DMP1 Activates Osteolytic Cycle in a Tumor Environment

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THESIS

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This thesis is dedicated to my parents Mr. John Karippacheril and Dr. Annie John and my husband Mr. Eapen P Chacko.

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LIST OF ABBREVATIONS

µl	Microlitre
%	Percentage
ADMATS1	Thrombospondin motifs-1
Ala	Alanine
ALP	Alkaline phosphatase
ARHR	Autosomal recessive hypophosphatemic rickets
Asp	Aspartic acid
ATP	Adenosine triphosphate
BAPTA-AM	1, 2-Bis (2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)
BMPs	Bone morphogenetic proteins
BMP1	Bone morphogentic protein 1
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
BMU	Basic multicellular unit
BSP	Bone sialoprotein
Ca^{2+}	Calcium ion
([Ca2+]i)	Intracellular calcium
CaR	Calcium receptor
Cbfa1	Core binding factor 1
cDNA	Complementary DNA
CTGF	Connective tissue growth factor
DAG	Diacylglycerol
DMP1	Dentin matrix protein 1
DMP2	Dentin matrix protein 2
DMSO	Dimethyl sulfoxide
DSP	Dentin sialoprotein
DSPP	Dentin sialophosphoprotein
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF23	Fibroblast growth factor-23
FST	Follistatin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GF- β1	Growth factor–β1
GPCRs	G protein-coupled receptors
Grb 2	Growth-factor-receptor-bound-2
GRP78	Glucose-regulated protein-78
GTP	Guanosine triphosphate

LIST OF ABBREVATIONS (continued)

h	Hour
H_2O_2	Hydrogen peroxide
HAP	Hydroxyapatite
Hh	Hedgehog
HRP	Horseradish peroxidase
HSP27	Heat shock protein 27
HSP 70	Heat shock protein 70
IGFs	Insulin-like growth factors
IHH	Indian Hedgehog
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-11	Interleukin-11
IP3	Inositol trisphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
kb	Kilobase
KDa	Kilodalton
KO	Knock out
LB	Luria Broth
MAP	Mitogen-Activated Protein
MAPK	Mitogen-Activated Protein Kinases
MAPKAPK2	Mitogen-activated protein-kinase-activated protein kinase 2
MAP3K	Mitogen-Activated Protein Kinases kinase kinase
M-CSF	Macrophage colony stimulating factor
MEPE	Matrix extracellular phosphoglycoprotein
Met109	Methionine 109
min	Minutes
MKK	Mitogen-Activated Protein Kinases kinase
MKK3	MAPK kinases3
MKK6	MAPK kinases6
Mm	Millimoles
MMP-2	Matrix metalloproteinase-2
MMP9	Matrix metalloproteinase-9
MMP13	Matrix metalloproteinase-13
M1V	Methionine with valine
ng	Nanogram
nm	Nanomoles
OC	Osteocalcin
OCN	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2

LIST OF ABBREVATIONS (continued)

PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP	Phosphophoryn
PTHrP	Parathyroid hormone-related protein
qPCR	Quantitative real time polymerize chain reaction qPCR
RANKL	Receptor Activator of Nuclear Factor kappa B Ligand
rDMP1	Recombinant DMP1
RGD	Arginine- Glycine- Aspartic acid
RNA	Ribonucleic acid
R-Smads	Receptor-activated Smads
RTKs	Receptor tyrosine kinases
shRNA	Short hairpin RNA
SIBLING	Small integrin-binding ligand N-linked glycoproteins
Smurfs	Smad ubiquitination regulatory factors
SnoN	Ski-related novel protein N
SOCs	Store-Operated Channels
SOS	Son of sevenless
TAK1	TGF- β activated protein kinase 1
TGF-β	Transforming growth factor- β
TGIF	TG-interacting factor
TGN	Trans-Golgi network
Thr-Gly-Tyr	Threoenine-glycine- tyrosine
thr-180	Threoenine
TNF-α	Tumor necrosis factors
TRAP	Tartrate-resistant acid phosphatase
tyr-182	Tyrosine
VEGF	Vascular endothelial growth factor

SUMMARY

Dentin matrix protein 1 (DMP1) is an acidic noncollagenous protein localized both intracellularly and in the mineralized matrix of bone and dentin. It is a multifunctional protein, involved in gene regulation (within the cell) and hydroxyapatite (HAP) nucleation (in the extracellular matrix (ECM)). DMP1 is endocytosed by preosteoblasts, triggering the calcium dependent and calcium independent signaling pathways resulting in a series of downstream events leading to osteoblast differentiation. These signaling events facilitate the activation of a calcium dependent stress-induced p38 MAP kinase and an calcium independent integrin mediated ERK1/2 MAP kinase pathway resulting in the expression of downstream target genes such as Runx2 and osteocalcin. Results from this study have shown that the osteoblast cells upon differentiation release receptor activator of nuclear factor kappa B ligand (RANKL) that stimulates the differentiation of precursor monocytes to osteoclasts.

The study also focuses on identifying the role of DMP1 in breast cancer cells that could enhance osteoclast formation thereby accelerating bone resportion. Thus, the study reveals a new mechanism by which DMP1 can activate the vicious cycle leading to the aggressive growth and behavior of the cancer cells. To address the mechanism by which DMP1 might contribute to the osteolytic process, DMP1 was overexpressed in the metastatic cancer cell line MDA MB231. Upregulation of markers like Runx2, MMP2, MMP9, RANKL, OPN, BSP, VEGF and the activation of Smad2/3 and MAP kinase pathways (p38 and ERK1/2 pathways) were observed.

Results from this project have shown that DMP1 secreted by the breast cancer cells into the extracellular environment could stimulate the differentiation of osteoblasts, leading to the secretion of factors that signal monocytes to differentiate to osteoclast thus resulting in bone metastasis. Overall, the results obtained from this study identify a new role for DMP1 in the differentiation of breast cancer tumor cells that may be directly related to their metastatic potential.

CHAPTER 1

INTRODUCTION

A. DMP1

Dentin matrix protein 1 (DMP1), a member of the small integrin-binding ligand N-linked glycoproteins (SIBLING) family, is a multifunctional noncollagenous protein localized specifically in the mineralized matrix of bone and dentin. This acidic noncollagenous protein was first discovered and cloned from a rat odontoblast mRNA library (George et al., 1993). The DMP1 gene has been mapped to 4q21 in humans and to 5q21 in mice (Hirst et al., 1997; Fisher et al., 2001). Although DMP1 is predominantly expressed in bone (George et al., 1993) and dentin (Fen et al., 2001; Toyosawa et al., 2001), the transcription of DMP1 is highly regulated. In the teeth, DMP1 is expressed in dental pulp cells, odontoblast, predentin, dentin and cementum (Feng et al., 2003). Presence of DMP1 mRNA was noted in chondrocytes and osteoblasts while the protein is highly expressed in osteocytes (Toyosawa et al., 2001; Feng et al., 2002). Recent studies have shown that DMP1 is expressed in non-mineralized tissue like brain, salivary glands and tumors of epithelial origin (Fisher *et al.*, 2004; Ogbureke and Fisher, 2004; Terasawa *et al.* 2004).

The cDNA for DMP1 is encoded by 6 exons of which the first 5 exons range from size 33 to 104 base pairs. Exon 6 is the largest and contains 80% of the coding information. The signal peptide is present in the exon 2. The exon 5 which is 45 p is spliced in some species. The tissue specificity of DMP1 expression

depends on intron 1 which is the largest (3791 kb to ~ 6 kb) while intron 4 is the smallest (162–189 base pairs). There are 2 promoter elements present in DMP1 with one located at the proximal end (−2.4 kb and +4 kb region) and the one distal (−2.4 kb and −9.6 kb region). The early stage of DMP1 expression is controlled by the proximal domain and the distal domain controls later stages of expression. Published reports have shown that these promoter activities are controlled by transcriptional factors that act in a stage specific manner (Lu et al., 2005).

The full length form of DMP1 has been cloned in mammalian and non mammalian systems. The molecular weight of native DMP1 is 61kDa (George et al., 1993) which is reported to be less than that of the recombinant rat DMP1 from bacteria (94 kDa) (Figure.1) (Srinivasan et al., 1999). It has been reported that DMP1 is proteolytically processed into N terminal 37kDa and C terminal 57kDa fragments. The processed C terminal fragment is functional in mineralization. Reports have shown that this 61kDa band is a processed fragment(s) of DMP1 which is equivalent to the 57kDa fragment (Qin et al., 2003a). The entire protein exists as random coil and is hydrophilic in nature. In addition, the distribution of N terminal DMP1 in the tooth is different from that of C terminal DMP1. The former is mainly located in the predentin, while the latter is primarily present in the mineralized dentin (Maciejewska et al., 2009a) The secreted form of the rat full length DMP1 consist of 473 amino acids with 22 Ser/Thr casein kinase I and II phosphorylation sites at the N terminal and 43 Ser/Thr casein kinase I and II phosphorylation sites at the C terminal (George at al., 1993). Most of these serines are phosphorylated by messenger independent kinases. The presence of 53 phosphates/mol and 3 putative

LPVARYQNTESESSEERTGNLAQSPPPPMAN
SDHTDSSESGEELGSDRSQYRPAGGLSKSA
GMDADKEEDEDDSGDDTFGDEDNGPGPEER
QWGGPSRLDSDEDSADTTQSSEDSTQENS
AQDTPSDSKDHHSDEADSRPEAGDSTQDSE
SEEYRVGGGSEGESSHGDGSEFDDEGMQSD
DPGSTRSDRGHTRMSSAGIRSEESKGDHEPT
STQDSDDSQEDSPEGQDPSSESSEEAGEPSQ
ESSSESQEGVASESRGDNPDNTSQTGDQRD
SESSEEDRLNTFSSSESQSTEEQGDSESNES
LSLSEESQESAQDEDSSSQEGLQSQSASRES
RSQESQSEQDSRSEENRDSDSQDSSRSKEE
SNSTGSTSSSEEDNHPKNIEADNRKLIVDAYHN
KPIGDQDDNDCQDGY

Figure 1:
DMP1 protein sequence

glycosylation sites has been reported to be present in DMP1 (Qin et al., 2003a). These phosphates might serve as sequestering groups for recruiting calcium ions. The presence of Arg- Gly- Asp (RGD motif) in the C terminal portion of the DMP1 protein plays a pivotal role in cell attachment and signaling (George et al., 1993; Wu et al., 2011). Studies have shown that proteinases may be involved in the processing of DMP1 and these enzymes are non tissue specific. Bone morphogentic protein 1 (BMP1) and tolloid like metalloproteinase are examples of the enzymes that play a role in the processing of DMP1 (Steiglitz et al., 2004).

Reports show that DMP1 is primarily localized in the nucleus of undifferentiated osteoblast where it acts as a transcriptional regulator for the activation of osteoblast specific genes like osteocalcin (Narayanan et al., 2003). Identification of glucose-regulated protein-78 (GRP78) as a cell surface receptor for DMP1 is particularly interesting as its induction is a protective response against several kinds of stress, including endoplasmic reticulum (ER) calcium ion (Ca^{2+}) depletion and accumulation of unglycosylated proteins. GRP-78, also known as Hspa5, is a member of the heat shock protein 70 (HSP 70) family and primarily acts a molecular chaperone in the endoplasmic reticulum. The use of small interfering RNA confirmed GRP-78 as the receptor for DMP1. Immunohistochemical analysis performed with biotin-labeled DMP1 confirmed the spatial co-localization of DMP1 and GRP-78. However, the binding of DMP1 to GRP-78 receptor is specific and saturable with a binding dissociation constant K_D=85 nm. Furthermore studies have shown that the cellular uptake of DMP1 occurs through endocytosis and the internalization of DMP1 is a caveolae-associated, receptor-mediated event. The

vesicles containing DMP1 are then transported intracellularly to the Golgi and the nucleus via the microtubules. Therefore, binding of DMP1 with GRP-78 receptor might be the mechanism by which DMP1 is internalized and transported to the nucleus during bone development (Ravindran et al., 2008).

The acidic nature of DMP1 is responsible for binding Ca^{2+} ions, thus playing an important role in mineral nucleation and growth of hydroxyapatite. During the initial phase of osteoblast maturation, signals from the extracellular matrix (ECM) result in the surge of intracellular calcium from the cytoplasm into the nucleus. The export of DMP1 from the nucleus during osteoblast maturation was found to be in response to the stimulus by the pool of nuclear Ca^{2+} ions thus triggering the phosphorylation of DMP1 by a nuclear isoform of casein kinase II. This phosphorylated DMP1 is then exported into the extracellular matrix where it regulates nucleation of hydroxyapatite. Thus DMP1 acts as an effective signaling modulator that initiates osteoblast differentiation by transcription in the nucleus and orchestrates mineralized matrix formation extracellularly, at later stages of osteoblast maturation (Narayanan et al., 2003). The pathways that connect the rise in intracellular $[Ca^{2+}]_i$ to osteoblast differentiation have not been elucidated yet. Also, the specific signaling pathways activated following DMP1 stimulus and osteoblast differentiation are not delineated yet.

Studies have shown that the C terminal DMP1 has high affinity to bind to fibrillar collagen thus enhancing the nucleate hydroxyapatite formation. The nucleation and growth process involves the transformation of metastable amorphous calcium phosphate phase into a thermodynamically stable hydroxyapatite (HAP)

crystal. Interestingly, the N terminal of DMP1 inhibited the formation of HAP crystals but is able to stabilize the amorphous form (Gajjeraman et al., 2007).

In order to study the biological function of DMP1 transgenic cell lines C3H10T1/2, MC3T3-E1 and RPC-C2A overexpressing DMP1 were created by using the adenoviral systems. Results show that they differentiate and form functional odontoblast-like cells. Expression of transcription factors like core binding factor 1 (Cbfa1), bone morphogenetic protein 2 (BMP2), and bone morphogenetic protein 4 (BMP4); early markers for extracellular matrix deposition like alkaline phosphatase (ALP), osteopontin (OPN), osteonectin (OCN), and osteocalcin (OC); and late markers like dentin matrix protein 2 (DMP2) and dentin sialoprotein (DSP) were expressed by the genetically engineered C3H10T1/2 cells. However, MC3T3-E1 and RPC-C2A overexpressing DMP1 cell lines tested by the adenoviral expression system failed to express these odontoblast-phenotypic specific genes (Narayanan et al., 2001).

In vitro experiments with recombinant N- and C-terminal polypeptides products of DMP1 demonstrated that the C terminal fragment of DMP1 can initiate differentiation of dental pulp stem/progenitor cells to a putative odontoblast phenotype. *In vivo* implantation of this fragment in a rat injured pulp model resulted in a rapid formation of a homogeneous dentin bridge covered by cells expressing DSP and DMP1 (Chaussain et al., 2009). However, little is known regarding the mechanisms by which DMP1 can regulate such diverse cellular responses.

DMP1 plays a critical role in osteogenesis and dentinogenesis. However, DMP1 knock out (DMP1 KO) mice had no major phenotypic abnormality in

dentin and bone during early development; on the other hand, there were defects in chondrocyte differentiation and cartilage formation. As age progressed profound defects in bone and dentin were noted (Ye et al., 2004). Genetic studies in mouse and human have demonstrated that the inactivation of DMP1 leads to osteomalacia (rickets) and dentin hypomineralization. Excess accumulation of osteoid in the bone and widening of predentin in the tooth are a few of the histopathological changes resulting from DMP1deficiencies (Ye et al., 2004).

Results from the DMP1 KO suggest the involvement of other compensatory mechanisms that might be responsible for bone and dentin formation during early development. Published reports have shown that the substitution of Asp 213 (Aspartic acid 213) (a residue at a cleavage site) by Ala 213 (Alanine 213) blocks the *in vitro* processing of mouse DMP1. Findings have indicated that the proteolytic processing of DMP1 is essential to the formation and mineralization of dentin, cementum and jaw bones (Qin et al., 2011).

Futhermore, studies have lead to the discovery of the novel disorder autosomal recessive hypophosphatemic rickets (ARHR), associated with the missense replacement of the initial methionine with valine (M1V) and the deletion of nucleotides 1484–1490 in DMP1. Patients with ARHR had elevated fibroblast growth factor-23 (FGF23) and carried a large, biallelic deletion that removed the majority of DMP1 resulting in the loss of DMP1 function. Further analyses showed that these M1V DMP1 mutants were not sorted to the trans-Golgi network (TGN) and secretory pathway, but rather filled the entire cytoplasm (Emily et al., 2009).

B. BONE

Bone is a complex type of connective tissue composed of several cell types which are continuously undergoing a process of renewal and repair. Bone is continuously destroyed and reformed in vertebrates in order to maintain bone volume and calcium homeostasis. The two closely coupled events are responsible for renewing the skeleton while maintaining its anatomical and structural integrity. The bone forming cells called osteoblast and the bone resorbing cells, called osteoclasts are the main players involved in bone formation. Osteoprogenitors mature and give rise to osteoblasts that residue in bone marrow. These osteoblasts differentiate to produce an unmineralized matrix called osteoid which later mineralizes. These cells terminally differentiate and are embedded in the mineralized matrix and gives rise to osteocytes. Osteoblasts produce bone matrix proteins including type I collagen, the most abundant extracellular protein of bone. Mesenchymal stem cells also reside in the bone cavity and are proposed to differentiate under the control of respective regulatory factors into a majority of marrow stromal cell lineages, including chondrocytes, osteoblasts, fibroblasts, adipocytes, endothelial cells and myocytes.

Bone formation occurs in three successive phases: the production and the maturation of osteoid matrix, followed by mineralization of the matrix. In normal adult bone, these processes occur at the same rate so that the balance between matrix production and mineralization is equal. Initially, osteoblasts produce osteoid by rapidly depositing collagen. This is followed by an increase in the mineralization

rate to equal that of collagen synthesis. In the final stage the rate of collagen synthesis decreases and mineralization continues until the osteoid becomes fully mineralized. Osteoporosis a common disease in which the reduction in skeletal mass due to an imbalance between bone resorption and bone formation.

Bone morphogenetic proteins (BMPs) and transforming growth factor-β (TGF-β) play critical roles in the differentiation of undifferentiated mesenchymal cells into osteoblasts (Katagiri et al., 2002; Zhou, 2011). For the purpose of *in vivo* studies osteoblasts have been isolated from calvaria of new born animals or from bone marrow that contains osteoprogenitor cells. MC3T3-E1 cells are a well-established preosteoblast cell line derived from mouse calvaria that maintain much of the tightly linked controls between proliferation and differentiation. Proliferation and differentiation of these cells to a fully functional osteoblastic lineage is regulated by various potent regulators, including various transcription factors, cytokines, morphogens, and secreted growth factors. Recent studies have elucidated the molecular mechanism by which these primary cells undergo differentiation process in the presence of inducing factors like BMP, members of TGF-β family leading to the activation of Smad pathway (Tang et al., 2008; Wang et al., 2010). Osteoblast differentiation through protein kinase A (PKA), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K) (Guntur &Rosen, 2011; Isowa et al., 2010; Park et al., 2004) signaling pathways, have been shown to play key roles in regulating bone remodeling. Runx2, osteopontin, osteoprotegerin (OPG), type I collagen, and alkaline phosphatase are found to be osteoblast-specific gene markers (Lo et al., 2011; Miyazaki et al., 2008; Siqueira et al., 2011). However,

studies are yet to be performed to study gene expression profile when stimulated by DMP1 and the signaling pathway involved in the differentiation of osteoblasts.

C. Calcium signaling

Calcium signaling is important for muscle contraction, cellular motility, regulating enzyme activity, ion pumps, and components of the cytoskeleton. The resting concentration of Ca^{2+} in the cytoplasm is normally maintained at 10–100 nM. This low concentration is maintained by active pumping of Ca^{2+} from the cytosol to the extracellular space. Signaling occurs when the cell is stimulated to release Ca^{2+} from intracellular stores, and/or when calcium enters the cell through plasma membrane ion channels (Berridge et al., 2003). Phospholipase C (PLC) pathway is the common signaling pathway that increases cytoplasmic calcium concentration. Cell surface receptors like G protein-coupled receptors and receptor tyrosine kinases activate the PLC enzyme. PLC hydrolyses the membrane phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP2) to form inositol triphosphate (IP3) and diacylglycerol (DAG), two classical second messengers. DAG activates the protein kinase C enzyme, while IP_3 diffuses to the endoplasmic reticulum, binds to its receptor (IP3 receptor), which is a Ca^{2+} channel, and thus releases $Ca^{2+}$from the endoplasmic reticulum. Depletion of calcium from the endoplasmic reticulum will lead to Ca^{2+} entry from outside the cell by activation of "Store-Operated Channels" (SOCs). Store-operated Ca^{2+} channels, which are activated in response to Ca^{2+} store depletion, control homeostasis between the extracellular Ca^{2+}reservoir and intracellular Ca^{2+} storage and control a wide range of cellular functions (Lewis, 2007). Calcium signaling is essential for the proliferation and differentiation of

osteoblasts. Earlier studies have shown that treating osteoblasts with parathyroid hormone or vitamin D_3 induces an increase in intracellular calcium ($[Ca^{2+}]_i$) by increasing the release of Ca^{2+} from the intracellular stores (Green et al., 1992; Dvorak & Riccardi,2004; Sharan et al., 2008). Published reports have shown that $[Ca^{2+}]_i$ increase is important in the activation of extracellular signal regulated kinase (ERK1/2) Mitogen-Activated Protein (MAP) kinase pathway in osteoblast cells (Riddle et al., 2008). The activation of P2X7 receptors in osteoblast requires $[Ca^{2+}]_i$ (Jorgensen et al.,2002) . Recent studies have shown that bone cells also express the calcium receptor (CaR) and evidence indicates the role of this receptor in their responses to the changing extracellular ionic environment (Sharan et al., 2008). The events that occur downstream of CaR activation are complex and are mediated via several different signaling pathways.

D. Mitogen-Activated Protein Kinases (MAPK) –p38 MAPK

One of the signaling pathways involved in osteoblast differentiation are the mitogen-activated protein kinases (MAPK) that affects a variety of intracellular responses like cell-cycle regulation, cell death, development, differentiation, senescence and tumorigenesis (Cuenda & Rousseau, 2007;Ono & Han ,2000). The family of MAPK consists of four characteristic subfamilies: the extracellular signal-regulated kinases1 and 2 (ERK1/2), c-jun, p38 MAPK and ERK 5. These serine/threonine kinases are thought to play an essential role in signal transduction by modulating gene transcription in the nucleus in reponse to changes in the cellular environment. p38 MAPK can be phosphorylated by many extracellular

stimuli through a classic MAPK kinase kinase (MAP3K)–MAP kinase kinase (MKK) pathway. Inflammtory stress, cytokines, lipopolysaccarides and osmotic stress are some examples of the stimulus (Cano & Mahadevan, 1995). p38 MAPK is inactive in the non-phosphorylated state, becomes rapidly activated by dual phosphorylation (threonine (thr-180) and tyrosine (tyr-182)) in the activation loop sequence Threonine-Glycine- Tyrosine (Thr-Gly-Tyr). In response to appropriate stimuli, threonine and tyrosine residues can be phosphorylated by three dual specificity MKKs/MAPKKs (MAPK kinases). The protein kinase MKK3/6 has been found upstream of the p38 pathway and also contribute in the activation of certain isoforms of p38 and is dependent upon cellular stimulus and cell type. Phosphorylation of all the four p38 MAPK family members is performed by MKK6, whereas MKK3 activates p38α, p38γ and p38δ, but not p38β. On the other hand, MKK4, an activator of the JNK (c-Jun N-terminal kinase) pathway can also phophorylated p38α. This phosphorylated p38 MAPK can activate a wide range of substrates that include transcription factors, protein kinases, cytosolic and nuclear proteins. Activated MAPKs requires phosphorylation on a flexible loop called as the phosphorylation lip or activation loop. Upon phosphorylation, a conformational reorganization occurs that relieves steric blocking and stabilizes the activation loop in an open and extended conformation thus facilitating substrate binding. Inhibitors of the ATP-binding site have most frequently been designed to interact with Methionine 109 (Met109), to stabilize inhibitor interaction with p38α. However, these ATP-mimicking inhibitors do not interact with the p38γ and p38δ isoforms, because they lack the methionine residue at position 109 required to stabilize the

interaction between the inhibitor and the ATP-binding site. Disruption of single p38β, p38α, p38δ genes, or double knockouts of p38γ and p38δ, results in viable fertile mice with no discernable phenotypic differences. Interestingly, ablation of one isotype has no apparent effect on the expression or activity of the other isotypes (Coulthard et al., 2009; Cuadrado & Nebreda, 2010; Ono & Han, 2000). In a recent study, Greenblatt *et al*. had evaluated the role of p38 signaling cascade in osteogenesis. Reports established that p38 MAPK signaling is essential for bone formation *in vivo* and Runx2 is a direct target of p38 MAPK.

E. MAPK –Extracellular signal regulated kinase (ERK1/2)

Extracellular signal regulated kinase is another member of the MAP kinase family and is composed of the ERK proteins that are the conserved products of the two genes namely the 44 kDa ERK1 (MAPKKK) and the 42 kDa ERK2 (MAPK). Apart from the two main protein products there exist other alternatively spliced forms such as the 46 kDa ERK1b in rodents, the ERK1c in primates and finally the ERK2b. Extracellular stimuli such as growth factors, cytokines, mitogens, hormones and oxidative or heat stress trigger a signal by interacting with the receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs)(Cobb & Goldsmith,1995). The activating signals are then transmitted to the receptors by recruiting the SOS (son of sevenless), a Ras-activating guanine nucleotide exchange factor through the adaptor protein growth-factor-receptor-bound-2 (Grb 2) to stimulate Ras and convert GDP to GTP. The activated Ras then initiates the interaction with downstream effector proteins.

GTP bound Ras recruits RAF to the membrane where it is activated by multiple phosphorylations and de-phosphorylations thus providing a proper environment for Raf signaling (Geyer & Wittinghofer, 1997; Zhang et al., 1993). Activated Raf then phosphorylates MEK1/2 (MAPKK) which then phosphorylates ERK1/2 (MAPKs) at the threonine and tyrosine residues. Phosphorylated ERK1/2 further regulates cellular activities ranging from gene expression, cell differentiation, gene transcription, movement, metabolism and programmed death. Studies have been performed with different scaffolds and inhibitors that coordinate ERK1/2 signaling, thus targeting this pathway for the treatment of cancer (Joe, 2008). ERK1 null mice have a generally normal phenotype while ERK2 null mice are embryonic lethal by E8.5 (Rose et al., 2010). ERK1/2 activation was found to stimulate osteoblast differentiation and skeletal development through the involvement of the transcription factor Runx2. Studies have reported that inhibition of MAPK in differentiated cells blocked osteoblast-specific gene expression (Ge et al., 2007).

F. TGF-β signaling pathway-Smad pathway

Smads are a group of related intracellular proteins critical for transmitting to the nucleus signals from the TGF-β and BMPs superfamily at the cell surface during the differentiation process. Signals are initiated upon binding of TGF-β superfamily members to cell-surface serine/threonine kinase receptors and are then propagated by the intracellular mediators known as Smads. The members of the TGF- β superfamily include TGF-β s, activins and bone morphogenic proteins

(BMPs). Binding of the ligand to its primary (type II) receptor, a constitutively active kinase, allows the recruitment, transphosphorylation and activation of the signaling (type I) receptor. This leads to a phosphorylation-dependent serine–threonine kinase activity thus resulting in the phosphorylation of cytoplasmic protein mediators of the Smad family. Upon phosphorylation, they form heteromeric complexes with Smad4, a common mediator for all Smad pathways. The ligand-specific, receptor-activated Smads (R-Smads), Smad1, Smad2, Smad3, Smad5 and Smad8, interact directly with, and are phosphorylated by, activated TGF-β receptors type I. Substrates of the BMP receptors are the Smad1, Smad5 and Smad8, whereas Smad2 and Smad3 are activated by both TGF-β and activin receptors. The resulting Smad heterocomplexes then translocate into the nucleus where they activate or repress transcription together with transcription factors so as to regulate target gene expression.

Regulatory mechanisms for the Smad signaling cascade have been extensively studied. Among the negative regulators of TGF-β signaling a third group of Smads, known as inhibitory Smads (Smad6 and Smad7), Smad ubiquitination regulatory factors (Smurfs), TG-interacting factor (TGIF) and Ski-related novel protein N (SnoN) are direct target genes for TGF-β signaling. Smad signaling was controlled by preventing phosphorylation and or nuclear translocation of receptor-associated Smads. Inhibition is also achieved by inducing receptor complex degradation through the recruitment of ubiquitin-ligases that leads to proteasomal degradation. Although R-Smads can be phosphorylated by ERK1/2, R-Smads can form complexes with Smad4, inhibiting translocation to the nucleus, and thereby regulating the signaling by TGF-β and BMPs (Javelaud & Mauviel A, 2005)

G. Bone Remodeling

Bone remodeling is often described as a cycle beginning with bone degradation and ending with bone deposition. In 1990, Frost defined this phenomenon as bone remodeling. In a normal adult, the factors that contribute to bone remodeling are microfractures, loss of mechanical loading, hormones, cytokines, calcium levels and inflammation. The remodeling cycle consists of three consecutive phases: resorption, reversal, and formation. The bone remodeling cycle begins with activation mediated by cells of the osteoblast lineage. Activation may involve the osteocytes, the lining cells, and the preosteoblasts in the marrow which are present within a functional and anatomic unit known as the basic multicellular unit (BMU). Osteocytes may act as mechanosensing cells and initiate the process when microfractures and loading are involved. Osteoblasts produce macrophage colony stimulating factor (M-CSF) and Receptor Activator of Nuclear Factor kappa B Ligand (RANKL), which bind to their respective receptors, c-fms and RANK, on preosteoclasts to bring about osteoclast differentiation and activation. The RANKL/RANK interaction results in activation, differentiation, and fusion of hematopoietic cells of the osteoclast lineage so that they begin the process of resorption. Osteoblasts also produce OPG, a decoy receptor to RANKL that curtails osteoclast activation (Figure.2). The ratio of RANKL to OPG determines the extent of the osteoclast activity, release of calcium from bone and bone degradation.

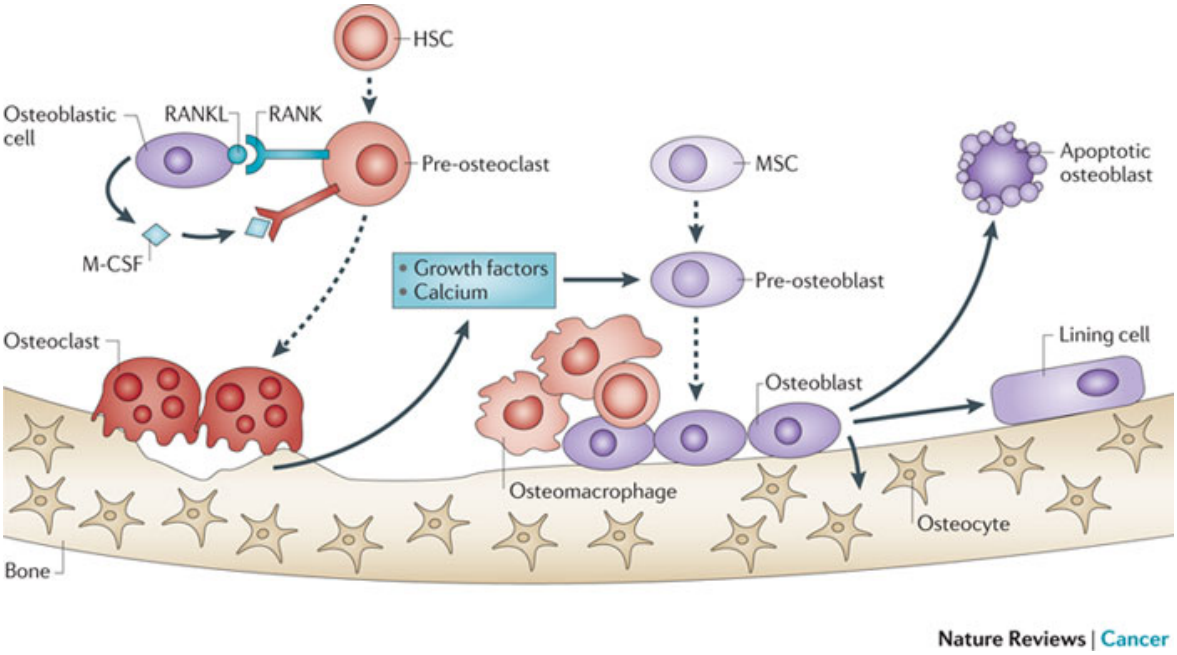


Figure 2:

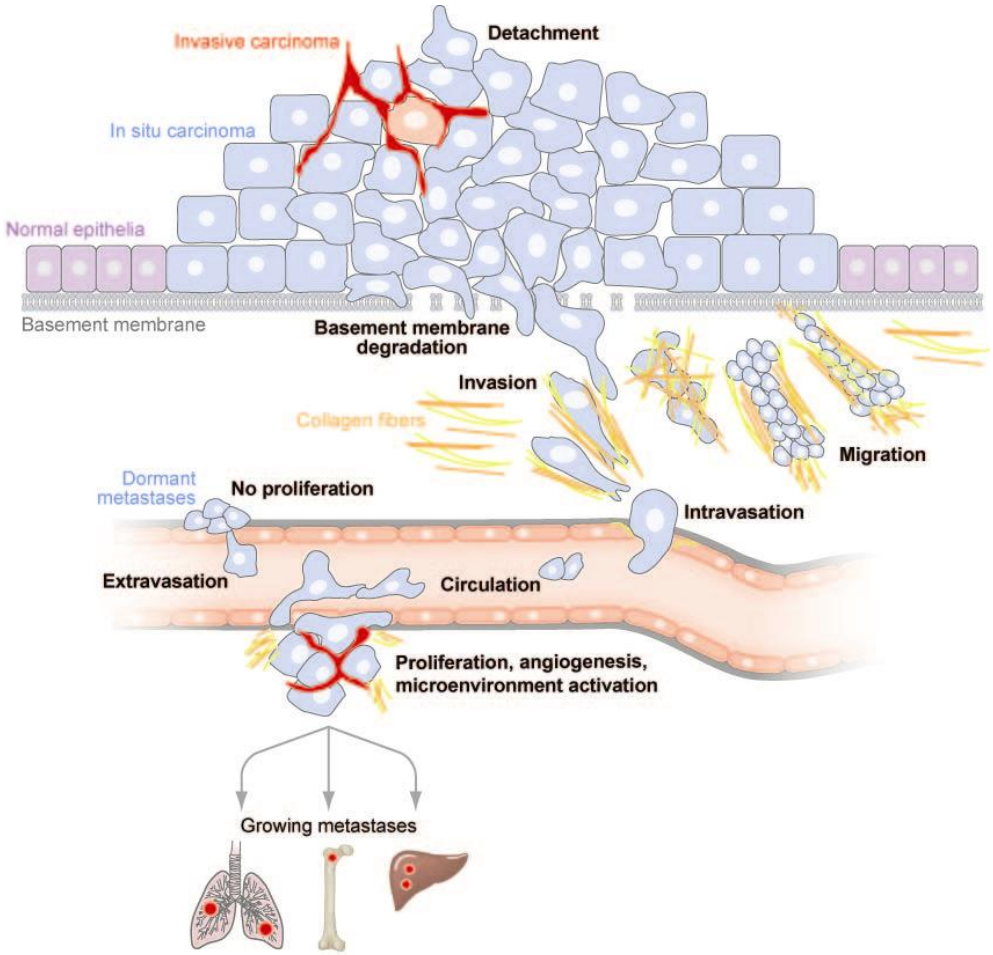
Bone Remodeling
Bone remodeling is a cyclic and continuous physiological process coordinated by the osteoblasts and osteoclasts. The balance of the trimolecular control factor complex composed of osteoprotegerin (OPG), RANKL (osteoprotegerin ligand) and RANK maintains physiologic bone remodeling

Resorption begins with the activation of the large multinucleated osteoclasts leading to their attachment to the bone surface thus creating a resorption lacuna, a sealed zone in which acid and proteolytic enzymes, such as cathepsin K, are released and degrade the bone matrix (Corisdeo et al., 2001). The reversal phase occurs when mononuclear cells appear on the bone surface. The preosteoblasts are recruited from the mesenchymal stem cell population and diffrentiate into osteoblasts. They follow the osteoclasts, depositing the bone matrix. Clusters of osteoblasts produce osteoid, composed of collagen, osteonectin, chondroitin sulfate and other non-mineral molecules, which matures and is then mineralized over several months (Hadjidakis & Androulakis, 2006). The final phase is the formation phase where the osteoblasts lay down bone until the resorbed bone is completely replaced by new. The completion of this phase will result in a surface that is covered with flattened lining cells. Finally the prolonged resting period begins until a new remodeling cycle is initiated.

H. Breast cancer

Breast cancer is the most common female cancer in the United States and the second most common cause of cancer death in women. Breast cancer exhibits a high propensity to metastasize to bone causing bone pain, pathological fractures and immobility. Successful metastasis of cancer cells from primary tumors to bone involves a number of steps such as the migration of tumor cells at the primary or lymph node sites, intravasation, migration in the circulation and extravasation of cancer cells followed by arrest in bone marrow leading to osteolytic

bone destruction and colonization of cancer cells in the bone (Yoneda & Hiraga, 2005). The cancer cells first detach from the primary tumor, invade the extracellular matrix and cross the endothelium thus entering the blood stream. This event is called as homing. Bone metabolic rate plays an important role in this process by increasing blood flow. Active bone resorption increases the concentration of growth factors and calcium release from bone which would in turn trigger the arrest of the cancer cell in vessels through an initial adhesion to vessel walls. The cancer cell then escapes the vasculature of bone into the surrounding tissue through a process called as extravasation. Appropriate signals in the extracellular matrix and the presence of concentration gradients of chemoattracting molecules enhance extravasation (Figure. 3). Migration of the cancer cells to a fertile niche suitable for their survival and proliferation is required. Cancer cells on reaching the bone environment usually enter a dormant stage. Some cells undergo apoptosis while others get local signals inhibiting apoptosis thus enhance bone metastasis. Once cancer cells harbor in the bone they begin proliferating, resulting in micrometastasis. The proliferation within micrometastases depends on the bone remodeling rate. Growth of tumors is limited to 1–2 mm in diameter due to the insufficient supply of nutrients to the tumor mass to support proliferation. Angiogenesis is required for further growth to take place which in turn depends on the tumor to initiate its immediate environment to induce angiogenesis. With effective angiogenesis, the tumor grows beyond the confines of the bone, perforating the cortical shell, and expanding into the surrounding soft tissues. At this time, breast cancer cells do not



Bacac M, Stamenkovic I. 2008.
Annu. Rev. Pathol. Mech. Dis. 3:221–47

Figure 3:
Steps in metastasis
Tumor cells at the primary or lymph node sites cross the basement membrane by degradation and invade the surrounding stroma by the process of intravasation. This is followed by its migration to reach the circulatory system. Metastasis of the organs occurs with the extravasation of cancer cells.

resorb bone, instead they rely on stimulation of osteoclasts cells that are physiologically responsible for bone destruction. The destructive effect of the tumor on bone leads to bone metastasis (Ooi et al., 2011).

Bone metastasis is common and has devastating effects on cancer patients. The frequency of bone metastasis is 70% in advanced breast cancer patients. The bone microenvironment is a unique and fertile soil for cancer metastasis, and tumor cells modify the bone microenvironment during cancer invasion and expansion through the recruitment and modulation of osteoclasts, osteoblasts, immune cells, vascular elements, bone matrix and neuronal processes. Skeletal metastases are classified into 2 types namely osteoblastic and osteolytic. These lesions result from an imbalance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Osteoblastic lesions, characteristic of prostate cancer, are caused by an excess of osteoblast activity relative to resorption by osteoclasts, leading to abnormal bone formation (Galasko et al., 1982).

Breast cancer cells preferentially metastasize to bone, leading to the formation of primarily osteolytic lesions. Osteolytic lesions are found in 80% of breast cancer patients with stage IV metastatic disease. These lesions are characterized by increased osteoclast activity and net bone destruction. Osteolytic lesions completely destroy the bone cortex allowing tumor cells to infiltrate and move into the surrounding tissues. The four components of a vicious cycle necessary for the initiation and development of metastatic lesions in the skeleton are the tumor

cells, osteoblasts, osteoclasts and bone extracellular matrix (Galasko et al., 1982). In 1889, Stephen Paget proposed the theory of “seed and soil” to describe the association of breast cancer cells with skeletal morbidity. He hypothesized that certain tumor cells (seeds) colonize selectively distant organs (soil) with a favorable environment facilitating survival and growth of the tumor cells. Breast cancer cells are the seeds which activate the bone cells, which in turn provide a fertile soil in which the cancer cells can grow.

I. Osteolytic cycle

A well studied mechanism in this vicious cycle involves osteoclast-secreted TGF-β. TGF-β is a crucial cytokine involved in the development and progression of bone metastasis. TGF-β stimulates the excessive production of parathyroid hormone-related protein (PTHrP) in tumor cells by a post transcriptional mechanism through the Smad and p38 MAPK pathways. The extracellular Ca^{2+} binds and activates a Ca^{2+} pump. This leads to the activation of osteoblasts to release RANKL and OPG. RANKL is a member of the TNF superfamily. Other factors that stimulate RANKL are tumor necrosis factors (TNF-α), Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-11 (IL-11) and prostaglandin E2 (PGE2) which are also released from tumor cells. Osteoblasts express OPG, which in turn counteracts the RANKL-RANK (receptor activator of NF-kB) binding. However, binding of RANKL with RANK, a transmembrane receptor of osteoclast precursors, leads to the differentiation of preosteoclast into mature, active osteoclasts. This leads to subsequent bone matrix degradation by MMPs and release of diverse growth factors

such as DGF, growth factor–β1 (GF- β1), fibroblast growth factor (FGF) and insulin-like growth factors (IGFs) resulting in the restart of the cycle stimulating tumor cells to release PTHrP. Signaling through these pathways promotes tumor cell proliferation and excessive PTHrP production, thus fueling osteoclastogenesis and osteolysis. Other important molecules which have shown a strong association with osteolytic metastases include CTGF (connective tissue growth factor), activin A, follistatin (FST), MMP1, a disintegrin and metalloproteinase with thrombospondin motifs-1 (ADMATS1) and heparanase/proteoglycans. Vascular endothelial growth factor (VEGF) is also another growth factor seen to play a potent role in angiogenesis and is strongly expressed in breast cancer metastasis (Fokas et al., 2007).

Reports have shown that patients with advanced-stage breast cancers frequently develop skeletal metastases, have elevated levels of TGF-β cytokines that promote osteoclast mediated bone destruction. Although TGF-β is a potent inhibitor of cell growth and functions as a tumor suppressor in the early stages of cancer, in advanced cancers, TGF-β has shown to promote tumor spreading by enhancing invasion and angiogenesis through TGF- β activated protein kinase 1 (TAK1) signaling pathway (Safina et al., 2011). Paracrine Hedgehog (Hh) signaling pathway also plays a potent role in the differentiation of osteoclast leading to osteoclastogenesis. Studies have shown that the Hh ligands secreted by breast cancer cells promote maturation of osteoclasts by the up-regulation of OPN, which in turn enhances osteoclastic activity by up-regulating cathepsin K and MMP9 (Das et al., 2011). Research has shown that elevated expression of the Notch ligand

Jagged1 is associated with breast cancer bone metastasis. Functional studies have delineated the activation of the Notch pathway in the bone microenvironment when tumor-derived Jagged1 promotes osteolytic bone metastasis (Sethi et al., 2011). Activation of TGF-β signaling pathways and its role in the production of osteolytic factors like interleukin-8 (IL-8) and interleukin-11 (IL-11) in breast cancer cells has been well documented. The up-regulation of the osteolytic factors in response to TGF-β1 has shown to involve both the Smad pathway and the p38 MAPK pathway, thus contributing to bone metastases (Gupta et al., 2011).

J. Runx2 in breast cancer

Studies have shown that Runx2 promotes breast tumor growth and its associated osteolytic lesions in the bone microenvironment. Breast cancer cells that have the potential to form bone metastasis, express several genes like Indian Hedgehog (IHH), parathyroid hormone-related protein (PTHrP), matrix metalloproteinase-13 (MMP13), matrix metalloproteinase-9 (MMP9) that are inturn regulated by Runx2 . Apart from the role of Runx2 in normal osteoblast proliferation, it has also shown to be the key components of the "vicious cycle". A well studied mechanism in this cycle involves osteoblast- secreted TGF-β, which enhances the local tumor cell expression of Parathyroid Hormone related Protein (PTHrP) thus leading to tumor growth and survival. This results in elevated osteoblastic expression of RANKL, leading to osteoclast activation, and increased osteolytic bone metastasis. This vicious cycle continues by the release of TGF-β by the resorbed bone and consequently increased PTHrP secretion. Furthermore, PTHrP

is regulated by Gli, a Hedgehog signaling factor, and is known that this pathway causes pathologic consequences in a variety of human tumors. Studies have shown that Runx2 interactions with Hedgehog signaling molecule Gli2 thus regulating TGF-β mediated activation of PTHrP. Binding of Runx2 to the IHH promoter leads to the activation of gene expression in cancer cells. This results in an increase of PTHrP levels in breast cancer cells thus leading to osteoclastogenesis *in vivo*. Interestingly, knock out of Runx2 in MDA MB231 breast cancer decreases cell migration, invasion and blocks the ability of the tumor cells to survive in the bone microenvironment (Pratap et al., 2005; Pratap et al., 2008).

K. SIBLINGs in breast cancer

SIBLINGs are a family of five integrin-binding glycophosphoproteins, located on chromosome 4 comprising osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). SIBLING proteins are small soluble, RGD motif containing secreted proteins that can act as modulators of cell adhesion as well as other autocrine and paracrine factors by their interaction with cell surface receptors such as integrins (Bellahcène et al., 2008).

OPN is the only SIBLING for which there is evidence of its role in cancer development and progression including invasion, metastasis and angiogenesis. Cancer cells bind to SIBLINGs and their various proteolytic fragments by both RGD-dependent and RGD-independent interactions. OPN binding to CD44v6 results in inside out signaling. Studies have shown that OPN and BSP can

accelerate the proliferation of breast cancer cells *in vitro.* The invasive potential of OPN and BSP are expressed at high levels in numerous cancers. OPN also enhances *in vitro* migration of various types of cancer cells. Enzymatic processing of SIBLINGs can play a pivotal role in altering invasion and migration properties of cancer cells. Studies have shown that OPN and BSP promote angiogenesis (Bellahcène et al., 2008).

The regulation of SIBLING genes has been best studied in osteoblastic differentiation. Runx2 is crucial for the regulation of genes that support bone formation and regulates OPN, BSP, DMP1 and DSPP expression (Bellahcène et al., 2008). Published reports have shown the association of Runx2 with tumor progression, invasion and metastasis (Pratap et al., 2011).

The expression of DMP1 has been recently investigated in breast, uterus, colon and lung carcinoma (Bellahcène et al., 2008). Although the RGD domain of DMP1 has been shown to mediate the adhesion and spreading of dental pulp cells *in vitro*, no data about DMP1-mediated cancer cell adhesion are available. DMP1 interacts with the specific splice variants of CD44 that are expressed by cancer cells. Published immunolocalization experiments also showed the localization of DMP1 in breast cancer (Bucciarelli et al., 2007). The degree of correlation between DMP1 and MMP9 was observed in lung cancer (Fisher et al., 2004). Interestingly, it has been seen that low DMP1 expression correlates with skeletal metastases. The expression of DMP1 in invasive and non invasive breast cancer cell lines has been reported (Bucciarelli et al., 2007). However, the function of DMP1 in bone and breast cancer has not been extensively studied.

In conclusion, Narayanan K *et al* have shown that the nuclear localization of DMP1 can lead to the transcription of genes specific for osteoblast differentiation. However, the signaling cascade activated during this process has not been investigated. Published studies have also shown that DMP1 is highly expressed in the mammary epithelium of breast cancer. Breast cancer cells have the potential to cause bone metastasis. The role of DMP1 in breast cancer cells in enhancing osteoclast formation and thereby accelerating bone resportion has not been studied. Thus, this thesis will focus to address the signaling pathways activated during osteoblast differentiation and also investigate the role of DMP1 in activating the vicious cycle in bone during metastatic breast cancer.

CHAPTER 2

SPECIFIC AIMS

AIM: 1. To investigate the role of DMP1 in the differentiation of preosteoblast cells to functional osteoblasts.

DMP1 is localized in the bone matrix and plays a regulatory role in the calcification of the extracellular matrix. Extracellular DMP1 can be internalized by binding to its receptor GRP-78 localized on the plasma membrane. DMP1 has been proposed to play a key regulatory role during osteoblast differentiation. In this aim, our focus is to delineate the role of DMP1 in activating MAPK signaling pathways during the differentiation of preosteoblasts.

AIM: 2. To investigate the role of DMP1 in metastatic breast cancer cell line MDA MB231.

Breast cancer cells have the potential to invade bone and cause bone metastasis. Published immunolocalization experiments have showed the localization of DMP1 in the mammary epithelium of breast cancer patients. The mechanism by which DMP1 functions in metastatic breast cancer cells has not been studied. Therefore, we would evaluate the signaling pathway activated by DMP1 and the downstream gene expression targets in human metastatic breast cancer cell line MDA MB231.

AIM: 3. To determine if over expressed breast cancer cells can activate osteoclasts

The components of a vicious cycle necessary for the initiation and development of bone osteolysis are the tumor cells, osteoblasts and the osteoclasts.

We hypothesize that DMP1 could activate metastatic breast cancer cells and thus play a role in activating the vicious cycle. Therefore experiments proposed in this specific aim would demonstrate the role of DMP1 in triggering the osteolytic cycle.

CHAPTER 3

EXPERIMENTAL DESIGN

1. *Does DMP1 play role in the mobilization of intracellular calcium? If so, from where does the calcium release occur and is the endocytosis of DMP1 necessary for the release of calcium?*

Intracellular calcium measured in MC3T3-E1 cells stimulated with DMP1 or denatured DMP1 or BSA and the use of 2-APB inhibitor or blocking of the receptor GRP-78 and RGD would answer the question.

2. *Does calcium –mediated signaling play a role in the differentiation of osteoblast cells?*

Real time PCR performed on DMP1 stimulated cells with or without SB203580 for Runx2, an early marker and osteocalcin, a late marker of osteoblast differentiation would confirm the role of the stress kinase p38 signaling pathway. Western blots and immunofluorescence performed with p38 pathway specific antibodies can confirm the role of the stress activated kinase. The use of BAPTA-AM inhibitor will validate the role of calcium in activating the p38 signaling pathway.

3. Can RGD in DMP1 activate an integrin -mediated signaling pathway?

RGD blocking peptide and the use of PD inhibitor on DMP1 stimulated cells would confirm the role of integrin in activating the ERK1/2 pathway.

4. *Do differentiated osteoblast cells secrete RANKL into the extracellular environment?*

Western blot performed with RANKL antibody on the media obtained from DMP1 stimulated MC3T3-E1 cells will answer this question.

5. *Do breast cancer cells express DMP1 and how does it have an effect on activating and differentiating preosteoblast cells?*

Western blot analysis using total cell lysate from MDA MB231 can detect the expression of DMP1 in breast cancer cell lines. Activation of signaling pathways in DMP1 over expressed cells can be analyzed by pathway specific antibodies. Secretion of DMP1 into the extracellular environment can be analyzed by immunoblotting media obtained from DMP1 over expressing metastatic cell lines.

6. *Does DMP1 over expressing breast cancer cells lead to the activation of osteoclast?*

Effect of the conditioned media on RAW 264.7 cells obtained from the co-culture of breast cancer cells and osteoblast cells could trigger the differentiation of osteoclast cells. TRAP staining and immunblot analsysis with TRAP antibody performed can confirm osteoclast activation.

7. *Do differentiated osteoclast cells during bone metastasis secrete TGF-β and does it have an effect on breast cancer cells?*

Analysis of the media from differentiated RAW 264.7 cells with TGF-β antibody and studying gene expression and pathways activated in TGF β–stimulated over expressed cells can answer this question.

CHAPTER 4

Materials and Methods

A. Pre osteoblast cells

1. Expression and Purification of DMP-1

The recombinant DMP1 protein was expressed in *Escherichia coli*. The coding region of rat DMP1 cDNA was amplified using a set of sequence-specific sense and antisense primers with BamH1 (Fermentas Inc., MD) and NotI (Fermentas Inc., MD) recognition sites on the 5′ ends of both the primers. The sense primer had the sequence: 5′ GCCGCCGGATCCCTCCCTGTCGCCAGATAC 3′ and the antisense primer: 5′ GCCGCCGCGGCCGCACTAGTAGCCATCTTGGCAATC 3′. The amplified mature DMP1 cDNA was cloned into the BamHI and NotI sites of the prokaryotic expression vector PGEX-4T-3 and transformed into the *E. coli* host JM109 strain (Promega., WI). Transformed colonies were grown in LB/ampicillin medium (BD Bioscience., MA) and the expression of fusion proteins was induced by adding 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fischer Scientific.,PA). The fusion protein was purified from bacterial lysates by Sepharose 4B-Glutathione affinity (BD Bioscience., MA).

Native DMP1 was a kind gift by Dr. Chunlin Qin, Baylor College of Dentistry, Dallas, TX.

2. Cell Culture

Mouse calvarial preosteoblast MC3T3-E1 cells (a kind gift of Dr. R. T. Franceschi, University of Michigan) were cultured in α-minimum Eagle's medium (Mediatech, Inc., VA) supplemented with 10% FBS (Mediatech, Inc., VA) and 1% penicillin/streptomycin (Invitrogen Corp., CA). They were seeded in 6-well plates. The cells were allowed to proliferate until it attained 70% confluence. Media were changed every 2 days. 12–16 h before the start of the experiment, the cells were cultured in α-minimum Eagle's medium supplemented with 1% FBS (basal medium). These cells were then stimulated with 250 ng/ml of rDMP1. The treated cells were then trypsinized, and RNA was extracted for RT-qPCR.

3. Protein Extraction

Total proteins were extracted from rDMP1-stimulated MC3T3-E1 using M-per reagent (Pierce Biotechnologies., MD). Total proteins were extracted using M-per reagent. About 150µl of the extraction buffer was added into each well and total proteins were isolated at 15, 30, and 45 min and 1, 1.5, and 2 h, respectively. Nuclear and cytoplasmic proteins were also extracted from MC3T3-E1 cells treated with rDMP1 using NE-Per reagent (Pierce Biotechnologies., MD).

4. Quantitative Real Time PCR

RNA was extracted according to the manufacturer's recommended protocol by using TRIzol (Invitrogen Corp., CA). RT-qPCR was performed with DNase I (Promega., WI)-treated RNA. A total of 1 µg of total RNA was reverse-transcribed for 90 min at 50 °C with Superscript III (Invitrogen., CA). Quantitative real time PCR (qPCR) analysis was then carried out using ABI StepOnePlus

instrument. Expressions of osteocalcin, Runx2, and GAPDH transcripts were analyzed by qPCR during its linear phase. The relative gene expression level was estimated by using the $2^{-\Delta\Delta}C_T$ method, where C_T value = log linear plot of PCR signal *versus* the cycle number. $\Delta C_T = C_T$ value of target gene $- C_T$ value of GAPDH. Primers were obtained from Qiagen.

5. *In Vitro* Assay for Induction of Terminal Differentiation

The mineralization microenvironment was induced by the addition of 10 mM β-glycerophosphate and 100 µg/ml ascorbic acid (Sigma–Aldrich., MO) along with 10 nM dexamethasone (Sigma–Aldrich., MO) in the basal media. Cells were stimulated with rDMP1 and total RNA, and total proteins were extracted at 7, 14, and 21 days.

6. Mineralization Assay by von Kossa Staining

von Kossa staining was used to determine the presence of phosphate. MC3T3-E1 cells were grown to 60% confluency in growth media. Cells were then stimulated with either DMP1 or DMP1 and SB203580 inhibitor (Enzo Life Sciences, Inc. NY) for 7, 14, and 21 days in mineralization media. The cells were fixed in formalin for 20 minutes, washed twice with distilled water and then stained with 1% silver nitrate solution (Sigma–Aldrich., MO) for 30 minutes.

7. Detection of Protein Phosphorylation by Western Blot Analysis

30 µg of the total proteins were resolved on a 10% SDS-polyacrylamide gel under reducing conditions. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membrane (Bio-Rad Laboratories, Inc.,

CA), blocked with 5% nonfat milk, probed with either anti-$G\alpha_q$ (1:500) (Santa Cruz Biotechnology., CA), anti-p38 (1:500 (Cell Signaling Technology., MA), anti-phospho-p38 (1:500) (Santa Cruz Biotechnology., CA), anti-phospho-HSP27 (1:500) (Cell Signaling Technology., MA), anti-MAPKAPK2 (1:500) (Santa Cruz Biotechnology., CA), anti-phospho-MAPKAPK2 (1:500) (Cell Signaling Technology., MA), anti-ERK1/2 (1:500) (Santa Cruz Biotechnology., CA), anti-phospho-ERK1/2 (1:500) (Santa Cruz Biotechnology., CA), anti-MEK1/2 (1:500) (Cell Signaling Technology., MA), anti-phospho-MEK1/2 (1:500) (Cell Signaling Technology., MA), or anti-Runx2 (1:500) (Abcam., MA) for 16 h at 4 °C. Blots were then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Chemicon International., MA). They were washed three times with PBS containing 0.05% Tween 20 and once with PBS. The bands were visualized by the ECL-Western blot reagent (PerkinElmer Life Sciences., MA). Each membrane was then carefully washed, treated for 5 min with a stripping buffer (Pierce., IL), and washed with PBS, and Western blot analysis was performed with anti-tubulin antibody (Sigma–Aldrich., MO) and HRP-conjugated goat anti-mouse IgG secondary antibody (Sigma–Aldrich., MO).

8. Blocking p38 MAPK and ERK1/2 Activation

Cells were cultured as described above and were treated with 10 μM of SB203580, an inhibitor specific for p38 MAPK or 15 μ*M* PD98059 (Enzo Life Sciences, Inc. NY) – an inhibitor specific for ERK1/2 MAP kinase. SB203580 or PD98059 was added to the DMP1 containing basal media and was preincubated for 30 min at 37 °C prior to the start of the experiment. Total RNA and cell lysates were

harvested at various time points and were analyzed by qPCR and Western blotting performed as described above.

MC3T3-E1 cells stimulated with rDMP1 were grown in mineralization media with or without SB203580 for 7, 14, and 21 days, and Western blot analysis were performed with anti-phospho-p38 antibody.

To study the role of intracellular calcium in activating the p38 MAPK pathway, cells were treated with BAPTA-AM (50 μM) (Invitrogen., CA) for 30 min and then stimulated with rDMP1 for various time points. Total protein was isolated and analyzed by Western blotting for p38 activation.

9. Cytosolic Ca^{2+} Measurement

Changes in the $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent dye Fura-2AM (Invitrogen., CA). MC3T3-E1 cells were grown to confluence on tissue culture glass coverslips and then washed two times with serum-free medium and incubated for 2 h at 37 °C in culture medium containing 1% FBS. Cells were again washed and loaded with 3 μM Fura-2AM for 30 min. After loading, cells were washed with HBSS, and the coverslips were transferred on a perfusion chamber and imaged using an Axio Observer D1 semi-motorized microscope (Carl Zeiss., France) equipped with a camera and a Fluar 40× oil immersion objective lens (Carl Zeiss., France). Light was provided by the DG-4 wavelength switcher (Princeton Instruments). A dual excitation at 340 and 380 nm was used, and emission was collected at 515 nm. The software AxioVision physiology module was used to acquire the images at a frequency of 1-s intervals,

and the data were analyzed off line. Regions of interest in individual cells were marked and excited at 340 and 380 nm with emission at 520 nm at 5-s intervals. In each experiment, 30–40 cells were selected to measure change in $[Ca^{2+}]_i$.

For blocking experiments, cells were treated with various agents as follows. MC3T3-E1 cells were exponentially grown and were pretreated with anti-GRP78 antibody (1:100) (gift from Sylvie Blond, Dept. of Pharmaceutical Biotechnology, University of Illinois., Chicago), GRGDNP blocking peptide (2 mM) for 30 min. The cells were then loaded with Fura-2AM for 30 min. rDMP1 (250 ng/ml) was then added to the cells, and cytoplasmic Ca^{2+} concentration was measured. Cells triggered with BSA (250 ng/ml) served as negative control.

10. Blocking Integrin and GRP78 Receptors and Detection of Phospho-p38 and Phospho-ERK1/2 Activation

MC3T3-E1 cells were grown to 70% confluency on tissue culture plates. GRGDNP blocking peptide (2 mM) was added to the cells and incubated for 60 min. The cells were then stimulated with DMP1 for 30 mins, and total proteins were extracted. Western blot was performed to determine phosphorylation of p38 kinase and ERK1/2.

To confirm that receptor mediated DMP1 entry is needed for p38 kinase activation, we added GRP78 antibody (1:100) to MC3T3-E1 cells and incubated for 60 min. The cells were then stimulated with DMP1 for 30 min, and total proteins were extracted. Western blot was performed to determine phosphorylation of p38 kinase.

11. Immunofluorescence

Activation of p38 and ERK1/2 signaling by DMP1 was analyzed by immunofluorescence. MC3T3-E1 cells were seeded on glass slides and treated with 250 ng/ml rDMP1 for 30 min, 1 and 2 h. The cells were then washed with 1× PBS and fixed with 4% paraformaldehyde for 30 min. Fixed cells were then rinsed three times with 1× PBS and then permeabilized with 0.05% Triton X-100 in PBS for 20 min. The cells were then washed with PBS and blocked with 5% BSA (Sigma – Aldrich., MO) in PBS for 1 h. After blocking, the cells were then incubated overnight with anti-phospho-p38 (1:100) (Santa Cruz Biotechnology., CA), anti-ERK1/2 (1:500) (Santa Cruz Biotechnology., CA) anti-phospho-HSP27 (1:100) (Cell Signaling Technology., MA) or with anti-phospho-ERK1/2 (1:100) (Santa Cruz Biotechnology., CA) and followed by 1 hr incubation with a fluorescein-conjugated goat anti-rabbit IgG (Sigma –Aldrich., MO). After washing with PBS, the cover glass was mounted using mounting media (Vector Lab., CA), and labeling was detected with an Axio Observer D1 fluorescence microscope equipped with Axiovision imaging software.

12. Identification of RANKL in the Extracellular Environment

MC3T3-E1 cells were stimulated with DMP1 for 24 hours in basal media. The media was then collected, dialyzed and lyophilized. Western blot was performed using anti- RANKL (1: 1000) (IMAGENEX., CA) antibody.

B. Breast Cancer

1. Cell Culture

Metastatic MDA MB231 human breast cancer cell line was cultured in RPMI 1640 media (Invitrogen., CA) containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded in 6 well plates and were allowed to proliferate until it attained 60% confluence. 12–16 h before the start of the experiment, the cells were cultured in RPMI 1640 media supplemented with 1% FBS (basal medium). These cells were then stimulated with 5 ng/ml of TGF- β (R and D systems, MN). The treated cells were then trypsinized, RNA and proteins were extracted for RT-qPCR and western blot analysis.

RAW 264.7 monocyte cells were grown in DMEM media (Invitrogen., CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

2. Plasmid Construction

DMP1 cDNA was PCR amplified and cloned into pSTBlue-1 (EMD Chemical., NJ) vector. cDNA for DMP1 were then subcloned into *KpnI/XhoI* sites of pcDNA3.1 vector (Invitrogen., CA).

3. Stable Transfections

Stable transfections of pcDNA 3.1 DMP1 plasmid or shRNA for DMP1 (Sigma-Aldrich., MO) in MDA MB231 cells were performed with Fugene HD (Roche., NJ) according to the manufacturer's protocol. Selections of the stable colonies were made with G418 sulfate (Sigma–Aldrich., MO) at 1μg/ml of medium. Overexpression and knock out efficiency of DMP1 was confirmed by Western blot analysis.

4. Real Time PCR

Total RNA was isolated from MDA MB231 cells (mock), MDA MB231 cells overexpressing DMP1(OE), shRNA-DMP1 mock cells or shRNA-DMP1 OE cells using RNA extraction kit (Qiagen., CA) as per manufacturer's protocol. Real time PCR was performed using primers for DMP, DSP, Runx2, MMP2, MMP9, RANKL, OPG and BSP (IDT DNA., IL).

5. Protein Extraction and Western Blot Analysis

Total proteins were isolated from mock cells, OE cells, shRNA-DMP1 mock cells or shRNA-DMP1 OE cells in the presence or absence of TGF- β using M-per (Pierce., IL). Western blot was performed using anti-DMP1 (1:200) (Laboratory made), anti-TRAP (1:500) (Santa Cruz Biotechnology., CA), anti-p38 (1:500) (Santa Cruz Biotechnology., CA), anti-phospho-p38 (1:500) (Santa Cruz Biotechnology., CA), anti-Smad2/3 (1:500) (Santa Cruz Biotechnology., CA), anti-phospho-Smad2/3 (1:500) (Santa Cruz Biotechnology., CA), anti-ERK1/2 (1:500) (Santa Cruz Biotechnology., CA) and anti-phospho-ERK1/2 (1:500) (Santa Cruz Biotechnology., CA).

6. Immunofluoresence

DMP1 over expressing MDA MB231 cells were seeded on cover glass and immunoflurosence were performed using anti-DMP1 antibody ((1: 2000), kind gift from Dr Qin., Texas).

7. Histology

Tissue sections from breast cancer patients and normal (were obtained from Worsham M, Henry Ford Health systems, Detroit) (n=6) were used for IHC

staining. The core tissue array slide was obtained from IMEGENEX (n=100). The paraffin sections were deparaffinized in xylene rehydrated in alcohol and rinsed three times (5 min each) in double distilled water before quenching of endogenous peroxidase activity (3% H_2O_2 in PBS at room temperature for 10 min). The sections were rinsed three times (5 min each) in PBS and blocked with 10% normal goat serum for 1 hour at room temperature. The sections were again rinsed in PBS solution and incubated with anti-DMP1 antibody (1:200) overnight at 4°C at dilutions respectively. A negative control was included by using an equal concentration of rabbit preimmune serum as primary antibody. After incubation with the primary antibody, the tissue sections were rinsed three times (15 min each) in PBS and incubated with a biotinylated rabbit or mouse anti-goat immunoglobulin (1:500) for 60 min at room temperature. Amplification of the antigen-antibody complex was achieved using avidin-biotin-peroxidase (ABC kit; Vector Laboratories., CA) for 60 min at room temperature. The color reaction was precipitated using DAB (Vector Laboratories., CA) for 1–5 min at room temperature. The tissue sections were counterstained with hematoxylin.

8. Invasion Assay

Mock cells, OE cells and shRNA-DMP1 OE were analyzed for invasion through Matrigel (BD Biosciences., NJ) according to the manufacturer's protocol. 1 x 10^5 cells/ml cells in basal medium were placed in Matrigel inserts and were allowed to migrate for 24 hrs at 37°C. With the help of a cotton swab the non migrating cells were removed from the top of the membrane. Migrated cells were

fixed and stained with a Hema 3 kit (Fisher Scientific., PA) as per manufacturer's protocol. Cells that migrated to the bottom side of the insert were counted manually.

9. Cell Proliferation Assay

For the cell proliferation assay, mock cells and OE cells were seeded in 96-well plates (10^4 cells / well) and grown to 60% confluence under regular growth conditions. Growth media was replaced with basal medium 24 h prior to the start of the experiment. Experiments were done in triplicates. After 48 h, cell proliferation assay was performed by adding 20 µl per well of the Cell Titer 96® Aqueous One Solution Reagent (Promega., WI), following incubation for 3 hours and then recording with a spectrophotometric plate reader (Bio-Tek instruments., USA). The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture.

10. Migration

Metastatic MDA MB231 cells (mock cells) or DMP1 overexpressed MDA MB231 cells (OE) were seeded on cover glass and were allowed to proliferate to reach 80 % confluency. A uniform streak was made on the monolayer culture with 10 µl pipette tips. The cells were then washed with PBS to remove detached cells. Immediately after wounding and 4 h later, each wound was photographed and the distance between the wound edges were observed.

11. Co-culture

To test the hypothesis that DMP1 in MDA MB231 cells can lead to the activation of osteoclast, we would co-culture MDA MB231 or DMP1 over expressed MDA MB231 cells with MC3T3-E1 osteoblastic cells. MC3T3-E1cells and breast

cancer cells were mixed and were seeded in a 100 mm tissue culture plates containing DMEM media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The media from the co-culture was collected after 24 hours. The co-culture media was mixed with fresh media of DMEM media containing 10% fetal bovine serum and 1% penicillin/streptomycin (conditioned medium) in a 50:50 ratio. The conditioned medium was then used for 7 days to differentiate RAW 264.7 (precursor monocytes) cells into osteoclast. Media was changed every other day.

12. TRAP Staining

RAW 264.7 cells were differentiated using conditioned media for 7 days. Osteoclasts were stained using TRAP kit (Sigma–Aldrich., MO according to manufacturer's protocol.

13. Treatment with SB203580 and PD98059

RAW 264.7 cells were cultured in conditioned media as described above and were treated with or without 10 μM of SB203580 (Enzo Life Sciences, Inc. NY), an inhibitor specific for p38 MAPK or 15 μ*M* PD98059 (Enzo Life Sciences, Inc. NY), an inhibitor specific for ERK1/2 MAP kinase. Total proteins were extracted and Western blot was performed with anti-TRAP antibody (Santa Cruz Biotechnology., CA).

14. Identification of DMP1 and TGF- β in the Extracellular Environment

Mock and OE cells were grown in serum free media for 2 days. The media was then collected, dialyzed and lyophilized. Western blot was performed using anti- DMP1 antibody (1: 500).

RAW 264.7 cells in the presence of conditioned media obtained from mock cells or OE cells. The media was collected after 7 days of differentiation, dialyzed and lyophilized. 20µl of the media was resolved on a SDS PAGE gel and immunoblotted with TGF-β antibody (1:500) (Santa cruz Biotechnology., CA).

15. Statistics

The western blots were quantified using the Image J software. When comparing two groups, the student's t-test was used. Data are means +/- SE. All experiments were done in triplicates.

CHAPTER 5

RESULTS

A. Purification of recombinant DMP1 protein

The recombinant DMP1 protein (rDMP1) expressed in *Escherichia coli* was resolved on 10% SDS-PAGE. The gel was stained with coomassie stain and rDMP1 corresponding to 95 kDa size was seen (Figure. 4).

B. Expression of DMP1 in mouse femur

To determine the expression of DMP1 during osteoblast differentiation, immunohistochemical analysis on long bone sections obtained from 4 week old mice was performed. DMP1 was highly expressed in endochondral bone (Figure. 5).

C. Differentiation of Osteoblasts

1. DMP1 stimulation results in mobilization of intracellular calcium in preosteoblasts

In the first set of experiments the effect of DMP1 on the mobilization of intracellular calcium $[Ca^{2+}]_i$ in MC3T3-E1 preosteoblast cells was investigated. Cells were loaded with the fluorescent calcium-sensitive dye Fura 2-AM for 30 minutes followed by DMP1 stimulation and measured for $[Ca^{2+}]_i$. In the absence of external Ca^{2+}, treatment of cells with DMP1 produced a 2-3 fold transient increase in $[Ca^{2+}]_i$

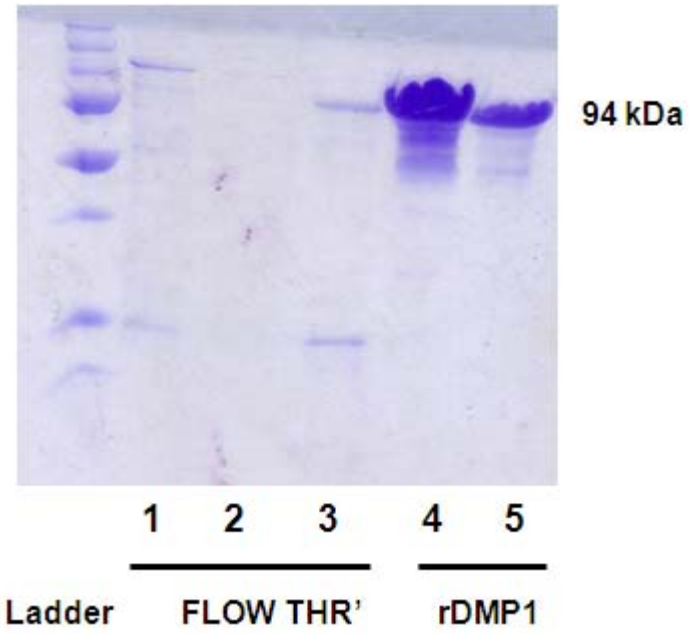


Figure 4:

Purification of recombinant DMP1 from *E.coli*
The recombinant DMP1 protein (rDMP1) expressed in *Escherichia coli* was resolved on a 10% SDS-PAGE. The gel was stained with coomassie. Lanes 1, 2 and 3 indicates the flow through material from the cell lysate after bound to the beads. Lanes 4 and 5 are the purified rDMP1 protein loaded at 30 µl and 20 µl respectively.

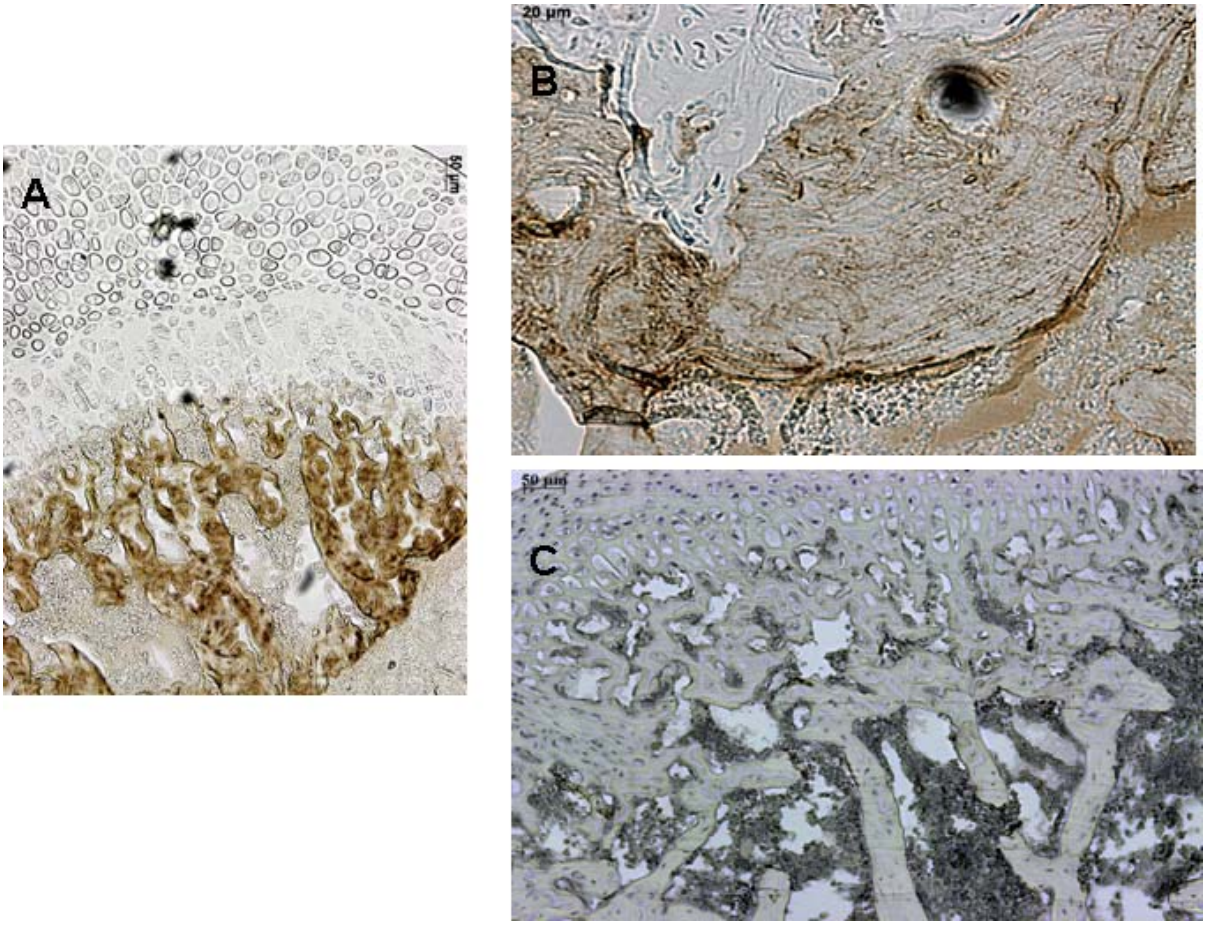


Figure 5:

Expression of DMP1 in mouse femur

Immunohistochemical analysis was performed with anti-DMP1 antibody on long bone section obtained from 4 week old mice. DMP1 was highly expressed in endochondral bone forming areas. The scale bar represents 50 microns **(A).** Magnified view of the endochondral bone showing highly stained areas of DMP1. The scale bar represents 20 microns **(B).** Rabbit secondary antibody control. The scale bar represents 50 microns **(C).**

when compared to unstimulated cells (Figure.6A). Interestingly, cells stimulated with heat denatured DMP1 or BSA (Figure. 6A) caused no observable changes in the $[Ca^{2+}]_i$ levels. In order to confirm that DMP1 was endocytosed by binding to GRP-78 receptor as reported previously, MC3T3-E1 cells were treated with anti-GRP78 antibody and then loaded with Fura 2-AM. Results (Figure 6B) demonstrate that DMP1 binding to GRP-78 was necessary for release of $[Ca^{2+}]_i$. Together, these data indicate that endocytosis of DMP1 by binding to GRP-78 receptor mediate the transient rise in $[Ca^{2+}]_i$.

The role of integrins in activating the store Ca^{2+} release was next explored. Results demonstrate that MC3T3-E1 cells treated with GRGDNP blocking peptide (2mM) for 30 minutes followed by stimulation with DMP1 (Figure. 6C) did not block intracellular Ca^{2+} release.

The study next investigated if release of Ca^{2+} from ER stores occurs through the calcium release channel, namely, IP_3Rs (inositol 1, 4, 5-triphosphate receptors). Therefore, cells were treated with 2-APB an IP_3R antagonist prior to the administration of DMP1. Results in figure 6D demonstrate that 2-APB completely inhibited the store-Ca^{2+}-release in preosteoblasts. This data demonstrates that 2-APB, an IP_3 receptor blocker, could suppress the intracellular Ca^{2+} changes elicited by DMP1 and strongly suggests Ca^{2+} release is mediated through IP_3R–gated stores in DMP1-stimulated preosteoblasts.

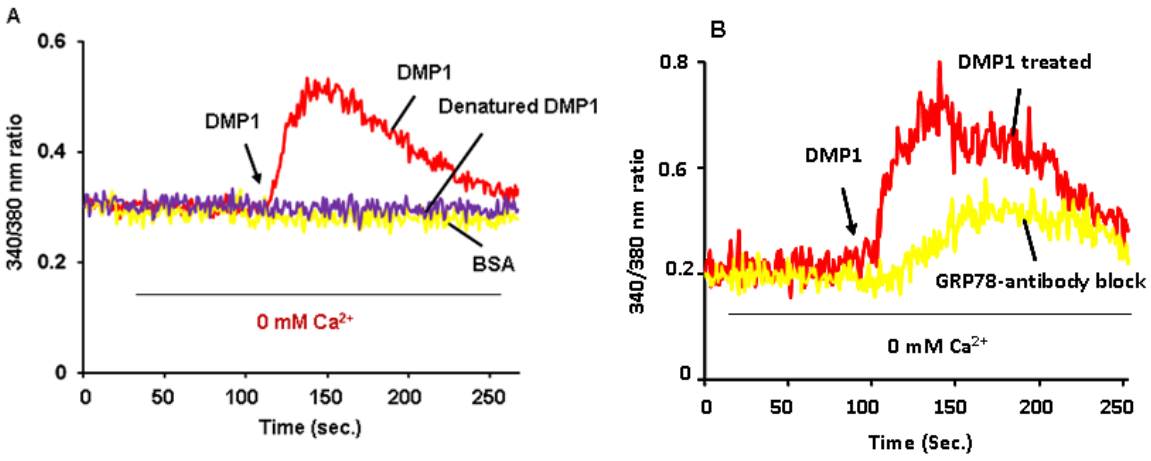


Figure 6:

Effect of rDMP1 on $[Ca^{2+}]_i$ in MC3T3-E1 cells.

DMP1-induced $[Ca^{2+}]_i$ was measured in MC3T3-E1 cells. Fura 2-AM loaded cells were washed 3 times, placed in Ca^{2+}- and Mg^{2+}-free HBSS and then stimulated with rDMP1 (250 ng/ml). Heat inactivated DMP1 protein was used to confirm the specificity and BSA (250ng/ml) was used as a control. The arrow indicates the time point when the cells were stimulated with DMP1 or heat inactivated DMP1 or BSA **(A).** The experiment was repeated 3 times and a representative plot is shown.

GRP78 is necessary for release of $[Ca^{2+}]_i$.
Cells were treated with anti-GRP78 antibody and then loaded with Fura 2AM and then stimulated with rDMP1 (250 ng/ml) as indicated. Blocking DMP1 internalization by anti-GRP-78 suppressed release of $[Ca^{2+}]_i$. The arrow indicates the time point when the cells were stimulated with DMP1 **(B).**

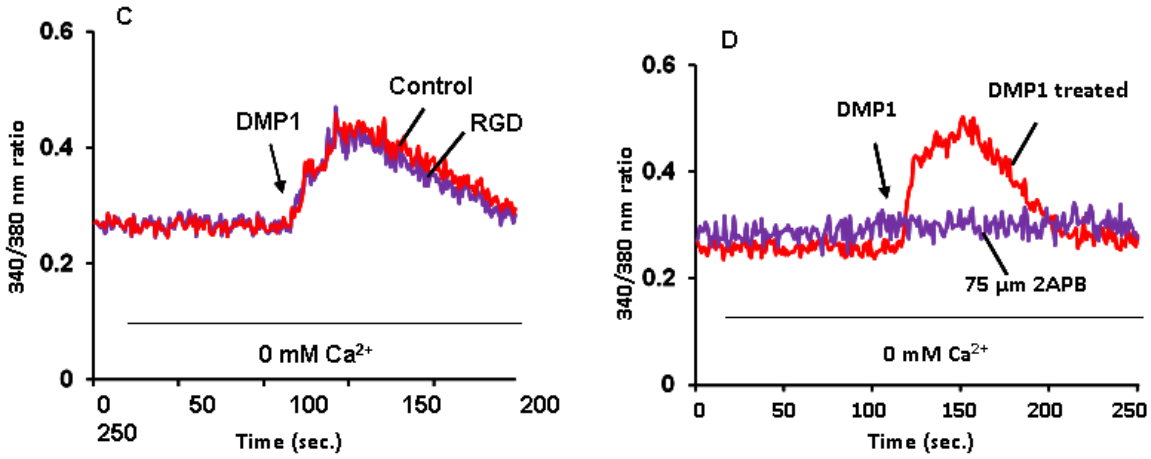


Figure 6:

RGD containing peptide does not inhibits DMP1 induced $[Ca^{2+}]_i$ release in MC3T3-E1 cells.
DMP1-induced $[Ca^{2+}]_i$ was measured in confluent MC3T3-E1 cells that were blocked with 2 mM of RGD containing peptide for 30 minutes prior to the addition of Fura 2-AM. Fura 2-AM loaded cells were washed 3 times, placed in Ca^{2+}- and Mg^{2+}-free HBSS and then stimulated with rDMP1 (250 ng/ml). The arrow indicates the time point when the cells were stimulated with DMP1. The experiment was repeated 4 times and a representative plot is shown **(C)**.

2-APB blocks DMP1-stimulated increase in $[Ca^{2+}]_i$ in MC3T3-E1 cells.
Cells pretreated with 2-APB (75 µM) for 2 hours were loaded with Fura-2 AM and placed in Ca^{2+} and Mg^{2+} free HBSS. The cells were then stimulated with rDMP1 (250 ng/ml) and then observed for Ca^{2+} store release. Cells with no 2-APB treatment served as control. Arrow indicates the time at which cells were stimulated with rDMP1. Experiments were repeated a minimum of 4 times. A representative plot is shown in this figure **(D)**.

2. Osteoblast differentiation can be mediated by DMP1 stimulation

To gain insight into the downstream effect of DMP1 on gene expression and osteoblast differentiation, MC3T3-E1 cells were treated with rDMP1 (250ng/ml) for 4 and 24 hours in basal medium. Quantitative real-time RT-PCR on RNA samples showed that DMP1 stimulation caused a 4 and 5 fold increase in Runx2 mRNA expression at 4 and 24 hrs respectively (Figure. 7A). Interestingly, Osteocalcin, a differentiation marker for osteoblast differentiation had a 6 and 8 fold mRNA expression at 4 and 24 hrs respectively (Figure. 7B). In order to gain insight into the role of DMP1 in terminal differentiation of osteoblasts, MC3T3-E1 cells (Figure. 7C, 7D) were grown in differentiation media for 7, 14 and 21 days in the presence of DMP1. Significant increase in osteocalcin gene expression was seen in MC3T3-E1 cells with cellular differentiation.

3. p38 MAP kinase signaling pathway is required for osteoblast differentiation

The next set of experiments was performed to investigate the signaling pathway mediating the increase in gene expression. As p38 MAP kinase can be activated by cellular stresses, MC3T3-E1 cells were treated with SB 203580 a specific inhibitor for the p38 MAP kinase. RT-qPCR results demonstrate that the mRNA expression of Runx2 and osteocalcin were markedly down regulated in the presence of the p38 inhibitor (Figure. 7). Interestingly, SB203580 inhibited gene expression during the terminal differentiation of osteoblast in both cell types (Figure. 7). To confirm that the down regulation was not mediated through DMSO a vehicle

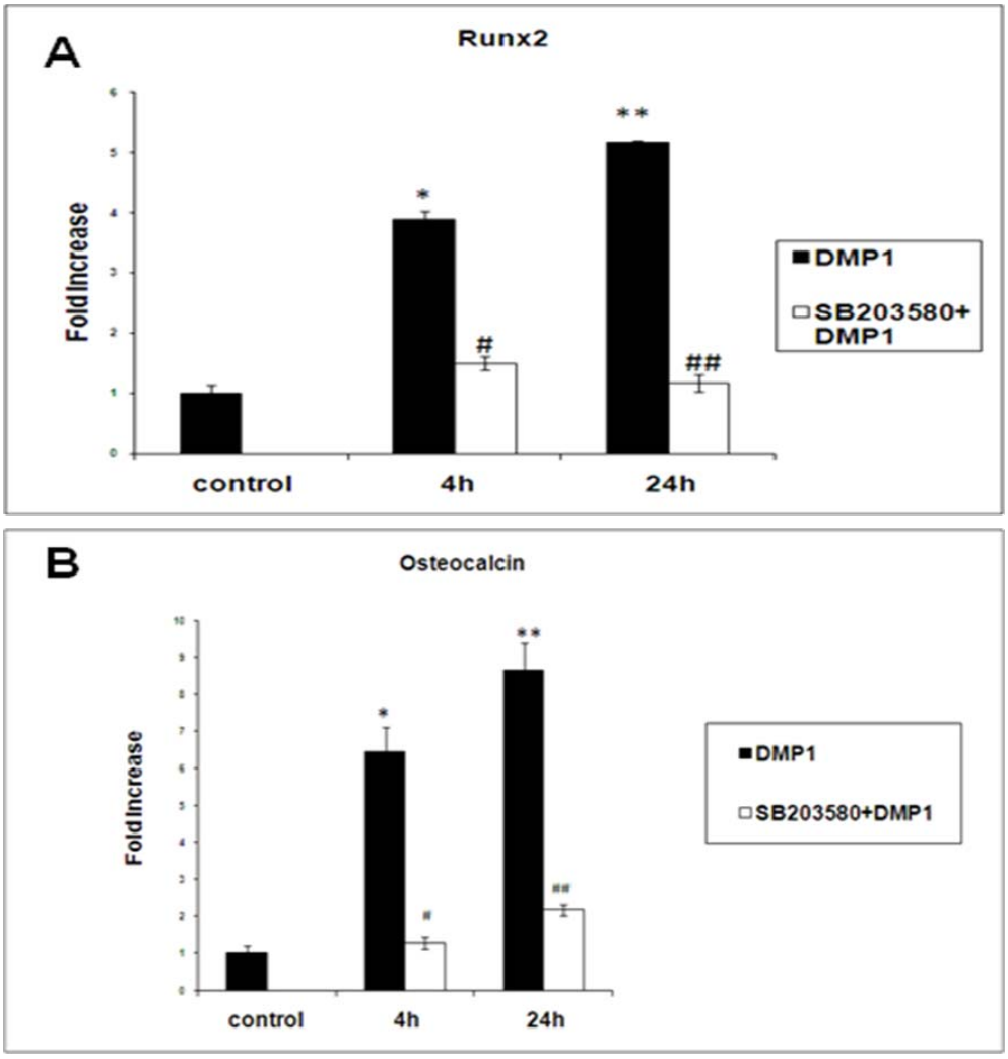


Figure 7:

Effect of DMP1 on osteogenic gene expression and their abrogation in the presence of p38 inhibitor SB203580

MC3T3-E1 cells were either left untreated (control) or starved for 24hr prior to stimulation with 250ng/ml rDMP1 peptide or with SB203580 and rDMP1 in basal medium for 4h and 24h. Total RNA was isolated and subjected to Real time PCR and analyzed for the expression of Runx2 **(A)** and Osteocalcin **(B).** Stimulation with DMP1 significantly up regulated the expression levels of Runx2 and Osteocalcin. Treatment with SB203580 a specific inhibitor for p38 MAP kinase activation down regulated gene expression. These results were normalized with the loading control GAPDH. *P< 0.05 compared to control. **P< 0.03 compared to control. #P< 0.01 compared to 4h DMP1 treatment. ## P< 0.05 compared to 24h DMP1 treatment. Experiments were performed in triplicates.

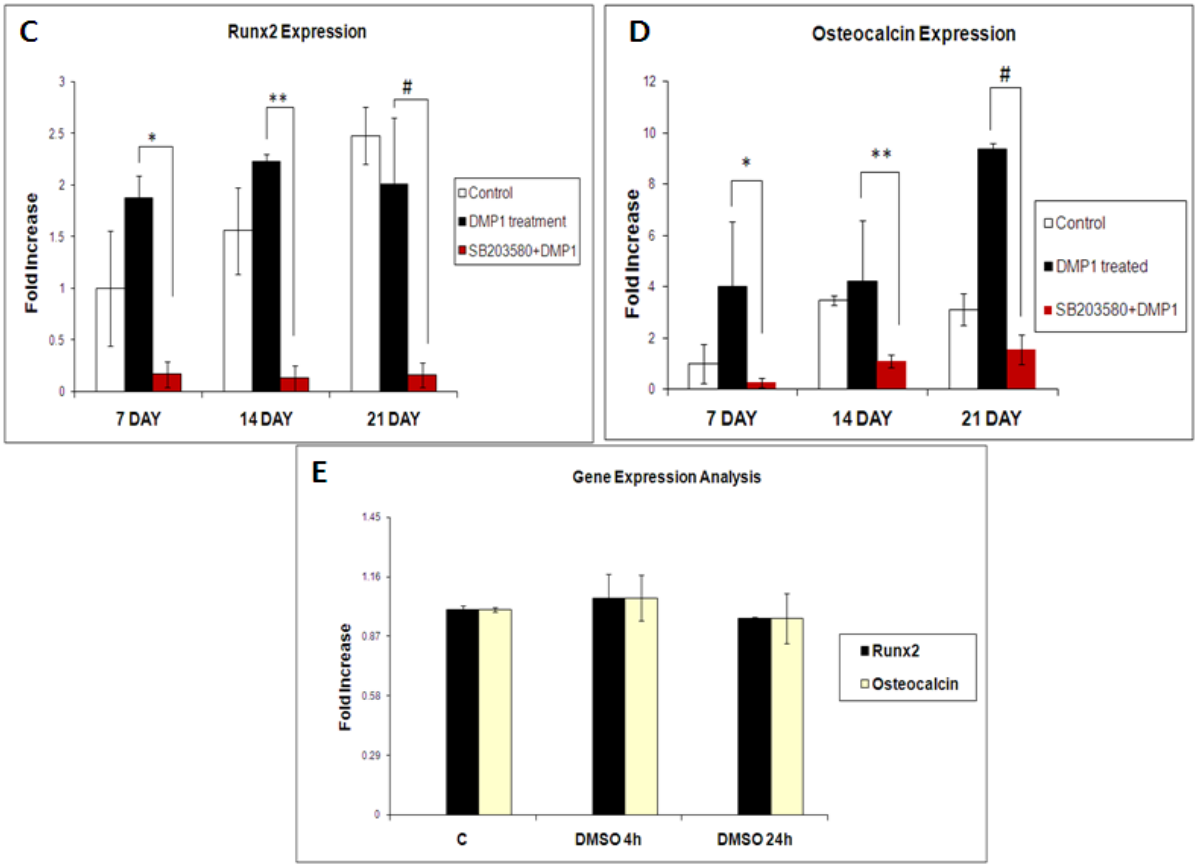


Figure 7 (condt):

Effect of p38 kinase stimulated by DMP1 on terminal differentiation of osteoblastDMP1 enhances terminal differentiation of osteoblast in MC3T3-E1 (**C** and **D**). Cells were treated with DMP1 for 7, 14 & 21 days in the presence of mineralization media and with or without SB203580. Relative amounts of mRNA for Runx2 **(C)** and Osteocalcin **(D)** were determined using real-time PCR. Values are normalized to the levels of GAPDH mRNA. *P< 0.05, **P< 0.01, # P< 0.002 in **(C).** *P< 0.001, **P< 0.01, # P< 0.03 in **(D).** DMSO had no effect on gene expression **(E).** n=6-9 samples.

used for dissolving SB203580 inhibitor, RT-qPCR was performed on mRNA extracted at 4 and 24 hrs from MC3T3-E1 cells stimulated with DMSO. Results demonstrate that MC3T3-E1 cells treated with DMSO had little or no effect on gene expression (Figure. 7E).Together, the data suggest that the augmented gene expression levels in DMP1 stimulated cells are mediated through p38 signaling pathways.

4. DMP1 induces nuclear localization and protein expression of Runx2

Increase in nuclear localization and cytoplasmic staining of Runx2 was observed when MC3T3-E1 cells were stimulated with rDMP1 for 4h, 24h and 48h (Figure. 8A). No nuclear localization was observed with pre-immune serum (Figure. 8B). These results confirm that DMP1 mediates the nuclear translocation of Runx2 protein. Western blot analysis performed on total cell extracts after DMP1 stimulation confirmed the upregulation of Runx2 protein levels (Figure. 8C).

5. Activation of p38 MAP kinase by DMP1 during "early" differentiation of osteoblasts

Studies were performed to determine if the stress-induced MAP kinase, p38, is activated early on during osteoblast differentiation. Western blot results presented in figure 9A demonstrate that DMP1 stimulation can activate p38 kinase (phosphorylated) in MC3T3-E1 cells. This activation is observed as early as 15 min and can be sustained for 2 hrs after DMP1 stimulation. Maximum levels of phosphorylation were observed at 2 hrs in MC3T3-E1 cells (Figure. 9A).

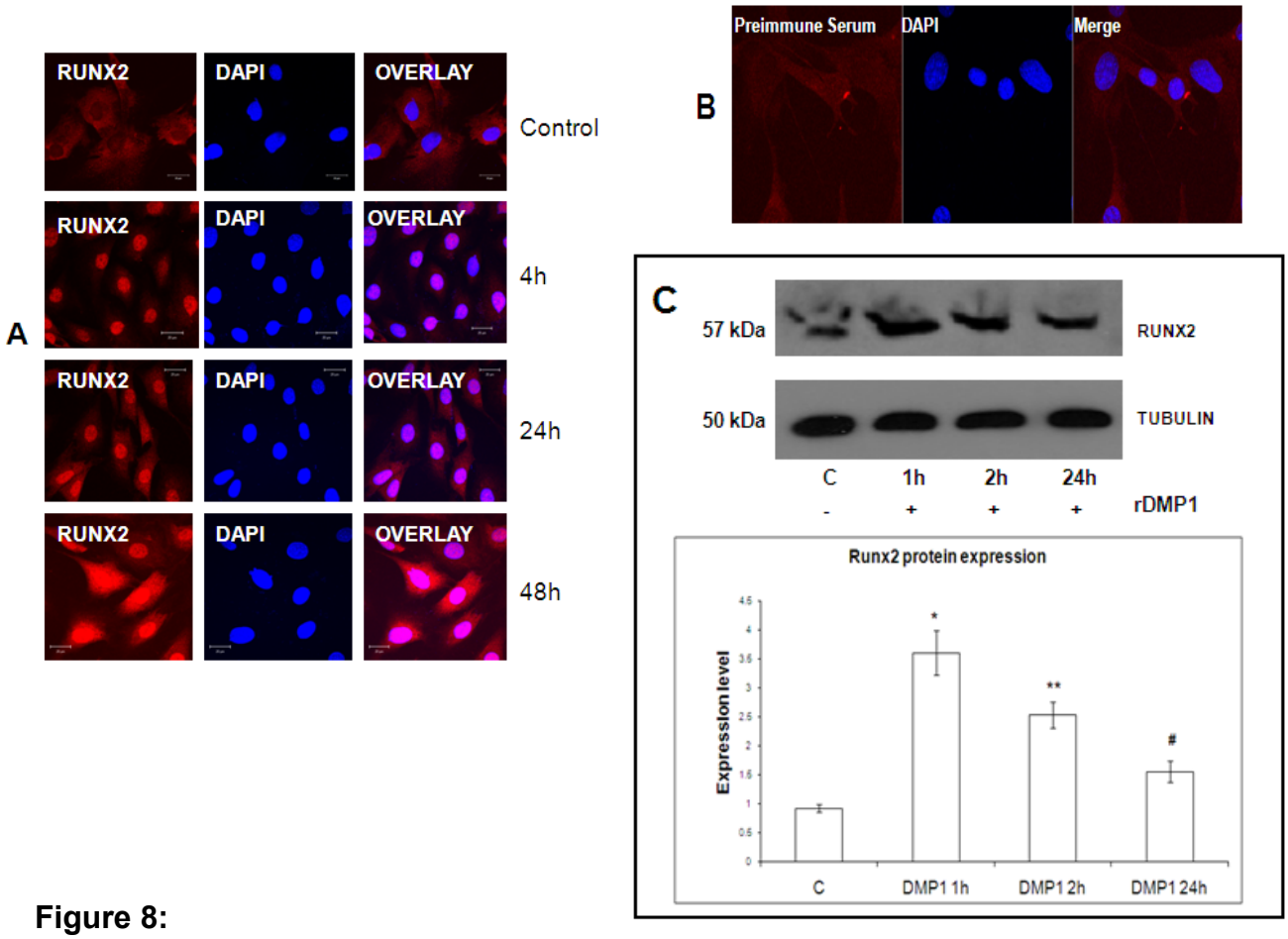


Figure 8:

DMP1 stimulates the nuclear translocation of Runx2.

Confocal image showing nuclear and cytoplasmic staining of Runx2 when stimulated with rDMP1 for 4h, 24h and 48h, while predominant cytoplasmic localization was present in unstimulated cells (**A**). MC3T3-E1 cells stained with control preimmune serum did not show nuclear translocation of Runx2 (**B**). The scale bar represents 20 microns.

Effect of DMP1 on Runx2 protein expression.

MC3T3-E1 cells were either left untreated (control) or starved for 24 h prior to stimulation with 250 ng/ml rDMP1 for 1, 2, and 24 h. Total protein was then isolated and subjected to Western blot analysis using mouse anti-Runx2 antibody **(C)**. *P< 0.01 compared to control. **P< 0.04 compared to 1h of DMP1 treatment. #P< 0.02 compared to control.

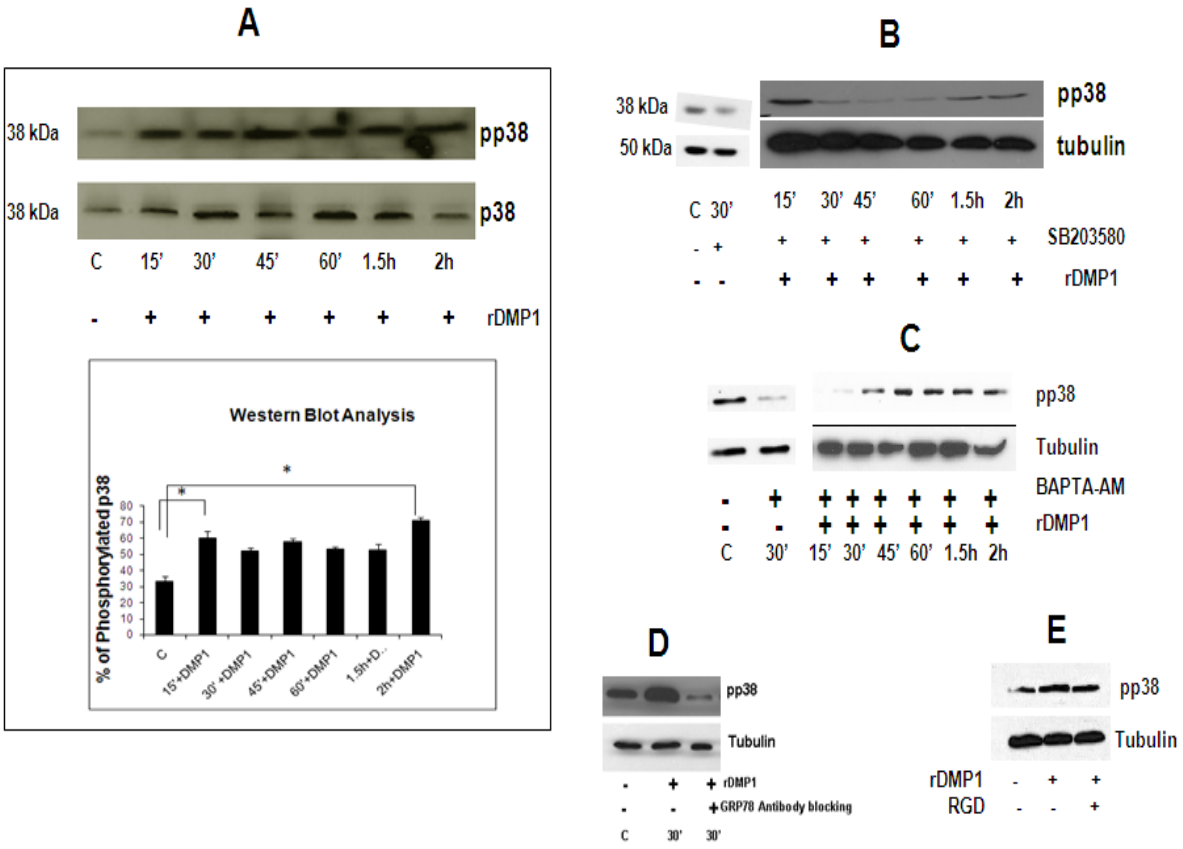


Figure 9:

rDMP1 stimulates phosphorylation of p38 MAP kinase in MC3T3-E1.

Cells in basal medium were untreated (control) or treated with rDMP1 (250ng/ml). Cell lysates were harvested and subjected to SDS-PAGE and western blot analysis performed with phospho- p38 and total p38 antibody. Stimulation by DMP1 phosphorylates p38 MAP kinase in MC3T3-E1 cells **(A).** *P< 0.03.

Activation of p38 kinase by DMP1 can be abrogated by treatment with BAPTA-AM, SB203580, anti-GRP-78 antibody and in the presence of RGD blocking peptide.

MC3T3-E1 cells were treated with p38 inhibitor 10μM SB203580 and stimulated with or without DMP1 and western blots were developed with anti-phospho- p38 antibody **(B).** MC3T3-E1 cells were treated with BAPTA-AM (50 μM) followed by stimulation with rDMP1**(C).** MC3T3-E1 cells were treated with GRP78 antibody (1:100) for 60 minutes **(D)** or with RGD blocking peptide (2mM) for 30 minutes **(E)** followed by stimulation with rDMP1. Total proteins were isolated and probed for anti-phospho-p38 antibody.

Unstimulated control cells showed no significant increase in p38 kinase activity. This data suggest the involvement of p38 MAP kinase in “early” osteoblast differentiation.

6. Activation of p38 kinase by DMP1 can be abrogated by treatment with SB203580, BAPTA-AM and anti-GRP-78 antibody

To examine the DMP1 mediated p38 kinase activation, MC3T3-E1 cells were pre-treated for specific time points with SB203580 a potent chemical inhibitor specific for the p38 MAP kinase. Immunoblot results using phospho- p38 antibody confirmed that the DMP1-induced increase in p38 phosphorylation was effectively blocked by SB203580 (Figure. 9B). To further characterize whether an increase in $[Ca^{2+}]_i$ was required for p38 activation, cells were treated with the Ca^{2+} chelator BAPTA-AM first and then stimulated with DMP1 and subjected to Western blot analysis. BAPTA-AM is inactive in the extracellular environment; however once this membrane permeable chelator enters cells, the AM group is cleaved by intracellular esterases to form active BAPTA. Results in figure 9C show that BAPTA-AM caused a considerable decrease in p38 activation, implicating that $[Ca^{2+}]_i$ was involved in downstream signaling .

Further, blocking endocytosis of DMP1 by anti-GRP78 antibody resulted in decreased p38 phosphorylation (Figure. 9D) indicative of the necessity for DMP1 binding to GRP-78 to mediate downstream p38 activation. Together, these findings suggest that DMP1 internalization increases cytosolic Ca^{2+} influx resulting in p38 MAP kinase activation.

7. Role of integrins in the activation of p38 kinase by DMP1 stimulation

To investigate if the RGD in DMP1 was involved in p38 activation through the integrins, studies were performed to block the integrin receptor by an RGD-containing peptide before DMP1 stimulation. Result in figure 9E show that blocking integrin receptor interactions had much less effect on p38 activation relative to GRP-78 interactions.

8. Activation of p38 kinase by native DMP1

To determine if native DMP1 plays an important role in the activation of p38, MC3T3-E1 cells were stimulated with native DMP1 for 30 minutes, 60 minutes and 2 hrs respectively. Activation of p38 MAP kinase was observed from 60 minutes (Figure. 9F).

9. Stimulation by DMP1 facilitates translocation of phospho- p38 from the cytoplasm to the nucleus in MC3T3-E1 cells

Studies were performed to examine whether activated p38 kinase was translocated to the nuclear compartment of preosteoblasts stimulated with DMP1. Confocal microscopy images in figure 10 indicated that in unstimulated cells phospho- p38 was uniformly distributed throughout the cytoplasm and sparsely in the nucleus. However, in DMP1 stimulated cells, phospho-p38 was localized in the nucleus as early as 30 min. Predominant nuclear localization was observed from 30 minutes. This suggests that preosteoblasts stimulated with DMP1 can activate p38 kinase which then translocates to the nucleus within 30 minutes.

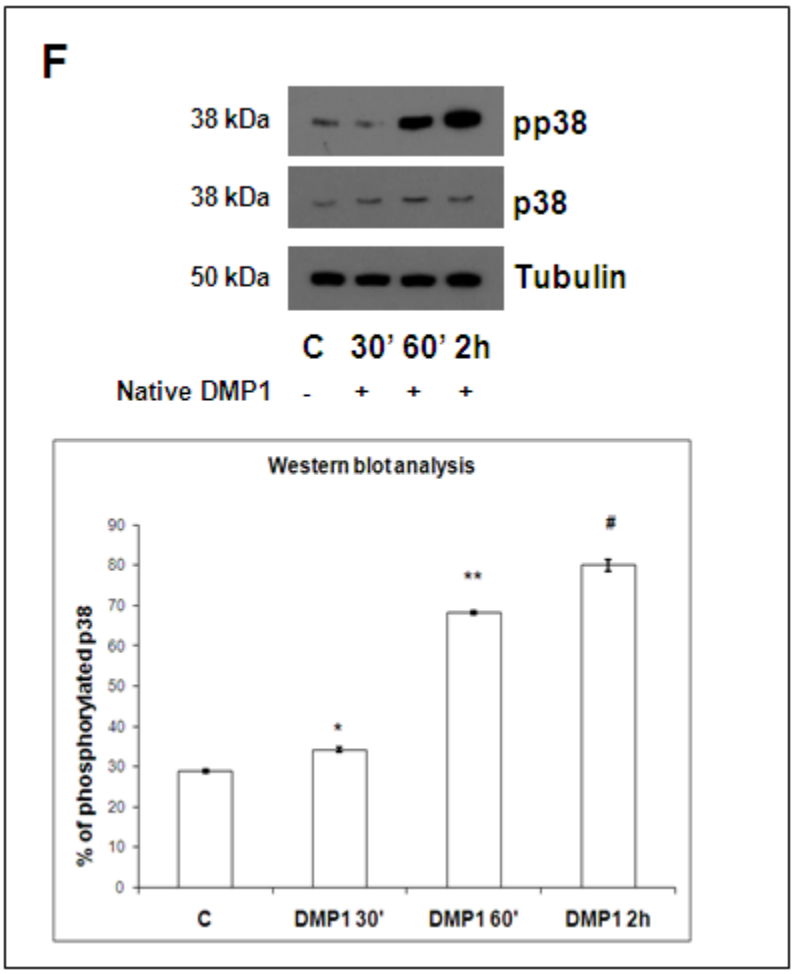


Figure 9 (condt):

p38 Map kinase activation by native DMP1 in MC3T3-E1 cells.

MC3T3-E1 cells were triggered with native DMP1. Total proteins were isolated and immunoblot was performed with phospho- p38, p38 and tubulin antibody **(F).** *P< 0.001 compared to control. **P< 0.02 compared to control. #P< 0.01 compared to control. Experiments were done in triplicates.

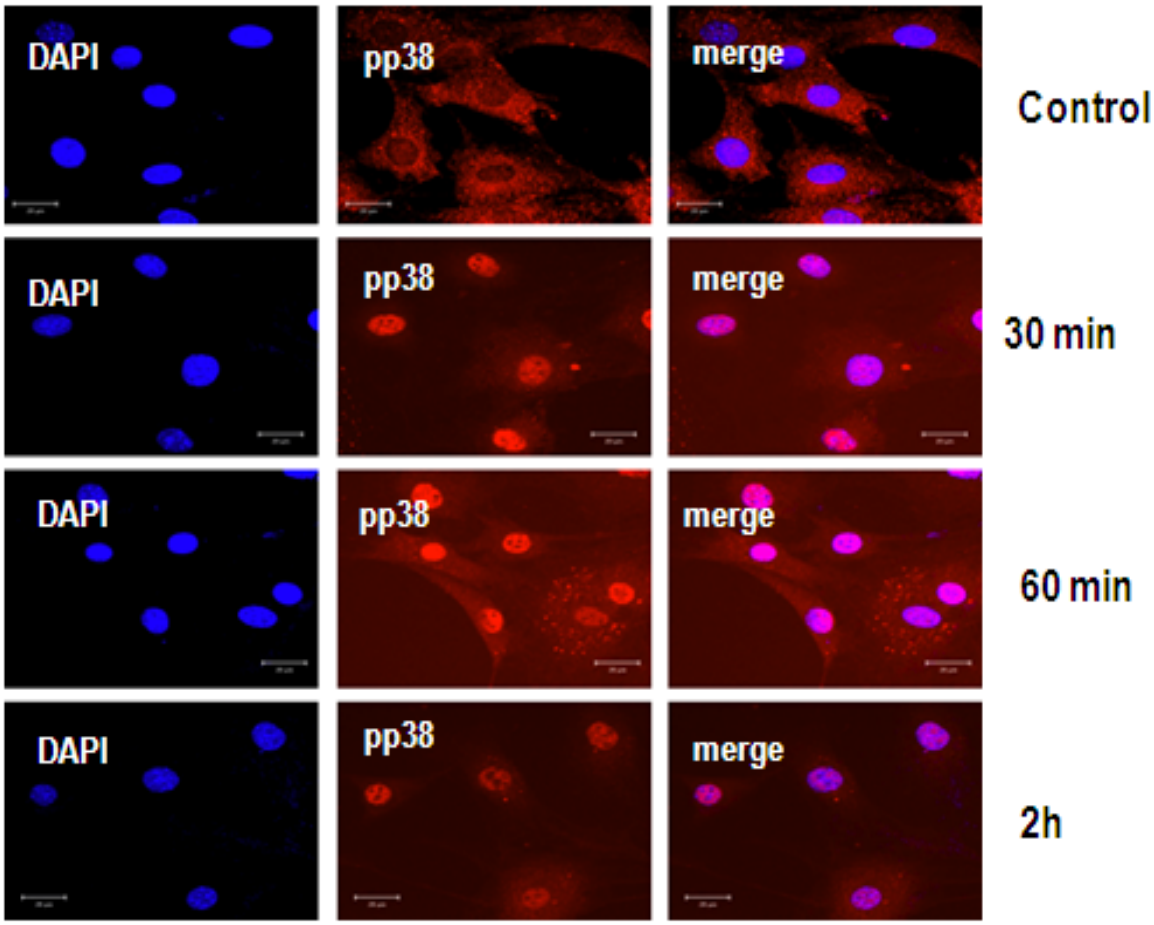


Figure 10:

DMP1 stimulates the nuclear translocation of phospho- p38

Immunofluorescence analysis showing localization of phospho-p38 (red) in MC3T3-E1 cells stimulated with rDMP1 at the indicated time points. DNA was stained with DAPI (blue). Note nuclear localization of phospho-p38 in the nucleus as early as 30 min. Overlap between DAPI and phospho- p38 images is depicted in “merge”**.** The scale bar represents 20 microns.

10. Effect of DMP1 on activation of pMAPKAPK2, downstream target of p38 MAP kinase signaling pathway

Having established the importance of p38 MAP kinase in regulating DMP1 mediated osteoblast differentiation, the next step was to identify downstream effectors of p38 MAP kinase. MAPKAPK2 (mitogen-activated protein-kinase-activated protein kinase 2) is a known substrate of p38 MAP kinase, therefore, the involvement in DMP1 mediated osteoblast differentiation was identified. Western blot analysis revealed that stimulation by DMP1 increased the levels of phosphorylated MAPKAPK2 from 15 minutes until 2 hrs in MC3T3-E1 cells (Figure. 11A). Importantly, inhibition of MAPKAPK2 activation was observed with SB203580, which blocked MAPKAPK2 phosphorylation (Figure. 11B). These results demonstrate that DMP1 stimulation leads to activation of p38 kinase which in turn leads to increased levels of phosphorylated MAPKAPK2 in preosteoblasts.

11. DMP1 stimulates protein activation and nuclear localization of HSP27

Heat-shock protein 27 (HSP27) is constitutively expressed in a variety of mammalian cell types and its expression increases in response to sublethal stresses including heat shock, heavy metal, toxins and oxidative stress. HSP27 has previously been reported to be phosphorylated by MAPKAPK2 downstream of p38 MAPK in the stress response pathway. Activation of HSP27 was therefore evaluated as a potential regulator of osteoblast differentiation. Therefore experiments were performed to determine if DMP1 stimulation can phosphorylate HSP27 in a time dependent manner. In figure, 12A expression of phospho- HSP27 increased after 15

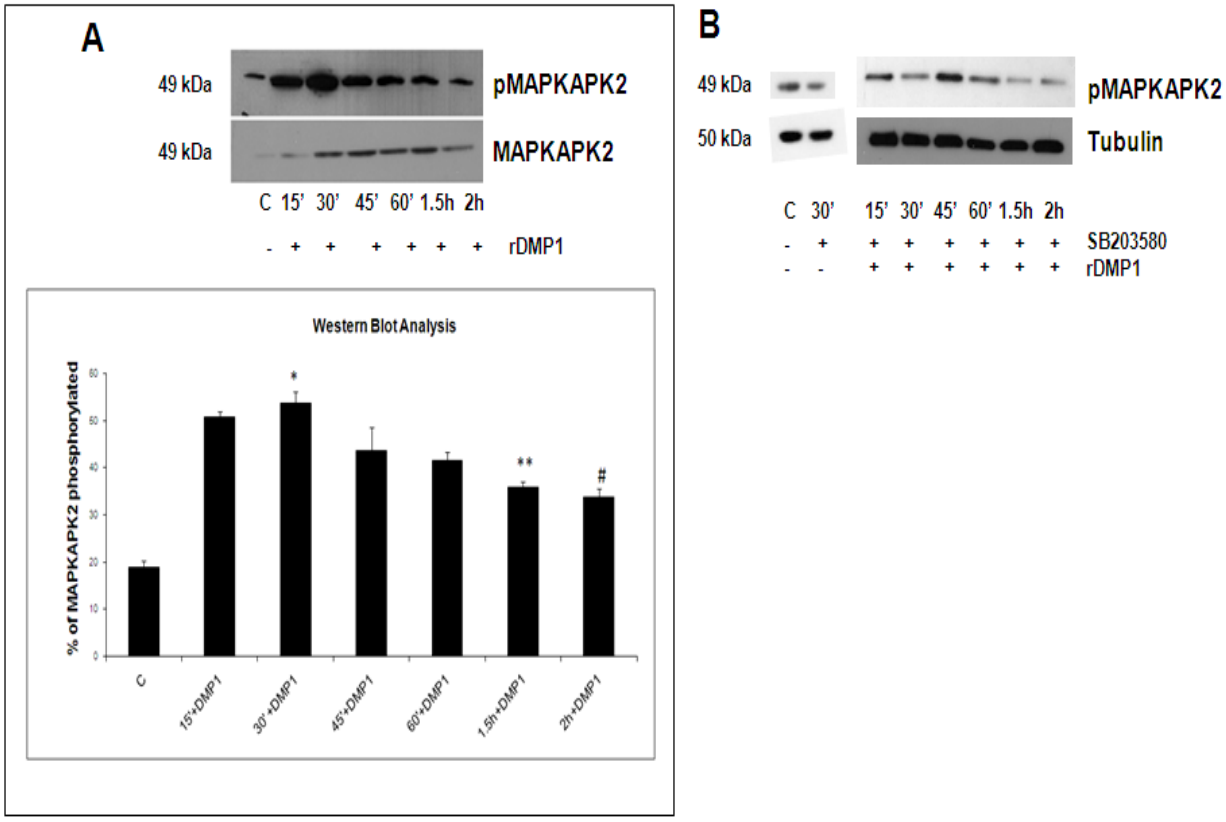


Figure 11:

DMP1 stimulation activates MAPKAPK2 which is one of the downstream targets of the p38 MAP kinase signaling pathway.

Confluent adherent MC3T3-E1 cells were treated without (control) or with DMP1 and western blot analysis was performed with anti-phospho-MAPKAPK2 and total MAPKAPK2. Phosphorylation of MAPKAPK2 was assessed following DMP1 stimulation **(A)**. Phosphorylation of MAPKAPK2 was assessed following treatment with SB203580 and then stimulated by DMP1 **(B)** *P< 0.01 compared to control. **, #P< 0.03 compared to control.

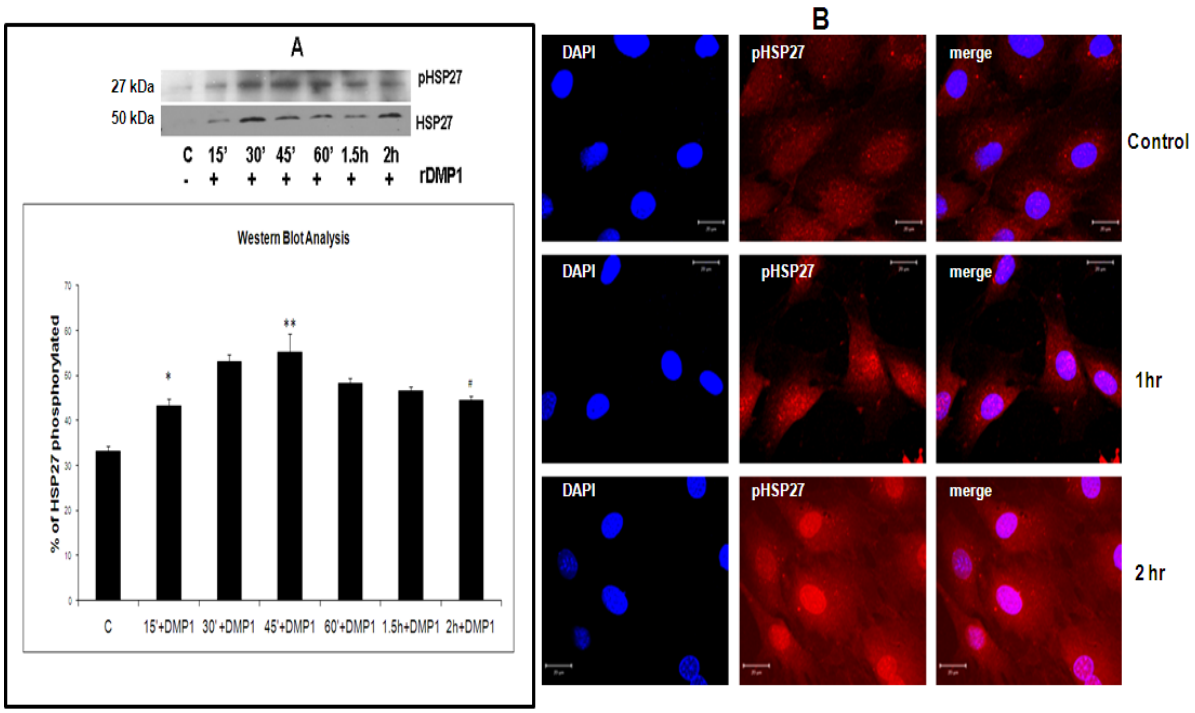


Figure 12:

Activation of HSP27 by DMP1 in MC3T3-E1 cells

Western blot was also performed with anti-phospho- HSP27 antibody after stimulating cells without (control) or with DMP1 . Equal amount of proteins were loaded as assessed by tubulin **(A)**. *P< 0.05 compared to control. **P< 0.003 compared to control. #P< 0.001 compared to control.
Confocal Microscopy images showing nuclear localization of HSP27 in DMP1 stimulated cells **(B)**. The scale bar represents 20 microns.

min of DMP1 treatment. This was further confirmed by the nuclear translocation of the phosphorylated form of HSP27 in DMP1 treated cells (Figure. 12B).

12. p38 MAP kinase signaling pathway is required for terminal differentiation of osteoblasts

Mineralized nodule formation is the hallmark of *in vitro* osteogenic differentiation. MC3T3-E1 cells were grown for 7, 14 and 21 days in mineralization media containing DMP1 or DMP1 and SB203580. The cells were then stained for mineralized matrix using von kossa staining technique. As expected, the cells grown in the presence of DMP1 and mineralization for 14 and 21 days stained positive for a mineralized matrix. However, mineralized nodule formation was suppressed in the presence of SB203580 (Figure 13A). Activation of phosphorylated form of p38 during the terminal differentiation process in MC3T3-E1 cells was determined (Figure.13B).

13. Role of G protein signaling in mediating downstream p38 activation

This study next sought to identify if the signals resulting from DMP1 endocytosis is transmitted intracellularly from the cell surface through the activation of G protein subunits. Specifically, the study explored the role of $G\alpha_q$-mediated p38 signaling on osteoblast differentiation as $G\alpha_q$ signaling played a crucial role in bone formation. Results in figure 14A show that stimulation by DMP1 results in increased $G\alpha_q$ expression.

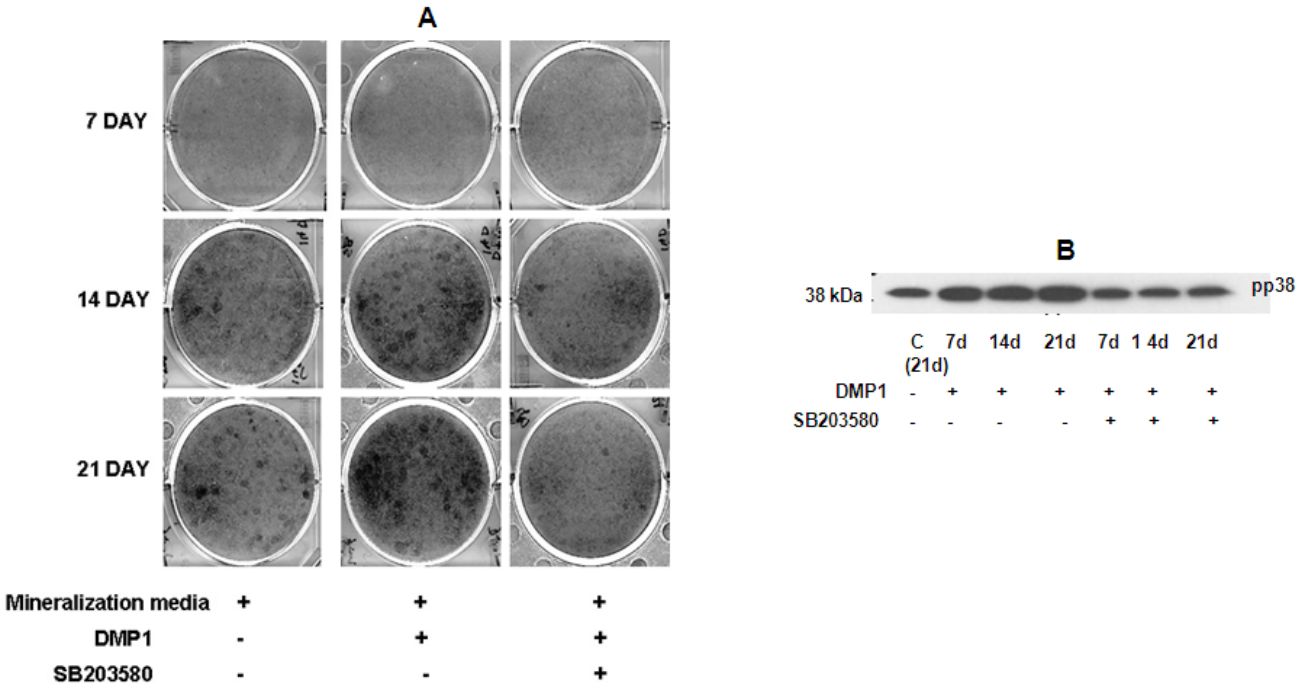


Figure 13:

Effect of p38 kinase stimulated by DMP1 on mineralized nodule formation

MC3T3-E1 cells were grown in mineralization media in the presence of DMP1 or SB203580 and DMP1 for 7, 14, and 21 days. Mineralized nodules were assayed with von kossa staining. DMP1 stimulated the formation of mineralized nodules at 14 and 21 days while SB203580 inhibitor suppressed nodule formation **(A).** Activation of p38 MAPK pathway is dependent on DMP1 signaling during terminal differentiation of osteoblast **(B).**

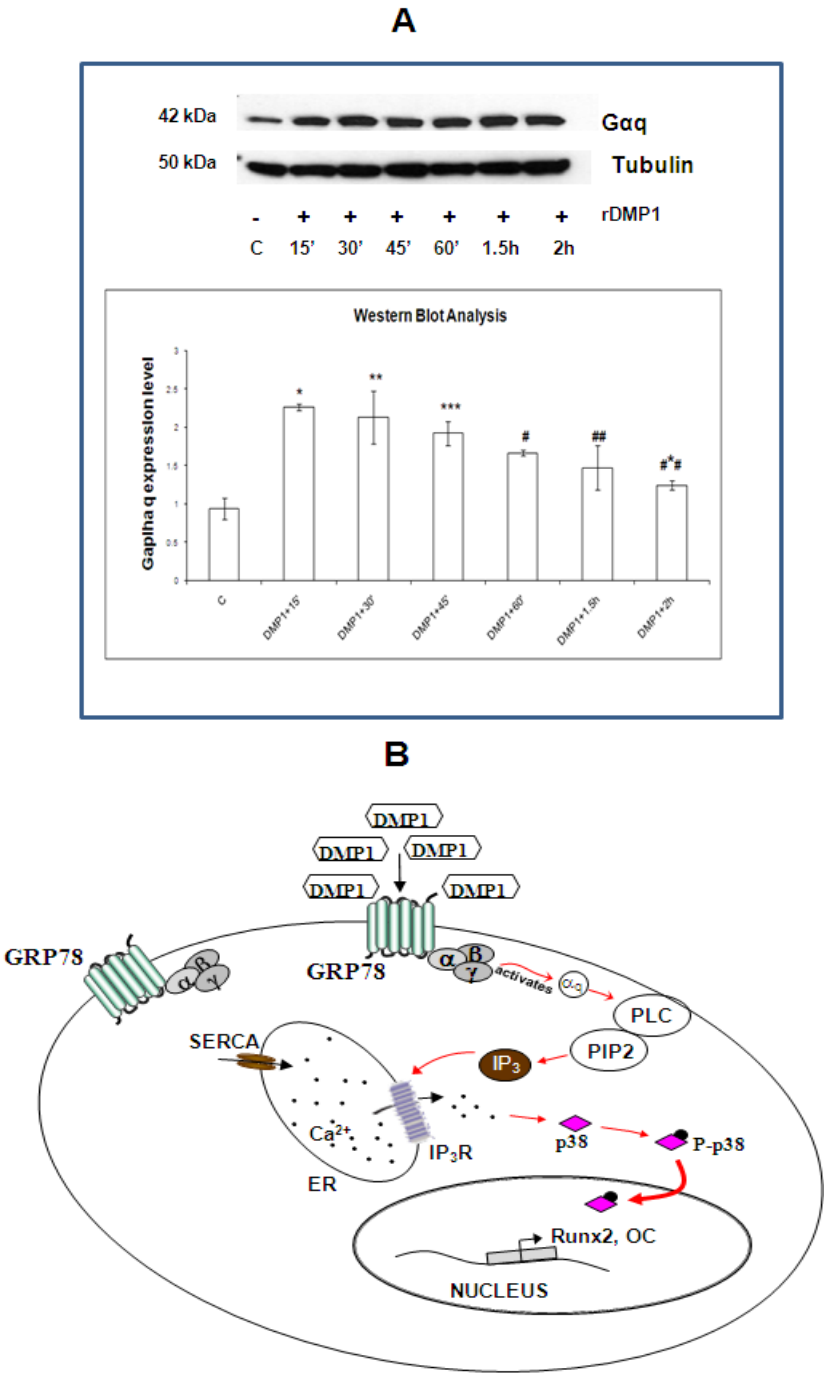


Figure 14:

Effect of DMP1 on the expression of Gαq11 and Hypothetical model depicting DMP1 stimulation of p38 activation.

Total proteins were extracted from DMP1 stimulated MC3T3-E1 at the indicated time points. Western blot analysis was performed with anti-Gαq11 antibody. Tubulin was used as the loading control **(A)**. P< 0.05 compared to control. Hypothetical model depicting the depletion of ER Ca^{2+} stores upon DMP1 endocytosis and the subsequent activation of p38 MAP kinase and downstream gene transcription **(B)**.

Overall, the experiments helped us to derive a hypothetical model where calcium mediated stress kinase activation by DMP1 promotes osteoblast differentiation by activating the p38MAP kinase pathway (Figure. 14B).

14. DMP1 Stimulation Induces the Expression of Runx2 and osteocalcin is also dependent on ERK1/2 MAPK pathway

Activation of Runx2 and osteocalcin gene expression on MC3T3-E1 cells stimulated with DMP1 has been seen. The study next sought to examine if the DMP1 stimulated expression of Runx2 and osteocalcin is dependent on the ERK1/2 pathway. MC3T3-E1 cells were treated with or without DMP1 in the presence of PD98059, an inhibitor for ERK1/2. Interestingly, it was seen that, Runx2 and osteocalcin gene expression with DMP1 stimulation was abrogated in the presence of PD98059 (Figure. 15). These results demonstrate that like DMP1-mediated p38 activation, ERK1/2 activation is also required for osteoblast differentiation.

15. DMP1 Stimulation Induces ERK1/2 Activation

The study next explored the phosphorylation state of ERK1/2 mitogen-activated kinase in preosteoblasts stimulated by DMP1. Western blot analysis performed on DMP1 stimulated cells at 30 min, 1 h, and 2 h showed sustained activation from 30 min to 2 h (Figure. 16A). An increase in phosphorylation at Thr 202/204 demonstrates the activation of ERK1/2 in response to DMP1 stimulation. When cells were treated with the ERK1/2 inhibitor PD98059, this activation was

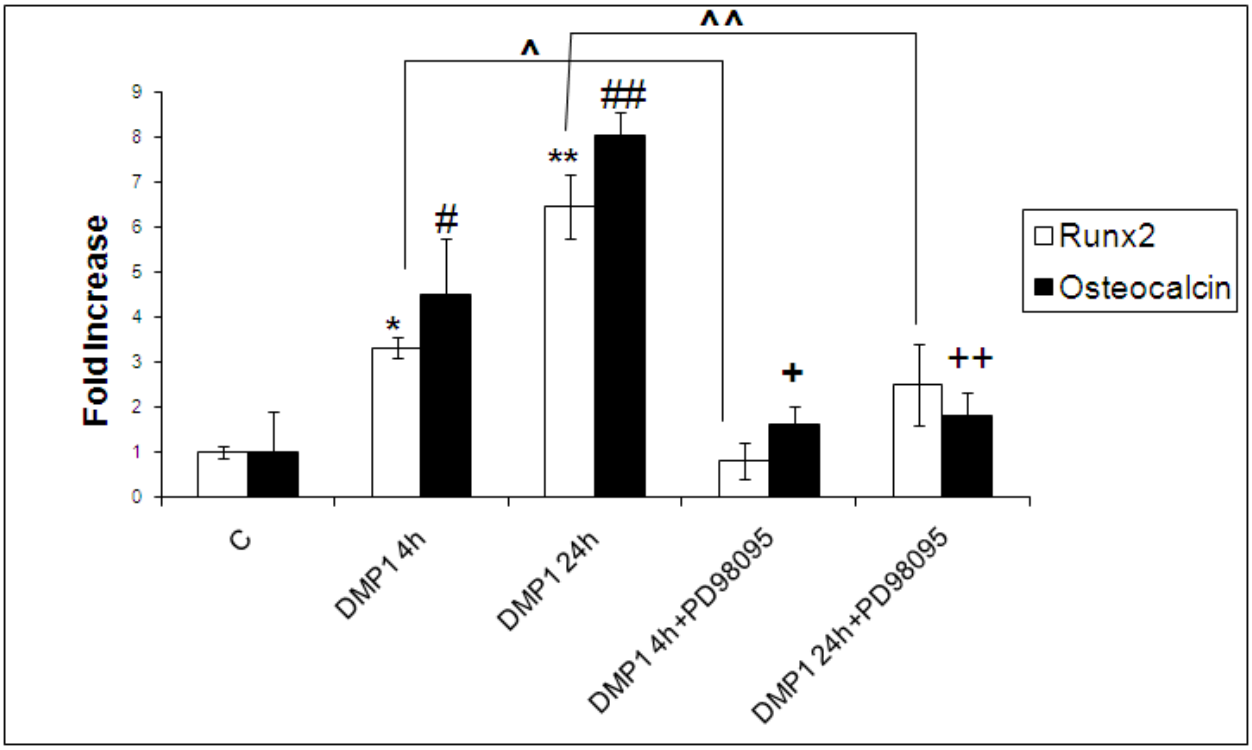


Figure 15:

Effect of DMP1 on Runx2 and osteocalcin mRNA expression and its abrogation in the presence of ERK inhibitor PD98095.

MC3T3-E1 cells were left untreated (control) in basal media for 24 h prior to stimulation with 250 ng/ml rDMP1 or with PD98095 for 4 and 24 h. Total RNA was isolated, subjected to real-time PCR, and analyzed for the expression of Runx2 and osteocalcin. These results were normalized with the loading control GAPDH.n=3-6. *P< 0.003 compared to control. **P< 0.02 compared to control. #, ##P< 0.05 compared to control. +P< 0.001 compared to DMP1 4h. ++P< 0.02 compared toDMP1 24h. ^P< 0.001 and ^^P< 0.002.

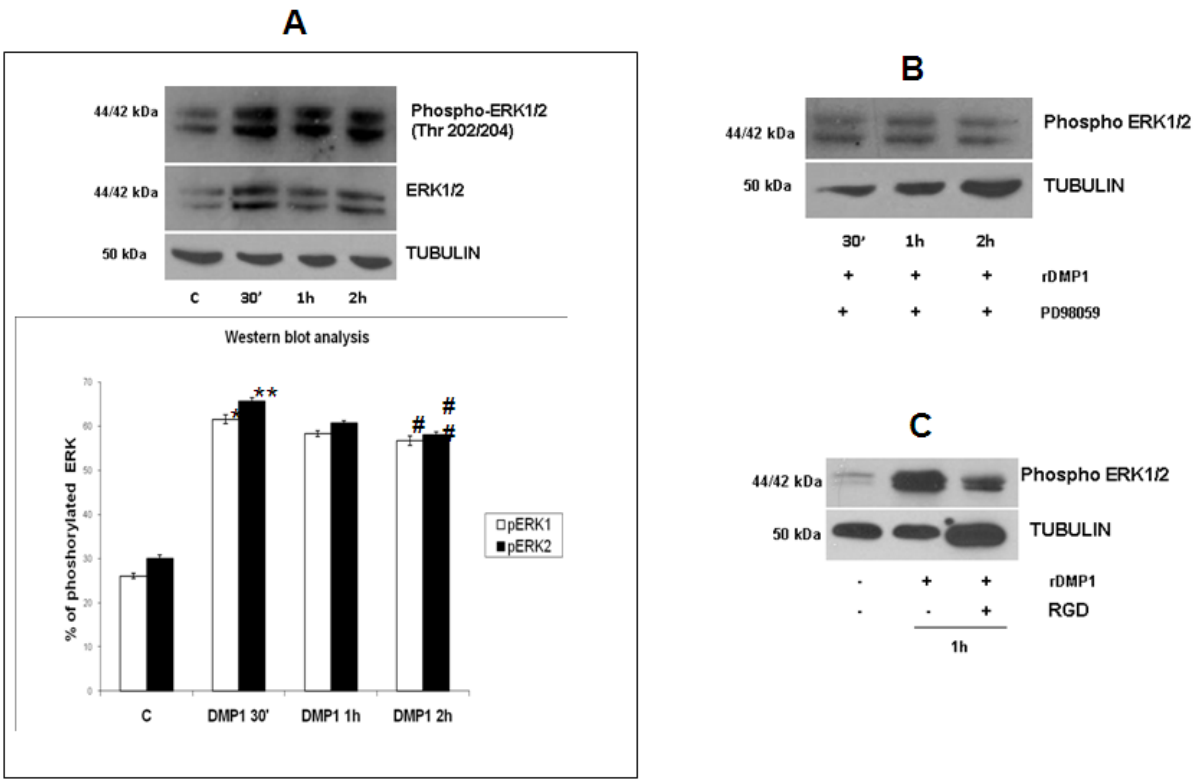


Figure 16:

DMP1 stimulates phosphorylation of the ERK1/2 MAP kinase in MC3T3-E1 cells. MC3T3-E1 cells in basal medium were untreated (control) or treated with rDMP1 (250 ng/ml) for 30 min, 1 h, and 2 h. Cell lysates were harvested and subjected to SDS-PAGE and Western blot analysis performed with phospho-ERK1/2 total ERK1/2 **(A).** Equal loading of the proteins was confirmed by stripping the blot and then probing it with tubulin. *P< 0.001 compared to control. **P< 0.04 compared to control. #P< 0.05 compared to control. ##P< 0.001 compared to control

DMP1-stimulated phosphorylation of ERK1/2 is abrogated in the presence of PD98059. Cell lysates obtained from control and MC3T3-E1 cells treated with the ERK1/2 MAP kinase inhibitor PD98059 and then stimulated with DMP1 for 30 min, 1 h, and 2 h were immunoblotted with phospho- ERK1/2 antibody. Tubulin was used as a loading control **(B).**

DMP1-stimulated phosphorylation of ERK1/2 is reduced in the presence of an RGD-blocking peptide. MC3T3-E1 cells were incubated with or without an RGD-blocking peptide and then stimulated with DMP1 for 1 h and immunoblotted with phospho- ERK1/2 antibody. Tubulin was used as a loading control **(C).**

suppressed (Figure. 16B), indicating that induction of osteoblast differentiation depends on ERK1/2 activation.

16. DMP1-Mediated ERK1/2 Activation in Preosteoblasts Is Mediated through the RGD Integrin-Binding Domain

DMP1 contains an RGD integrin-binding domain; therefore, experiments were performed to investigate the role of integrin-mediated intracellular signaling. An RGD-blocking peptide was used to study its effect on the activation of ERK1/2. The Western blot analysis presented in figure 16C shows that blocking the integrin-binding domain with an RGD peptide did suppress ERK1/2 activation. The ability of DMP1 in the presence of the RGD peptide to suppress ERK1/2 activation suggests a role for integrins in ERK1/2 activation. However, blocking with RGD peptide had little or no effect on p38 activation.

17. DMP1-Mediated ERK1/2 Activation Occurs through MEK1/2

To test whether activation of MEK, an upstream regulator, is required for ERK activation, Western blot analysis was performed. The results in figure 16D show the activation of MEK1/2 in response to DMP1 stimulation. This data suggests that the activation of MEK is more pronounced in DMP1-stimulated cells when compared with the control. MEK1/2 activation is characterized by activation by phosphorylation at 2 activation loop residues, i.e. Ser 217 and 221.

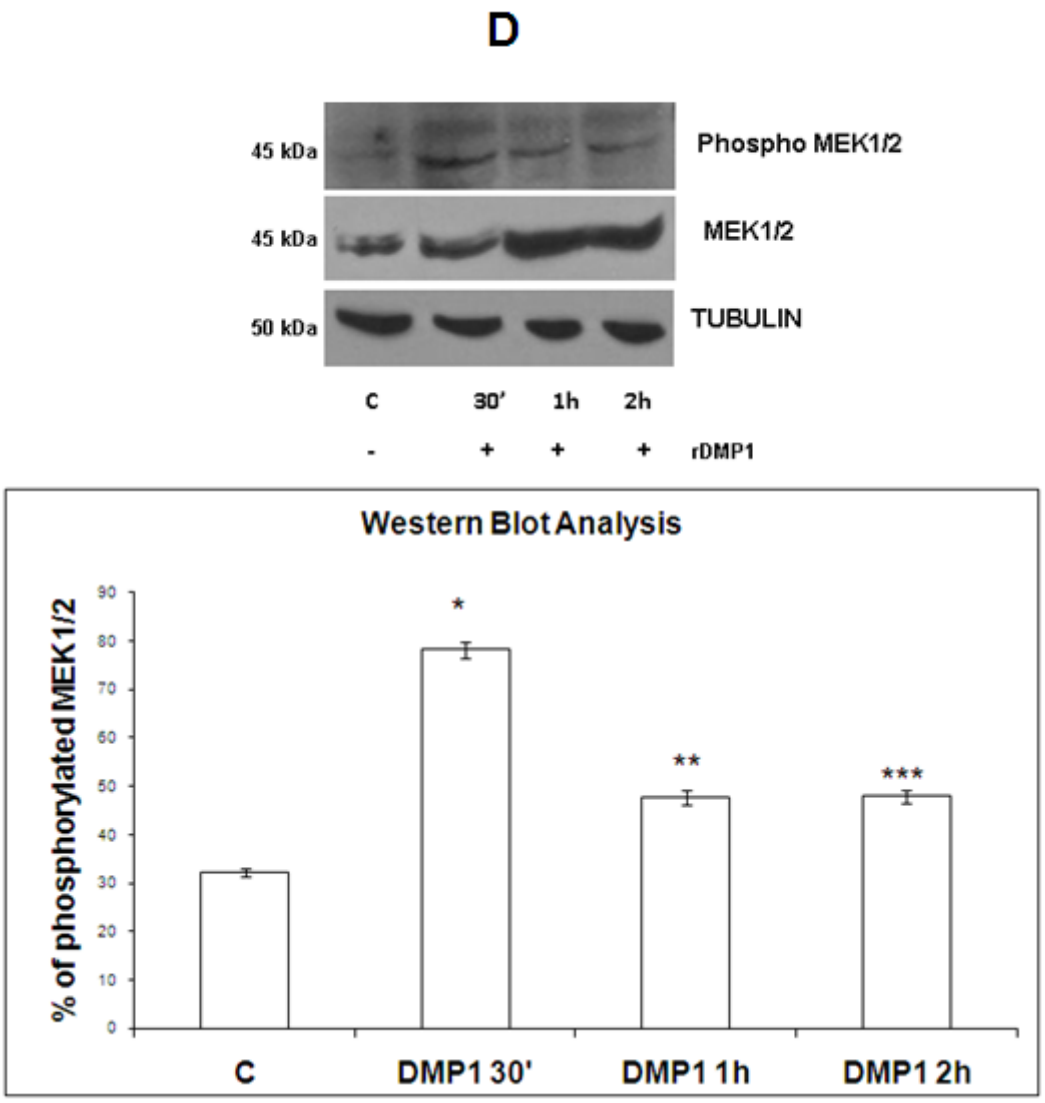


Figure 16 (condt):

DMP1 stimulates phosphorylation of the MEK1/2 MAP kinase in MC3T3-E1 cells.

MC3T3-E1 cells in basal medium were untreated (control) or treated with rDMP1 (250 ng/ml) for 30 min, 1 h, and 2 h. Western blot analysis performed with phospho-MEK1/2 antibody **(D)**. Equal loading of the proteins was confirmed by stripping the blot and then probing it with tubulin. *P< 0.003 compared to control. **P< 0.01 compared to control. ***P< 0.05 compared to control.

18. Translocation of Phospho-ERK1/2 to the Nucleus of Preosteoblasts upon DMP1 Stimulation

Confocal analysis of DMP1-stimulated MC3T3-E1 cells stained for phospho-ERK1/2 showed nuclear localization within 30 min and intense staining by 2 h (Figure. 17), while unstimulated cells showed diffuse cytoplasmic staining. This is consistent with the immunoblotting analysis shown in figure 16A which demonstrates an increase in the phosphorylation of ERK1/2 in MC3T3-E1 cells.

Thus, the study has shown that DMP1 can stimulate the differentiation of osteoblast through RGD mediated activation of ERK1/2 MAP kinase pathway (Figure. 18).

19. Secretion of RANKL by osteoblast into the extracellular environment

Experiments were next performed to test the hypothesis that RANKL is secreted by fully differentiated osteoblast cells when stimulated with rDMP1. MC3T3-E1 cells were stimulated with rDMP1 for 24 hours in basal media. The media was collected, dialyzed and lyophilized. 20µl of the medium was resolved on a SDS PAGE gel and immunoblotted with anti- RANKL antibody. Western blot results confirm the secretion of RANKL by differentiated osteoblasts (Figure.19).

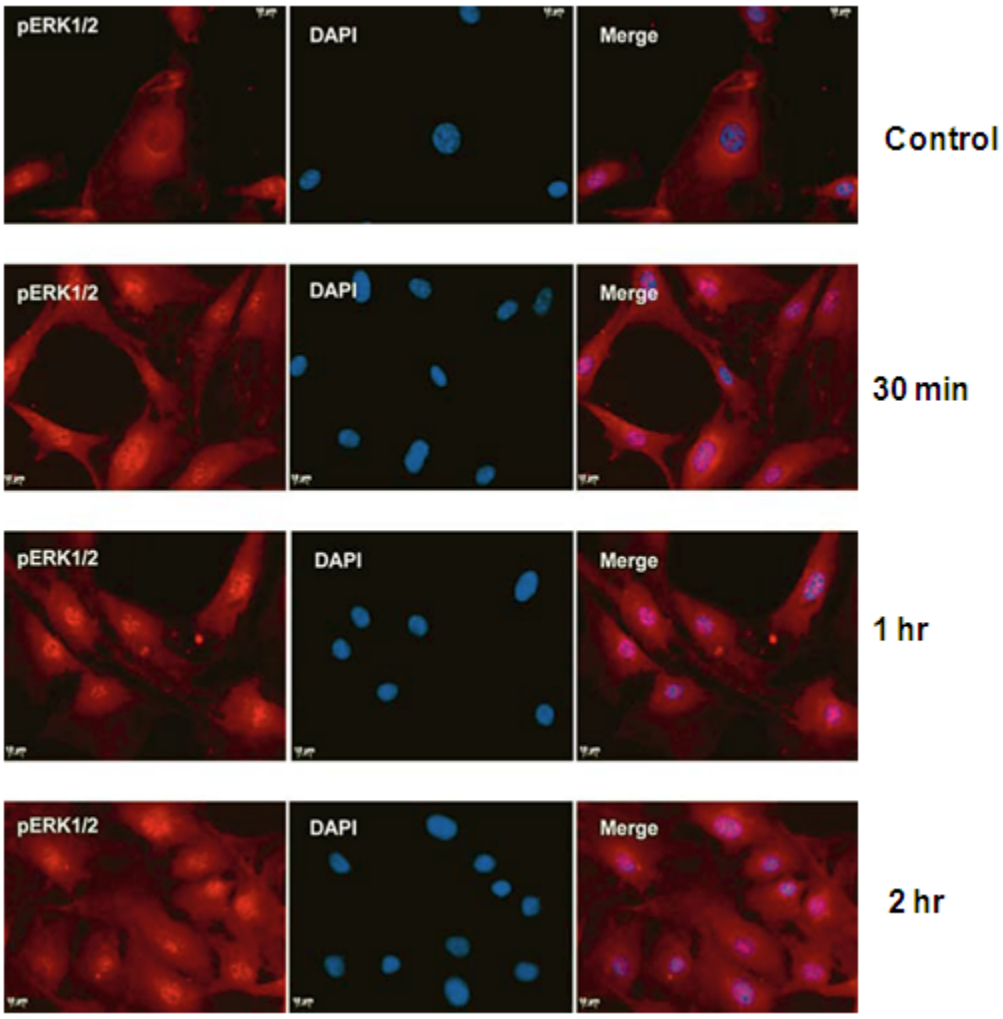


Figure 17:

DMP1 stimulation leads to the accumulation of phospho-ERK1/2 in the nucleus of MC3T3-E1 cells.

MC3T3 cells were incubated with anti-phospho-ERK antibody after stimulation with DMP1 for 30 min, 1 h, and 2 h followed by incubation with secondary antibody. Cells were subjected to confocal imaging. The confocal image shows the subcellular localization of phospho- ERK1/2. It is important to note that in the control unstimulated cells phospho-ERK1/2 was predominantly cytoplasmic while the intensity of the nuclear staining increased from 30 min to 2 h. Scale bars = 10 μm.

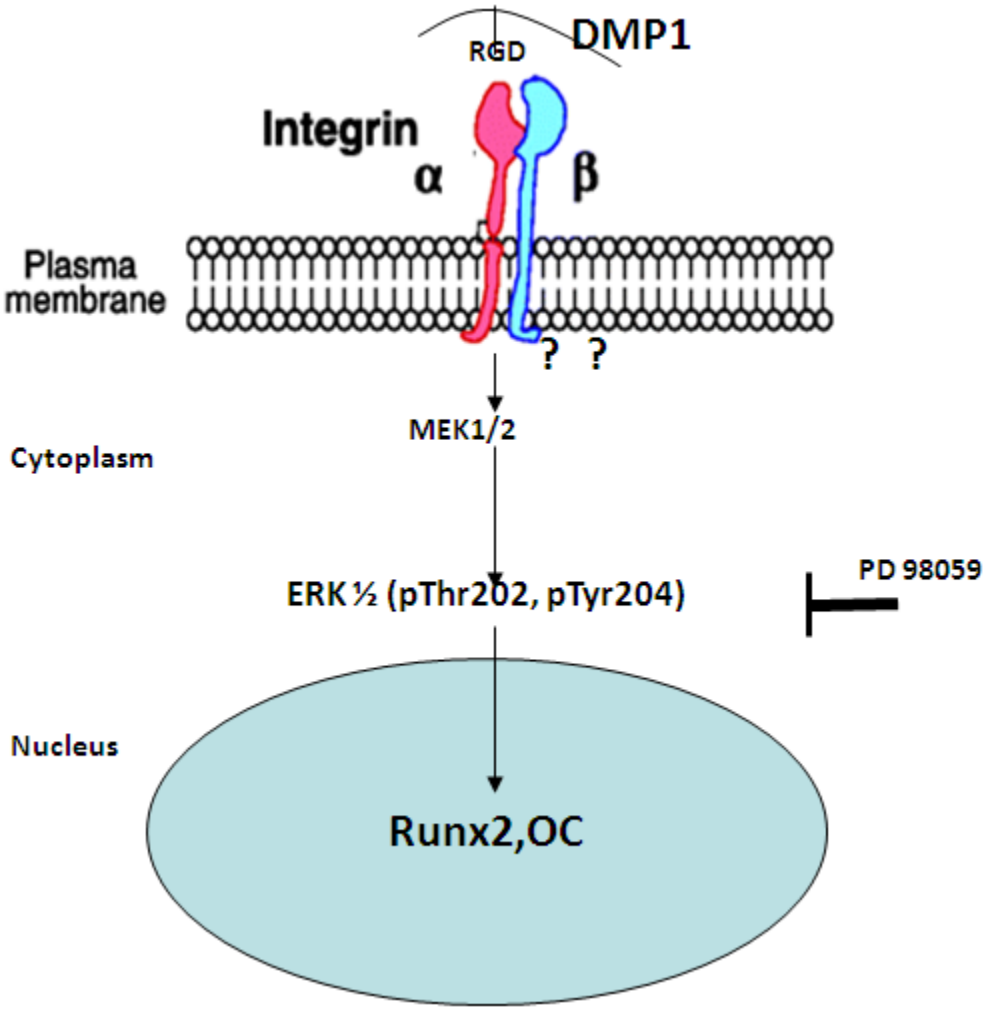


Figure 18:

Hypothetical Model depicting DMP1 stimulation of ERK1/2 activation and gene transcription.

RGD mediated endocytosis of DMP1 leads to the activation of the ERK1/2 MAP kinase pathway and subsequent downstream gene transcription.

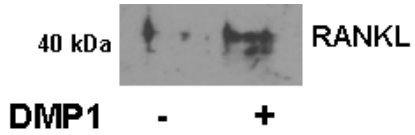


Figure 19:

Identification of RANKL in the secretome of osteoblast cells.

Serum free media from DMP1 stimulated MC3T3-E1 cells were collected, dialyzed and lyophilized. To identify the presence of RANKL in the secreted pool of proteins Western blot was performed using anti- RANKL antibody.

D. SIGNALING IN BREAST CANCER

1. Immunohistochemical staining of DMP1 in metastatic breast cancer patients

Results from the studies have shown the signaling pathways activated by DMP1 in bone. However, the expression and role of DMP1 in breast cancer mediated bone metastasis is yet to be investigated. For this purpose, the study first sought to investigate the expression of DMP1 in breast cancer in human breast cancer sections using anti-DMP1 polyclonal antibody. Interestingly, there was profound nuclear staining observed in the patient section (Figure. 20B) when compared to the control (Figure. 20A). This localization pattern suggests that DMP1 is highly expressed in metastatic breast cancer patients.

2. DMP1 expression in different stages of metastatic breast cancer patient

The progression of breast cancer has been broadly divided into 2 stages namely early and advanced. However, the conventional method of classification had 4 stages ranging from stage 0 to stage IV. Stage 0 is described as the non invasive breast cancer such as the ductal carcinoma *in situ*. Stage I is the invasive breast cancer with tumor measuring up to 2 cm with no lymph nodes involved. Stage II has A and B subdivisions where the cancer cells are localized to the lymph nodes. For stage IIA the tumor size is 2 cm and Stage IIB the tumor size is more than 2 cm. Stage III has 3 subdivisions with cancer in the axillary lymph nodes (stage IIIA), chest wall or skin (stage IIIB) and collar bone (stage IIIC). Stage

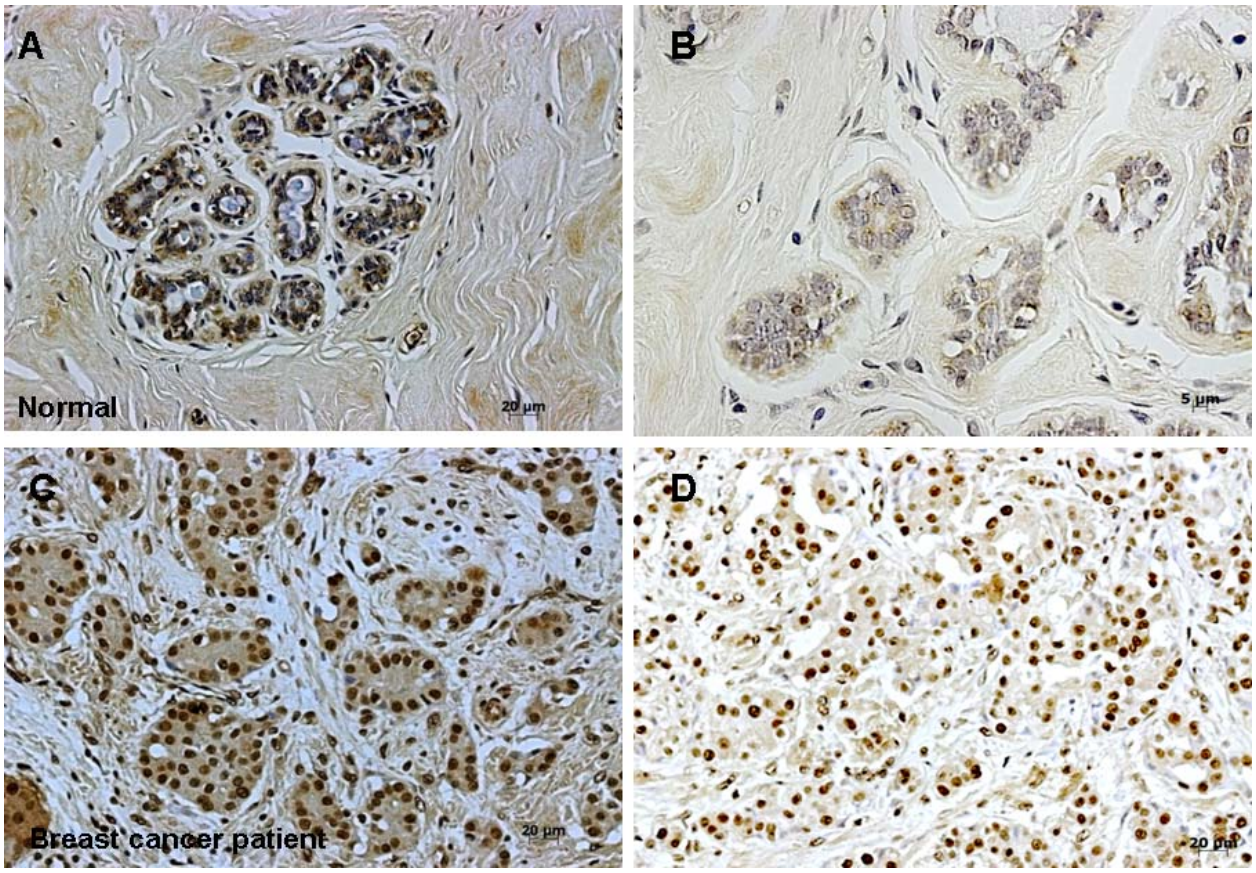


Figure 20:

Immunodetection of DMP1 in human breast cancer.

Paraffin-embedded tissue sections were immunostained with anti- DMP1 antibody and counterstained with hematoxylin. Section shows low intensity DMP1 immunostaining in the stroma and epithelial cells of normal patient **(A). (B)** represents the magnified image of figure A. High intensity of DMP1 immunostaining and nuclear localization of DMP1 was seen in epithelial cells of breast cancer patient with invasive ductal carcinoma **(B). (D)** represents the magnified image of figure B. Scale bar is 20 µm.

IV described invasive breast cancer that has spread lungs, skin, bones, liver or brain. To examine the stage at which DMP1 is expressed in breast cancer patients' immunohistochemistry with DMP1 antibody on human breast cancer tissue array was performed. There was not much of DMP1 staining noted in Stage I (Figure. 21a) when compared to the Stage II (Figure. 21b). High intensity of DMP1 staining was seen in Stage III with 60% of tumor in *situ* (Figure. 21c).

3. Expression pattern of DMP1 in different cell lines

The study next examined the expression pattern of DMP1 in MCF10A (Normal mammary epithelial cells), MDA MB231 (Metastatic invasive cells) and MCF-7 (Non invasive cells). Total proteins were extracted from MCF10A, MDA MB231 and MCF-7. Western blot was performed and DMP1 expression was examined in these breast cancer cell lines. Results from immunoblot confirmed that MDA MB231 expressed higher levels of DMP1 (Figure. 22 A2) than MCF10A (Figure. 22 A1) or MCF-7 (Figure. 22 A3). Interestingly, processing of DMP1 into its N terminal and C terminal fragment was observed in the metastatic breast cancer cells. The isolation of nuclear and cytoplasmic proteins from MDA MB231 clearly shows the localization of the N terminal fragment to be in the cytoplasm while the C terminal fragment is nuclear localized (Figure. 22B).

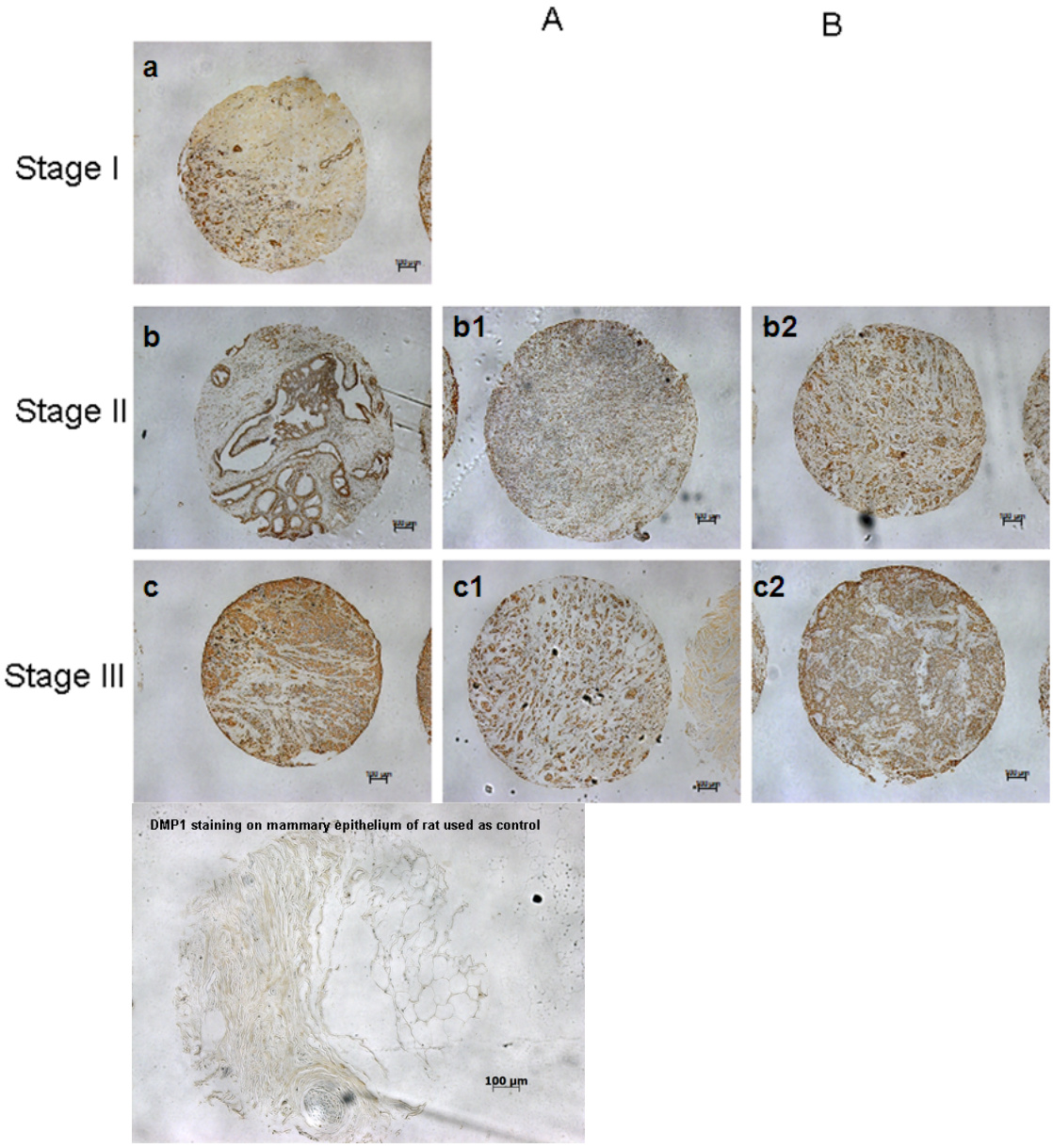


Figure 21:

Detection of DMP1 in different stages of breast cancer.

Immunostaining for DMP1 was performed on paraffin embedded breast cancer tissue array sections. Varying intensity of DMP1 stain was observed at Stage I with tumor in situ and lymphocytic **(a),** Stage II with tumor in situ and apocrine metaplasia **(b,b1,b2)** and Stage III with tumor in situ and tumor invasive **(c,c1,c2). A** and **B** represents the subdivisions to each stage. The scale bar represents 50 microns.DMP1 staining performed on mammary epithelium of rat was used as control. The scale bar represents 100 microns. [Note: There was no control core present in this tissue array. Hence the normal section from Figure 20(A) is used as the control].

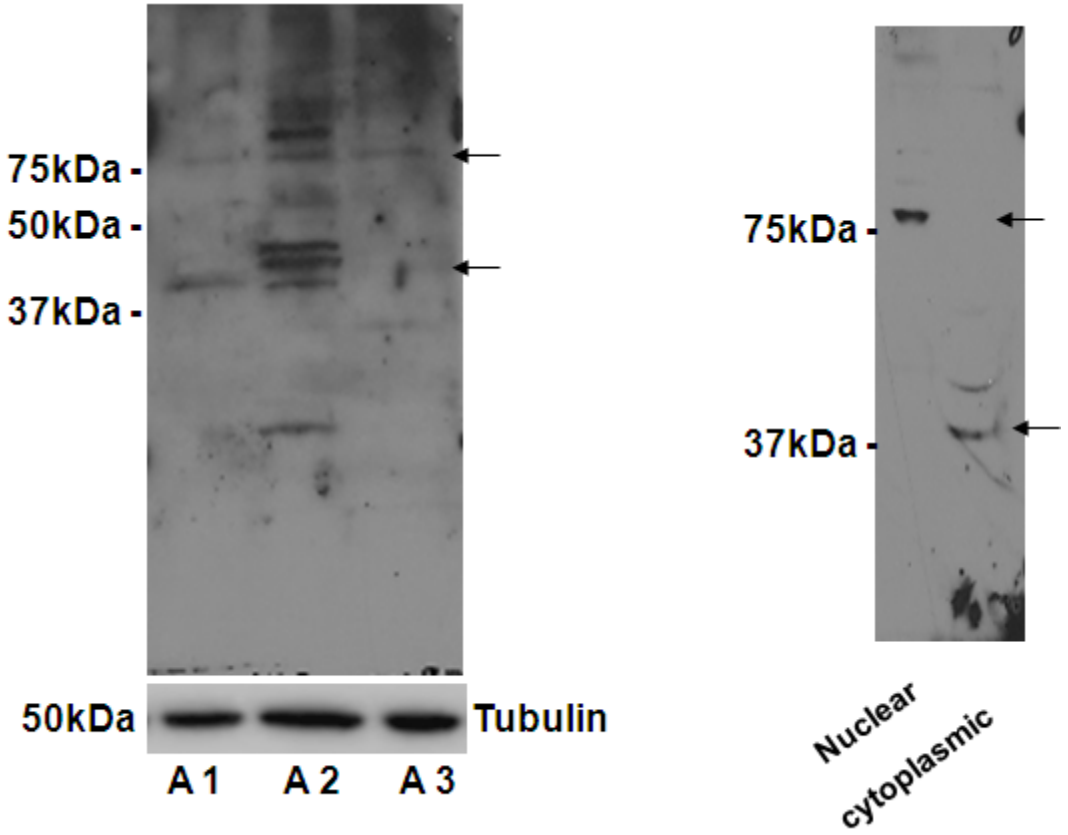


Figure 22:

Expression pattern of DMP1 in normal mammary epithelial cells, metastatic breast cancer cells and invasive breast cancer cell lines.

Total proteins were isolated from normal mammary epithelial cells (MCF10A) **(A1),** metastatic breast cancer cells (MDA MB231) **(A2)** and invasive breast cancer cell lines (MCF7) **(A3)**. Proteins were isolated from the nuclear and cytoplasmic compartment of MDA MB231 cells **(B)**. 35 µg of the proteins were resolved on a 10% SDS PAGE gel and immunoblotted with DMP1 antibody.

4. Overexpression and silencing of DMP1 in metastatic breast cancer cell line

To confirm the role of DMP1 in the progression of breast cancer, MDA MB231 cells were transfected with pcDNA 3.1 DMP1 plasmid. Stable transfection was performed with Fugene HD. After selection with G418, RNA and total proteins were isolated. Real time PCR was performed using rat DMP1 primers. Figure 23A shows high gene expression of DMP1 in the overexpressed cells (OE). It has to be noted that there was no gene expression for DMP1 on cells transfected with empty vector (mock cells) when rat DMP1 primes were used. The overexpression of DMP1 in cell extracts was detected by Western blotting with affinity-purified DMP1 antibody (Figure. 23B). Total proteins were extracted from mock and OE cells and immunoblotted against anti-DMP1 antibody. DMP1 expression level was high in OE cells when compared to mock cells. This would ascertain that the cells were transfected with the DMP1 construct. Proteins from mock cells served as the control Immunofluorescence was performed with anti-DMP1 antibody on OE cells to confirm the overexpression of DMP1 (Figure. 23C). Nuclear localization of DMP1 was observed in OE cells.

To gain insight into the role of DMP1 in the activation of osteolytic process, knocking out DMP1 in OE and mock cells was performed. OE and mock cells were transfected with lentiviral containing shRNA for DMP1 (shRNA-DMP1) using Fugene HD. Stable cell lines were made after selection with G418. shRNA – DMP1 used in the study targeted human DMP1 and rat DMP1. Hence real time PCR was performed with human and rat DMP1 primers. Results in figure 23A shows

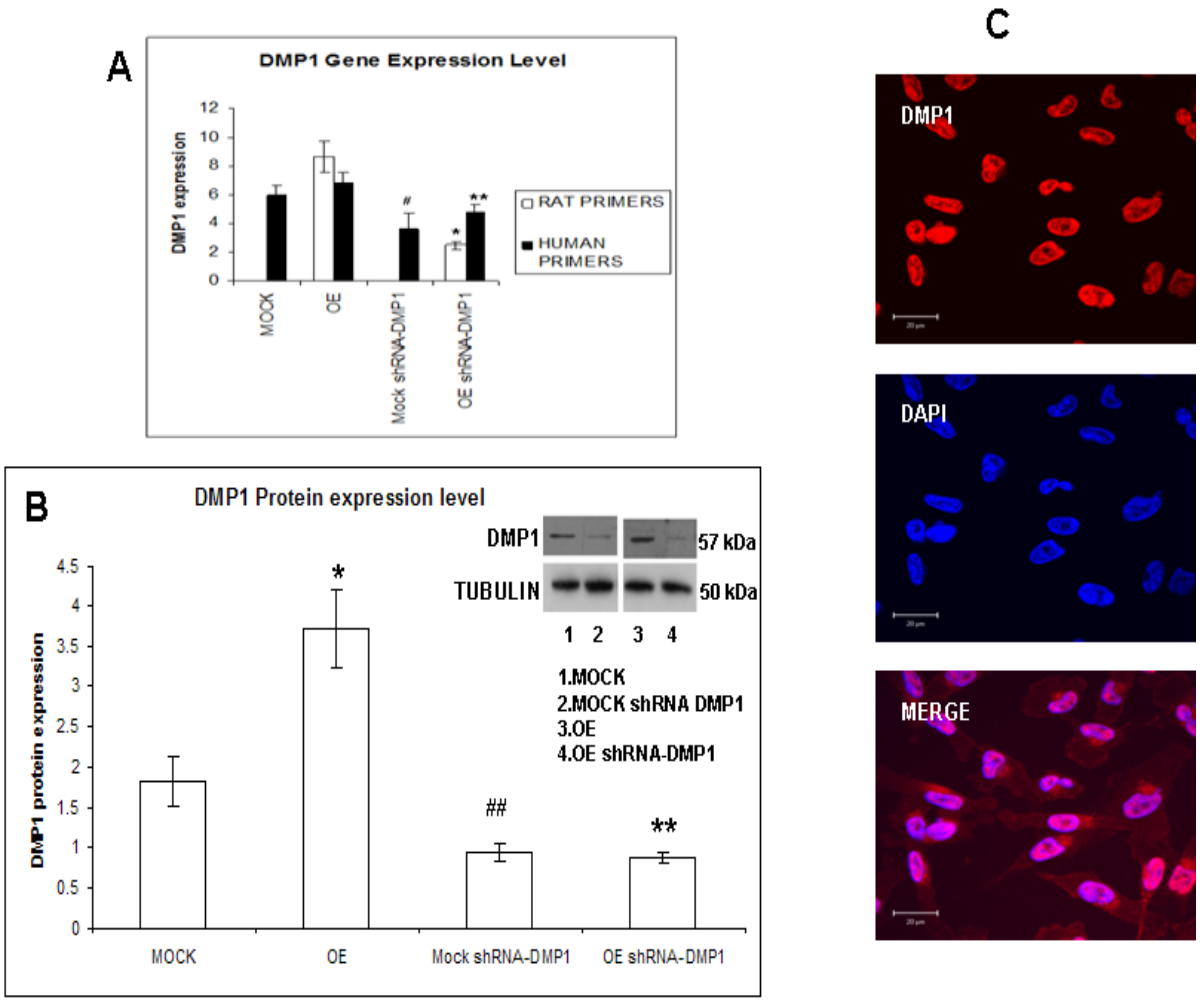


Figure 23:

Demonstration of the overexpression and knockout of DMP1 in metastatic breast cancer cell lines.

Metastatic breast cancer cell line MDA MB231 cells were transfected with mock plasmid (Mock) (pcDNA3.1) or pcDNA3.1 DMP1 plasmid (OE) or shRNA-DMP1 lentiviral particle. Stable selection was made with G418 sulfate. The expression level of DMP1 was analyzed by either real time PCR with rat and human primers **(A)** (#P< 0.05 compared to mock. *P< 0.003 and **P < 0.01 compared to corresponding OE control) or Western blot **(B)** (*P< 0.01 compared to mock. **P< 0.003 compared to OE and ##P < 0.02 compared to mock) or immunofluoresence with DMP1 antibody on OE cells **(C).** “Mock” represents a stable cell line expressing the “empty” vector.

that shRNA was able to knock down rat and human DMP1 gene expression in OE cells.Total proteins were isolated from the shRNA-DMP1 cells and were immunoblotted with DMP1 to confirm knockout of the gene (Figure. 23B).

5. Secretion of DMP1 into extracellular environment

The study next explored the hypothesis that secretion of DMP1 by OE cells into the extracellular environment could have an effect on osteogenic signaling. Mock and OE cells were grown in serum free media for 2 days. The media was then collected, dialyzed and lyophilized. Western blot analysis was performed using anti- DMP1 antibody. Analysis of serum free media from metastatic cell line revealed the secretion of DMP1 into the extracellular environment (Figure. 24). This further confirmed the hypothesis that the secreted DMP1 might have an effect on osteogenic signaling which in turn could play a pivotal role in activating the metastatic pathway.

6. DMP1 influences invasion and not migration

Breast cancer cell migration and invasion are the two critical steps in bone metastasis. Invasion assay was performed using matrigel invasion assay with mock cells and OE cells. Our results demonstrate that OE cells were highly invasive in nature *in vitro* when compared to mock cells. Further experiments were performed to study the consequences of DMP1 depletion in MDA MB231 cells. For this purpose invasion assay using shRNA-DMP cells was performed. Interestingly, results from

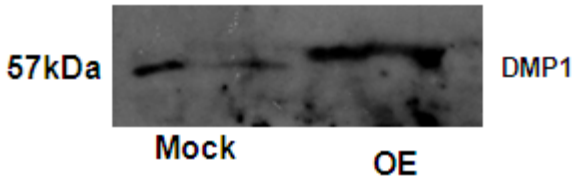


Figure 24:

Identification of DMP1 in the secretome of metastatic breast cancer cells.

Serum free media from Mock and OE cells were collected, dialyzed and lyophilized. To identify the presence of DMP1 in the secreted pool of proteins. Western blot was performed using anti- DMP1 antibody.

figure 25A shows that knocking down of DMP1 abrogates invasion of the metastatic breast cancer cells.

Wound migration assay was performed to study the migratory capacities of the OE cells and mock cells. MDA MB231 cells (mock) and overexpressing cells (OE) were grown to 100% confluency and a uniform wound was created. Phase-contrast microscopic pictures were taken at 0h, 8h and 24h time points respectively. Results from the migration assay clearly demonstrate that, as time progressed the distance between the wound edges created in the mock cells were much less than the overexpressing cells (Figure. 25B). Interestingly, at 24 hr time point there was no wound observed in the mock cells while the wound edges were still evident in the OE cells. Taken together, these results support the fact that DMP1 influences invasion and abrogates migration *in vitro*.

7. Role of DMP1 in proliferation of metastatic breast cancer cells

Having confirmed that DMP1 mediates differentiation and invasion of metastatic breast cancer cells experiments were performed to demonstrate that DMP1 participates in cellular proliferation. Cell proliferation assay using mock and OE cells were performed. Results from the assay in figure 26 demonstrate that DMP1 has significant effect on cellular proliferation of OE cells from day 1 to day 4 respectively. Overall, the data further confirms that DMP1 plays a pivotal role in differentiation, invasion and proliferation.

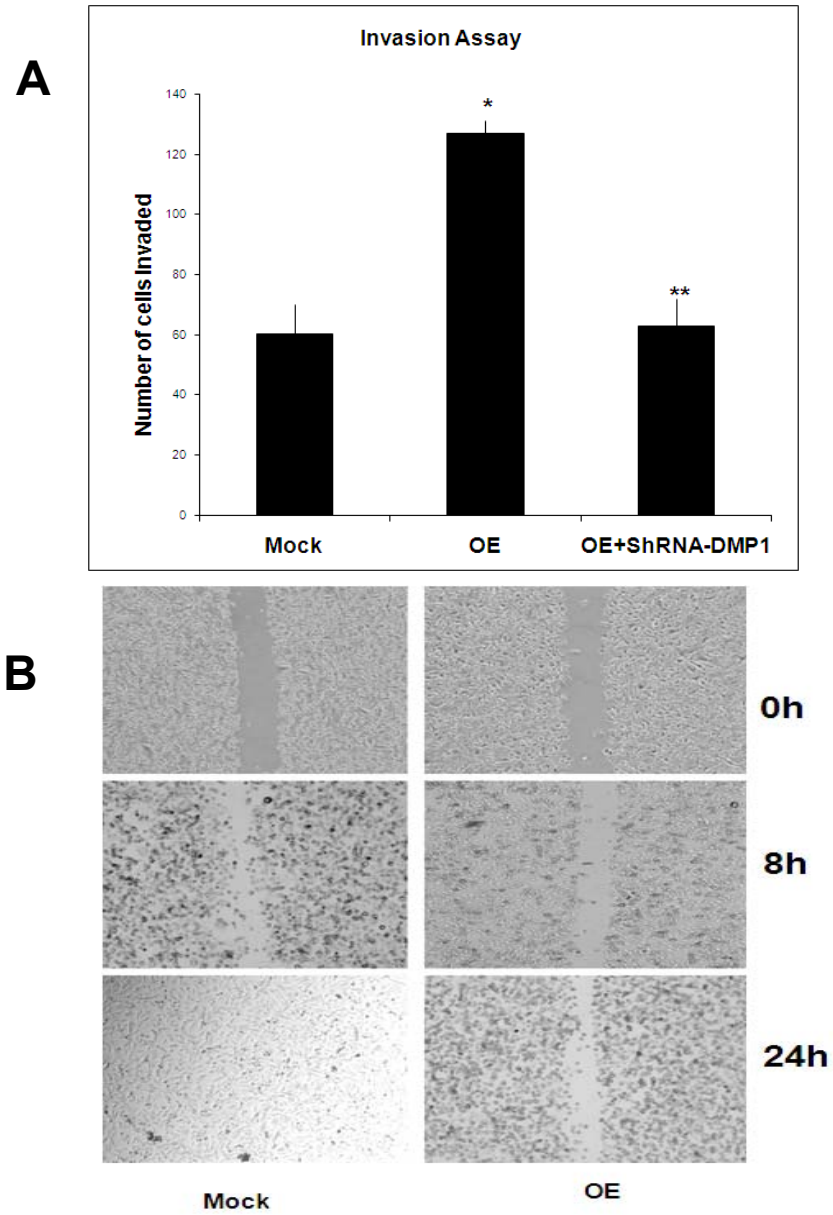


Figure 25:

DMP1 influences invasion and not migration of metastatic breast cancer cells. MDA MB231 cells containing the empty vector or DMP1 were plated on Matrigel and invasion assay was performed. Cells were allowed to migrate and the migrated cells were stained with Hema stain. 5 random fields of the stained cells were counted. MDA MB231 overexpressing cells infected with shRNA-DMP1 were also used to perform the invasion assay **(A)**. *P< 0.0001 compared to mock. **P < 0.003 compared to OE. n=9

Mock cells or OE cell layers were wounded with a sterile pipette tip. The wounded cells were then re-incubated for 8hrs and 24hrs at 37 ^{0}C as described in *Material and Methods*. The wound size remained the same from the start of the experiment to the end point in OE cells while the wound size decreased in the mock cells. Photographs were taken at 0hr with a phase-contrast microscope. Experiments were done in triplicate **(B).**

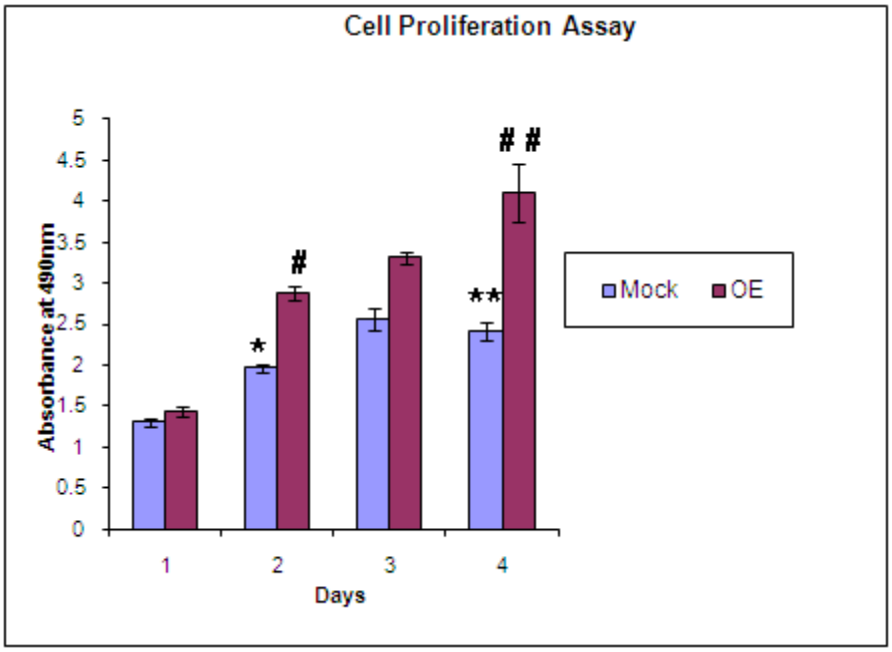


Figure 26:

Effect of DMP1 on proliferation.

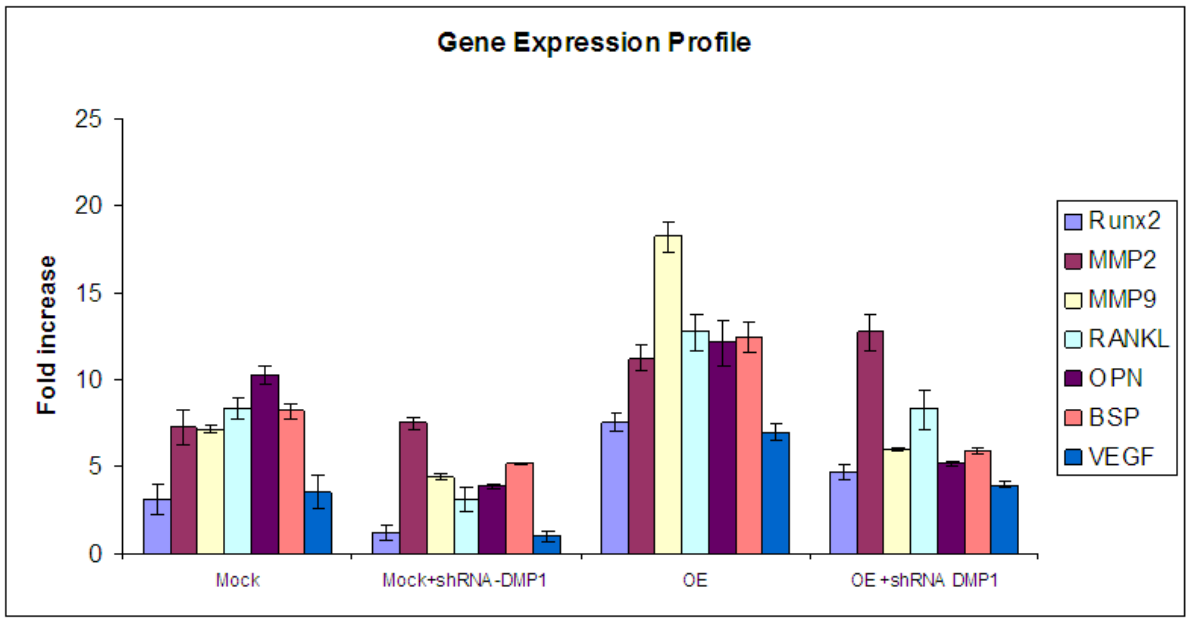
MDA MB231 mock cells and OE cells were plated in monolayer. Cell proliferation assay was performed from day 1 to day 4 as described in materials and methods. Absorbance was read at 490nm. There was significant difference in proliferation between the OE and mock cells. OE cells were highly proliferatory in nature. *P< 0.001 compared to mock day 1, **P < 0.0002 compared mock day 1, #P< 0.05 compared to OE day 1 and ##P< 0.001 compared to OE day 1. n=6

8. Gene expression profile in metastatic breast cancer cell line

To gain insight into the DMP1 mediated gene expression in breast cancer cells, RNA was isolated from mock and OE cells. Quantitative real-time RT-PCR on RNA samples showed that DMP1 overexpression caused a significant increase in an early marker of differentiation -Runx2, Matrix metalloproteases- MMP2 and MMP9, ligand for RANK-RANKL, SIBILING proteins like –osteopontin (OPN), bone sialoprotein (BSP) and angiogenic growth factor-vascular epithelial growth factor (VEGF) mRNA expression respectively (Figure. 27). Next, the study explored the specificity of DMP1 mediated increase in gene expression was investigated. To confirm this, real time RT-PCR on the shRNA-DMP1 cells was performed. Interestingly, Runx2, MMP9, RANKL, OPN, BSP and VEGF genes were down regulated in cells transfected with shRNA-DMP1. However, there was no suppression of MMP2 mRNA expression. Together, the data suggest that the MMP2 might be an indirect target of DMP1.

9. Signaling pathways activated by DMP1

To gain further insight into the DMP1 mediated activation of signaling pathways, total proteins were extracted from mock and OE cells and immunobloted against the phosphorylated form of p38, ERK1/2 and Smad2/3. Activation of the MAP kinase and Smad2/3 pathways were observed in OE cells. The specificity of the DMP1 mediated pathway activation was confirmed by knocking down DMP1 with lentiviral shRNA-DMP1.Total proteins were extracted from OE, mock and shRNA-DMP1 transfected cells at 24 hrs and immunoblot analysis was performed with



Runx2	P< 0.005
MMP2	P< 0.0001
MMP9	P< 0.03
RANKL	P< 0.05
OPN	P< 0.05
BSP	P< 0.01
VEGF	P< 0.002

Figure 27:

Overexpression of DMP1 and gene expression analysis

Total RNA was isolated from G418-resistant cell line overexpressing DMP1 (OE), Mock, shRNA-DMP1 OE and shRNA-DMP1 mock cells. 1µg of RNA was transcripted to cDNA and real time PCR was performed with SYBR labeled primers for Runx2, MMP2, MMP9, RANKL, OPN, BSP and VEGF. Glyceraldehyde-3-phosphate dehydrogenase was used as the house keeping gene. Table indicated the p values of OE + shRNA DMP1 when compared to OE. n=9.

phospho-p38, phospho-Smad2/3 and phospho-ERK1/2 antibodies. Results from Western blots demonstrate an inhibition of the MAP Kinase pathway in the shRNA-DMP1 cell lines (Figure. 28). Interestingly, no abrogation of the Smad2/3 pathway observed (Figure. 28). Overall, these results demonstrate the role of DMP1 in the activation of MAP kinase pathway. However the study concludes that the activation of Smad2/3 pathway is indirectly mediated by DMP1.

10. TGF-β influences gene expression in over expressed metastatic cell lines

During bone metastasis, breast cancer cells secrete factors that stimulate osteoclast-mediated bone resorption, which releases active factors from the bone matrix, particularly TGF-β. This secreted TGF-β has shown to have an effect on the breast cancer cells thus stimulating them to be highly metastatic in nature. In order to mimic the effect of secreted TGF-β on breast cancer cells, OE and mock cells were stimulated with TGF-β and gene expression analysis was performed. Up regulation in Runx2, MMP9, RANKL, OPN, BSP and VEGF mRNA expression was observed respectively (Figure. 29). Interestingly, these genes were down regulated in TGF-β stimulated cells transfected with shRNA-DMP.

11. TGF-β stimulates DMP1 mediated activation of MAP Kinase pathway

Studies have proved that TGF-β is a potent activator of most signaling pathways that could lead to differentiation, survival and proliferation. The study next determined if TGF-β mediated differentiation pathways are activated in the presence of DMP1. Western blot results presented in figure 30 demonstrate TGF-β mediated

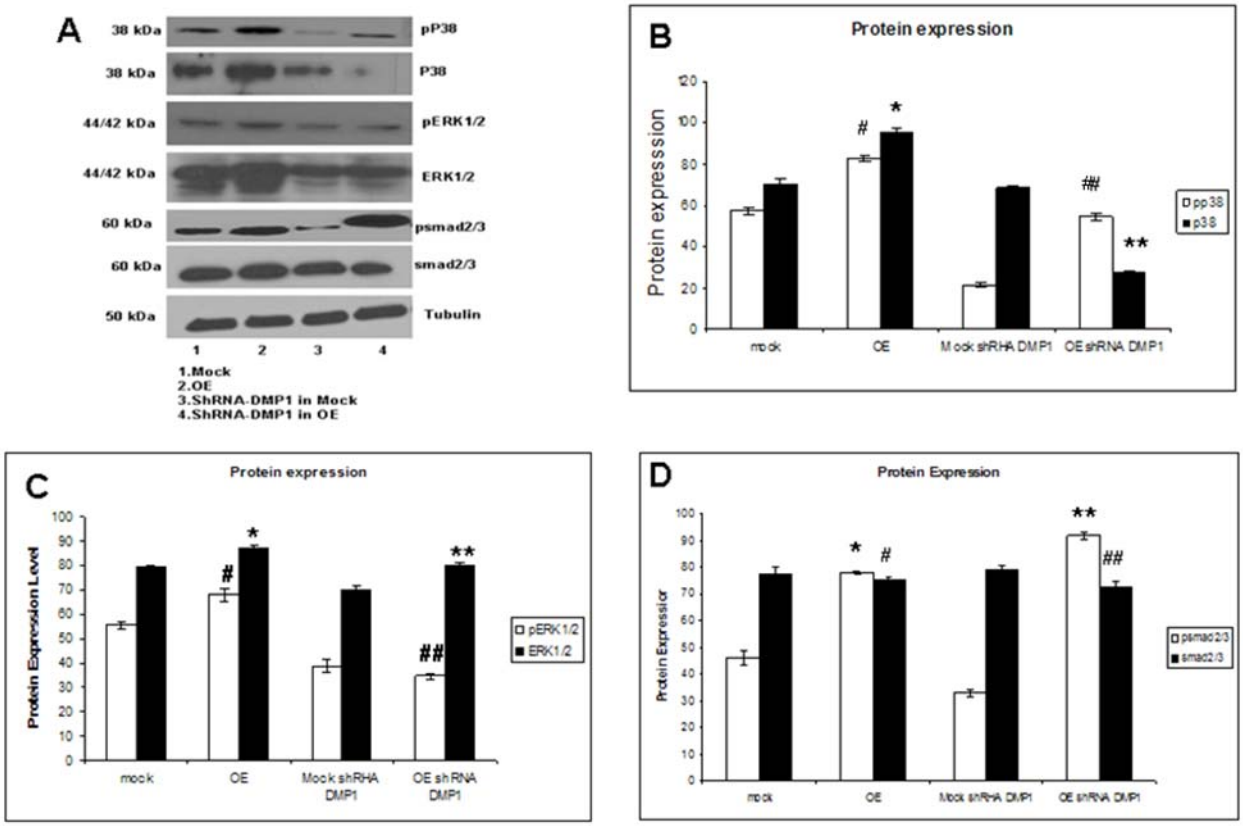
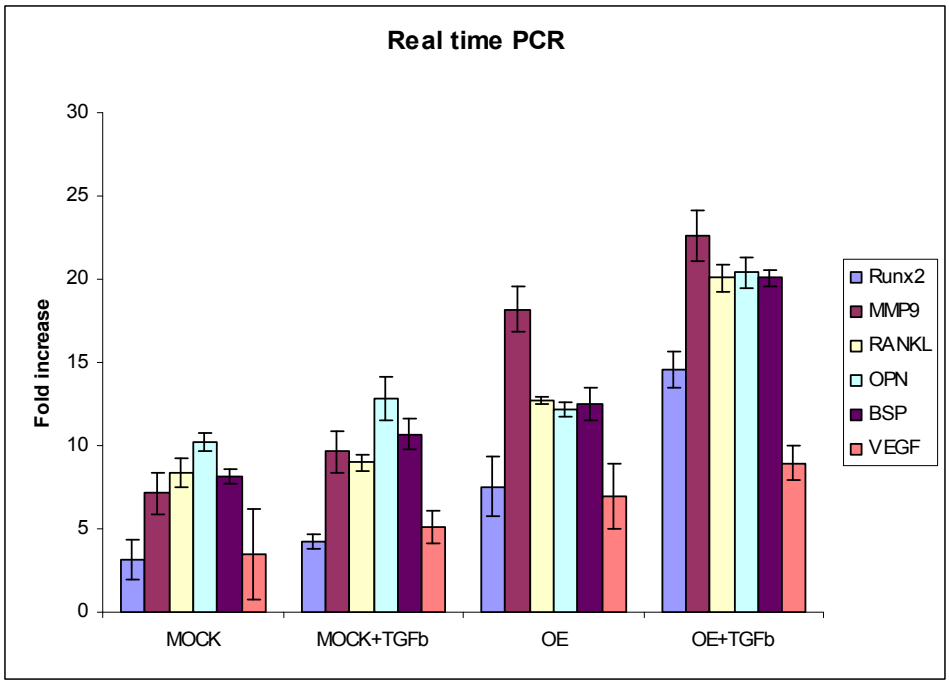


Figure 28:
Signaling pathways activated by DMP1
MDA MB231 cells were transfected with pcDNA 3.1 empty vector (Mock) or DMP1 construct (OE). Cell lines overexpressing DMP1 and mock cells were infected with lentiviral shRNA-DMP1 as described in *Materials and Methods.* Total proteins were isolated after 24hours and Western blot was performed with phospho- p38, phospho-ERK1/2 and phospho-smad2/3 **(A).** The graphs **(B), (C) and (D)** represent the quantification of the blots in (A)**. (B)** #P< 0.03 compared to pp38 of mock,*P < 0.001 compared to p38 of mock, ##P< 0.03 compared to pp38 of OE and **P< 0.05 compared to p38 of OE. **(C)** #P< 0.05 compared to pERK1/2 of mock,*P < 0.02 compared to ERK1/2 of mock, ##P< 0.004 compared to pERK1/2 of OE and **P< 0.05 compared to ERK1/2 of OE. (**D)** *P< 0.02 compared to psmad2/3 of mock, #P < 0.05 compared to smad2/3 of mock, **P< 0.05 compared to psmad2/3 of OE and ##P< 0.002 compared to smad2/3 of OE.



Runx2	P< 0.05
MMP9	P< 0.002
RANKL	P< 0.001
OPN	P< 0.005
BSP	P< 0.05
VEGF	P< 0.05

Figure 29:

TGF-β influences gene expression in over expressed metastatic cell lines

Total RNA was isolated from TGF-β stimulated OE, Mock, shRNA-DMP1 OE and shRNA-DMP1 mock cells. 1µg of RNA was transcripted to cDNA and real time PCR was performed with SYBR labeled primers for Runx2, MMP2, MMP9, RANKL, OPN, BSP and VEGF. Glyceraldehyde-3-phosphate dehydrogenase was used as the house keeping gene. Table indicated the p values of OE + TGF- β when compared to OE. n=9.

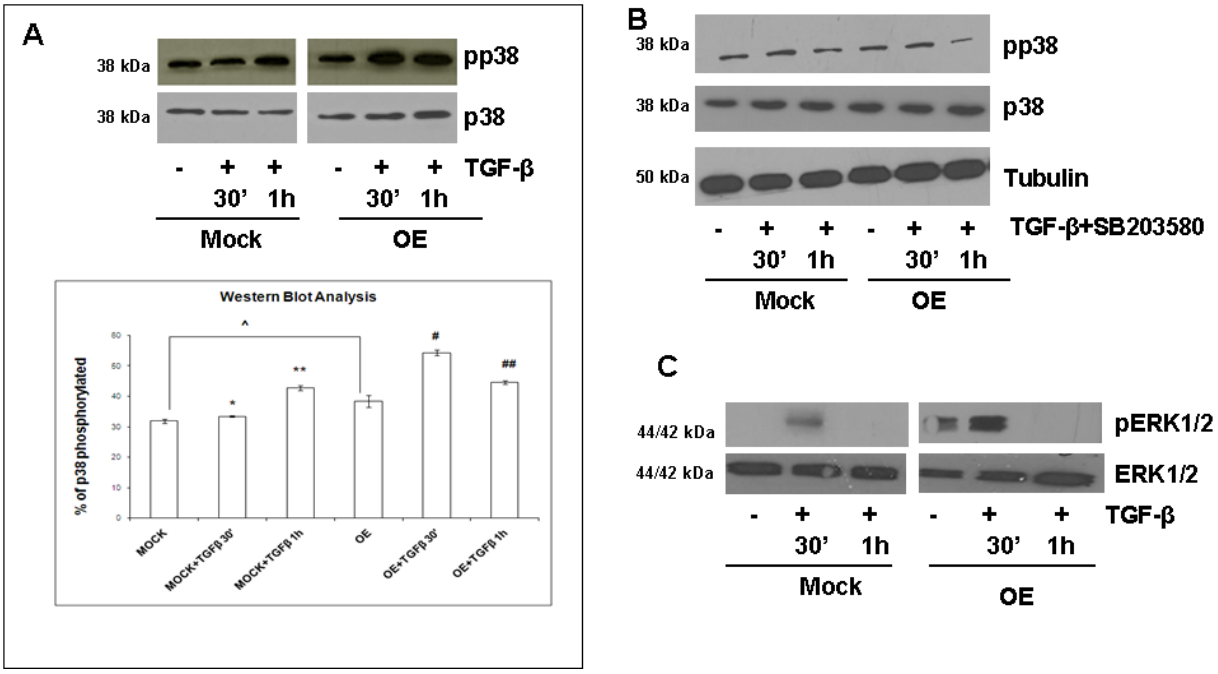


Figure 30:

DMP1 mediated p38 MAP kinase signaling pathway activated in metastatic breast cancer cell lines

MDA MB231 cells were transfected with pcDNA 3.1 empty vector (Mock) or DMP1 construct (OE). Mock or OE cells were stimulated with or without TGF-β for 30 minutes and 1 hour. Total proteins were isolated and immunoblotted with anti-p38 antibody and phospho-p38 antibody **(A).** ^P< 0.05, *P<0.01 and **P<0.05 compared to mock, #P < 0.02 and ##P< 0.05 compared to OE.

Abrogation of p38 MAP kinase activation by SB203580

Total proteins were isolated from OE and Mock cells that were triggered with or without TGF-β, in the presence or absence of SB20358 a pharmacological inhibitor for the p38 MAP kinase pathway. Western blot assay was performed with phospho-p38. Equal loading of proteins were confirmed by stripping the blot and reprobing it with tubulin antibody **(B).**

DMP1 mediated upregulation of ERK1/2 MAP kinase pathway.

To confirm the role of ERK1/2 MAP kinase pathway in OE and mock cells, total proteins were obtained from cells that were stimulated with or without TGF-β. Western blot assay was performed with ERK1/2 and phospho-ERK1/2 antibody **(C).**

activation of the MAP kinase pathway (p38 and ERK1/2) in OE cells when compared to the mock cells. The specificity of the p38 MAP kinase pathway was further confirmed in the presence of SB20358, a pharmacological inhibitor for p38 MAP kinase pathway. Total proteins were isolated from OE and mock cells at 30 minutes and 1h. Results from Western blot analysis depict a down regulation of p38 activation in OE and mock cells in the presence of SB20358 (Figure. 30B). Taken together, these results suggest the activation of p38 and ERK1/2 MAP kinase pathway in metastatic breast cancer cells.

E. OSTEOLYSIS OF THE BONE

1. DMP1 overexpressing breast cancer cells triggers osteoclast activation

To test the hypothesis that secreted DMP1 by human breast cancer cells can enhances osteoclast formation, a 1 day co-culture of mock cells or OE cells with MC3T3-E1 osteoblastic cells was performed (Figure. 31). The medium from this co-culture was then mixed with fresh media in a 50:50 ratio (conditioned media) and was used to differentiate RAW 264.7 (precursor monocytes) cells to osteoclasts. Media was changed every other day for 7 days. Tartrate-Resistant Acid Phosphatase staining (TRAP), an osteoclast marker to determine the activation of osteoclast was performed (Figure. 32). Fully differentiated osteoclast cells were seen when RAW 264.7 cells were grown in the conditioned media from OE cells (Figure. 32F). Total proteins were also extracted and western blot was performed

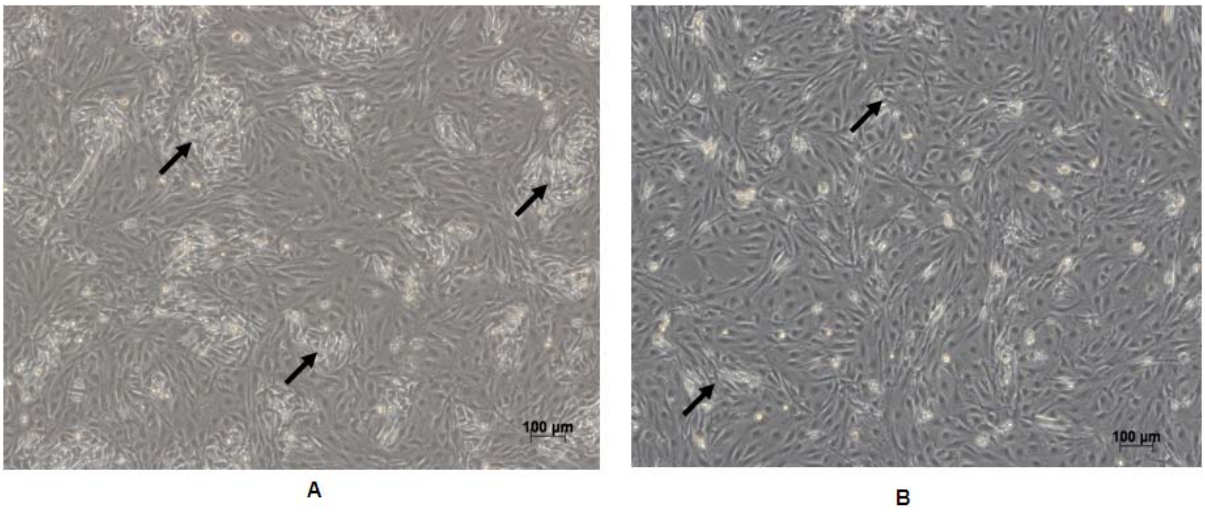


Figure 31:

Co-culture of metastatic breast cancer cell lines with osteoblast cells.

Phase contrast microscopic picture of the co-culture of MDA MB231 (mock) or DMP1 over expressed MDA MB231 cells (OE) with MC3T3-E1 osteoblastic cells. MC3T3-E1cells and metastatic breast cancer cells were mixed and were allowed to grow at 37^0C incubator for 24 hours. The solid arrows indicate the distribution pattern of the mock cells and OE cells in the presence of MC3T3-E1 cells. The mock cells formed small islets **(A)** while the OE cells were evenly distributed between the MC3T3-E1 cells **(B).**

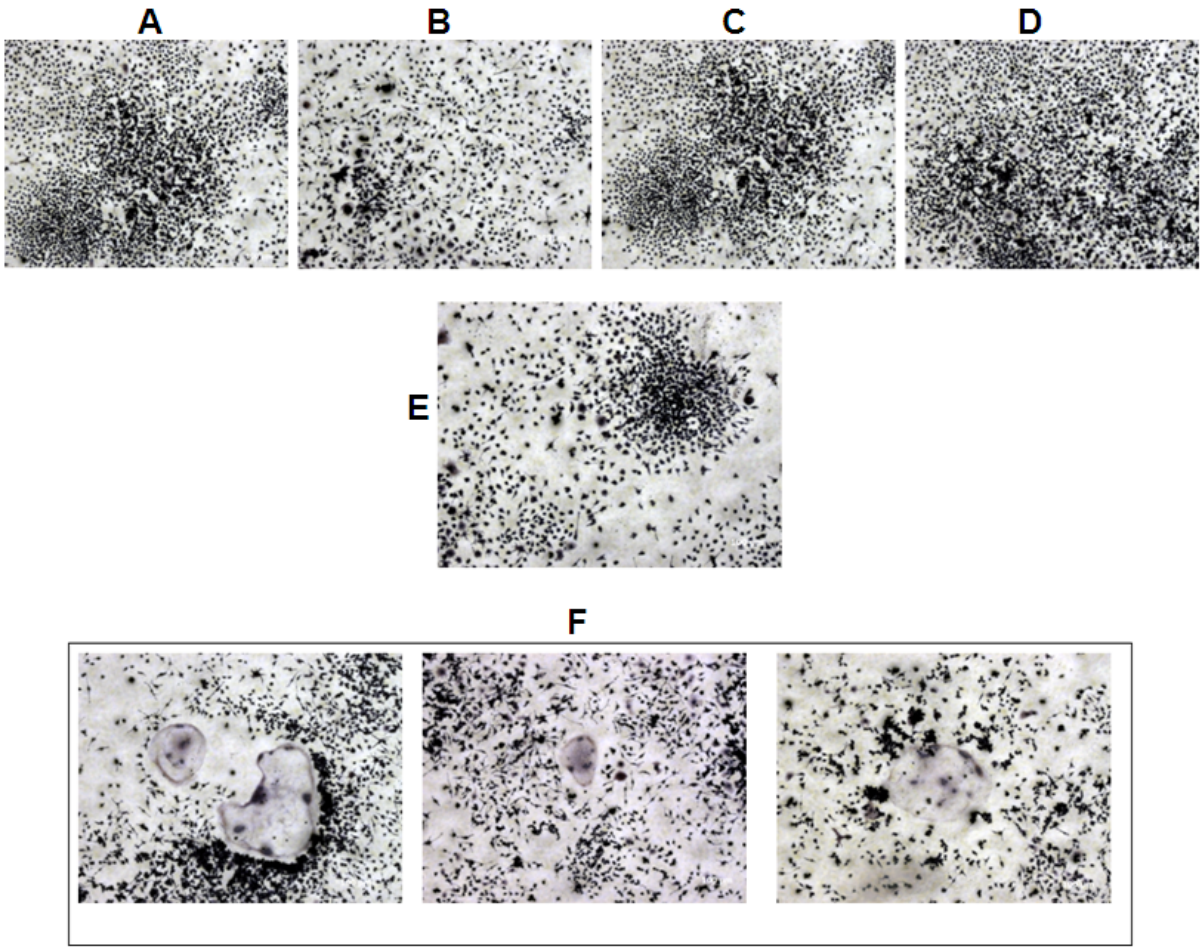


Figure 32:

DMP1 accelerates the formation of osteoclast in *vitro*

RAW 264.7 cells were grown in conditioned medium from co-culture of MC3T3-E1 with mocks cells or OE cells. Tartrate-Resistant Acid Phosphatase staining (TRAP) was performed as per the protocol. RAW 264.7 cells grown in DMEM media **(A)**, MC3T3-E1 cells in DMEM/F-12 media(B), Mock cells **(C)** or OE cells **(D)** in RPMI 1640 media and RAW 264.7 cells grown in conditioned media from mock cells**(E)** were used as controls. TRAP positive osteoclast cells were seen when RAW 264.7 cells were grown in the co-culture media from MC3T3-E1 cells and OE cells **(F).**

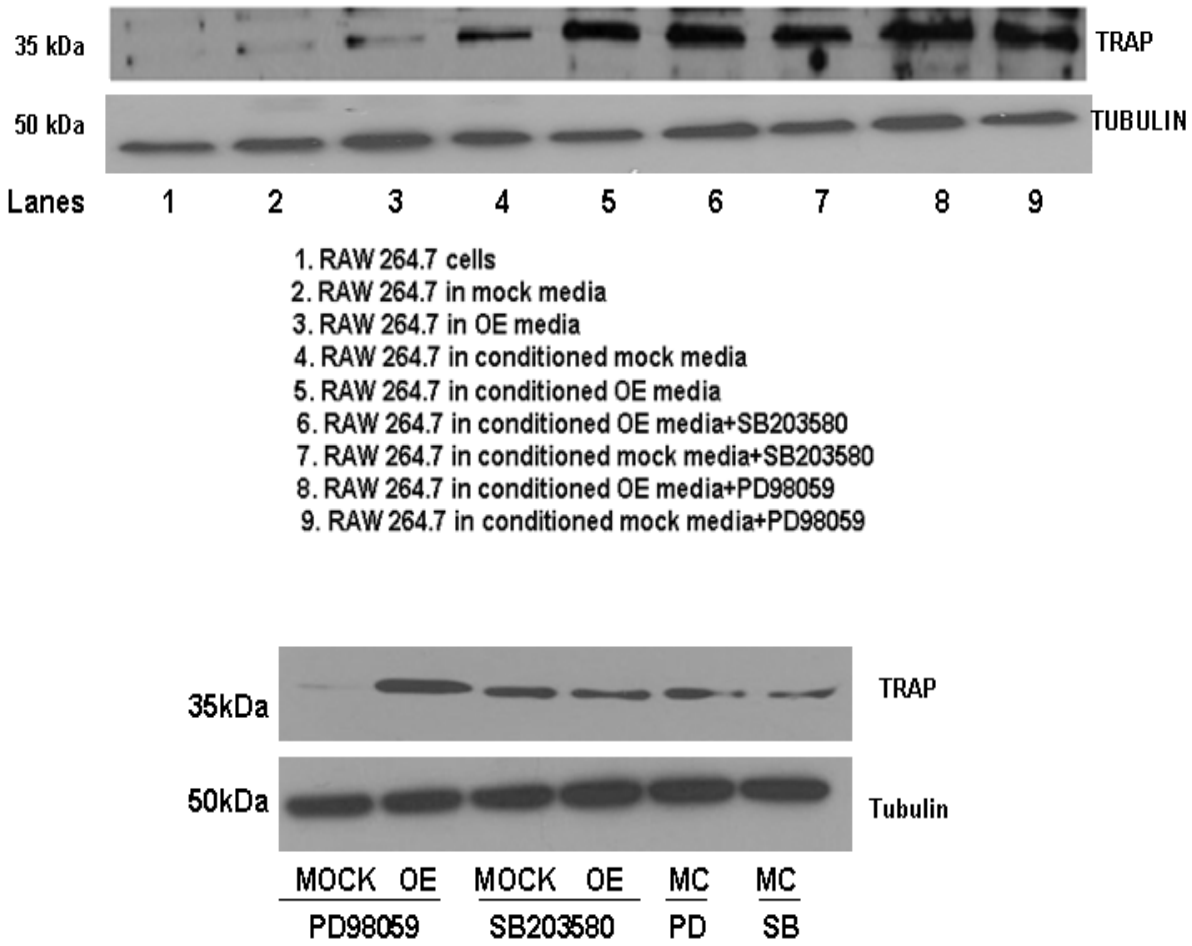


Figure 33:

DMP1 activated osteolytic cycle is mediated by the MAP kinase pathway.

Total proteins were also extracted from RAW 264.7 cells that were grown in conditioned medium of mock or OE cells and were treated with or without 10 µM of SB203580 (Biomol), an inhibitor specific for p38 MAPK or 15 µ*M* PD98059 (Biomol), an inhibitor specific for ERK1/2 MAP kinase. Western blot was performed with anti-TRAP antibody. RAW 264.7 cells grown in media obtained from mock cells, OE cells and MC3T3-E1 cells were used as controls. High expression of TRAP was observed in the RAW 264.7 cells that were grown in conditioned media of OE cells when compared to that of mock cells. Treatment of both the inhibitors along with the conditioned media OE enhanced the differentiation of osteoclast cells *in vitro.* However, treatment of RAW 264.7 cells with media from OE cells containing the PD98059 inhibitor stimulates the differentiation of precursor monocytes to osteoclast cells.

with anti-TRAP antibody (Figure. 33). High expression of TRAP was observed in the RAW 264.7 cells that were grown in conditioned media of OE cells when compared to RAW 264.7 cells grown in conditioned media of mock cells. Overall, these results support the fact that conditioned media of OE cells on RAW 264.7 cells can promote differentiation of RAW 264.7 cells to osteoclast.

2. DMP1 activated osteolytic cycle is mediated by the MAP kinase pathway

It has been shown that SB203580 (p38 pathway inhibitor) and PD98059 (ERK1/2 pathway inhibitor) can inhibit osteoblast differentiation. The studies hypothesize that this inhibition of osteoblast differentiation might trigger the osteoclast thus leading to the activation of vicious cycle. Thus the involvement of MAP kinase pathway in the activation of osteolytic cycle was studied by treating RAW 264.7 cells with SB203580 (p38 pathway inhibitor) or PD98059 (ERK1/2 pathway inhibitor) in the presence of conditioned media. Total proteins were extracted and immunoblotted with anti-TRAP antibody. High expression of TRAP staining was noted in the RAW 264.7 cells grown in the conditioned media of OE cells (Figure. 33). Interestingly, the treatment of either of the inhibitors along with the conditioned media of OE cells enhanced TRAP expression. The study further focused on investigating the role of osteoblast or breast cancer in mediating the differentiation process of osteoclast cells. RAW 264.7 cells were treated with media from OE or mock or MC3T3-E1 in the presence of SB203580 or PD98059 for 7 days. Total proteins were isolated from the stimulated RAW 264.7 cells and immunoblot analysis was performed with anti-TRAP antibody. The results from

Western blot analysis suggest that treatment of RAW 264.7 cells with media from OE cells containing the PD98059 inhibitor can stimulate the differentiation of precursor monocytes to osteoclast cells. On the other hand, differentiation of osteoclast could also be achieved through the activation of p38 pathway only in the presence of the conditioned media from OE and SB203580. Thus the role of the inhibitors in inhibiting osteoblast differentiation and accelerating the differentiation of osteoclast *in vitro* is evident.

3. Secretion of TGF-β by osteoclast into the extracellular environment

Experiments were next performed to test the hypothesis that the TGF-β secreted by the osteoclast stimulates DMP1 mediated differentiation of metastatic breast cancer cells. To study this hypothesis, differentiation of RAW 264.7 cells in the presence of conditioned media obtained from mock cells and OE cells was performed. The media was collected after 7 days of differentiation, dialyzed and lyophilized. 20µl of the media was resolved on a SDS PAGE gel and immunoblotted with TGF-β antibody. Large precursor protein of TGF-β was seen in the Western blot of RAW 264.7 cells that were differentiated in the presence of conditioned media from OE cells (Figure. 34). This further confirmed the hypothesis that this secreted form of TGF-β can activated the DMP1 mediated vicious cycle in metastatic breast cancer cells thus leading to the osteolysis of bone.

Figure 34:

Presence of TGF-β in the extracellular environment of osteoclast cells.

RAW 264.7 cells were grown in the presence of conditioned media obtained from mock cells and OE cells. The media was collected after 7 days of differentiation, dialyzed and lyophilized. 20µl of the media was resolved on a SDS PAGE gel and Western blot was performed with TGF-β antibody.

CHAPTER 6

DISCUSSION

This study shows the binding of DMP1 with GRP-78 is necessary for intracellular calcium release. It is known that calcium ions can act as a ubiquitous second messenger. Previous studies have shown that Ca^{2+} could serve as a signal for DMP1 export (Narayanan et al., 2003). Studies have shown that during the maturation of osteoblast, Ca^{2+} influx into the nucleus from the cytoplasm results in the phosphorylation of DMP1 by casein kinase II. This leads to the export of DMP1 into the extracellular environment (Narayanan et al., 2003). However, experiments with specific inhibitors helped to clearly study and understand the release of Ca^{2+} from internal stores such as the endoplasmic reticulum. Studies performed with an inositol triphosphate (IP3)R antagonist (2-aminoethoxydiphenyl borate; 2APB) showed that these elevated intracellular calcium transients are the result of calcium release from endoplasmic reticulum stores. As DMP1 contains an RGD domain, the possibility that integrins might facilitate the endocytosis of DMP1 and store-Ca^{2+}-release was explored. Blocking integrin receptors with an RGD peptide did not have any significant effect on calcium release and other downstream signaling events. Thus, this study demonstrates that DMP1 stimulation results in the release of calcium from intracellular stores and this could be perceived as a cellular stress. The role of DMP1 in the regulation of phosphate homeostasis has been documented (Gajjeraman et al., 2007). Knockout models of DMP1 have shown predominant

skeletal defects confirming the role for DMP1 in osteoblast differentiation and mineralization (Feng et al., 2006)

The release of calcium from the ER results in signals that are transduced into intracellular responses. These signaling mechanisms that are pathway specific can convey information across individual cells and between connected cells thus leading to differentiation of cells to a specific lineage. Osteoblasts handle large amounts of calcium during bone formation, and how the osteoblasts deposit mineral is a mystery. The pathways that connect the rise in $[Ca^{2+}]_i$ to osteoblast differentiation have not been elucidated yet. Furthermore, this study investigated whether DMP1-mediated ER Ca^{2+}depletion, resulting in an increase in the intracellular free calcium ion concentration, could activate the stress-induced MAPK, p38, and mediate osteoblast differentiation. Previous studies have shown the activation of MAP kinase pathway during the differentiation of DMP1 overexpressed cells into odontoblastic lineage (Narayanan et al., 2001). However, the activation of MAP kinase pathway has been shown to be the potential signaling pathway that promotes differentiation in neuronal cells (Burden, 2000). Activation of p38 MAPK has been reported to be necessary in the differentiation of a variety of cell types, including adipocytes, chondrocytes, osteoclasts, and neurons (Hu et al., 2003).

The stress-induced MAPK, p38, can be activated in preosteoblasts by DMP1 stimulation. This up-regulation was seen as early as 15 min suggesting the activation of p38 at early stages of osteoblast differentiation. The chemical inhibitor SB230580, known to inhibit the phosphorylated form of p38, confirmed the findings.

Published studies demonstrate the role of p38 isoforms in osteoblastic differentiation (Hu et al., 2003). Three different isoforms of p38 MAPK exist, namely p38α, p38β, and p38γ (Hu et al., 2003). The results from this study have been found to be in accordance with other groups that have shown that SB203580 can inhibit the phosphorylated form of p38α and p38β, although the dominant negative form of p38 inhibits p38α (Hu et al., 2003). Thus, DMP1 can specifically activate p38α isoform and facilitate osteoblast differentiation. Experiments results obtained from the treatment with BAPTA-AM resulted in the attenuation of p38 phosphorylation, confirming the role of Ca^{2+} in downstream signaling.

Propagation of p38-mediated phosphorylation and activation of several downstream protein kinases are necessary to elicit the cellular response. MAPKAPK2 is known to be a p38 MAPK downstream signaling protein (Stokoe et al., 1993). Interestingly, MAPKAPK2 is observed to be activated with DMP1 stimulation and inhibited in the presence of the inhibitor SB203580. Miguel *et al.* (Miguel et al., 2005) have demonstrated that the osteogenic growth peptide could activate MAPKAPK2 in osteoblasts by phosphorylation and the transcriptional activity of the cAMP-response element-binding protein. Indirect activation of MAPKAPK2 by vascular growth factor was inhibited by SB203580 in endothelial cells (Kobayashi et al., 2006). Activation of a multilayered protein kinase cascade downstream of p38 is known to control a broad range of cellular functions.

Hsp27, a small heat shock protein, functions in protein folding. However, Hsp27 is also known to intervene in the modulation of differentiation, which in turn is dependent on the phosphorylation state of Hsp27. Hsp27 has

previously been reported to be phosphorylated by MAPKAPK2 downstream of p38 MAPK in the stress response pathway (Stokoe et al., 1992). Results from this study demonstrate that DMP1 can up-regulate HSP27 protein expression in preosteoblasts. Activation of Hsp27 was therefore an indication of its function as a potential regulator of osteoblast differentiation. Published reports have shown a transient increase in the expression of Hsp27 during the transition of the cellular states between proliferation and differentiation in several *in vitro* cellular systems (Davidson & Morange, 2000; Walsh et al., 1999). This result corroborates well with published reports on Hsp27 expression during development of the craniofacial bones, suggesting that this protein could be involved in the balance between differentiation and apoptosis, by modulating the viability of osteoblasts and chondrocytes (Leonardi et al., 2004).

Thus, the role of DMP1 in the activation of p38 MAP kinase pathway leading to the differentiation of preosteoblast cells to fully differentiated osteoblast is clear. Mature and fully differentiated osteoblast secrete mineralized matrix. Mineralization of the bone matrix is a well orchestrated process by osteoblast during which calcium phosphate crystals are laid down on the fibrous collagenous matrix. Results from the study depict that DMP1 promotes the formation of mineralized nodule *in vitro*. Interestingly, the nodule formation was suppressed in the presence of SB203580. This study therefore concluded the role of p38 during the terminal differentiation process in MC3T3-E1 cells when stimulated with DMP1.

DMP1 mediated activation of signaling pathways is not only restricted to the p38 MAP kinase pathway. We have seen the ability of DMP1 to induce the

phosphorylation and activation of ERK1/2. ERK1/2, a member of the MAP kinase pathway represents an important signaling mechanism in the osteoblast maturation process. This study also identifies that the stimulation of preosteoblasts by DMP1 leads to the activation of ERK1/2. The ERK pathway is one of the best studied MAPK pathways in mammals (Dhillon et al., 2007; Gayer et al., 2010). ERK1/2 activation regulates proliferation, differentiation, survival, migration, angiogenesis, and even chromatin remodeling through the phosphorylation of both cytoplasmic and nuclear targets including phosphatases, transcriptional factors, and cytoskeletal proteins. In the canonical ERK1/2 pathway, receptor tyrosine kinases are activated by specific ligands and trigger guanosine triphosphate (GTP) loading of the Ras protein which can then recruit the Raf kinases (A-Raf, B-Raf, and cRaf) (Robertson et al., 2010). These kinases consecutively phosphorylate and activate MEK (MEK1 and MEK2), ultimately leading to the activation of ERK1/2. In addition to the canonical pathway, ERK1/2 has been shown to be activated by a variety of pathways depending on the individual ligand, cell surface receptor, and cell type. Although MAP kinases participate in different signaling pathways, they may be functionally related and coactivated in response to certain stimuli. Furthermore, this study demonstrates that the phosphorylation state of ERK1/2 is mediated through integrins as blocking the RGD domain with a specific blocking peptide abrogated ERK phosphorylation. Upstream regulators of ERK1/2 are Raf and MEK1/2. Results from this study showed activation of MEK1/2 in osteoblasts stimulated with DMP1 when compared with control cells, indicating that ERK activation mediated by DMP1 is mediated through the MEK/ERK signaling pathway. Report has

shown the regulation of bone specific genes is via the integrin mediated activation of MAP kinase pathway (Jadlowiec et al., 2004).

An interesting observation in this study is that stimulation of preosteoblasts with DMP1 induced Runx2 and osteocalcin expression by activating the p38 and ERK1/2 MAP kinase pathway. Runx2 is a transcription factor essential for osteoblast differentiation, maturation, and bone formation during embryonic development. Induction of Runx2 gene transcription is a requirement for osteoblast differentiation *in vivo*. Furthermore, Runx2 regulates the expression of several osteoblast specific proteins and extracellular matrix protein genes (Komori, 2010). Overexpression of Runx2 in nonosteogenic cells such as C3H10T1/2 cells and skin fibroblasts induced them to express osteoblast-related genes (Ducy et al., 1997; Komoro et al., 1997). Antisense oligonucleotides for Runx2 down regulated the expression of osteoblast-related mRNAs in ROS17.2/8 osteoblastic cells (Ducy et al., 1997). Reports also demonstrate that antisense oligonucleotides for Runx2 inhibited osteoblast differentiation, including formation of bone nodules *in vitro* (Banerjee et al., 1997). Several studies have shown that ERK1/2 activation stimulates Runx2 activation and osteoblast differentiation (Ge et al., 2007; Raucci et al., 2008; Franceschi et al., 2009; Jun et al., 2010). Thus, Runx2 regulates the expression of major bone matrix proteins during the early stage of osteoblast differentiation, but it is not essential to maintain these gene expressions in mature osteoblasts.

Earlier studies have shown that in the nucleus DMP1 can act as a transcriptional component for activation of osteoblast-specific genes like osteocalcin

(Narayanan et al., 2001). Published studies showed that osteocalcin, an osteoblast-specific product is encoded by the *Bglap2* (bone γ-carboxyglutamate protein) gene **(**Keertik et al., 2010). Reports suggest that Foxo1, a forkhead transcription factor indirectly down-regulates osteocalcin expression by inhibiting Runx2, a major upstream transcriptional activator of the *Bglap2* gene in osteoblasts (Shengyong et al., 2011). During osteogenesis, osterix plays a pivotal role in directing osteochondral progenitor cells down the osteoblast lineage by upregulating Osteocalcin (OC) expression (Corinne et al., 2011). However, results form our studies have shown that the upregulation in Runx2 and OC gene expression was abrogated by SB203580 and PD98059 confirming the role of p38 and ERK1/2 MAP kinase pathway in the differentiation of osteoblast.

This study also establishes the molecular connection between the MEK/ERK signal transduction pathway and osteoblast differentiation mediated by the integrin-binding RGD domain of DMP1. Overall, the data suggests the possibility of cross talk between the p38 MAPK and ERK pathways facilitating osteoblast differentiation and bone remodeling.

Bone remodeling is a continuous process where the bone is resorbed by the osteoclasts and is deposited by the osteoblast in a coordinated fashion. The deregulation in the balance between the osteoblasts and osteoclasts results in osteoporosis, rheumatoid arthritis and osteolytic metastasis. Metastasis to the bone is one of the most common and severe complications of breast cancer. When breast cancer cells metastasize to the bone, thus activates osteoclasts resulting in osteolytic lesions (Pratap et al., 2008). However, there exists data showing direct

evidence for cancer cell osteoclast interaction, less is known about the interaction of tumor cell with osteoblast at the site of metastasis.

Having gained insight into the mechanism of DMP1 activation in osteoblast we further sort to explore the potential role of DMP1 in breast cancer cells. Apart from the published report showing high intensity of immunohistochemical staining of DMP1 in breast cancer patients (Bucciarelli et al., 2007) not much is known about DMP1 in breast cancer. For this purpose, overexpression of DMP1 in metastatic breast cancer cells was performed to study the activated signal transduction pathways which lead to the stimulation of osteoblast differentiation thus activating osteoclast. This process could result in bone metastasis. Data from this study have shown that DMP1 overexpression causes a significant increase in Runx2, MMP2, MMP9, RANKL, OPN, BSP and VEGF mRNA expression. However reports have shown that factors such as MMPs, chemokine receptor 4 (CXCR4), VEGF, and connective tissue growth factor target metastatic tumor cells to bone and can facilitate survival within the bone microenvironment (Guise et al.,2006; Kang et al., 2003). Studies have shown that the expression of Hedgehog (Hh) ligands by breast cancer cells can initiate a cross-talk directly with osteoclasts thus promoting osteoclast differentiation and increase in resorption activity accompanied by increased expression of OPN and MMP9 (Das S et al.,2011). The ectotopic expression of Runx2 in human breast cancer cells has been extensively studied (Barnes et al., 2003). OC, BSP, MMP-9 and 13, and VEGF are some of the genes that are expressed in breast cancer cells and are also regulated by Runx2 (Liu et al., 2002; Sung et al., 1998;Thomas et al., 1999; Winding et al., 2002). Furthermore,

knockdown of DMP1 in metastatic breast cancer cell lines resulted in significant reduction in the gene expression of the above mentioned genes except for MMP2. Although MMP-2 and MMP-9 share structural and catalytic similarities, studies have suggested that the transcription of MMP-2 and MMP-9 may be independently regulated due to distinct arrays of *cis*-acting elements in the promoter (Sato et al., 1993). This study for the first time demonstrates that DMP1 like Runx2 could play a role in activating the osteolytic cycle in bone.

Phosphorylation by kinases is a major event for many cellular responses. To understand the molecular mechanism involved in DMP1 overexpressing breast cancer cell lines, the study investigates the signal transduction pathways activated in metastatic breast cancer cell line MDA MB231. Activation of p38, ERK1/2 and Smad 2/3 in the overexpressed cells were observed. Further, inhibition of p38 MAP kinase signaling in cancer cells was observed in the presence of SB20359. Studies have shown that TGF-β increases PTHrP secretion from MDA MB231 cells by signaling through both Smad2/3 and p38 MAP kinase pathways (Kakonen et al., 2002). TGF-β is stored in bone matrix (Hauschka et al., 1986) and is released locally in active form during osteoclastic resorption (Pfeilschifter et al., 1987), having an effect on cancer cells. In order to dissect the signaling pathways mediated by TGF-β, DMP1 overexpressing cells were stimulated with TGF-β. Results from this study revealed that DMP1 signaling in the presence of the cytokine in breast cancer cells leads to the upregulation of the MAP kinase pathway and Smad 2/3 pathways. TGF-β induced ERK activation has been shown for different cell types including fibroblast and epithelial cells (Giehl et al., 2000; Yue

& Mulder, 2000: Ellenrieder et al., 2001). Runx2 mediated activation of Smad 2/3 pathway has been documented in metastatic breast cancer cell lines (Pratap et al., 2006). Recent reports have shown that knockdown of Smad 4 expression in breast cacner cells prevents bone metastasis (Kang et al., 2005; Deckers et al.,2006). Results suggest that along with DMP1, TGF-β exerts a significant effect on the progression of breast cancer bone metastasis. However, knockdown of DMP1 resulted in a significant decrease in the activation of these pathways. Overall, this study indicates a causal role for DMP1 signaling in promoting differentiation and proliferation of breast cancer cells.

One of the most important properties of metastatic cells is their ability to degrade and move through the extracellular ground substances. Cell migration, invasion and proliferation are closely associated with cancer metastasis. In the present study, DMP1 overexpressed cells were highly invasive and proliferative in nature. In contrast, DMP1 inhibited migration in metastatic breast cancer cells. Studies performed on animal models indicate that Runx2 overexpression in metastatic breast cancer cell line is highly invasive and migratory in nature (Pratap et al., 2008). A plausible explanation for the contradictory results would be due to the artificial growth environment *in vitro* and the absence of certain factors that are present *in vivo,* which might play a pivotal role in migration. Results from the present study also suggest that metastatic breast cancer cells secrete DMP1 as a potent factor into the extracellular environment thus directing the tumor cell invasion towards bone undergoing remodeling. DMP1 in the extracellular environment can indirectly play a potent role in enhancing osteoclast activity. Results from this study

strongly support a model in which DMP1 signaling plays a significant role in breast cancer bone metastasis.

To further explore the relationship between breast cancer and differentiated osteoblasts in activating the osteolytic cycle in bone a co-culture model using MC3T3-E1 cells and MDA MB231 cells was established. Results from the *in vitro* study showed differential pattern of arrangement of cancer cells in the presence of osteoblasts. This data suggest that the osteoblast and cancer cells influenced each other under co-culture conditions. Published reports have shown that osteoblast-conditioned medium reportedly promotes breast cancer cell proliferation (Mercer et al., 2004). This study typically employed cell culture systems in which DMP1 overexpressing breast cancer cells were co-cultured with osteoblasts. To more closely mimic the *in situ* environment of bone-cancer in activating osteoclasts, conditioned media obtained from the co-culture of osteoblasts and breast cancer cells were used to stimulate precursor monocytes (RAW 264.7) to differentiate into osteoclasts.

Tumor induced osteolysis is mediated by the osteoclasts. Multinucleation, an essential step in osteoclast differentiation, is a prerequisite for its efficient bone-resorbing ability. Mononuclear osteoclasts fuse repeatedly to form giant multinucleated osteoclasts, which after the polarization of the membrane and organization of the cytoskeleton result in mature bone-resorbing osteoclasts. The murine macrophage cell line RAW 264.7 cell expresses RANK and can readily differentiate into functional osteoclast cells in the presence of RANKL (Hsu et al., 1999). TGF-β has been shown to increase RANK expression on murine monocytic

cells and to stimulate osteoclast differentiation (Yan et al., 2001). Recently, reports have shown that TGF-β as well as RANKL is necessary for osteoclast formation (Fuller et al., 2000). It has also been reported that the interaction of RANK and RANKL increases survival of mature osteoclast *in vivo* and *in vitro (*Lacey et al., 2000). Studies have shown that the use of siRNA for RANK signaling can control osteoclast mediated bone resorption (Wang et al., 2010). Mice lacking RANK or RANKL are deficient in osteoclasts and lack osteoclastogenesis (Li et al., 2000). Based on the results, the presence of TRAP positive multinucleated cells was observed when RAW 264.7 cells were cultured in the presence of conditioned media of DMP1 overexpressing breast cancer cells. Thus the conditioned medium of the DMP1 overexpressing breast cancer cells enhances the efficacy of the breast cancer cells to elicit osteoclast differentiation and resorptive activity. The study then further sought to investigate the role of p38 and ERK1/2 MAP kinase signaling pathway in accelerating osteoclast formation. Published reports have shown that MEK specific inhibitors can accelerate differentiation of RAW 264.7 cells to osteoclast (Hotokezaka et al., 2002).This study therefore mimic the *in vivo* condition by performing experiments on RAW 264.7 cells using MAP kinase inhibitors like SB230580 and PD98059 in the presence of the conditioned media obtained from DMP1 overexpressed cells. The data demonstrate that these inhibitors also have an impact on osteoclasts. Results from the studies clearly show enhanced TRAP expression when p38 and ERK1/2 MAP kinase pathway are inhibited. In conclusion, the inhibition of DMP1 signaling in pre-osteoblasts using chemical inhibitors for MAP kinase pathway would accelerate the ability of pre-osteoclasts to respond to the

stimulatory effects of DMP1 overexpressing breast cancer cells, indicating that DMP1 signaling is vital to osteoclast activity. Our results have shown that the activated osteoclast released the matrix-bound TGF-β which in turn acts on the breast cancer cells thus activating the vicious cycle.

Overall, this study indicates the role of DMP1 signaling in promoting breast cancer cell-mediated osteolytic activity. Results from this study demonstrate that stimulation of preosteoblasts with DMP1 results in depletion of ER Ca^{2+} stores and a rise in cytosolic Ca^{2+}. This perturbation of Ca^{2+} homeostasis elicits a stress-like response, resulting in the activation of the p38 MAPK signal transduction cascade. On the other hand the study also shows the activation of integrin mediated ERK1/2 MAP kinase pathway. Upon activation, the MAP kinase can coordinate the expression of downstream target genes like Runx2 and OC to regulate osteoblast differentiation. The net result is the osteoblast maturation process. The differentiated osteoblastic cells produce and enhance the secretion a variety of mediators that modulate the differentiation of osteoclast precursors, a critical step for bone resorption to occur. Among them is the cytokine receptor activator of NF-κB ligand (RANKL) which is essential for osteoclastogenesis. The cytokine exerts specific effects on precursor monocytes and constitutes a part of the cellular microenvironment. The membrane-bound RANKL expressed by osteocytes then binds to receptor RANK present on the surface of the mononuclear osteoclast progenitors resulting in the fusion of monocytes into multinucleated cells. Furthermore, results obtained from this study also show that TGF-β released from the activated osteoclast, triggers the MAP kinase and Smad2/3 pathways in

metastatic breast cancer leading to the osteolysis of bone (Figure. 35). The study thus describes a new function for DMP1 besides its classical role as a hydroxyapatite nucleator.

CHAPTER 7

FUTURE STUDIES

This project identified the role of DMP1 in promoting the differentiation of pre-osteoblast cells into functional osetoblast cells by calcium mediated stress p38 MAP kinase and by the integrin mediated ERK1/2 MAP kinase activation. During this process the upregulation of MAP kinase dependent osteoblast-associated transcription factors like Runx2 and osteocalcin were observed. The secretion of RANKL by the activated osteoblast stimulates the differentiation of osteoclast. This results in osteoclastic bone resorption that causes the release and activation of growth factors TGF-β, which is stored in mineralized bone matrix. TGF-β can function at multiple stages that include recruiting stem cells and promoting stem cell renewal, coupling osteoclastic bone resorption to bone formation and inhibiting osteoblast differentiation. Results from this study have shown that the secreted TGF-β can stimulate the breast cancer cells. Moreover, the results obtained in this study identified a new role for DMP1 in the proliferation and invasion of breast cancer cells that may be directly related to their metastatic potential. To further evaluate the direct consequence of DMP1 overexpression and knock down in breast cancer cell–mediated osteolysis *in vivo*, future studies are to be performed by administrating subcutaneous and intra tibial injections of MDA MB231 over expressing DMP1 and shRNA-DMP1 cells in SCID mice. The bone loss would be determined by radiography for 3 weeks after injections. For this purpose the study demands the use lentivrial constructs of DMP1 for the overexpression in MDA

MB231 cells. TRAP staining will be performed to study the cellular changes in the tumor-bone microenvironment.

Future studies would be performed to study the gene regulation and activation of other signal transduction pathways like Sonic Hedgehog and Notch in breast cancer cells.

APPENDICES

APPENDICES A

Subject: RE:
From: "Bacac Marina" <Marina.Bacac@chuv.ch>
Date: Sun, October 2, 2011 4:05 am
To: "Eapen, Asha Sarah" <ashasara@uic.edu>
Cc: "Stamenkovic Ivan" <Ivan.Stamenkovic@chuv.ch>
Priority: Normal
Options: View Full Header | View Printable Version | Download this as a file | View Message Details | Add to Address Book

Dear Sarah,
it is fine for us.
Good luck with your thesis!
Marina

From: Eapen, Asha Sarah [ashasara@uic.edu]
Sent: 28 September 2011 05:26
To: Bacac Marina
Subject:

Dear Dr Bacac,
I am writing to request permission to use the following material from your publication (Metastatic cancer cell.Bacac M, Stamenkovic I.Annu Rev Pathol. 2008;3:221-47.) in my thesis.

This material will appear as originally published. Unless you request otherwise, I will use the conventional style of the Graduate College of the University of Illinois at Chicago as acknowledgment.

A copy of this letter is included for your records.

Thank you for your kind consideration of this request.

Sincerely,
Asha Sarah Eapen
PhD student
Anatomy and Cell Biology,
UIC, Chicago

APPENDICES B

From: "Laurie McCauley" <lauriemccauley@gmail.com>
Subject: Re: Request for permission to use your published material in Ph.D thesis
Date: Mon, September 26, 2011 5:22 pm
To: "Eapen, Asha Sarah" <ashasara@uic.edu>
Cc: "Kathy Weilbaecher" <kweilbae@im.wustl.edu>

Dear Asha:

I'm sure it will be fine
Best wishes for your thesis work and career goals!

Laurie McCauley

****
Laurie K. McCauley, DDS, PhD
William K. and Mary Anne Najjar Professor
Professor and Chair
Department of Periodontics and Oral Medicine
University of Michigan School of Dentistry
1011 N. University Ave.
Ann Arbor, MI 48109-1078 TEL: +1(734)-647-3206
mccauley@umich.edu
****

On Mon, Sep 26, 2011 at 5:57 PM, Eapen, Asha Sarah <ashasara@uic.edu> wrote:

> Dear Dr McCauley,
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> publication (Cancer to bone: a fatal attraction.
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> Jun;11(6):411-25. Epub 2011 May 19.) in my thesis.
>
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> otherwise, I will use the conventional style of the Graduate College of
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>
> A copy of this letter is included for your records.
>
> Thank you for your kind consideration of this request.
>
> Sincerely,
> Asha Sarah Eapen
> PhD student

APPENDICES C

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originally published. Unless you request otherwise, I will use the conventional
style of the Graduate College of the University of Illinois at Chicago as acknowledgment.

A copy of this letter is included for your records.

Thank you for your kind consideration of this request.

Sincerely,
Asha Sarah Eapen
PhD student
Anatomy and Cell Biology,
UIC, Chicago

APPENDICES D

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Chicago, Illinois 60612-7227

August 22, 2011

Anne George
Oral Biology
M/C 690

Dear Dr. George:

The protocol indicated below has been reviewed in accordance with the Institutional Biosafety Committee Policies of the University of Illinois at Chicago on 7/14/2011 **with the following condition.** *The protocol was not initiated until final clarifications were reviewed and approved on* ***8/11/11. Protocol expires 3 years from the date of review (7/14/14).***

Title of Application: Characterization of DMP1, 2, 3 and 4 Dentinphosphoproteins

IBC Number: 11-030

Condition of Approval: The enclosed report indicates the training status for bloodborne pathogen (BBP) training. Only those personnel who have been trained and whose training has not expired are approved for work that may involve exposure to bloodborne pathogens. Please note that federal regulations require yearly training for BBP.

Highest Biosafety Level: 2

You may forward this letter of acceptable IBC verification of your research protocol to the funding agency considering this proposal. **Please be advised that investigators must report significant changes in their research protocol to the IBC office via a letter addressed to the IBC chair prior to initiation of the change. If a protocol changes in such a manner as to require IBC approval, the change may not be initiated without IBC approval being granted.**

Thank you for complying with the UIC's Policies and Procedures.

Sincerely,

Randal C. Jaffe, Ph.D.
Chair, Institutional Biosafety Committee

RCJ/mbb

Enclosures

Cc: IBC file, Amsaveni Ramachandran

CITED LITERATURE

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Publications:

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- Mechanical loading inhibits the inflammatory response in articular cells. (Andonian C., Deschner, J., **John, A**., Rath-deschner, B., Agarwal, S.)

- Possible role of IL-10 in TMJ repair by motion-based therapies. (Rath-Deschner, B., Deschner, J., **John, A**., Agarwal,S.)

- Mechanical strain regulates N-cadherin and Wnt7a in articular cells.(Sjostrom, D., Deschner, J., Rath-Deschner ,B., **John, A**., Agarwal., S.)

- Inhibition of Il-1 stimulated RANK-L expression by biomechanical strain. (Wypasek, E., Deschner, J., Ferretti, M., **John, A**., Rath-deschner, B., Agarwal,S.)

Cyclic Strain alters inflammatory phenotype of meniscal cells in rats.36th Brazilian Orthopaedic and Traumatology Congress. 2004. Rio de Janeiro-Brazil (Ferretti, M., Deschner, J., **John, A**., Rath-deschner, B., Cohen, M., Agarwal, S.)