 domains [183]. Also it has been reported that the LIM domains 2 and

3 of Zyxin interact with Endoglin. Thus there is prospect of existence of a multi protein that could modulate various protein–protein interactions.

The TGF β receptors

TGF β RII and ALK5 have been shown to interact with Endoglin, leading to phosphorylation of Endoglin in the cytosolic domain. [187] Recent reports show that phosphorylation of Endoglin interacts differently with constitutively active ALK1 as opposed to constitutively active ALK5. Endoglin interacts with ALK1 as a direct kinase substrate and thus has preferential phosphorylation on the threonine residues. Also data indicates that the carboxyl terminal PDZ domain containing RGD motif is critical for control of phosphorylation of Endoglin. [188]

Chapter 8

RESULTS

8.1 Introduction

To investigate how the peptide stimulation affected expression of fat markers in C2C12 cells we selected TCF21 a white fat marker and PRDM16 a brown fat marker. Also to delineate the temporal behavior of adipocyte marker expression we performed RT-PCR. While we also investigated BMPR1b expression, expression of Endoglin, a coreceptor for the BMP signaling pathway was also examined.

8.1.1 Expression of Endoglin in C2C12 cells is regulated by stimulation of cells by CK2.1 only

To look at some other key players involved in the downstream signaling mediated by the peptides, we looked at Endoglin which has been studied extensively for its role in angiogenesis, migration and invasion in various cell lines. Endoglin is a co receptor in the BMP signaling pathway and has the potential to modulate and control the various downstream events.

C2C12 cells treated with various concentrations of the peptides were tested for Endoglin by RT-PCR. We found that cells treated with different concentrations (50nm, 100nm and 500nm) of peptide CK2.1 express Endoglin.

8.1.2 Stimulation of cells by peptides CK2.2 AND CK2.1 leads to expression of PRDM16 and TCF21

A recent study in human adipocyte derived cells showed that cells depleted of Endoglin have the potential to signal for osteogenesis whereas cells replete with Endoglin inhibit osteogenesis by downregulating the TGF β pathway.

In our current study to look at the effect of Endoglin on adipogenesis, we performed RT-PCR for markers of brown adipose tissue (BAT) and white adipose tissue (WAT).

PRDM16 which is a widely accepted marker for BAT was shown to be upregulated in cells treated with both peptides CK2.2 and CK2.1. Thus it seems that adipogenesis mediated by peptide CK2.1 is regulated through Endoglin.

(Fig: 9A, 9B, 9C)

8.1.3 Time course RNA isolation shows that cells stimulated with CK2.2 express PRDM16 at day1 and day2 while those stimulated with CK2.1 do not.

C2C12 cells were treated with CK2.2 and CK2.1 peptides separately for 1 and 2 days. Post stimulation RNA was isolated via Triazol method and cDNA was generated by reverse transcription method. To analyze the time points when the peptides CK2.1 and CK2.2 express brown fat, we looked at PRDM16. We found that cells treated with 40nM CK2.2 expressed PRDM16 day1 onwards while cells treated with only CK2.1 did not. (Fig 9D) Endoglin and TCF21 expression were also looked at. CK2.1 and CK2.2 stimulated cells were not seen to be upregulating Endoglin or white fat. (Results not shown)

8.1.4 Treatments with Rapamycin upregulates PRDM16 expression in C2C12 cells

As mentioned earlier MCK2.2 induces adipogenesis in C2C12 cells through MEK/ERK and the p38 pathway while Rapamycin has no effect on adipogenesis. So we wanted to treat C2C12 cells with Rapamycin and look at adipogenesis.

When cells were treated with 30nM Rapamycin and stimulated with CK2.2 for 3 days and post harvesting of RNA and subsequent production of cDNA, we found that cells expressed PRDM16, the quintessential brown fat marker. Thus suggesting that adipogenesis mediated by MCK2.2 as well as the peptide CK2.2 is through the same route. Cells treated with CK2.1 were also shown to be expressing PRDM16 in presence of Rapamycin. (Fig: 9E)

8.1.5 Treatments with MEK inhibitor or p38 inhibitor does block adipocyte formation in C2C12 cells

As previously mentioned, C2C12 cells were treated in the same way with 5µg MEK inhibitor and 1µg p38 inhibitor as well as with 40nM of either of the CK2.2 or CK2.1 peptide.

PCR analysis of the cDNA obtained from the stimulated cells showed no expression of TCF21 or PRDM16 or Endoglin. The inhibitors thus blocked adipogenesis in cells. (Fig:9F and 9G)

8.1.6 Endoglin expression downregulates osteogenesis in C2C12 cells

Since the study in adipocyte derived cells as mentioned previously, suggested that osteogenesis is downregulated in cells with low Endoglin expression, we wanted to look at the effect of Endoglin on osteogenesis in C2C12 cells.

C2C12 cells were treated with BMP2 or peptides or transfected with Endoglin DNA both the long form and short form and also a mutant form that has portion of the cytoplasmic tail removed. Cells were next fixed and stained with AgNO₃ for mineralization.

Our results show that both the forms of Endoglin as well as the mutant inhibit osteogenesis. Also as compared to peptide CK2.3 which has been proved to induce significant mineralization even in the absence of BMP2, Endoglin downregulated osteogenesis. Additionally for peptide CK2.2, mineralization was also inhibited by Endoglin. Although the data suggests that the inhibition of osteogenesis mediated by CK2.2 by the different forms of Endoglin and the mutant are different. Similarly osteogenesis mediated by CK2.1 is also inhibited by Endoglin and the mode of downregulation is different for each form and the mutant of Endoglin. (Fig: 10A B, C, D)

8.1.7 Endoglin expression upregulates adipogenesis in C2C12 cells

Next we wanted to look at adipogenesis induced by Endoglin post stimulation by the peptides. So in the same way C2C12 cells plated in 24 well plates were treated with BMP2 or transfected with Endoglin DNA both the long form and short form and also a mutant form that has portion of the cytoplasmic tail removed (ΔC). Cells were next fixed with 4% paraformaldehyde and stained with Oil Red O. Analysis was then performed for red lipid droplets by ImageJ.

Our analysis shows that the L -form of Endoglin induces significantly lower adipogenesis as compared to cells treated with either BMP2 or the mutant form of Endoglin. Our results thus seem to suggest that it is the cytoplasmic domain of Endoglin that inhibits adipogenesis in C2C12 cells. On the other hand the mutant form of Endoglin ΔC that is devoid of the cytoplasmic domain alone induces adipogenesis in C2C12 cells. (Fig: 11A)

8.1.8 Adipogenesis induced by CK2.1 is independent of Endoglin.

To look at adipogenesis induced in C2C12 cells stimulated with CK2.1 and transfected with Endoglin DNA, the cells were again plated in 24 well plates, and stimulated with the CK2.1 and transfected with Endoglin DNA. 4 days post stimulation cells were fixed with 4% paraformaldehyde and stained with Oil Red O.

Analysis by ImageJ shows that Endoglin has no influence on CK2.1 mediated adipogenesis. Also the mutated Endoglin lacking the cytoplasmic domain does not have any effect on adipogenesis induced via stimulation by CK2.1. Thus our results seems to suggest that adipogenesis induced via CK2.1 is independent of Endoglin and possibly Endoglin and its mutants act upstream of CK2.1 signal. (Fig: 11B)

8.1.9 CK2.3 has no effect on adipogenesis induced by Endoglin.

To understand adipogenesis induced by CK2.1 and simultaneous transfection by Endoglin DNA and its mutant, similar experiments as before were performed. After staining by Oil Red O analysis of the wells were done by ImageJ.

Our previous results show that CK2.3 does not induce significant adipogenesis as compared to BMP2. Thus when C2C12 cells were transfected with Endoglin DNA and simultaneously stimulated with CK2.3 we found no significant adipogenesis even with ΔC . Thus this proves that Endoglin and its mutants function upstream of the signaling cascade and CK2.3 downstream counteracts adipogenesis induced by Endoglin and its mutants. (Fig: 11D)

Chapter 9

DISCUSSION

Adipocyte differentiation is a critical event that involves a plethora of genes and proteins and their receptors although all the details in the adipocyte differentiation are yet to be determined.[189, 190] (Fig:7) Our earlier studies have revealed that treatment with peptides CK2.2 and CK2.1 stimulated adipocyte formation. Thus we were interested to look at the role of BMP2 signaling pathway in fat formation. PCR results revealed that when C2C12 cells were treated with peptide CK2.2 for three days, there was an upregulation in expression for BMPR1b as well as PRDM16, a known marker for brown fat. Again in cells treated with CK2.1, besides PRDM16, TCF21 a marker for white fat was also upregulated. Interestingly BMPR1b expression was lacking and instead Endoglin, the BMP co-receptor was expressed. A time course experiment however showed the expression of Endoglin occurs only on the third day while on the first and second day cells treated with CK2.2 showed expression of only PRDM16. (Fig: 9D)But BMPR1b did not express itself in cells treated with CK2.2 although PRDM16 expression as observed. (Fig: 9D) Thus it can be proposed that initially at least adipogenesis via CK2.2 is being mediated by BMPR1a.Since previous studies have linked BMPR1b upregulation to adipogenicity in cells,[147] we propose that adipocyte formation in C2C12 cells treated with CK2.2 could be triggering the BMPR1b expression, Our previous studies with MCK2.2 had also revealed that

adipogenesis in C2C12 cells is facilitated via the MEK and p38 pathways and independent of mTOR pathway. (Moseychuk et al; unpublished). Thus our results with Rapamycin (inhibitor of mTOR) corroborate the fact that the inhibitor has no effect on adipogenesis. Post three days of treatment with 30nM Rapamycin and with either of peptides CK2.2 or CK2.1 did not obstruct brown fat expression in the cells. (Fig:9E) while treatment with MEK inhibitor or p38 inhibitor showed no Endoglin, white fat (TCF21) or brown fat (PRDM16) expression. (Fig: 9F and 9G)

Several pathways like Smad, H-Ras and MAPK have implicated the role of Endoglin in various cancer cell lines.[155, 163, 191-194] Endoglin also plays key roles in adhesion, migration and invasion of cells. [192, 195, 196] Endoglin has been associated with TGF β receptors where it plays key roles in modulating downstream signaling to maintain cellular pathophysiology. Recently it was demonstrated that human adipocyte derived stromal cells (hASCs) rich in Endoglin (CD105) can induce fat formation while hASCs lacking CD105 do not.[197].In the current study, for the first time we show that Endoglin has the potential to downregulate osteogenesis and up regulate adipogenesis in C2C12 cells. For this study a mutant of Endoglin as well as the short form of Endoglin, S-Endoglin has also been used to elucidate its role in maintaining cellular homeostasis. The mutant variety used in this study is ΔC that is lacking the 47 residue cytoplasmic tail.

Our findings show that osteogenesis mediated by the peptides CK2.1, CK2.2 and CK2.3 are all downregulated by Endoglin. (Fig: 10B, 10C and

10D). Also Endoglin alone can downregulate osteogenesis and this is significantly lower than that induced by BMP alone. (Fig: 10A)

We find that when cells are transfected with a plasmid encoded for ΔC Endoglin, it alone can induce adipogenesis significantly higher than BMP while the L- form Endoglin that has the full cytoplasmic tail, alone also induces fat formation significantly over that in untransfected cells. (Fig: 11A) A possible explanation for this could be the PDZ domain (SSMA) containing cytoplasmic tail of Endoglin is inhibiting the enhanced lipid droplet formation. Alternatively when the C2C12 cells are treated with the peptide CK2.1 and transfected with L-Endoglin, or S-Endoglin or the mutant ΔC , there is no effect on adipogenesis as observed via Oil Red O staining. CK2.1 alone induces adipogenesis in C2C12 cells as we have seen earlier. With different isoforms and mutant variety of Endoglin used in the study, we observe no effect on adipogenesis mediated via CK2.1. Although it remains to be seen if there is any direct interaction of Endoglin with CK2 or if there is any change in phosphorylation status of Endoglin post transfection, it is interesting to note that signaling via Endoglin could be occurring downstream of CK2.1.(Fig:11B) As mentioned earlier CK2.3 does not induce adipogenesis but is a mediator of osteogenesis. However when observing the effects of Endoglin and CK2.3 in C2C12 cells, it is seen that fat formation is independent of Endoglin. (Fig: 11D) CK2.3 induces an osteoblast differentiation program in the C2C12 cells and Endoglin could be playing a role in overruling this differentiation program in favor of the adipocytes. Adipogenesis data suggests CK2.2 also has no effect on Endoglin mediated adipocyte generation. (Fig: 11C) Our PCR results show that CK2.2

induces adipogenesis through brown fat formation in the absence of Endoglin but we find expression of BMPR1b in cells. As mentioned earlier BMPR1b has been linked to adipogenic behavior exhibited by cells and thus this suggest BMPR1b could be a mediator of adipogenesis in C2C12 cells via CK2.2.

A new model for adipogenesis can be thus explained that could elucidate the role of Endoglin in adipogenesis. The peptides seem to stimulate adipogenesis through different pathways and seem to be doing it in a time-dependent fashion.

9.1 Future Work

The study with Endoglin opens up a whole new avenue which can be explored further in a number of ways. My studies have primarily dealt with Endoglin expression in the stromal cell line. It would be very interesting to see how Endoglin expression varies in other in vitro work in mice or rats. Even looking at Endoglin action in human osteoporotic bone cells would be a step forward. Since Endoglin is coming up to be a potential up regulator of adipogenesis in cells, effect of siRNA against Endoglin can also be a possible therapeutic to see if Endoglin downregulation has any effect on osteogenesis or not. Also since I was not able to do any work in protein level, expression of Endoglin and associated proteins can be looked at via Western Blot. This again would be an interesting study as it would bring forward if there any additional proteins associated or not. Role of Endoglin in adipogenesis is still in the fledgling status and thus there are numerous avenues that can be probed.

Chapter 10

CONCLUSION

10.1 Summary and Prospects

Thus overall this study suggests that adipogenic and osteogenic control in the BMP signaling pathway is a highly complex sequence of events that not only involves other signaling cascades as ERK or mTOR but also novel regulatory proteins as Endoglin. Role of Endoglin in the BMP signaling pathway is still under scrutiny and the exact interactions or the mechanistic aspects are yet to be determined. But the trend of Endoglin to downregulate osteogenesis in favor of adipogenesis definitely opens up an interesting chapter. Looking at further roles of Endoglin like its interaction with CK2, or involvement in associated pathways or its localization with the different receptors could help answer many critical questions about osteoporosis.

Osteoporosis and related fractures are a matter of growing concern in the modern society. Unavailability of effective cures makes this study with Endoglin all the more interesting. It opens up a wide field of study to elucidate further the mode of maintenance of bone homeostasis by Endoglin.

Fig 1

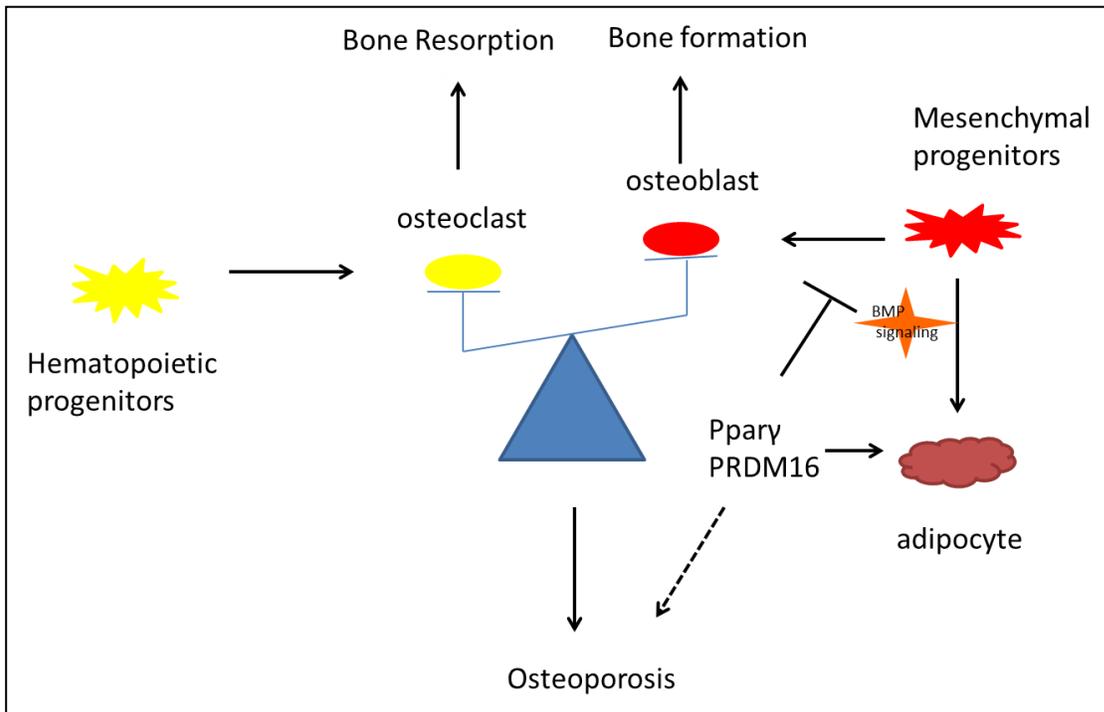


Figure 1: Osteoporosis is caused by an imbalance in the bone formation and bone resorption process. While osteoclasts are derived from hematopoietic progenitors, mesenchymal progenitors give rise to osteoblasts. The imbalance also causes formation of fats in bones that can be detected by presences of markers as Ppar γ or PRDM16. BMP signaling is believed to be playing a critical role in maintaining this homeostasis in bones.

Fig 2

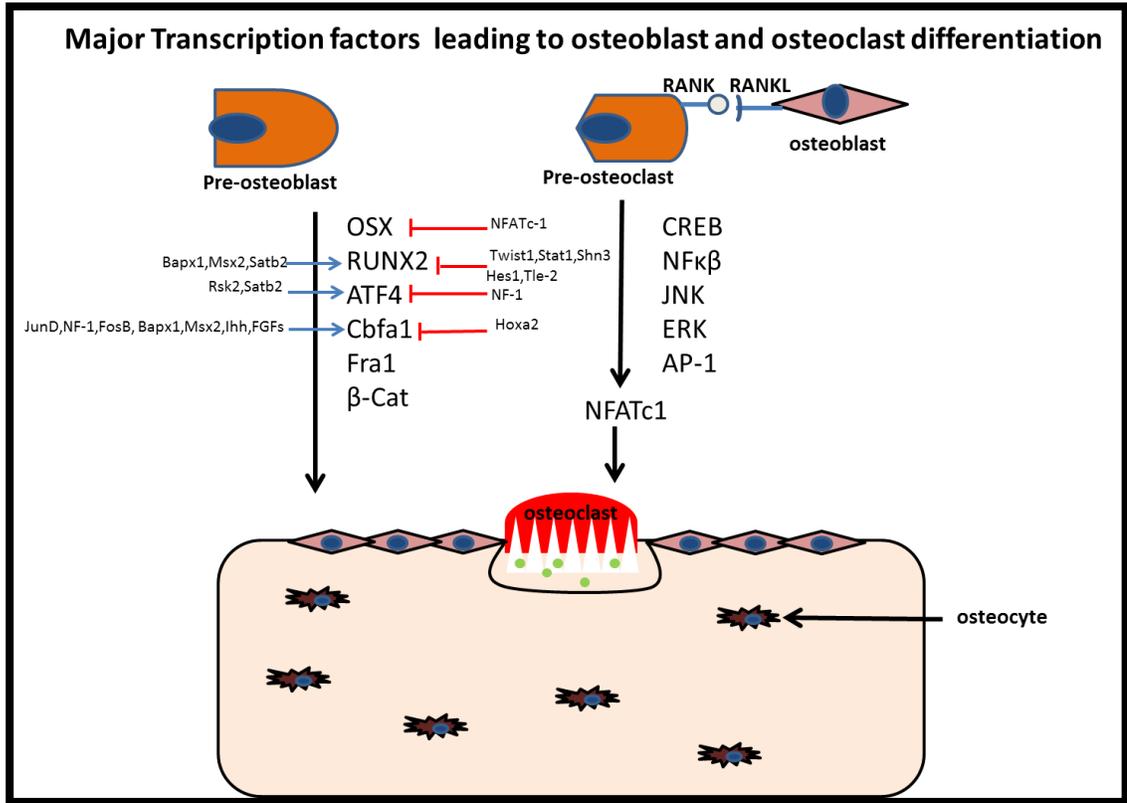


Figure 2: Principal transcription factors involved in the differentiation of osteoblasts and osteoclasts.

Fig 3

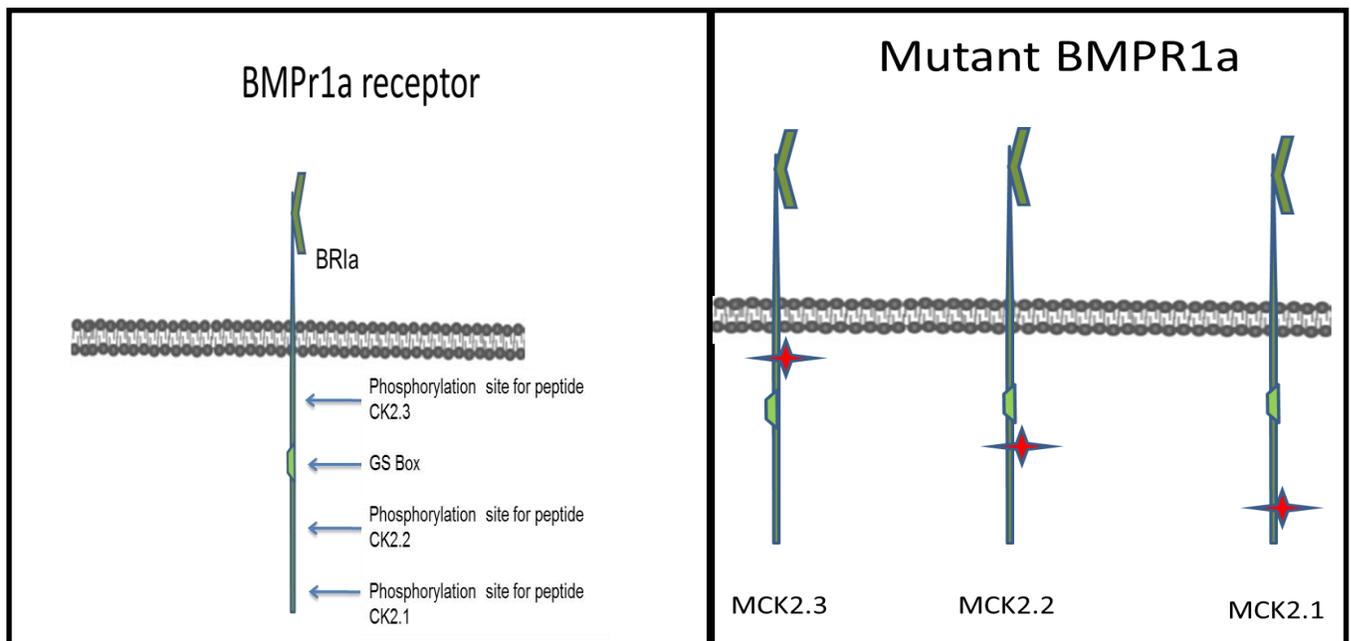


Figure 3: BMPR1a receptors consist of three potential CK2 interaction sites that are designated as CK2.1, CK2.2 and CK2.3. In between sites CK2.3 and CK2.2 there is a glycine–serine (GS) rich box that facilitates the phosphorylation of the receptor for downstream signaling. Mutants of BMPR1a were synthesized that have single point mutations at any one of these three sites and they were names MCK2.1, MCK2.2 and MCK2.3 after the sites they were mutated at CK2.1, CK2.2 and CK2.2 respectively. Red star indicates mutations in the BMPR1a receptor.

Fig 4A

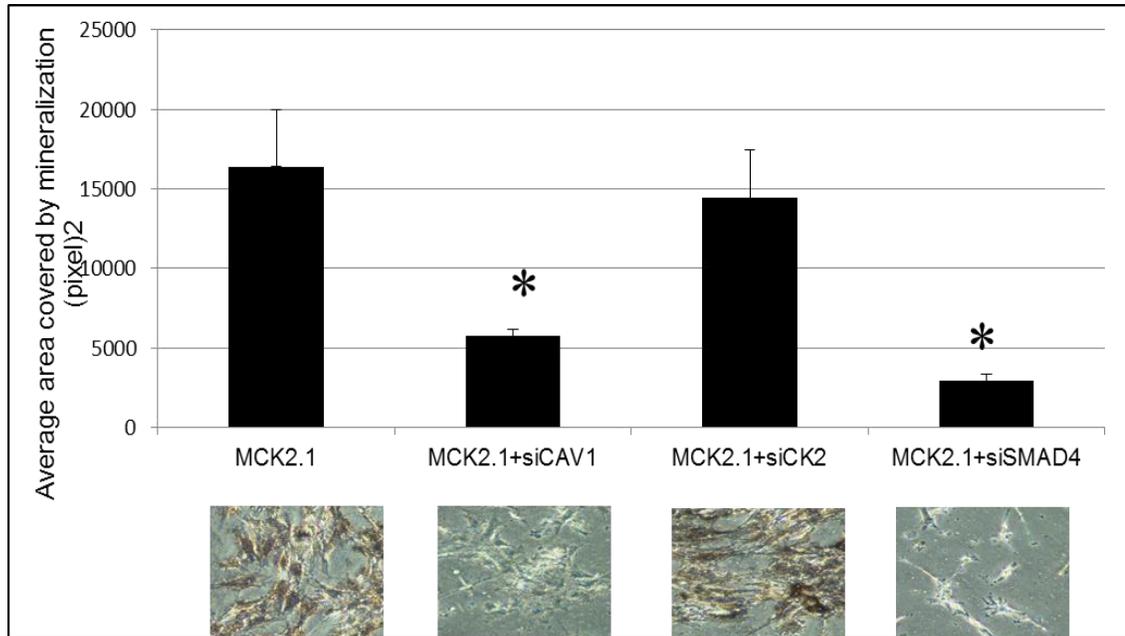


Figure 4A: Mineralization area covered by cells transfected with MCK2.1 and stimulated with siCAV1 and those transfected with MCK2.1 and stimulated with siSmad4 was significantly lower as compared to the positive control. Anova and Tukey's test results confirm that cells transfected with MCK2.1 and stimulated with siCK2 however shows comparable mineralization to the positive control. Lower panel shows representative wells for each treatment.

Fig 4B

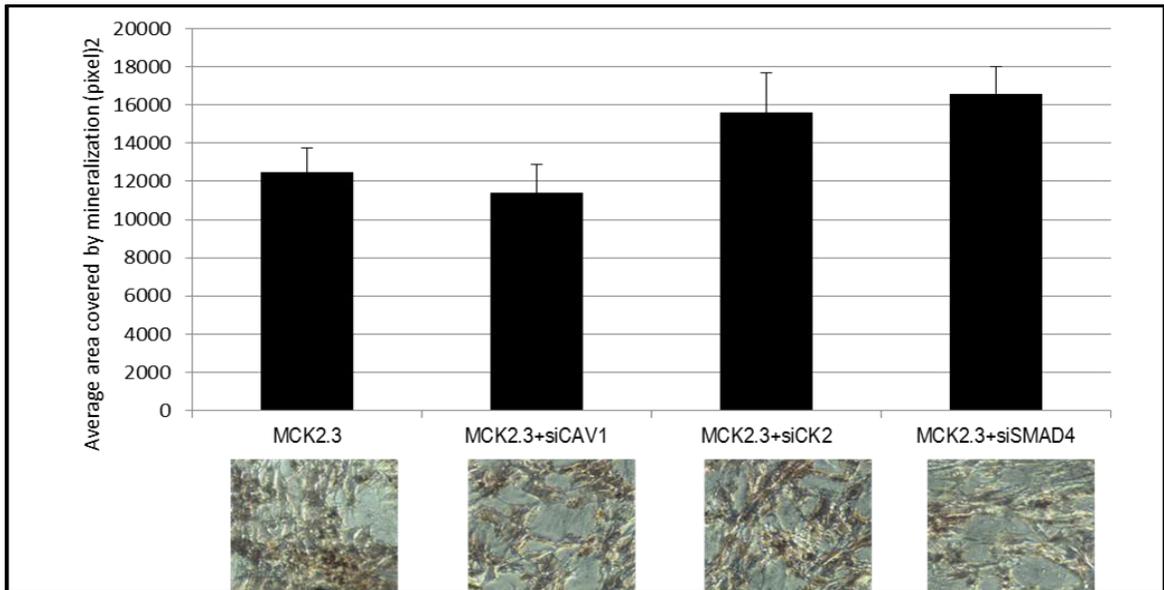


Figure 4B: Statistical analysis shows that mineralization area covered by cells transfected with MCK2.3 and treated with either siSmad4 or siCAV1 or siCK2 have no effect on mineralization. All the wells showed mineralization comparable to that of the well transfected with MCK2.3 alone. Standard Anova and Tukey's test were used to confirm the results. Lower panel shows representative wells for each treatment.

Fig 5A

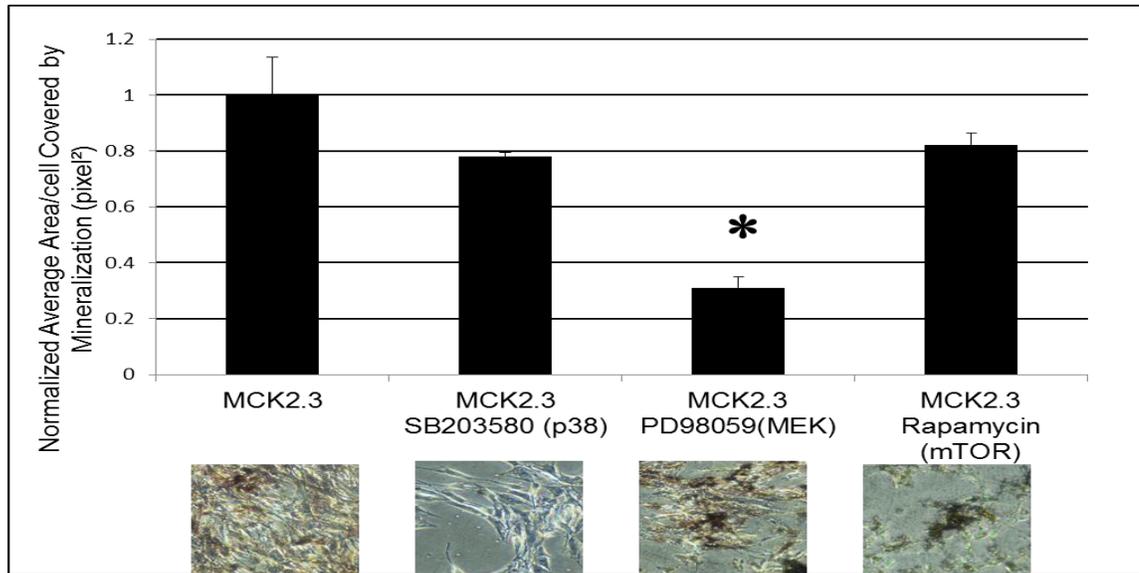


Figure 5A: Mineralization area covered by cells transfected with MCK2.3 and treated with either 5nM p38 inhibitor or 30nM Rapamycin (mTOR inhibitor) is not significantly different from that in cells transfected with MCK2.3 alone. However cells transfected with MCK2.3 and treated with MEK inhibitor show significantly lower mineralization compared to the positive control. Anova and standard Tukey's test were used for statistical analysis. Lower panel shows representative wells for each treatment.

Fig 5B

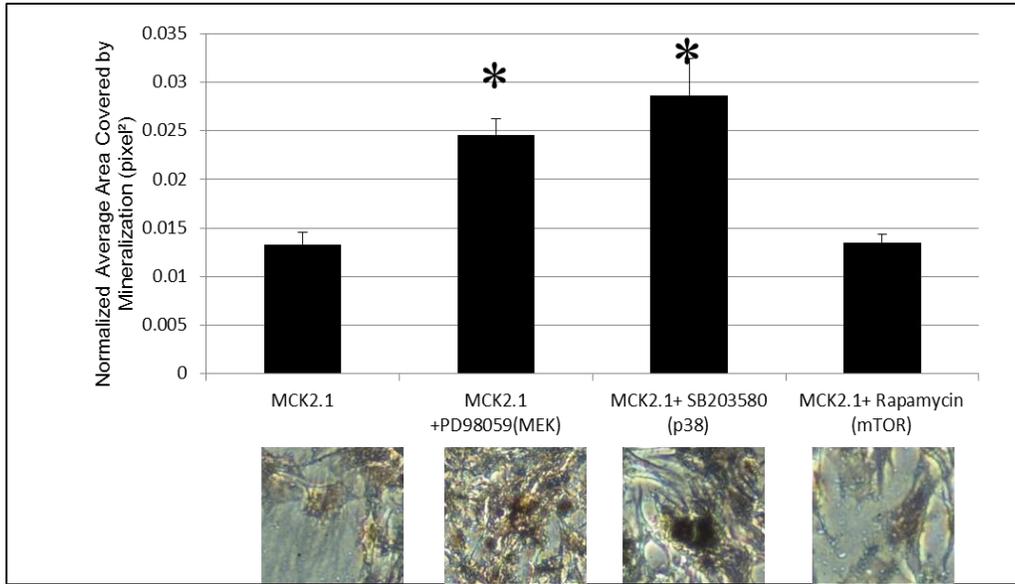


Figure 5B: Mineralization area covered by cells transfected with MCK2.1 and treated with MEK inhibitor, p38 inhibitor or Rapamycin. None of the inhibitors had any inhibitory effect on mineralization while MEK inhibitor and p38 inhibitor actually significantly increased mineralization. Standard Tukey's Test and Anova have been used for statistical analysis. Lower panel shows representative wells for each treatment

Fig 6

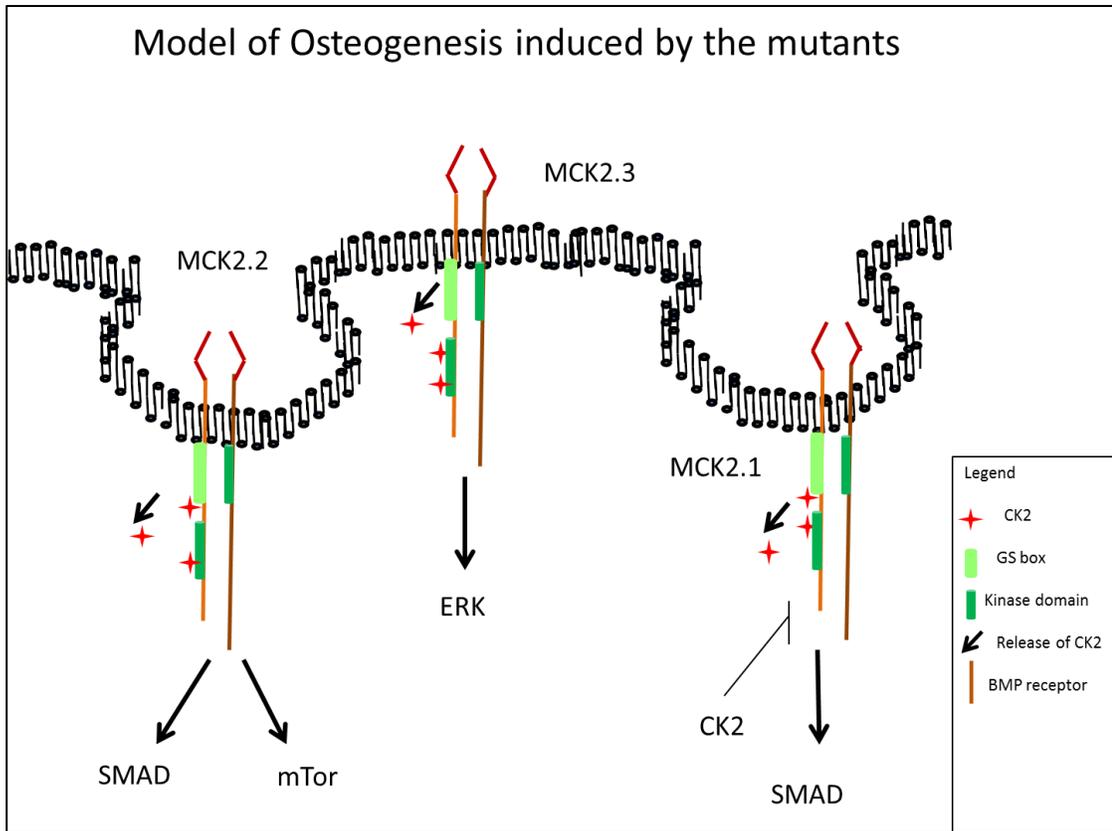


Figure 6: Model for the mutants used in the present study. MCK2.3 and MCK2.1 both signal via caveolae while MCK2.2 does not. MCK2.3 induces osteogenesis in C2C12 cells via the ERK/MEK pathway while both MCK2.2 and MCK2.1 signal through SMAD. It is proposed that the CK2 could be playing an inhibitory role downstream of SMAD.

Fig 7

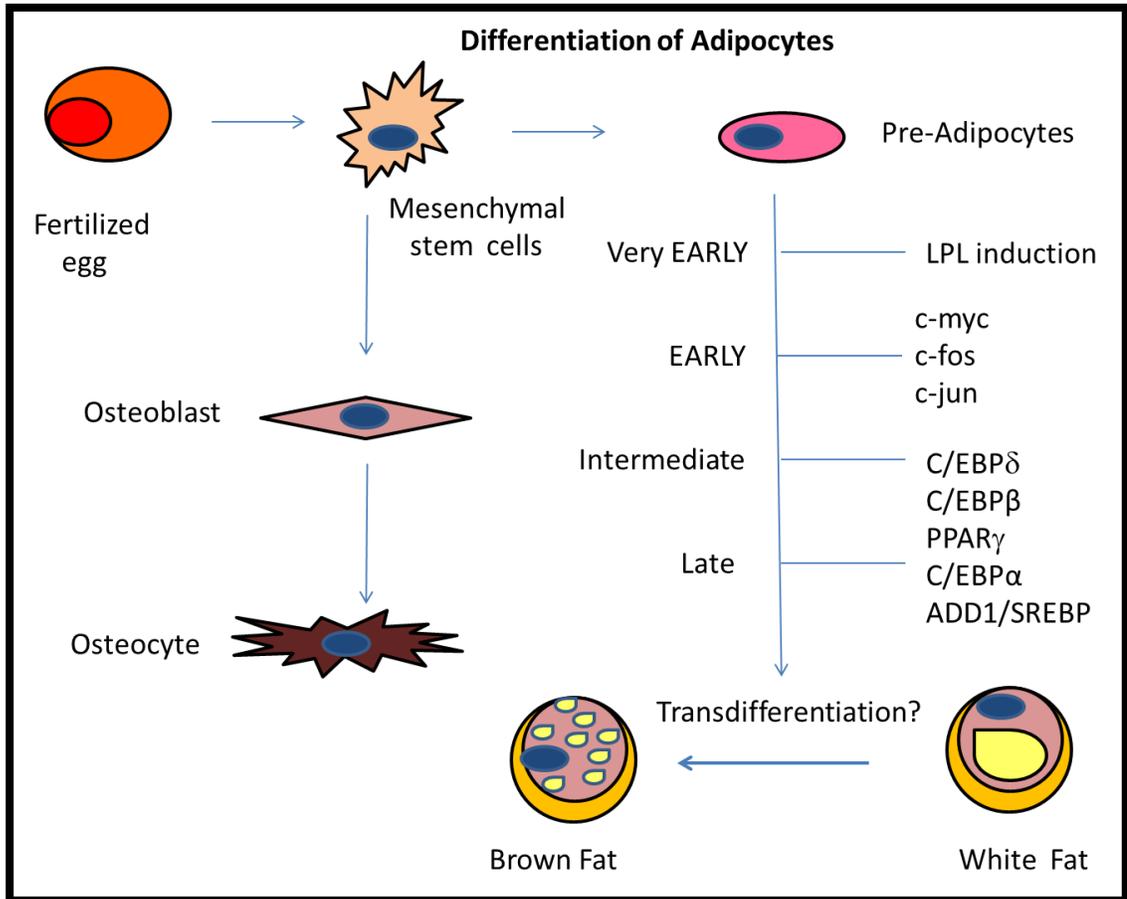


Figure 7: Transcription factors involved in differentiation of adipocytes

Fig: 8A

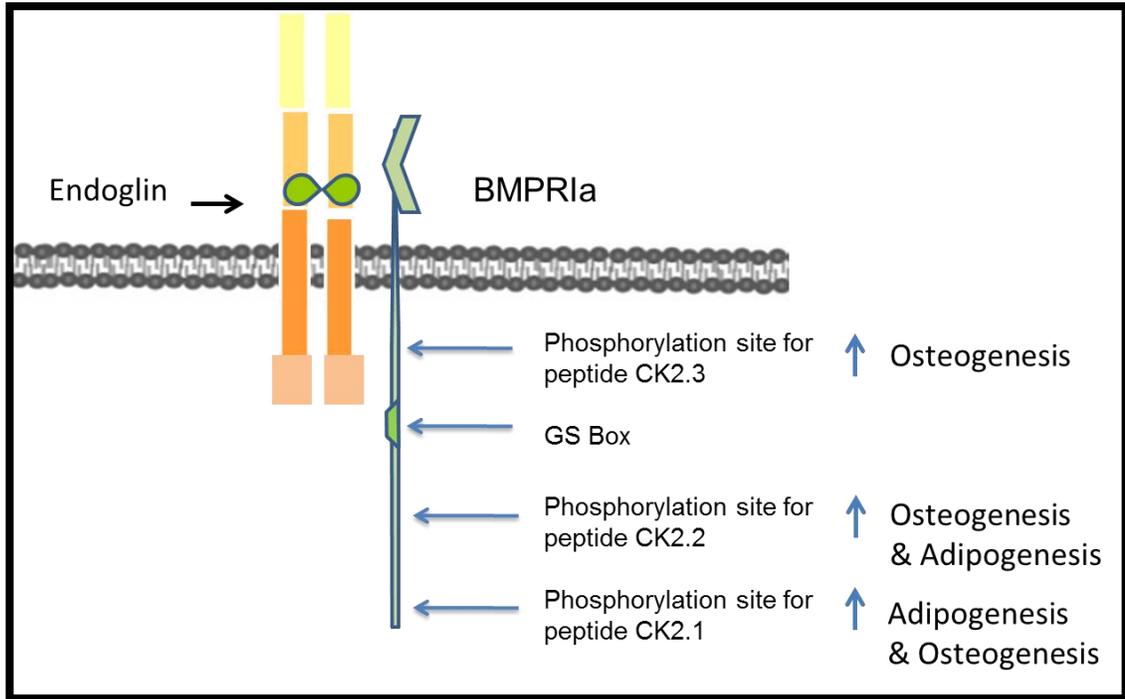


Figure 8A: Endoglin as a key control in the mechanism of adipogenic control in the BMP signaling pathway

Fig 8B

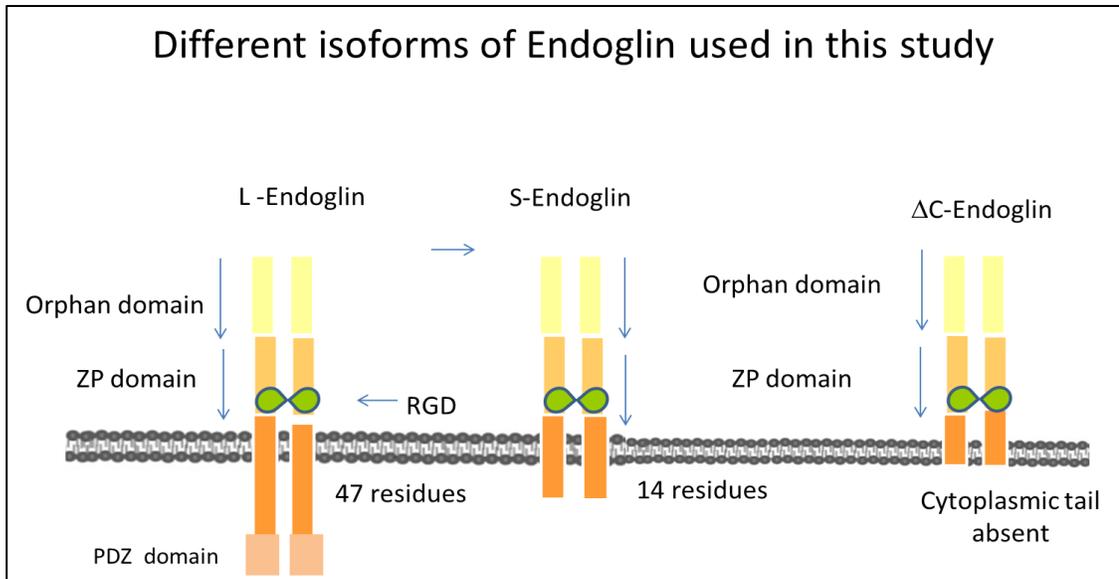


Figure 8B: Endoglin is normally present in two isoforms; L-Endoglin (long form) with a 47 residue cytoplasmic tail and S-Endoglin (short form) that has a 14 residue cytoplasmic tail. Endoglin also consists of an orphan domain and a Zona Pellucida (ZP) domain, a tripeptide RGD domain (Arginine-Glycine-Aspartate) and critical PDZ domain. In addition we have also used a Δ C mutant that lacks the cytoplasmic tail altogether.

Fig 9A

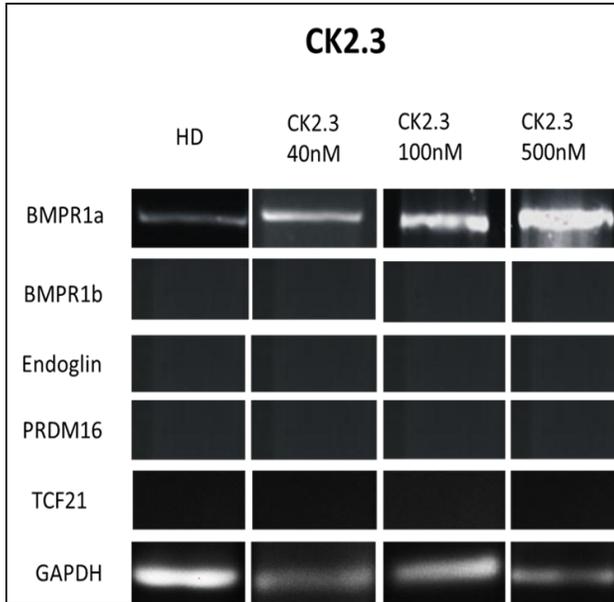


Fig9B

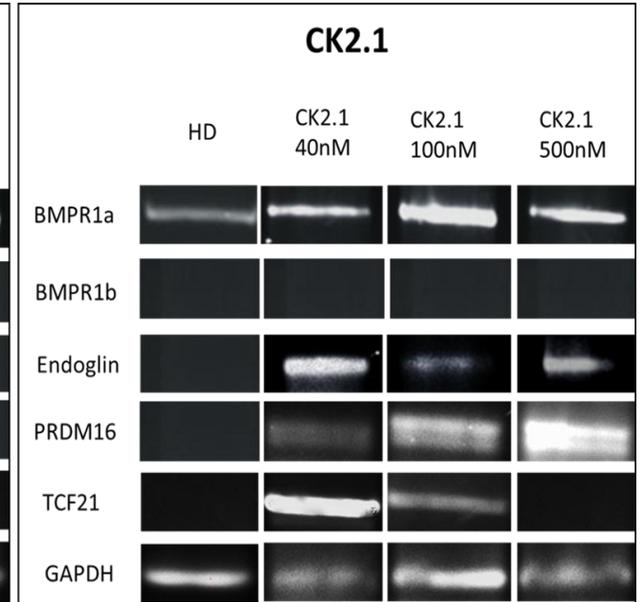


Fig 9C

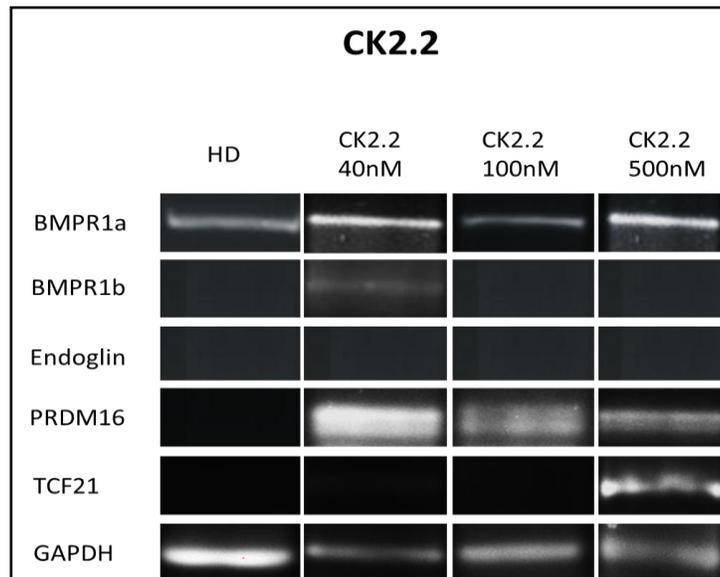


Figure 9A, 9B, 9C: RT-PCR results show constitutive expression of BMPR1a when treated with 40nM,100nM and 500nM CK2.1, CK2.2 and CK2.3 (9A,B and C).Endoglin expression is upregulated in cells treated with all three concentrations of CK2.1 (9B).PRDM16 a brown fat marker used in this study is shown o upregulated in cells treated with both CK2.1 and CK2.2 (9B and C) while TCF21, a white fat marker is expressed only in cells stimulated with CK2.1and high concentration of CK2.2.(9B and C) GAPDH is used as control.

Fig 9D

	CK2.1 Day1	CK2.2 Day1	CK2.1 Day2	CK2.2 Day2
BMPR1a				
BMPR1b				
Endoglin				
TCF21				
PRDM16				

Figure 9D: PRDM16 is upregulated in cells treated with 40nM CK2.2 peptide for Day1 and Day2 while no PRDM16 upregulation is seen in cells treated with CK2.1 at Day2

Fig 9E

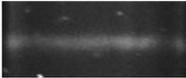
	Rapamycin +CK2.1	Rapamycin +CK2.2
BMPR1a		
TCF21		
PRDM16		
Endoglin		

Figure 9E: C2C12 cells were treated with Rapamycin (inhibitor of mTOR) and subsequently with 40nM CK2.2 and 40nM CK2.1 for 3 days. PRDM16 upregulation in cells treated with Rapamycin and CK2.1 and CK2.2

Fig 9F

	MEK inhibitor +CK2.1	MEK inhibitor +CK2.2
BMPR1a		
TCF21		
PRDM16		
Endoglin		

Fig 9G

	P38 inhibitor +CK2.1	P38 inhibitor +CK2.2
BMPR1a		
TCF21		
PRDM16		
Endoglin		

Figure 9F, 9G: C2C12 cells when treated with MEK inhibitor (9F) and p38 inhibitor (9G), no expression of TCF21, PRDM16 or Endoglin are observed.

Fig10A

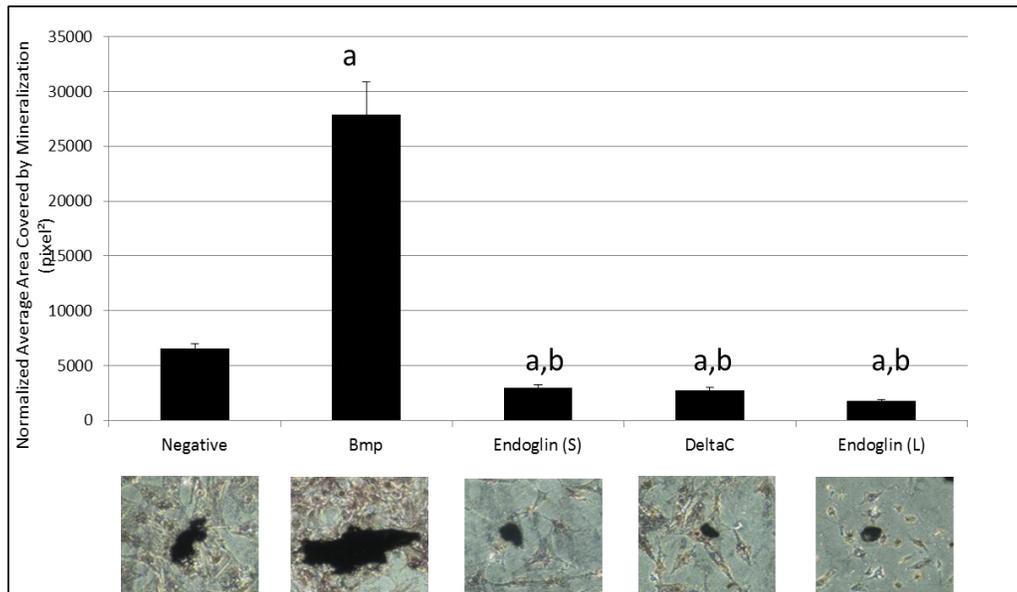
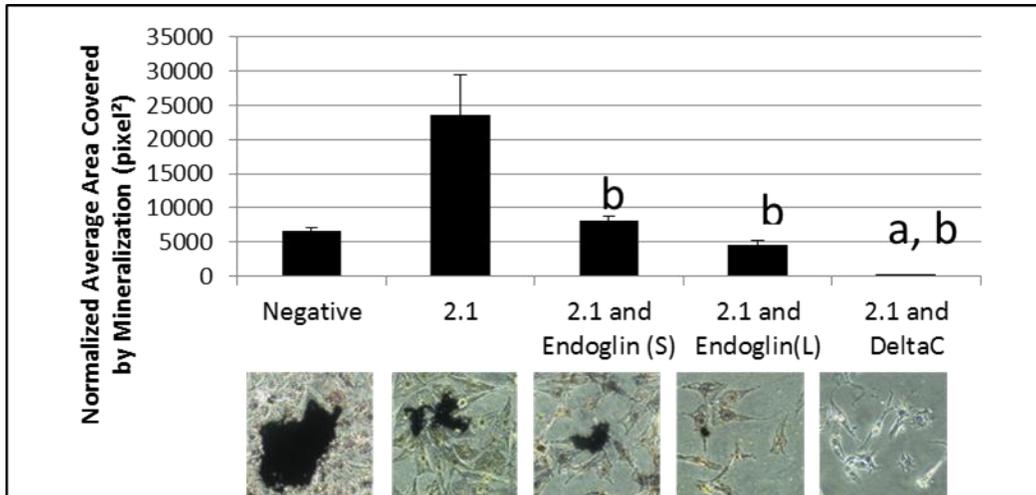
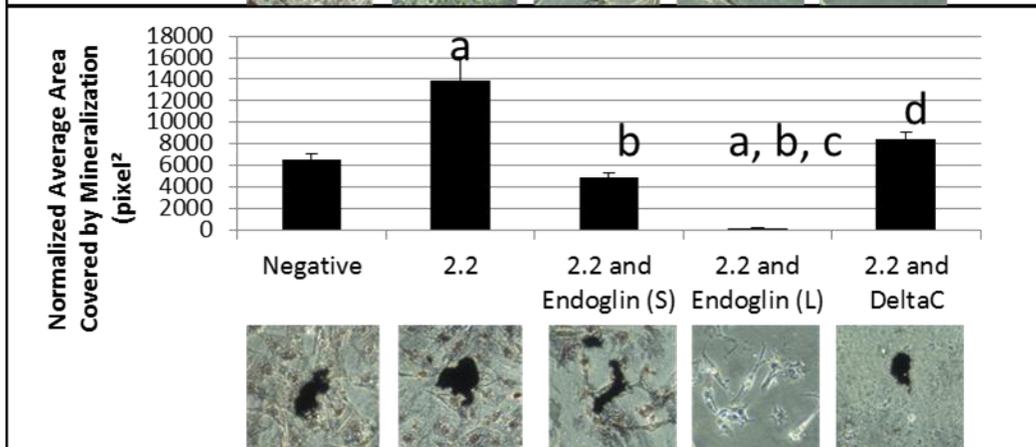


Figure 10A: C2C12 cells were treated with 1 μ g Endoglin, S-Endoglin and L-Endoglin dna. After 3 days, cells were fixed and stained with Van Kossa for mineralization. Cells treated with 40nM BMP were used as positive control and untransfected cells were used as negative control. Analysis by ImageJ shows osteogenesis is significantly reduced by all isoforms of Endoglin as compared to both positive and negative control. “a” is significantly different with respect to Negative while “b” denotes significance with respect to BMP treated cells. Standard Tukey’s test and Anova were used for statistical analysis. Lower panel shows representative wells for each treatment

10B



10C



10D

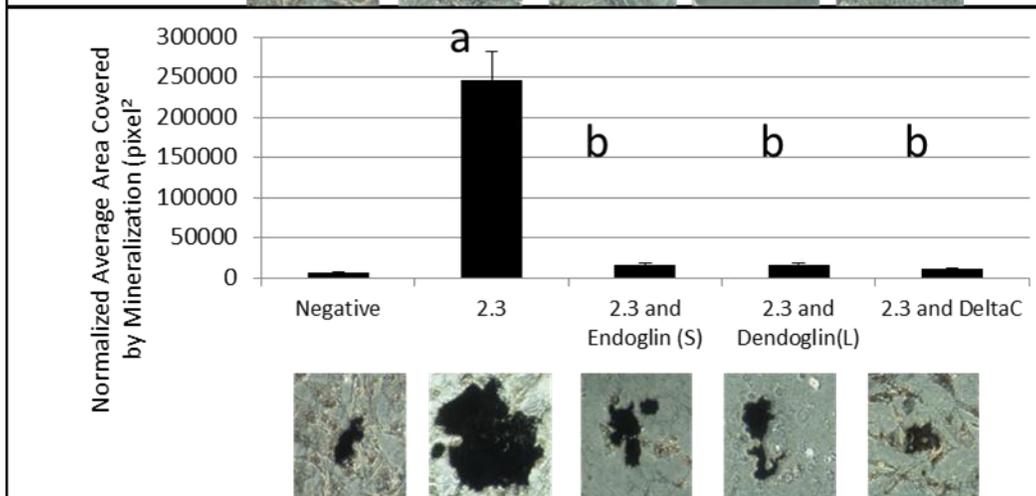


Figure 10B, 10C, 10D: C2C12 cells were transfected with 1 μ g Endoglin, S-Endoglin and L-Endoglin dna subsequently treated with 40nM CK2.1 (10B), 40nM CK2.2 (10C) and 40nM CK2.3 (10D). After 3 days, cells were fixed and stained with AgNo₃ for mineralization. Cells treated with 40nM BMP were used as positive control and untransfected cells were used as negative control. Analysis by ImageJ shows Endoglin downregulates osteogenesis mediated by all three peptides. “a” is significantly different with respect to negative while “b” denotes significance with respect to BMP treated cells. Standard Tukey’s test and Anova were used for statistical analysis. Lower panel shows representative wells for each treatment.

Fig 11A

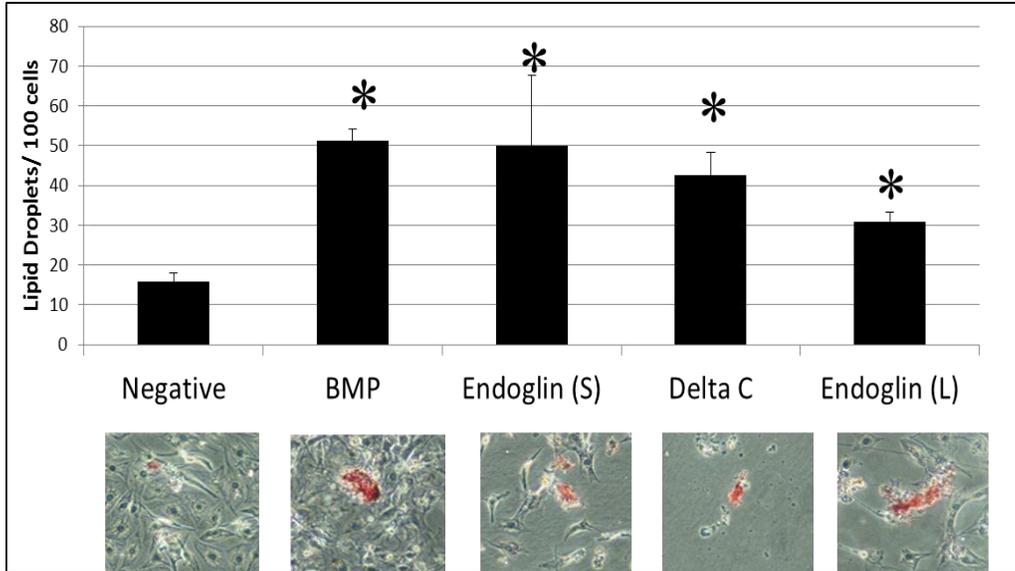
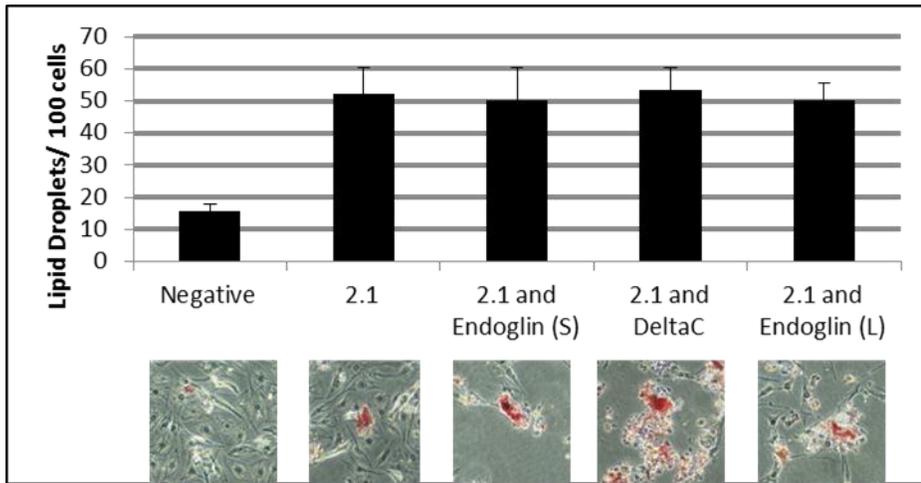
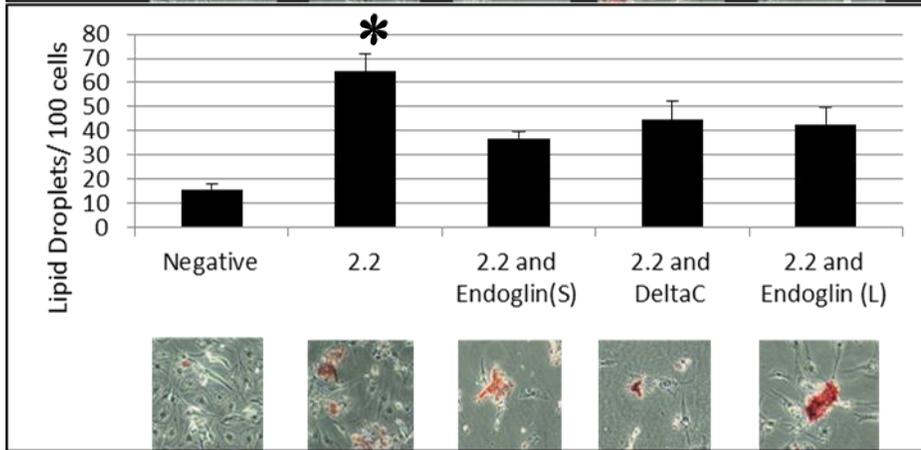


Figure 11A: C2C12 cells were treated with 1 μ g Endoglin, S-Endoglin and L-Endoglin. After 3 days, cells were fixed and stained with Red Oil O for lipid droplet formation. Cells treated with 40nM BMP were used as positive control and untransfected cells were used as negative control. Analysis by ImageJ shows adipogenesis is significantly upregulated by all isoforms of Endoglin as compared to that in untransfected cells. Standard Tukey's test and Anova were used for statistical analysis. Lower panel shows representative wells for each treatment

11B



11C



11D

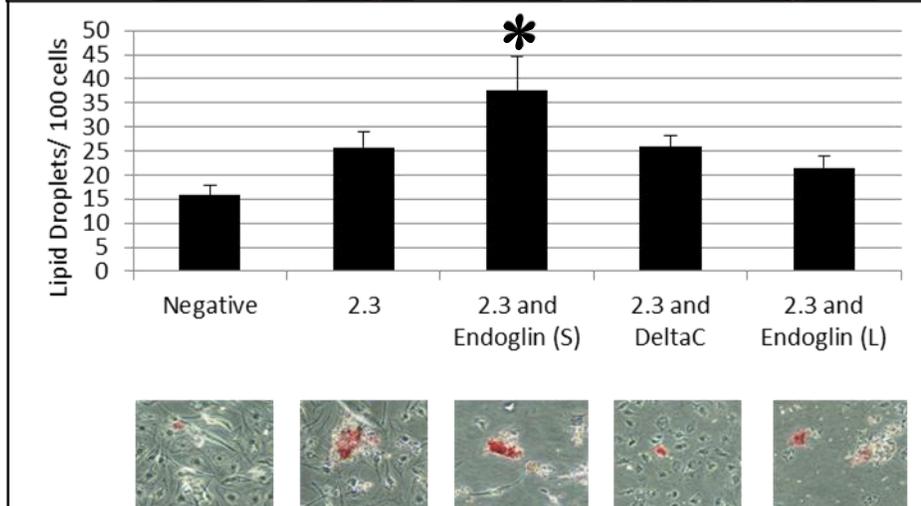


Figure 11B, 11C, 11D: C2C12 cells were transfected with 1 μ g Endoglin, S-Endoglin and L-Endoglin and subsequently treated with 40nM CK2.1 (11B), 40nM CK2.2 (11C) and 40nM CK2.3 (11D). After 3 days, cells were fixed and stained with Red Oil O for lipid droplet formation. Cells treated with 40nM BMP were used as positive control and untransfected cells were used as negative control. Analysis by ImageJ shows Endoglin has no effect on CK2.1 mediated adipogenesis (11B). Adipogenesis mediated by CK2.2 is also unaffected by Endoglin (11C). Standard Tukey's test and Anova were used for statistical analysis. Lower panel shows representative wells for each treatment

Fig 12A

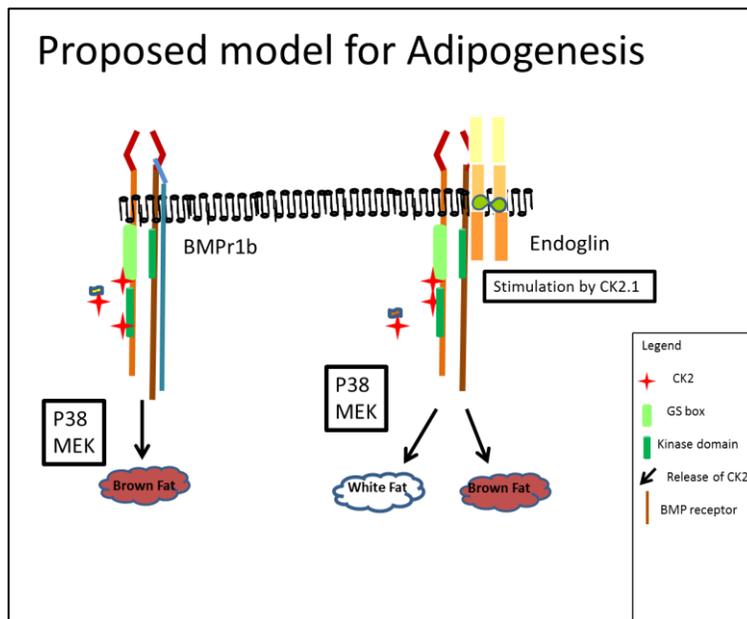


Figure 12A: Model for Endoglin and BMPR1b mediated adipogenesis

Fig 12B

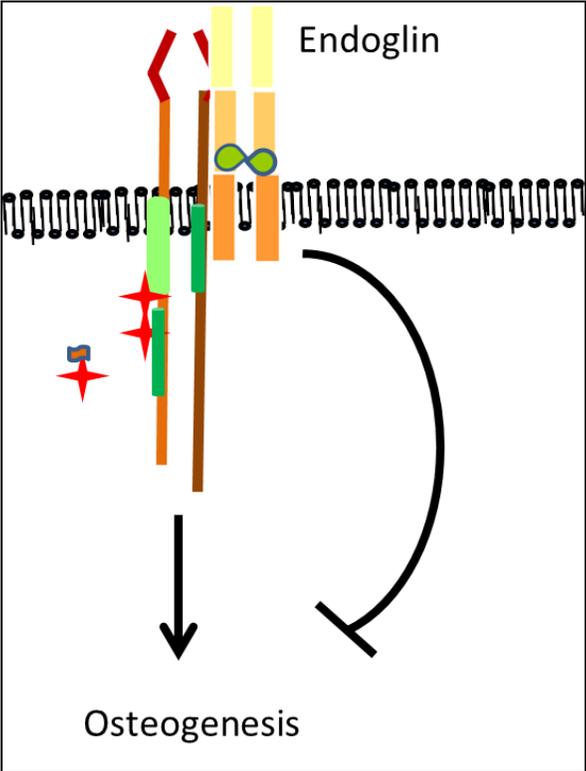


Figure 12B: Model for Endoglin mediated osteogenesis

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